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Guidebook to Protein Toxins and Their Use in Cell Biology

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A computer system will be available from April 1997 to accompany *Guidebook to protein toxins and their use in cell biology*

Due to the rapid pace of biological research, the editors and publishers of this book believe it is important that its readers are kept informed of recent developments on these proteins. For this purpose, we have established a computer database that can be accessed through the worldwide web. This database will not include the full entries shown in this book; instead the authors have been asked to add, periodically, any new information on their protein that has been published since they wrote their original entry. Authors will be asked to deposit their updates from April 1997.

The update system can be accessed using any of the standard tools for browsing the worldwide web, such as Netscape or Mosaic. The URL for information relating to this book is *http://www.oup.co.uk/guidebooks/toxins.* For information on other Sambrook & Tooze Guidebooks, start from the Oxford University Press home page at *http://www.oup.co.uk/* and follow the links to the Guidebooks series.

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Preface

Poisons are chemical scalpels for the dissection of physiological processes
(Claude Bernard (1866) *Leçons sur les propriétés des tissus vivants*, p. 177, Paris)

Many prokaryotes, animals, and plants produce toxins that exert their toxic, sometimes lethal, activity toward other living organisms. In several cases, the same organisms produce more than one toxin at the same time. Toxins can be considered a sort of biological warfare that somehow 'helps' the toxin-producing species in their struggle for life. Toxins are believed to increase the chance of survival and/or proliferation and/or spreading of a particular organism in its particular environment. While it is simple to understand the advantages conferred by certain toxins contained in the snake or spider venoms, this is not always the case for plants or microorganisms. However, this is to be attributed to our present lack of knowledge of the ecology and evolutionary history of the toxin-producing species, rather than to a lack of 'significance' or 'usefulness' of the toxin.

Hence, natural toxins (to distinguish them from those designed and produced by humans) are the product of a long-term co-evolution of species sharing the same ecological niche. In this process, toxins have been shaped and modelled to selectively hit a key molecular target of the host or of the prey. Somehow, in the course of evolution, toxins have *'learned which are the most essential or the most vital processes of living organisms'*. Moreover, *'they have learned how to affect selectively the key molecule(s) of a given processes'*. In the light of neo-Darwinism, we can fully substantiate and appreciate the conclusion derived by Claude Bernard from his classical physiological studies on the action of healing and other toxic substances: toxins can be used as very precious chemical scalpels which help the biologist to reach processes and targets not accessible to the anatomist's scalpel. In its essence, this conclusion is still valid and it is the basis of the present volume in the Guidebook series.

Animal toxins

One may notice that all animal toxins are directed to a cell surface protein involved in an essential cell function. Nearly all of them are ligands of ion channels which are inactivated upon toxin binding, and interfere with neuronal or muscular functions (Parts 7–13). This is related to the fact that toxin-producing animals use venom to attack and immobilize the prey or the potential predator. Blocking the synapse, the specialized anatomical structure at the basis of the function of integrated organized animals, leads to the immobilization/paralysis of the injected animal. Perhaps owing to the need for rapid action, these animal toxins are designed to act at the cell surface. Figure 1 summarizes the most important ion channels attacked by protein toxins.

Bacterial toxins

In this respect, bacteria are very different. They do not have such needs of rapidity of action. Moreover, they co-evolve with their hosts much more rapidly than animal species. Hence, they have been able to develop a variety of sophisticated strategies of survival and of modifications of host physiology in order to promote their own multiplication and spread. The study of these strategies has given rise to the emergence of a new discipline, which has been termed recently 'Cellular Microbiology'. Some of these strategies involve the use of protein toxins that attack the host cells in a variety of different ways. Figure 2 summarizes pictorially the fundamental aspects of a cell that are affected by bacterial toxins. A large group of toxins alters the plasma membrane integrity in such a way as to alter the membrane permeability barrier (Part 1). They do so with different mechanisms, but the end result is, in any case, the death of the cell. Membrane pore formation is being exploited in cell biology to gain access to the cell interior. Nearly all remaining bacterial protein toxins act inside the cell. These toxins usually consist of two parts: one involved in cell binding and penetration (termed B), and one domain (termed A) displaying intracellular enzymic activity. None of them enter the cell from the plasma membrane. Rather, they make use of the endocytic pathways elaborated by cells to intake ligands and nutrients and to digest away from the surface un-needed ligands and/or receptors, as well as bound materials. They bind to cells via the B part and are endocytosed, sometimes only at specialized portions of the cell surface, such as the apical membrane or the presynaptic membrane terminals. They are then taken up inside the lumen of intracellular compartments. Consequently, different toxins travel different cellular highways or minor roads, depending on the particular receptor they are bound to and on the cell type. They turn toxic only when they become translocation competent, i.e. they become capable of translocating domain A across the membrane into the cytosol. This is the least understood of the four steps of the cell intoxication process.

On the basis of the present knowledge, we can distinguish two group of bacterial toxins: those having a

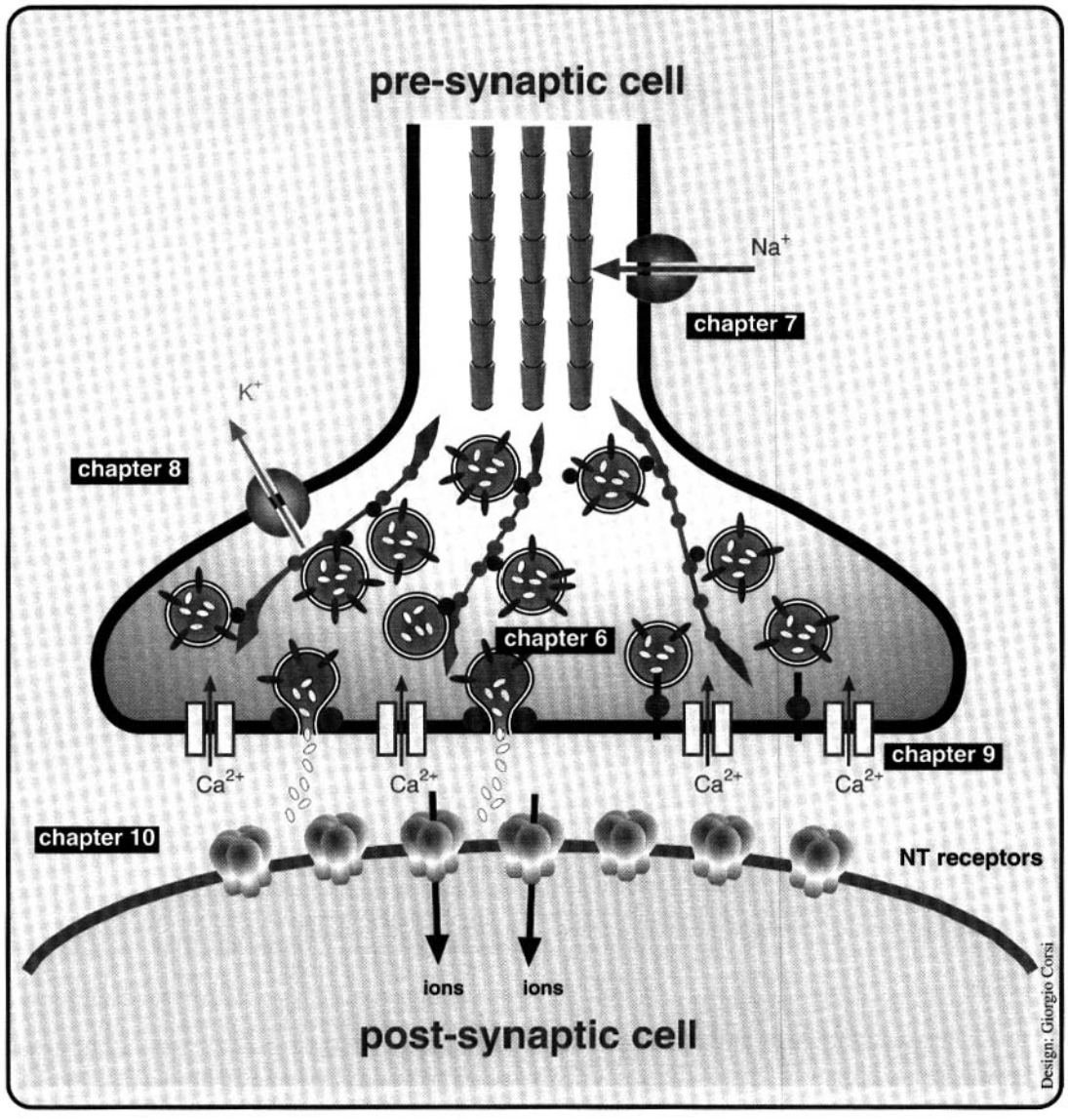

Figure 1. Scheme of a synapsis showing the most important ion channels and secretion patterns that are targets of protein toxins, and the chapters where they are described.

B part composed of two domains (diphtheria toxin, clostridial neurotoxins, etc.) and those having an oligomeric B domain (cholera toxin, shiga toxin, etc.). The first group of toxins enters the cell cytosol via an acidic intracellular compartment. There is evidence that low pH triggers a conformational change that makes these proteins able to insert in the lipid bilayer and to mediate the transfer of the catalytic A domain to the cytosol. The second group of toxins does not depend on low pH for their entry into the cytosol, and there is evidence that the reduction of the single disulfide bond that links A to the B oligomer is sufficient to cause a change in the solubility properties of the A chain, so that it becomes able to partition into the lipid bilayer. Unfortunately, there is no information on the subcellular compartment where reduction takes place. Its identification would expand the use of these toxins as markers of intracellular reducing events. Once in the cytosol, these toxins interfere with a number of key cell events. Some toxins attack various components of the protein synthesis machinery (Part 3),

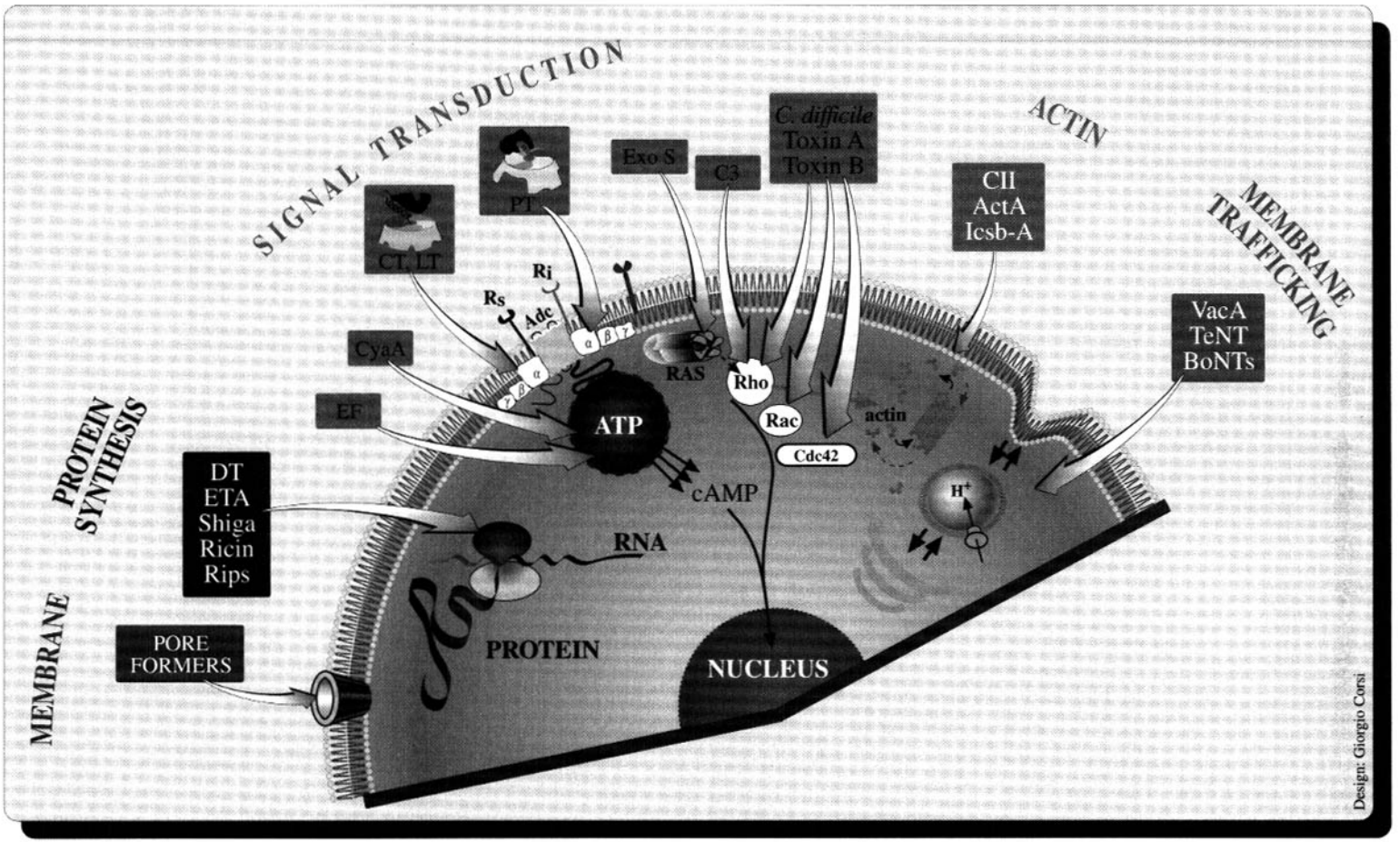

Figure 2. Schematic representation of bacterial toxins and their targets.

other toxins interfere in different ways with actin polymerization (Part 4). A group of toxins is specific for the trimeric G proteins that control several events of signal transduction or they increase directly the cellular c-AMP level (Part 2). Other toxins interfere with the immune and/or inflammatory response (Part 5), and another group of toxins affects different steps of vesicular trafficking inside cells (Part 6).

About this guidebook

Several toxins are well known in terms of their cellular activity, but their cellular targets remain to be identified (anthrax lethal factor, α-latratoxins, phospholipase snake toxins etc.). In general terms, the study of the mechanism of action of these toxins is important in two respects: (a) to understand the molecular pathogenesis of the disease in which the toxin is involved; (b) to learn about the molecular mechanism underlying the fundamental physiological process attacked by the toxin. Once this is known, the toxin can be used as a tool in the study of cell biology. This book focuses on the use of protein toxins as tools in cell biology. Accordingly, some toxins with poorly defined cellular effects are not included. At the same time, some toxins whose molecular targets are still to be identified, but which are expected to be very useful in the near future, are included. The aim of such a book is that of providing information and bibliographic references on the use of a large array of known protein toxins in the study of the molecular mechanisms involved in the particular process considered. In some cases, the understanding of the mechanism of action of a toxin has provided novel therapeutic approaches to diseases affecting a particular function.

Natural toxins display an incredible variety of complexity, ranging from the simple formic acid of ants to bacterial proteins composed of thousands of amino acids. This book only considers protein or peptide toxins, essentially because of our restricted competence and to keep the volume within a reasonable size. We have organized the book in parts, which begin with an introduction to the cellular protein or process affected by a given group of toxins. Each part includes an entry for each different toxin, or group of toxins, with that particular target molecule (i.e. calcium channels) or cellular function (i.e. assembly of actin cables). The book does not mention all known protein toxins, rather, it considers them with respect to the interest of the cell biologists and groups toxins performing the same action within the same entry. Given the large number of known protein toxins and the fact that new ones are continuously being discovered, we may have failed to include appropriate entries. We apologize for such an inconvenience. We will be grateful to those who signal to us novel entries of potential interest to the cell biologist to be included in future editions of this Guidebook.

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Siena and Padova, R. R.
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Membrane permeabilizing toxins

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Introduction

Among the wide variety of toxins which bind and kill cells quite a large proportion have the plasma membrane as a target. Whatever their origin, structure, and size, varying from small peptides to large sophisticated oligomeric proteins, all perform a similar function: they permeabilize cells. The action of all the toxins assembled in this part of the book results from a physical interaction with membrane components. They are defined as direct lytic factors as opposed to other toxins having enzymatic properties and able to chemically degrade the membrane.

The first primordial function of the plasma membrane is to maintain all materials, from macromolecules to small solutes, selectively in the cell, i.e. the membrane is equipped in order to very selectively and efficiently regulate permeability. Consequently, it is not surprising that many bacterial toxins act on the plasma membrane and have evolved to change efficiently the first critical property required for living. Indeed, any unbalanced entry, or efflux of ions or small molecules should be opposed by selective pumps and channels in order to recover, as soon as possible, the physiological conditions by active transport. But if the nonselective fluxes induced by toxins overcome the capabilities of the cells to recover their equilibrium quickly, due either to an important transitory change, 'a burst', or to a permanent leakage through a 'hole', cells will generally die. This often results from the entry of ions inducing metabolic disorders, like Ca^{2+} entry, causing entry of water which swells the cell in order to compensate the osmotic pressure due to the presence of concentrated polyelectrolytes inside the cell. Conversely, the escape of vital compounds, again ions and small metabolites, can be lethal. Then all the following toxins have the same effect, they only differ in the way they perform it, in their efficiency in inducing the deleterious imbalance of permeability, and in the detailed structure and mechanism causing an open structure through which lethal leakage will occur.

In order to generate defects, channels, or holes in the membrane, protein toxins should provide a pathway for water and small solutes. Whatever the detailed structures, all such proteins must be amphipathic: one part should be lining a water filled domain, whereas others should be in contact with lipid chains and/or apolar segments of integral membrane proteins. This amphipathicity can be achieved at different levels:

1. By secondary amphipathic structural segments like helices or β-sheets, as for the peptidic toxins (see entry for δ-toxin p. 13) or porins (Weiss and Schulz 1992).Toxins are dominated by the requirement of being amphipathic.The peptidic toxins are more soap-like, weakly specific, self-associated both in buffer and in the membrane and can even solubilize lipids by forming reversed disc-like structures (Cornut *et al.* 1993; Saberwal and Nagaraj 1994).
2. By tertiary amphipathic structures for sophisticated Janus type proteins. The constituting amphipathic helices are packed with an apolar core when in buffer, and with an hydrophilic one when embedded into lipids (Van der Goot *et al.* 1991).
3. By quaternary amphipathic structures of proteins, which oligomerize in buffer by burying their apolar faces, and conversely oligomerize in the membrane with a polar hole or central channel. A variant of such a situation was recently proposed from the X-ray structure of annexins (Luecke *et al.* 1995). The aerolysin structure provides the first example of a β-sheeted oligomeric channel, whose oligomerization takes place only in the membrane (Parker *et al.* 1994).

Increasing the size of toxins generally allows an increase in the selectivity for a more unique target, either a lipid such as SLO and thiol-activated toxins (see corresponding entries), or a defined protein as documented for α-toxin (Walker *et al.* 1992). This also allows water solubility to be maintained by burying the membrane active area and avoiding too severe aggregation in buffer, which will decrease the affinity and availability of the toxin for its target.

Whatever the toxin, different steps which govern their cytotoxicity can be identified:

1. Toxins should interact selectively or be able to cross the thick glycosylated outer part of most cells.
2. Then toxins quickly bind to a selected membrane protein and/or lipids with high affinity. This results generally in an increased concentration of toxins more or less reversibly adsorbed on to the membrane. Most of the hydrophobic effects are already recovered and conformational changes take place concomitantly.
3. Toxins then reorient and penetrate into the membrane. Such a step is governed by the membrane's properties, it is therefore strongly dependent on the physical state of the lipids: the more defects in packing, the more easily toxins penetrate. This step is slow, it can take minutes to form a new structure (Yianni *et al.* 1986), and is strongly temperature-dependent as is well documented for SH-activated toxins (Alouf and Geoffroy 1991).

4. Embedded toxins oligomerize differently or aggregate in order to expel the polar or charged domains from lipid contact. This generally requires lateral diffusion, i.e. fluid membranes. For the larger toxins quite specific interactions occur between the subunits leading to a single well-defined structure and properties like those of α-toxin (Ostolaza *et al.* 1993). But oligomerization could be rather nonselective and lead to high molecular weight aggregates as in the case of SH-activated toxins (see entries for SLO p. 5 and others). The progressive change of the channel size is also documented for the peptidic toxins (see entry for δ-toxin, p. 13). Crosslinking of such toxins drastically increases their effects (Vogel *et al.* 1995).

Most of the toxins are produced and/or stored in a protoxin inactive form. The activation step varies, it could be a cleavage of an N_{term} acidic peptide like for melittin, or a C_{term} proteolysis as in alveolysin, but the *Escherichia coli* hemolysin activation results from an acylation. All such steps increase the affinity for the membrane which appears to be essential for activity. High cytotoxicity and high affinity for the membrane seem to be correlated, for instance the cytotoxic peptides have to be present in 10^5 to 10^6 copies in the target cell to get lysis, their LD_{50} are rather high 10^{-8} to 10^{-7} M, their nonstereo-specific binding is governed by the water/membrane partition coefficient (Fisher *et al.* 1994; King *et al.* 1994). In contrast, thiol-activated toxins have a higher affinity and clear stereospecificity for cholesterol (see entry p. 7). Indeed, one of the highest affinities is that of *E. coli* hemolysin, which has probably the highest selectivity and then efficiency (see entry p. 18).

At sublytic concentrations most of the toxins already induce changes in many cellular properties. Such effects can be defined as secondary, and are generally accounted for by an increased permeability with special effects due to Ca^{2+} entry. This results in production of eicosanoid compounds through activation of phospholipases, secretions, or inhibition of other enzymes. Moreover, the best documented synergistical activation of lipases implies direct interactions between toxins and the substrate and products of the reaction, but until now no direct toxin–enzyme contacts have been established. This leads to the synergism between the direct lytic factors described in this part and the indirect lytic ones which are for example inflammatory toxins (see Part 5) and signal transductory ones (see Part 2).

Finally, despite quite impressive progress being made on the structures and modes of action of the toxins, we do not as yet understand the molecular laws for the cell selectivity out of a few significant cases like cholesterol-sensitive toxins. Generally, we do not have any clear explanation for increased sensitivity of some eukaryotic cells and the way membrane from mammals, or bacteria, responds differently from species to species to the same toxin attack. The comparison of toxins and physiological channels will also highlight the differences and common features (Bladon *et al.* 1992).

References

Alouf, J. E. and Geoffroy, C. (1991). The family of antigenically related cholesterol-binding, sulphydryl-activated, cytolytic toxins. In *Bacterial protein toxins* (ed. J. E. Alouf and J. H. Freer), pp. 147–86, Academic Press, London.

Bladon, C. M. , Bladon, P., and Parkinson, J. A. (1992). δ-Toxin and analogue as peptide models for protein ion channels. *Biochem. Soc. Trans.*, **92**, 862–4.

Cornut, I., Thiaudiere, E., and Dufourcq, J. (1993). The amphipathic helix in cytotoxic peptides. In *The amphipathic helix* (ed., R. M. Epand), pp. 173–219, CRC Press, Boca Raton, Florida.

Fisher, T. J., Prendergast, F. G., Ehchardt, M. R., Urbauer, J. L., Wand J., Sedarous, S. S., *et al.* (1994). Calmodulin interacts with amphipathic peptides composed of all D-amino acids. *Nature*, **368**, 651–3.

King, T. P., Wade, D., Coscia, M. R., Mitchell, S., Kochoumian, L., and Merrifield, B. (1994) Structure–immunogenicity relationship of melittin, its transposed analogues and D-melittin. *J. Immun.*, **152**, 1124–1131.

Luecke, H., Chang, B. T., Mailliard, W. S., Schlaepfer, D. D., and Haigler, H. T. (1995). Crystal structure of the annexin XII hexamer and its implications for bilayer insertion. *Nature*, **378**, 512–15.

Ostolaza, H., Bartholome, B., Ortiz de Zarate, I., de la Cruz, F., and Goni, F. M. (1993). Release of lipid vesicle contents by bacterial protein toxin α-haemolysin. *Biochim. Biophys. Acta*, **1147**, 81–8.

Parker, M. W., Buckley, J. T., Postma, J. P. M., Tucker, A. D., Leonard, K., Pattus, F., *et al.* (1994). Structure of Aeromonas toxin proaerolysin in its water soluble and membrane-channel states. *Nature*, **367**, 292–5.

Saberwal, G. and Nagaraj, R. (1994). Cell-lytic and antibacterial peptides that act by perturbing the barrier function of membranes: facets of their conformational features, structure–function correlations and membrane-perturbing abilities. *Biochim. Biophys. Acta*, **1197**, 109–31.

Van der Goot, F. G., Gonzàles-Mañas, J. M., Lakey, J. H., and Pattus, F. (1991). A 'molten globule' membrane insertion intermediate of the pore-forming domain of colicin A. *Nature*, **354**, 408–10.

Vogel, H., Dhanapal, B., Pawlak, M., Meseth, U., Eggleston, I. M., and Mutter, M. (1995). Membrane channel forming properties of melittin-TASP. In *Peptides 1994*, (ed. H. S. L Maia) pp. 449–50, ESCOM, Leiden.

Walker, B., Krishnasastry, M., Zorn, L., Kasianowicz, J., and Bayley, H. (1992). Functional expression of the α-hemolysin of *Staphylococcus aureus* in intact *Escherichia coli* and cell lysates. *J. Biol. Chem.* **267**, 10902–9.

Weiss, M. S. and Schulz, G. E. (1992). Structure of porin refined at 1.8 Å resolution. *J. Mol. Biol.*, **227**, 493–509.

Yianni, Y. P., Fitton, J. E., and Morgan, C. (1986). Lytic effects of melittin and δ-lysin from *Staphylococcus aureus* on vesicles of DPPC. *Biochim. Biophys. Acta*, **856**, 91–8.

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Streptolysin (*Streptococcus pyogenes*)

SLO is a 61 kDa protein produced by Streptococcus pyogenes *A and C. SLO binds specifically to cholesterol in target membranes. Bound toxin molecules associate with each other to form large, arc- and ring-shaped polymers that insert into the bilayer to produce transmembrane pores of up to 35 nm diameter.*

Streptolysin O (SLO) is produced by beta-hemolytic streptococci. It belongs to the group of thiol-activated toxins, a large family of homologous cytolysins which are secreted by gram-positive bacteria and share extensive homology (Alouf and Geoffroy 1991). All of these toxins bind to cell membranes containing cholesterol and then polymerize to form pores of up to 35 nm diameter (Bhakdi *et al.* 1985). The largest stretch of homology among all known thiol-activated toxins is situated about 40 amino acids upstream of the C-terminus and contains the single cysteine residue of the molecule. Chemical modification of this cysteine residue abrogates the ability of the toxins to bind to cell membranes (Iwamoto *et al.* 1987); thus the C-terminus plays an essential role in binding. At present, no other features of toxin function could be clearly assigned to parts of the protein sequence. The SLO gene has been cloned and sequenced (Kehoe *et al.* 1987; data bank accession numbers: M18638, P21131).

Streptococcal cysteine-protease cleaves the native, 61 kDa toxin close to its N-terminus to yield a 55 kDa truncate with unaltered pore-forming activity (Pinkney *et al.* 1995). The 61 kDa and 55 kDa toxin forms are present in streptococcal culture supernatants. Binding of SLO occurs very rapidly and in an essentially non-saturable fashion even at low temperature and without ionic requirements. After a rate-limiting nucleation step of second order, oligomerization proceeds by successive addition of monomers (Palmer *et al.* 1995). Electron microscopy reveals arc-shaped intermediate stages spanned by a free edge of membrane lipids (Bhakdi *et al.*

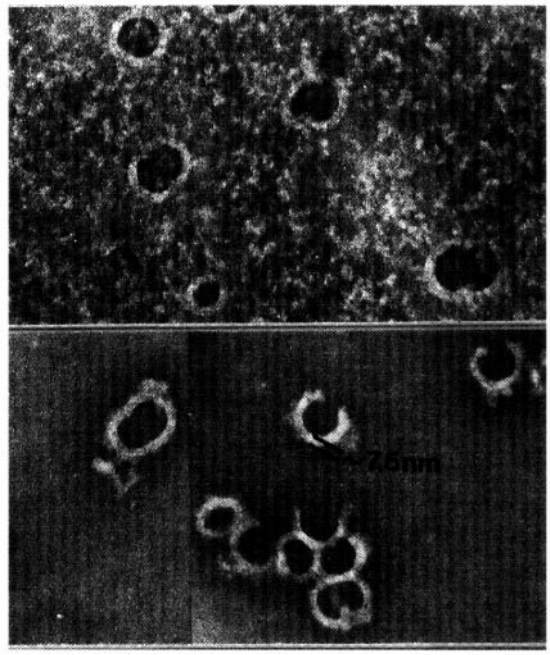

Figure 2. Top: Negatively stained erythrocyte membrane lysed by streptolysin O (SLO) showing curved rods (25–100 nm long and approximately 7.5 nm wide with inner radius of curvature of 16–18 nm. Most rods are approximately semicircular, often joined in pairs at their ends. Dense accumulations of stain are seen at the concave side of the rods. Bottom: Negative staining of isolated SLO oligomers, showing numerous curved rod structures identical to those found in toxin-treated membranes.

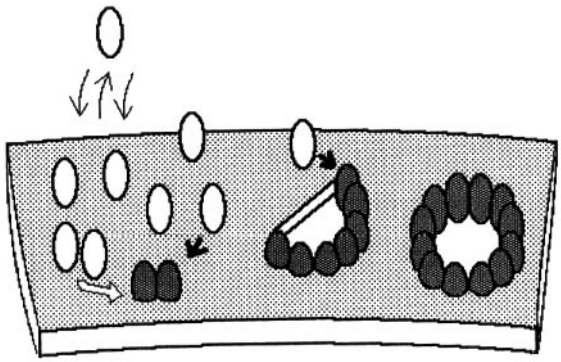

Figure 1. Schematic representation of streptolysin O oligomerization.

→: reversible association of monomeric streptolysin with membranes containing cholesterol.

⇒: nucleation step, two monomers react to form a stably membrane-embedded start complex.

→: oligomerization proceeds by successive addition of monomers, giving rise to arc- and finally ring-shaped complexes (consisting of 25 of 50 monomers).

1985), suggesting that transmembrane pores arise concomitant with oligomer growth. The strong dependence of this process on temperature and toxin concentration allows for good control of experimental cell permeabilization.

Purification and sources

SLO can be isolated from streptococcal culture supernatants by ion exchange chromatography (Bhakdi *et al.* 1984) and chromatography on SH-Sepharose (Alouf and Geoffroy 1988). The purification product usually comprises native and N-truncated SLO, both forms display the same functional activity. A protocol for obtaining recombinant SLO from *E. coli* in fusion with maltose-binding protein has been established (Weller, manuscript in preparation). The commercial products may contain considerable amounts of impurities including proteases. Purified SLO is stable when stored lyophilized at –70 °C.

Toxicity

Functional activities are most conveniently assessed by determining the hemolytic titer against rabbit erythrocytes. Tests should be performed in the presence of 2 mM DTT and 0.1 per cent albumin. A toxin concentration of approximately 2–3 ng/ml causes 50 per cent lysis of a rabbit erythrocyte suspension (1.25×10^8 cells/ml). The sensitivity of nucleated cells towards SLO varies (Walev *et al.* 1995), as do the domains of polarized cells. For example, the apical side of MDCK cells is approximately 10 times more resistant than the basal side (Pimplikar *et al.* 1994). The cause of these varying sensitivities is largely unknown but may be due to formation of nonfunctional polymers (Walev *et al.* 1995). Functional activity in nucleated cells is most conveniently tested by determining the toxin concentration required to render the majority of cells trypan-blue or propidium iodide-positive. Measurements of LDH release or of ATP-depletion may also be employed.

SLO does not damage intact skin or mucous membranes. Use of the toxin hence does not present problems to operators. Systemic toxic reactions and organ dysfunction are induced upon intravasal administration or local application to organs. The LD_{50} for mice is 8–25 μg/kg (Smyth and Duncan 1978). All adults have antibodies against SLO that neutralize its activity in the application range of < 1 μg/ml (Aguzzi *et al.* 1988; Falconer *et al.* 1993).

Use in cell biology

SLO is being widely used to produce large pores in cell membranes, allowing the introduction of macromolecules to the cytoplasm (Ahnert-Hilger *et al.* 1989; Bhakdi *et al.* 1993). When applied to intact cells, SLO will be quantitatively absorbed to cholesterol and trapped in the plasma membrane; hence, intracellular membranes are spared (Esparis Ogando *et al.* 1994). The most reliable way to ensure a selective action on the plasma membrane is to expose cells to the toxin at low temperature; binding occurs rapidly and virtually quantitatively, whereas polymerization is retarded (Alouf and Geoffroy 1991; Rapp *et al.* 1993). Thereafter, permeabilization is effected by raising the temperature. At this stage, macromolecules that are to be applied to the cytoplasm (e.g. antibodies) can be added to the medium. When cells are suspended in appropriate media, a variety of machineries *continue to operate over quite a prolonged time period.* Examples include trafficking of membrane vesicles, and import of proteins and peptides into peroxisomes (Wendland and Subramani 1993) and into the endoplasmic reticulum (Tan *et al.* 1992; Momburg *et al.* 1994). In the future, it will probably become possible to permeabilize intracellular membranes, e.g. by microinjecting SLO into cells. A recent report, yet to be confirmed, contends that it is also possible to transiently permeabilize cells, allowing introduction e.g. of antisense oligonucleotides under retention of viability (Harvey *et al.* 1994). Finally, polymerization-defective SLO mutants are being constructed that may become useful as non-cytotoxic probes for localizing membrane cholesterol.

References

Aguzzi, F., Rezzani, A., Nespoli, L., Monafo, V., and Burgio, G. R. (1988). Anti-streptolysin O titer, fifty-five years after Todd: a reappraisal of its clinical significance. *Boll. Ist. Sieroter. Milan.*, **67**, 162–4.

Ahnert-Hilger, G., Bader, M. F., Bhakdi, S., and Gratzl, M. (1989). Introduction of macromolecules into bovine adrenal medullary chromaffin cells and rat pheochromocytoma cells (PC12) by permeabilization with streptolysin O: inhibitory effect of tetanus toxin on catecholamine secretion. *J. Neurochem.*, **52**, 1751–8.

Alouf, J. E. and Geoffroy, C. (1988). Production, purification and assay of streptolysin O. In *Microbial toxins: tools in enzymology* (ed. S. Harshman), pp. 52–9, Academic Press, San Diego.

Alouf, J. E. and Geoffroy, C. (1991). The family of antigenically related, cholesterol-binding ('sulphhydryl-activated') cytolytic toxins. In *Sourcebook of bacterial protein toxins.* (ed. J. E. Alouf and J. H. Freer), pp. 147–86, Academic Press, London.

Bhakdi, S., Roth, M., Sziegoleit, A., and Tranum-Jensen, J. (1984). Isolation and identification of two hemolytic forms of streptolysin-O. *Infect. Immun.*, **46**, 394–400.

Bhakdi, S., Tranum-Jensen, J., and Sziegoleit, A. (1985). Mechanism of membrane damage by streptolysin-O. *Infect. Immun.*, **47**, 52–60.

Bhakdi, S., Weller, U., Walev, I., Martin, E., Jonas, D., and Palmer, M. (1993). A Guide to the use of pore-forming toxins for controlled permeabilization of cell membranes. *Med. Microbiol. Immunol.*, **182**, 167–75.

Esparis Ogando, A., Zurzolo, C., and Rodriguez Boulan, E. (1994). Permeabilization of MDCK cells with cholesterol binding agents: dependence on substratum and confluency. *Am. J. Physiol.*, **267**, C166–76.

Falconer, A. E., Carson, R., Johnstone, R., Bird, P., Kehoe, M., and Calvert, J. E. (1993). Distinct IgG1 and IgG3 subclass responses *to two streptococcal protein antigens in man: analysis of* antibodies to streptolysin O and M protein using standardized

subclass-specific enzyme-linked immunosorbent assays. *Immunology*, **79**, 89–94.
Harvey, A. N., Costa, N. D., and Savage, J. R. (1994). Electroporation and streptolysin O – a comparison of poration techniques. *Mutat. Res.*, **315**, 17–23.
Iwamoto, M., Ohno Iwashita, Y. and Ando, S. (1987). Role of the essential thiol group in the thiol-activated cytolysin from Clostridium perfringens. *Eur. J. Biochem.*, **167**, 425–30.
Kehoe, M. A., Miller, L., Walker, J. A., and Boulnois, G. J. (1987). Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. *Infect. Immun.*, **55**, 3228–32.
Momburg, F., Roelse, J., Hammerling, G. J., and Neefjes, J. J. (1994). Peptide size selection by the major histocompatibility complex-encoded peptide transporter. *J. Exp. Med.*, **179**, 1613–23.
Palmer, M., Valeva, A., Kehoe, M., and Bhakdi, S. (1995). Kinetics of streptolysin O self-assembly. *Eur. J. Biochem.*, **231**, 388–95.
Pimplikar, S. W., Ikonen, E., and Simons, K. (1994). Basolateral protein transport in streptolysin O-permeabilized MDCK cells. *J. Cell Biol.*, **125**, 1025–35.
Pinkney, M., Kapur, V., Smith, J., Weller, U., Palmer, M., Glanville, M., *et al.* (1995). Different forms of streptolysin O produced by *Streptococcus pyogenes* and by *Escherichia coli* expressing recombinant toxin: cleavage by streptococcal cytein protease. *Infect. Immun.*, **63**, 2776–9.
Rapp, S., Soto, U., and Just, W. W. (1993). Import of firefly luciferase into peroxisomes of permeabilized Chinese hamster ovary cells: a model system to study peroxisomal protein import in vitro. *Exp. Cell Res.*, **205**, 59–65.
Smyth, C. J. and Duncan, J. L. (1978). Thiol-activated (oxygen-labile) cytolysins. In *Bacterial toxins and cell membranes* (ed. J. Jeljaszewicz and T. Wadström), pp. 129–83, Academic Press, London.
Tan, A., Bolscher, J., Feltkamp, C., and Ploegh, H. (1992). Retrograde transport from the Golgi region to the endoplasmic reticulum is sensitive to GTP gamma S. *J. Cell Biol.* **116**, 1357–67.
Walev, I., Palmer, M., Valeva, A., Weller, U., and Bhakdi, S. (1995). Binding, oligomerization, and pore formation by streptolysin O in erythrocytes and fibroblast membranes: detection of nonlytic polymers. *Infect. Immun.*, **63**, 1188–94.
Wendland, M. and Subramani, S. (1993). Cytosol-dependent peroxisomal protein import in a permeabilized cell system. *J. Cell Biol.*, **120**, 675–85.

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Cholesterol binding toxins (*Streptococcus, Bacillus, Clostridium, Listeria*)

This family of 50 to 60 kDa pore-forming bacterial toxins (sulfhydryl-activated cytolysins) comprises 19 structurally and antigenically related, chromosomally encoded single-chain soluble proteins endowed with potent lethal and lytic properties resulting from the disruption of the cytoplasmic membrane of eukaryotic cells and that of certain cell organelles. Membrane cholesterol appears as a specific binding site of these toxins and is thought to contribute to their oligomerization in target membranes.

The 19 toxins identified to date are produced by gram-positive aerobic or anaerobic, sporulating or non-sporulating bacteria from the genera *Streptococcus, Bacillus, Clostridium*, and *Listeria* (Smyth and Duncan 1978, Alouf and Geoffroy 1991) listed below in Table 1.

Except for PLY, which is intracytoplasmic, the other toxins are secreted in culture medium during bacterial growth. Among the toxin-producing bacteria, only *Listeria* are intracellular pathogens which grow and release their toxins in host phagocytes or possibly in other cells.

The lethal (cardiotoxic) and cytolytic properties of the toxins are suppressed by oxidants or by thiol-group blocking agents and restored by reducing agents. The toxins share common epitopes and elicit in humans or in animals neutralizing and precipitating cross-reacting antibodies. The biological properties are irreversibly lost in the presence of very low concentrations of cholesterol and other related 3β-hydroxysterols which interfere with toxin binding on target cells (Alouf and Geoffroy 1991).

A considerable progress in our understanding of structure–activity relationship resulted from the cloning, sequencing, and expression of *tox* genes of 8 out of the 19 toxins of the family.

Table 1

Bacterial genus	Species	Toxin name	Established or suggested[b] toxin and gene acronyms	
Streptococcus	*S. pyogenes*	Streptolysin O[a]	SLO	*sio*
	S. pneumoniae	Pneumolysin[a]	PLY	*ply*
	S. suis	Suilysin[a]	SUI	*sui*
Bacillus	*B. cereus*	Cereolysin O[a]	CLO	*clo*
	B. alvei	Alveolysin[a]	ALV	*alv*
	B. thuringiensis	Thuringiolysin O[a]	TLO	*tlo*
	B. laterosporus	Laterosporolysin	LSL	*lsl*
Clostridium	*C.tetani*	Tetanolysin*	TTL	*ttl*
	C. botulinum	Botulinolysin	BTL	*btl*
	C. perfringens	Perfringolysin O[a]	PFO	*Pfo*
	C. septicum	Septicolysin O	SPL	*spl*
	C. histolyticum	Histolyticolysin O	HTL	*htl*
	C. novyi A (oedematiens)	Novyilysin	NVL	*nvl*
	C. chauvoei	Chauveolysin	CVL	*cvl*
	C. bifermentans	Bifermentolysin	BFL	*bfl*
	C. sordellii	Sordellilysin	SDL	*sdl*
Listeria	*L. monocytogenes*	Listeriolysin O[a]	LLO	*llo/lisA*
	L. ivanovii	Ivanolysin[a]	ILO	*ilo*
	L. seeligeri	Seeligerolysin	LSO	*lso*

Some strains of streptococci of groups C and G also produce streptolysin O.
[a]Native toxins reported to be purified to apparent homogeneity [b]Author's proposal.

The deduced number of amino acid residues and mol. wt. of mature toxins are the following: SLO (538 AA, 60151 Da; databank accession number M18638; see preceding entry p. 5), PLY (471 AA, 52800 Da, databank accession number: M17717; Walker *et al.* 1987), PFO (472 AA, 52469 Da; databank accession number: M36704; Tweten 1988*b*), ALV (469 AA, 51766 Da; databank accession number M62709; Geoffroy *et al.* 1990), LLO (504 AA, 55842 Da; databank accession number M24199; Mengaud *et al.* 1988; Domann and Chakraborty 1989), ILO (505 AA, 55961 Da; databank accession number X60461; Haas *et al.* 1992), and LSO (505 AA, 56371 Da; databank accession number X60462; Haas *et al.* 1992) Cereolysin gene *(clo)* has been cloned and expressed in *E. coli* and *B. subtilis* (Kreft *et al.* 1983) but to our knowledge no sequence has been so far reported. A considerable AA sequence homology (stronger at the C-terminal part) was found. It was more pronounced when structurally related AA were taken into account. At the nucleotide sequence level, the homology was lower although detectable, indicating that *tox* genes have undergone extensive divergence from a common ancestor (Boulnois *et al.* 1991).

An 11 AA sequence (—ECTGLAWEWWR—) was the longest common motif conserved in each protein (except for single amino acid change in seeligerolysin). It contained the unique Cys residue of the toxins (except for ILO which possesses a second Cys residue beyond the consensus sequence closer to the C-terminus). The thiol group of the Cys residue of the conserved undecapeptide was logically considered as an 'essential' group required for lytic activity as supported by its abrogation by thiol blocking agents. This contention was not supported by site-directed mutagenesis undertaken on PLY, SLO, and LLO (see Michel *et al.* 1990; Boulnois *et al.* 1991; Sheehan *et al.* 1994). Changing the Cys residue to either alanine, serine, or glycine did not affect or only reduced lytic activity suggesting that there is no absolute requirement for the thiol group in the *in vitro* activity of the toxins. In contrast, the overall structure of the motif appears important (at least in part) for interaction with cholesterol and pore-formation. On the other hand, the use of genetically truncated recombinant PLY showed that the deletion of the six C-terminal AA reduced binding by 96 per cent as also found for PFO (Owen *et al.* 1994).

The genetic regulation of toxin expression has been particularly investigated for LLO and PFO. The former was shown to be positively regulated by a 27.1 kDa protein encoded by *prfa* gene the deletion of which besides LLO affects at least four other virulence factors (Schwan *et al.* 1994; Sheehan *et al.* 1994). PFO expression was also under positive control of a regulatory gene *(pfor)* which also affected the expression of clostridal collagenase and hemagglutinin (Shimizu *et al.* 1994).

Purification and sources

The native toxins purified so far are isolated to apparent homogeneity by standard protein chemistry techniques from culture supernatants (except for PLY obtained from disrupted pneumococci) of appropriate toxin-producing strains, grown under culture conditions specific for each bacterial species described in the hereafter cited publications. Most procedures comprise a combination of crude material concentration (ultrafiltration and or salting-out by ammonium sulfate), then ion-exchange column chromatography, followed by gel molecular

sieving and (or) hydrophobic (low- or high-pressure) column chromatography. Covalent thiopropyl gel chromatography has been widely used taking advantage of toxin cysteinyl residue. Appropriate purification techniques have been described for PLY (Kanclerski and Möllby 1987; Rubins *et al.* 1994), PFO (Tweten 1988*a*), ALV (Geoffroy and Alouf 1983), LLO (Geoffroy *et al.* 1987; Kreft *et al.* 1989), ILO (Kreft *et al.* 1989; Vazquez-Boland *et al.* 1989), BTL (Haque *et al.* 1992), and suilysin (Jacobs *et al.* 1994). The production and purification of fully bioactive recombinant PFO expressed in *E.coli* (Tweten 1988*a*) or of PLY expressed in this micoorganism (Mitchell *et al.* 1989; Rubins *et al.* 1994) and *B.subtilis* (Taira *et al.* 1989) has been reported. The toxins are not commercially available to our knowledge.

The pH optimum for the lytic activity of the various toxins so far studied falls between 6.5 and 7.4 except for LLO with a pH optimum about 5 (no activity at pH 7.0) which may reflect the acid nature of the phagolysosome where it acts (Geoffroy *et al.* 1987; Sheehan *et al.* 1994).

Toxicity

Microgram quantities of the toxins are lethal for mice, rabbits, and other laboratory animals. The LD_{50} (i.v. route) ranges from 0.2 to 0.8 μg (Smyth and Duncan 1978; Geoffroy *et al.* 1987).

Hemolytic activity on sheep or rabbit erythrocytes ranges from 0.5 to 2×10^6 hemolytic units/mg of protein (Alouf and Geoffroy 1991). The use of the toxins is safe for operators following normal safety recommendations for bacterial manipulations.

Use in cell biology

The lytic properties of the toxins and their specific binding of cholesterol on to eukaryotic cells and organelle membranes have been used for the permeabilization of these structures for the analysis of various cell functions and metabolism, the introduction of exogeneous molecular effectors and the generation of limited lesions in various cells for the isolation of membrane receptors intracellular enzymes or organelles which could not be easily obtained by other methods (see Alouf and Geoffroy 1991; Berthou *et al.* 1992; Launay *et al.* 1992; Ahnert-Hilger *et al.* 1993).

References

Ahnert-Hilger, G., Stecher, B., Beyer, C., and Gratzl, M. (1993). Exocytotic membrane fusion as studied in toxin-permealized cells. *Methods Enzymol.*, **221**, 139–49.

Alouf, J. E. and Geoffroy, C. (1991). The family of antigenically-related, cholesterol-binding (sulfhydryl-activated) cytolytic toxins. In *Sourcebook of bacterial protein toxins* (ed. J. E. Alouf and J. H. Freer), pp. 147–86, Academic Press, London.

Berthou, L., Corvaia, N., Geoffroy, C., Mutel, V., Launay, J. M., and Alouf, J. E. (1992). The phosphoinositide pathway of lymphoid cells: labeling after permeabilization by alveolysin, a bacterial sulfhydryl-activated cytolysin. *Eur. J. Cell. Biol.*, **58**, 377–82.

Boulnois, G. J., Paton, J. C., Mitchell, T. J., and Andrew, P. W. (1991). Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. *Mol. Microbiol.*, **59**, 2611–16.

Domann, E. and Chakraborty, T. (1989). Nucleotide sequence of the listeriolysin gene from a *Listeria monocytogenes* serotype 1/2a strain. *Nucleic Acids Res.*, **17**, 6406.

Geoffroy, C. and Alouf, J. E. (1983). Selective purification by thiol–disulfide interchange chromatography of alveolysin, a sulfhydryl-activated toxin of *B. alvei*. *J. Biol. Chem.*, **255**, 9968–72.

Geoffroy, C., Gaillard, J. L., Alouf, J. E., and Berche, P. (1987). Purification, characterization and toxicity of the sulfhydrl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. *Infect. Immun.*, **55**, 1641–46.

Geoffroy, C., Mengaud, J., Alouf, J. E., and Cossart, P. (1990). Alveolysin, the thiol-activated toxin of *Bacillus alvei*, is homologous to listeriolysin O, perfringolysin O, pneumolysin, and streptolysin O and contains a single cysteine. *J. Bacteriol.*, **172**, 7301–5.

Haas, A., Dumbsky, M., and Kreft, J. (1992). Listeriolysin genes : complete sequence of ilo from *Listeria ivanovii* and of lso from *Listeria seeligeri*. *Biochim. Biophys. Acta.*, **1130**, 81–4.

Haque, A., Sugimoto, N., Horiguchi, Y., Okabe, T., Miyata, T., Iwanaga, S., *et al.* (1992). Production, purification, and characterization of botulinolysin, a thiol-activated hemolysin of *Clostridium botulinum*. *Infect. Immun.*, **60**, 71–8.

Jacobs, A. A. C., Loeffen, P. L. W., Van Den Berg, A. J. G., and Storm, P. K. (1994). Identification, purification and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. *Infect. Immun.*, **62**, 1742–8.

Kanclerski, K. and Möllby, R. (1987). Production and purification of *Streptococcus pneumoniae* hemolysin (pneumolysin). *J. Clin. Microbiol.*, **25**, 222–5.

Kreft, J., Berger, H., Härtlein, M., Müller, B., Weidinger, G., and Goebel, W. (1983). Cloning and expression in *Escherichia coli* and *Bacillus subtilis* of the hemolysin (cereolysin) determinant from *Bacillus cereus*. *J. Bacteriol.*, **155**, 681–9.

Kreft, J., Funke, D., Haas, A., Lottspeich, F., and Goebel, W. (1989). Production, purification and characterization of hemolysins from *Listeria ivanovii* and *Listeria monocytogenes* Sv4b. *FEMS Microbiol. Lett.*, **57**, 197–202.

Launay, J. M., Geoffroy, C., Mutel, V., Buckle, M., Cesum, A., Alouf, J. E., *et al.* (1992). One-step purification of the serotonin transporter located at the human platelet plasma membrane. *J. Biol. Chem.*, **267**, 11344–51.

Mengaud, J., Vincente, M. F., Chenevert, J., Moniz Pereira, J., Geoffroy, C., Giquel-Sanszey, B., *et al.* (1988). Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. *Immun.*, **56**, 766–72.

Michel, E., Reich, K. A., Favier, R., Berche, P., and Cossart, P. (1990). Attenuated mutants of the intracellular bacterium *Listeria monocytogenes* obtained by single amino acid substitutions in listeriolysin O. *Mol. Microbiol.*, **4**, 2167–78.

Mitchell, T. J., Walker, J. A., Saunders, F. K., Andrew, P. W., and Boulnois, G. J. (1989). Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties. *Biochim. Biophys. Acta.*, **1007**, 67–72.

Owen, R. H. G., Boulnois, G. J., Andrew, P. W., and Mitchell, T. J. (1994). A role in cell-binding for the C-terminus of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*. *FEMS Microbiol. Lett.*, **121**, 217–22.

Rubins, J. B., Mitchell, T. J., Andrew, P. W., and Niewoehner, D. E. (1994). Pneumolysin activates phospholipase A in pulmonary artery endothelial cells. *Infect. Immun.*, **62**, 3829–36.

Schwan, W. R., Demuth, A., Kuhn, M., and Goebel, W. (1994). Phosphatidylinositol-specific phospholipase C from *Listeria monocytogenes* contributes to intracellular survival and growth of *Listeria innocua*. *Infect. Immun.*, **62**, 4795–803.

Sheehan, B., Kocks, C., Dramsi, S., Gouin, E., Klarsfeld, A. D., Mengaud, J., *et al.* (1994). Molecular and genetic determinants of the *Listeria monocytogenes* infectious process. *Curr. Top. Microbiol. Immunol.*, **192**, 187–216.

Shimizu, T., Ba-Thein, W., Tamaki, M., and Hayashi, H. (1994). The vir gene, a member of a class of two-component response regulators, regulates the production of perfringolysin O, collagenase, and hemagglutinin in *Clostridium perfringens*. *J. Bacteriol.*, **176**, 1616–23.

Smyth, C. J. and Duncan, J. J. (1978). Thiol-activated (oxygen-labile) cytolysins. In *Bacterial toxins and cell membranes* (ed. J. Jeljaszewicz and T. Wadström), pp. 129–83, Academic Press, London.

Taira, S., Jalonen, E., Paton, J. C., Sarvas, M., and Runeberg-Nyman, K. (1989). Production of pneumolysin, a pneumococcal toxin, in *Bacillus subtilis*. *Gene*, **77**, 211–8.

Tweten, R. K. (1988a). Cloning and expression in *Escherichia coli* of the perfringolysin O (thetatoxin) gene from *Clostridium perfringens* and characterization of the gene product. *Infect. Immun.*, **565**, 3228–34.

Tweten, R. K. (1988b). Nucleotide sequence of the gene for perfringolysin O (theta-toxin) from *Clostridium perfringens*: significant homology with the genes for streptolysin O and pneumolysin. *Infect. Immun.*, **56**, 3235–40

Vazquez-Boland, J.-A., Dominguez, L., Rodriguez-Ferri, E.-F., and Suarez, G. (1989). Purification and characterization of two *Listeria ivanovii* cytolysins, a sphingomyelinase C and a thiol-activated toxin (ivanolysin O). *Infect. Immun.*, **57**, 3928–835.

Walker, J. A., Allen, R. L., Falmagne, P., Johnson, M. K., and Boulnois, G. J. (1987). Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. *Infect. Immun.*, **55**, 1184–9.

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α-Toxin (*Staphylococcus aureus*)

α-Toxin is a 33 kD protein secreted by most pathogenic strains of S. aureus. *It binds with both high and low affinity to the membrane of the target cell where it assembles into an oligomeric pore. Pore formation triggers secondary events like eicosanoid production, secretion, endonuclease activation, and cytokine release. Eventually it causes colloid-osmotic lysis of red blood cells, swelling and death of nucleated cells.*

Staphylococcal α-toxin is an exotoxin with hemolytic, cytotoxic, dermonecrotic, and lethal activity (Thelestam and Blomqvist 1988; Bhakdi and Tranum-Jensen 1991). It is secreted by most pathogenic strains of *S. aureus* (commonly Wood 46) as a water-soluble polypeptide of 33 kD (293 amino acid residues, sequence accession number to the Swiss Prot databank: P09616 HLA-STAAU; corrections were published (Walker *et al.* 1992)). Its relevance as a virulence factor has been firmly established, at least in animal models. As outlined in Fig. 1, this toxin oligomerizes on the surface of mammalian cells (and liposomes) to form a membrane-embedded oligomer of about 220 kD, which appears in the electron microscope as a hollow cylinder protruding from the plane of the bilayer. It forms crystalline 2D layers on lipid membranes of either natural or artificial origin. From such arrays, a low-resolution three-dimensional map of the oligomer was obtained (Olofsson *et al.* 1988), suggesting it was a hexamer. However, more recent low-resolution X-ray analysis of microcrystals (Gouaux *et al.* 1994) provided evidence that the oligomer is actually an heptamer like the one formed by aerolysin from *Aeromonas hydrophila*.

α-Toxin causes membrane damage to a variety of cells including red blood cells (RBC), platelets, and white cells. RBC damage proceeds in distinct steps: binding to the cell membrane, ion leakage, and, eventually, lysis with release of larger molecules. Nucleated cells can survive its action if adequately protected, e.g. by divalent cations, particularly Zn^{2+} (Bashford *et al.* 1986). Binding occurs either with low affinity to the lipid phase (with a preference for phosphatidylcholine mixed with cholesterol), or with high affinity to a protein receptor present in a few copies on some sensitive cells (e.g rabbit RBC, human platelets, and monocytes, Hildebrand *et al.* 1991). Independent of the binding affinity, damage always requires the assembly of the oligomer. Before inducing cell death, pore formation triggers a number of secondary events: eicosanoid production, secretion, activation of endonucleases, and release of cytokines, which are all explained by a toxin-induced Ca^{2+} influx into cells still possessing an intact cytosolic protein machinery. Early apoptotic effects, at a stage at which the pore is small and permeant only to Na^+, have been detected (Jonas *et al.* 1994). On planar lipid membranes, composed of purified phospholipids, α-toxin forms a water-filled anion-selective pore, with a diameter of around 1 nm (Menestrina 1986). Similar pores were detected in patch-clamped Lettre cells (Korchev *et al.* 1995).

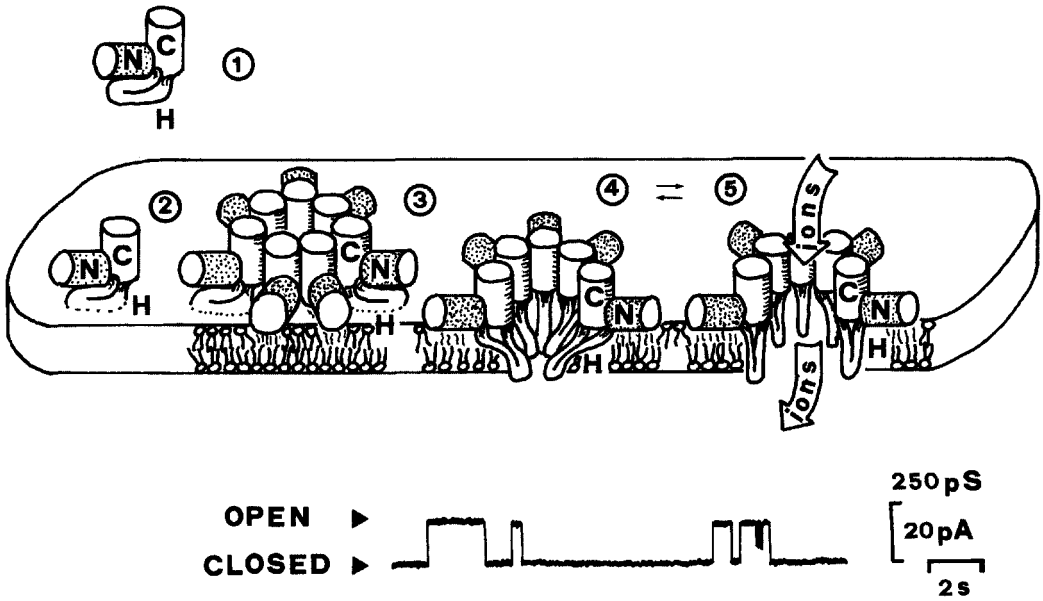

Figure 1. Steps leading to the formation of a pore by *Staphylococcus aureus* α-toxin, as resulting from structural, biochemical and, genetic studies.

1. Soluble α-toxin is monomeric and comprises two regions (N-terminal and C-terminal) connected by a glycine-rich flexible hinge (H).

2. The monomer binds in a temperature-independent step to the cell membrane either via a high affinity protein receptor, or, more commonly, via ubiquitous low affinity acceptors (phosphatidylcholine and cholesterol).

3. Absorbed α-toxin monomers oligomerize, via a temperature dependent surface diffusion, to form a non-lytic amphiphilic hexamer.

4. The oligomer can further insert into the lipid matrix of the cell membrane, generating a transmembrane channel precursor which behaves as an integral protein and is resistant to external (or membrane bound) proteases

5. The pore may open allowing the passage of ions and small molecules (up to molecular weight 2000). In artificial membranes the opening of the pore is visualized by an increase of the ionic current (lower trace). The open and closed states of the pore (i.e. states 4 and 5) are in equilibrium and the probability of it being open depends on several factors like pH, divalent cations, and applied voltage.

Table 1 The family of α-toxin and other related leukotoxins from *Staphylococcus aureus*

Toxin	Acronym	Mol. weight (kD)	Identity[a](%)	Similarity[a](%)	Reference[b]
α-toxin	Hla	33	–	–	1
F-component					
α-lysin B	HlgB	34	30.4	12.3	3
leukocidin F	LukF	34	29.4	11.9	2
leukocidin F-R	LukF-R	34	28.0	14.0	2
leukocidin F-PV	LukF-PV	34	26.3	15.7	4
S-component					
α-lysin A	HlgA	32	24.9	11.7	3
α-lysin C	HlgC	32	21.0	14.0	3
leukocidin S	LukS	32	20.6	14.0	2
leukocidin S-R	LukS-R	32	20.6	14.0	2
leukocidin S-PV	LukS-PV	32	21.7	14.0	4

[a] Identity and similarity of the mature forms are referred to α-toxin and were calculated after alignment according to Dayhoff MDM-78 matrix method.
[b] References: 1, Gray and Kehoe 1984; 2, Hunter *et al.* 1993; 3, Cooney *et al.* 1993; 4, Prévost *et al.* 1995.

S. aureus leukocidins and γ-lysins share sequence homology with α-toxin (Cooney *et al.* 1993), suggesting they form a family (Table 1). See also the entry on leukocidins and gamma lysins (p. 94).

Purification and sources

α-Toxin purification should be as fast and thorough as possible to avoid subsequent damage by endogenous proteases. Popular procedures use, after ammonium sulfate precipitation, adsorption chromatography on controlled-pore glass followed by ion-exchange (Cassidy and Harshman 1976), or cation-exchange chromatography followed by size-exclusion (Lind *et al.* 1987). α-Toxin can be purchased from Behringwerke (Marburg, Germany) and List.

Toxicity

LD_{50} in mice is 50 μg/kg or 1.5 pmoles/kg. Hemolytic activity, on the most sensitive rabbit red blood cells, should be in the range 40 000 HU/mg (tested by addition of 1 volume of 2.5×10^8 red cell/ml for 1 hour at 37 °C). After 4 hours at room temperature it can reach 100 000 HU/mg. Human red blood cells are typically 400 times less sensitive

Use in cell biology

α-Toxin is used to selectively permeabilize cells to small molecules (below the cut-off of the pore) while excluding large molecules such as cytoplasmic proteins (above that cut-off). For example, its ability to let Ca^{2+} ions enter the cell while leaving intact the cytoplasmic enzyme cascades and machineries has been exploited to study the minimal requirements for exocytosis (Ahnert-Hilger *et al.* 1985). Since that time a large number of papers reported a similar usage, reviewed by Bhakdi *et al.* (1993). Other applications can be foreseen. For example, in patch-clamp electrophysiology the use of the *perforated patch* as an alternative to the whole-cell configuration is becoming diffuse. In this variant the patch under the pipette is not broken but rather made very permeable by using the channel-forming antibiotic nystatin. Electrical access is gained, but (due to the small molecular weight cut-off of the nystatin pores) internal proteins are retained inside the cell. α-Toxin could be used in place of nystatin providing an alternative choice of pore diameter and selectivity. Relevant for both applications is the possibility to fine tune the size of the α-toxin pores and/or to switch them on and off via different stimuli after direct mutagenesis and modifications of the molecule. Many such possibilities have been reviewed recently (Bayley, 1994) and include opening by a flash of light or by limited proteolysis and closing by Zn^{2+}. The fact that α-toxin self assembles in 2D crystals is potentially relevant for the production of biosensors.

References

Ahnert-Hilger, G., Bhakdi, S., and Gratzl, M. (1985). Minimal requirements for exocytosis: a study using PC 12 cells permeabilized with staphylococcal alpha-toxin. *J. Biol. Chem.*, **260**, 12730–4.

Bashford, C. L., Alder, G. M., Menestrina, G., Micklem, K. J., Murphy, J., and Pasternak, C. A. (1986). Membrane damage by haemolytic viruses, toxins, complement and other agents: a common mechanism blocked by divalent cations. *J. Biol. Chem.*, **261**, 9300–8.

Bayley, H. (1994). Triggers and switches in a self-assembling pore-forming protein. *J. Cell. Biochem.*, **56**, 177–82.

Bhakdi, S. and Tranum-Jensen, J. (1991). Alpha-toxin of *Staphylococcus aureus*. *Microbiol. Rev.*, **55**, 733–51.

Bhakdi, S., Weller, U., Walev, I., Martin, E., Jonas, D., and Palmer, M. (1993). A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes. *Med. Microb. Immunol.*, **182**, 167–75.

Cassidy, P. and Harshman, S. (1976). Purification of staphylococcal alpha-toxin by adsorption chromatography on glass. *Infect. Immun.*, **13**, 982–6.

Cooney, J., Kienle, Z., Foster, T. J., and O'Toole, P. W. (1993). The gamma-hemolysin locus of *Staphylococcus aureus* comprises three linked genes, two of which are identical to the genes of the F and S component of leukocidin. *Infect. Immun.*, **61**, 768–71.

Gouaux, J. E., Braha, O., Hobaugh, M. R., Langzhou, S., Cheley, S., Shustak, C., and Bayley, H. (1994). Subunit stoichiometry of staphylococcal α-toxin in crystals and on membranes: a heptameric transmembrane pore. *Proc. Natl. Acad.Sci. USA*, **91**, 12828–31.

Gray, G. S. and Kehoe, M. (1984). Primary sequence of the alpha-toxin gene from *Staphylococcus aureus* Wood 46. *Infect. Immun.*, **46**, 615–18.

Hildebrand, A., Pohl, M., and Bhakdi, S. (1991). *Staphylococcus aureus* α-toxin, dual mechanism of binding to target cells. *J. Biol. Chem.*, **266**, 17195–200.

Hunter, S. E. C., Brown, J. E., Oyston, P. C. F., Sakurai, J., and Titball, R. W. (1993). Molecular genetic analysis of beta-toxin of *Clostridium perfringens* reveals sequence homology with alpha-toxin, gamma toxin, and leukocidin of *Staphylococcus aureus*. *Infect. Immun.*, **61**, 3958–65.

Jonas, D., Walev, I., Berger, T., Liebetrau, M., Palmer, M., and Bhakdi, S. (1994). Novel path to apoptosis: small transmembrane pores created by staphylococcal alpha-toxin in T lymphocytes evoke internucleosomal DNA degradation. *Infect. Immun.*, **62**, 1304–12.

Korchev, Y. E., Alder, G. M., Bakhramov, A., Bashford, C. L., Joomun, B. S., Sviderskaya, E. V., *et al.* (1995). *Staphylococcus aureus* alpha-toxin-induced pores: channel-like behavior in lipid bilayers and clamped cells. *J. Membrane Biol.*, **143**, 143–51.

Lind, I., Ahnert-Hilger, G., Fuchs, G. and Gratzl, M. (1987). Purification of alpha-toxin from *Staphylococcus aureus* and application to cell permeabilization. *Anal. Chem.*, **164**, 84–9.

Menestrina, G. (1986). Ionic channels formed by *Staphylococcus aureus* alpha-toxin: voltage dependent inhibition by di- and trivalent cations. *J. Membrane Biol.*, **90**, 177–90.

Olofsson, A., Kaveus, U., Thelestam, M., and Hebert, H. (1988). The projection structure of α-toxin from *Staphylococcus aureus* in human platelet membranes as analyzed by electron microscopy and image processing. *J. Ultrastruct. Mol. Struct. Res.*, **100**, 194–200.

Prévost, G., Cribier, B., Couppie, P., Petiau, P., Supersac, G., Finck-Barbançon, V., *et al.* (1995). Panton-Valentine

leucocidin and gamma-hemolysin from *S. aureus* ATCC 49775 are encoded by distinct genetic loci and have different biological activities. *Infect. Immun.*, **63**, 4121–9.
Thelestam, M. and Blomqvist, L. (1988). Staphylococcal alpha-toxin – recent advances. *Toxicon*, **26**, 51–65.
Walker, B., Krishnasastry, M., Zorn, L., Kasianowicz, J., and Bayley, H. (1992). Functional expression of the α-hemolysin of *Staphylococcus aureus* in intact *Escherichia coli* and in cell lysates. Deletion of five C-terminal amino-acids selectively impairs hemolytic activity. *J. Biol. Chem.*, **267**, 10902–9.

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δ-Toxin (*Staphylococcus aureus*) and melittin (*Apis mellifera*)

δ-Toxin and melittin (Mel) are 26 residue long peptides, secreted by Staphylococcus aureus *strains and representing about 50 per cent of the dry weight of bee venom, respectively. Both are pleiotropic toxins with no sequence homology, despite this they share most of their biological properties. The first target is the plasma membrane, but the toxins also act on a wide variety of intracellular elements whether membranes, proteins, or nucleic acids. Their behaviour is dominated by their amphipathic character, they are surface active, have rather nonspecific affinity for interfaces, and are classified as 'membrane invading' or 'direct lytic' peptides.*

At first glance the sequence of Mel (Table 1) looks like that of a soap with an apolar segment followed by the strongly basic C_{term} hexapeptide. Careful inspection shows the presence of several polar or less hydrophobic residues, namely $Gly_{1,3}$, Lys_7, $Thr_{10,11}$, Pro_{14}, and Ser_{18} which result in an amphipathic sequence. This is more clearly seen for δ-toxin where comparison of the known sequences from different strains shows a conservative

Table 1 Sequences of the different δ-toxins and melittins and some synthetic binary analogues code: hydrophobic residue; + hydrophilic residue; ~ indifferent. From Fitton *et al.* 1980; McKevitt *et al.* 1990; Habermann 1980; de Grado *et al.* 1982; Blondelle and Houghten 1992; Comut *et al.* 1994

	1				5					10					15					20						26
δ toxin	M	A	Q	D	I	I	S	T	I	G	D	L	V	K	W	I	I	D	T	V	N	K	F	T	K	K_{Coo}
S. aureus human																										
S. aureus canine			A							V	E	F			L		A	E			E			I		
S. epidermidis			A																					K	K_{Coo}	
binary reading	o	o	o/+	+	o	o	+	o/+	o	~	+	o	o	+	o	o	o	+	+	o	+	+	o	o/+	+	+
melittin	G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	R	K	R	Q	QNH_2
A. mellifera																										
A. dorsata					I					S																
A. florea					I					A					T							N			K	
Analogue 1 de Grado *et al.*	L	L	Q	S	L	L	S	L	L	Q	S	L	L	S	L	L	L	Q	W	L	K	R	K	R	Q	ONH_2
binary reading	~	o	~	o	o	o	+	o	o	+	+	o	o	~	o	o	o	+	o	o	+	+	+	+	+	+
L_9K_9 Blondelle *et al.*	AcL	K	L	L	K	K	L	L	K	K	L	K	K	L	L	K	K	LNH_2								
$L_{10}K_5$ Cornut *et al.*	K	L	L	K	L	L	L	K	L	L	L	K	L	L	K											

polar/apolar character at each position (Table 1). Synthetic analogues with a similar periodic polar/apolar alternation, despite massive changes in the residues, are active (de Grado *et al.* 1982; Alouf *et al.* 1989). This gives sense to a simplified reading of the sequence in a binary hydrophilic/hydrophobic code. The periodicity all along the sequences nicely fits that of an α-helix, and will result in a typical secondary amphipathic structure. This has proved to be the minimal requirement to get strongly hemolytic peptides, since Leu and Lys copolypeptides properly sequenced in order to generate an amphipathic α-helix (Table 1) mimic the natural toxins (Blondelle and Houghten1992; Cornut *et al.* 1994).

In the crystal Mel shows α-helical segments I $\rightarrow$ 10 and 14 $\rightarrow$ 26 with an angle of about 120° due to Pro_{14}. Antiparallel helices are packed with all apolar residues buried in the core of a tetramer (Terwilliger and Eisenberg 1982). The structure of δ-toxin is still unsolved, but energy calculations allow the lattice to be filled with antiparallel α-helices, which generate planar oligomers with one polar face, the opposite one being apolar (Raghunathan *et al.* 1990). When in their aggregated state, in organic solution and when bound to lipids, both toxins are rather similarly α-helical, i.e. about 70 to 80 per cent (Dempsey 1990); for δ-toxin it corresponds to a single helix from Ile_5 to Phe_{23} (Lee *et al.* 1987; Tappin *et al.* 1988).

The amphipathicity of the toxins leads to self-association equilibria in buffer and in membrane media. At low concentration, monomeric peptides are almost structureless in buffer but almost totally α-helical in low dielectric organic solvents and membranes. Increasing concentration, ionic strength, or pH stabilizes oligomers in water with a concomitant folding in the α-helix up to 60–70 per cent (Cornut *et al.* 1993). The hydrophobic effect stabilizes Mel tetramers, favored by phosphate and sulfate. For the δ-toxin, self-association is much more drastic, it occurs in the μM range and association numbers strongly increase up to very large asymmetric aggregates. Out of the hydrophobic burying of the apolar face, self association is increased by intermolecular ion pairing through Asp/Lys residues on the polar face of the α-helices (Thiaudière *et al.* 1991).

Both toxins interact with lipids and membranes with partition coefficients larger than 10^5. While Mel has a selectivity for negatively charged lipids, δ-toxin has similar affinities whatever the lipid charge (Freer 1986; Cornut *et al.* 1993). Such a binding to lipids supports the fact that several 10^6 toxins can bind per erythrocyte (Tosteson *et al.* 1985). Despite a long-running controversy about the location on the membrane, it is now clear that toxins bind essentially parallel to the membrane surface and not as a transmembrane helical rod (Cornut *et al.* 1996). An increase in the membrane bound concentration of toxins leads to self-association of the peptides and increased perturbation of bilayer structure, whose thickness decreases. This ends up fragmenting lipidic membranes into discoidal or micellar structures (Dufourcq *et al.* 1986). The length and physical state of the acyl chains, the net charge, and the presence of cholesterol modulate the lytic efficiency on vesicles (Faucon *et al.* 1995; Monette and Lafleur 1995; Pott and Dufourc 1995). In the presence of an applied potential the two toxins form channels interpreted as barrel–stave aggregates of the amphipathic helices. Increasing association numbers explains the levels of conductance which can vary from a few tens of pS up to several nS for the larger channels (Sansom 1991). The potential-dependent channel activity does not correlate strictly with lysis; one can get channels without lysis and vice versa (Stankowski *et al.* 1991; Kerr *et al.* 1995).

Biological activities

δ-Toxin and Mel are cytotoxins active on most of the eukaryotic cells. Their hemolytic activity is a colloid osmotic process, i.e. cells first become permeable to small solutes, particularly to ions, and only after swelling hemoglobin does leak (Tosteson *et al.* 1985). The size of permeants increases differently for the two toxins, on fibroblastes Mel induces faster growing holes compared to δ-toxin (Thelestam and Möllby 1979). When cell lysis occurs, up to 10 per cent of the lipids can be solubilized, which looks like the quantitative solubilization of pure lipid bilayers (Katsu *et al.* 1989). Mel binds also to proteins facing outside and induces their aggregation (Van Veen and Cherry 1992). Nevertheless, this should play some role; the parallel between lysis of lipid vesicles and cells strongly supports a similar mechanism. The net charge of the toxins is a critical parameter in lysis; the lytic activity and the strong amphipathic and basic character of the peptides are paralleled (Cornut *et al.* 1993). Cell lysis can be opposed by a variety of compounds such as polyanions, amphipathic proteins like calmoduline, or glycophorin, which either compensate for the osmotic pressure or efficiently bind to the toxins, whether in solution or on the membrane sites (Tosteson *et al.* 1985; Cornut *et al.* 1993).

δ-Toxin is not antibacterial (Dhople and Nagaraj 1993), while Mel inhibits the growth of bacteria in the range of 1–10 μM, acting both on *gram* + and *gram* –, though in this case it does not lyse *E. coli* unless the outer membrane has been removed (Katsu *et al.* 1989). Mel also inhibits the growth of mycoplasma (Cornut *et al.* 1995). Numerous analogues of Mel, chimeric melittin-cecropin peptides (Cornut *et al.* 1993) and the simplified and shorter LiKj amphipathic peptides (Blondelle and Houghten 1992; Cornut *et al.* 1995) proved to be active. Analogues and fragments of δ-toxin with net positive charge proved to become antimicrobial (Dufourcq, Beven, Siffert, Wroblewski in preparation; Dhople and Nagaraj 1995). Then besides an apolar character, the peptides must have a net positive charge in order to be antimicrobial.

Both toxins interfere with numerous enzymatic pathways (Kasimir *et al.* 1990). Their synergism with phospholipase A_2 (PLA_2) is well documented. Short fragments or analogues of Mel, which are not lytic, increase significantly the PLA_2 activity (Grandbois, Dufourcq and

Salesse, submitted; Hingaro *et al.* 1995). Melittin proved to activate intrinsic PLA_2 in intact cells, mammalian and bacterial ones, and organs, as well as phospholipase C and lipases (Fletcher and Jiang 1993).

Purification and sources

δ-Toxin is secreted in the medium by *Staphylococcus aureus* strains at the end of the exponential phase of growth. It can be adsorbed on hydroxylapatite and then eluted at high phosphate concentration. Further purification by HPLC can be performed to get the purest peptide (Birkbeck and Freer 1988; Tappin *et al.* 1988). Synthetic δ-toxin can be used to eliminate any risk of contamination by much more powerful toxins (Alouf *et al.* 1989).

Mel is available from many manufacturers, Serva Feinbiochemicals, Calbiochem, Fluka, or Sigma, it can also be purified from crude bee venom by HPLC. Whatever the origin, special attention should be paid to eliminate PLA_2 contamination, which can lead to severe artefacts especially when using large amounts of toxin compared to the lipid content and/or when looking at long time effects (Dufourcq *et al.* 1984). Synthetic melittin, already available from Bachem, is very helpful in getting rid of such artefacts.

Toxicity

LD_{50} of Mel for human erythrocytes (10^7 cells/ml) is 1.2 μg/ml, that of δ-toxin is significantly higher. The lethal dose of Mel for mice is 4 mg/kg (Habermann 1972).

Alternative names

δ-Toxin is also referred as δ-haemolysin, δ-hemolysin, or δ-lysin. Melittin spelling is not appropriate since the toxin comes from *Apis mellifera,* but it is too widely used to be changed now.

References

Alouf, J. E., Dufourcq, J., Siffert, O., Thiaudière, E., and Geoffroy, C. (1989). Interaction of δ-toxin and synthetic analogues with erythrocytes and lipid vesicles. *Eur. J. Biochem.*, **183**, 381–90.

Birkbeck, T. H. and Freer, J. H. (1988). Purification and assay of staphylococcal δ-lysin. *Meth. Enzymol.*, **165**, 16–23.

Blondelle, S. E. and Houghten, R. A. (1992). Design of model amphipathic peptides having potent antimicrobial activities. *Biochemistry*, **31**, 12688–94.

Cornut, I., Thiaudière, E., and Dufourcq, J. (1993). The amphipathic helix in eytotoxic peptides. In *'The amphipathic helix'*, (ed. R. M. 'Epand), pp. 173–219, CRC Press, Loc.

Cornut, I., Büttner, K., Dasseux, J. L., and Dufourcq, J. (1994). The amphipathic α-helix concept, application to the *de novo* design of ideally amphipathic Leu, Lys peptides more hemolytic than melittin. *FEBS Lett.*, **349**, 29–33.

Cornut, I., Büttner, K., Beven, L., Duclohier, H., and Dufourcq, J. (1995). Minimal requirements for the design of highly cytotoxic peptides. In *'Peptides 1994'* (ed. H. L. S. Maïa), pp. 666–7, Escom, Leiden.

Cornut, I., Desbat, B., Turlet, Y. M., and Dufourcq, J. (1996). *In situ* study by P structure and orientation of amphipathic peptides at the air-water interface. *Biophys. J.*, **70**, pp. 305–12.

De Grado, W. F., Musso, G. F., Lieber, M., Kaiser E. T. and Kèzdy, F. J. (1982). Kinetics and mechanism of hemolysis induced by melittin and a synthetic anologue. *Biophys. J.*, **37**, 329–38.

Dempsey, C. E. (1990). The action of melittin on membranes. *Biochim. Biophys. Acta*, **1031**, 143–161.

Dhople, V. H. and Nagaraj, R. (1993). δ-Toxin, unlike melittin, has only hemolytic and no antimicrobial activity. *Biosc. Rept.*, **13**, 245–50.

Dhople, V. H. and Nagaraj R. (1995). Analogs having potent automicrobial and hemolytic activities with minimal changes from an inactive 16-residue fragment of δ-toxin. *Prot. Engineer.*, **8**, 315–18.

Dufourcq, J., Dasseux, J. L. and Faucon J. F. (1984). A review of melittin–phospholipid systems. In *Bacterial protein toxins*, pp. 127–38, Academic Press, London.

Dufourcq, J., Faucon, J. F., Fourche, G., Dasseux, J. L., Le Maire, M., and Gulik-Krzywicki, T. (1986). Morphological changes in bilayers induced by melittin: vesicularization, fusion, discoidal particles. *Biochim. Biophys. Acta*, **859**, 33–48.

Faucon, J. F., Bonmatin, J. M., Dufourcq, J., and Dufourc, E. J. (1995). Acyl chain length dependence in the stability of melittin–phosphatidylchloline complexes. *Biochim. Biophys. Acta.*, **1234**, 235–43.

Fitton, J. E., Dell, A., and Shaw W. V. (1980). Aminoacid sequence of δ-haemolysin of *Staphylococcus aureus. FEBS Lett.*, **115**, 209–12.

Fletcher, J. E. and Jiang, M. S. (1993). Possible mechanisms of action of snake venom cardiotoxins and bee venom melittin. *Toxicon*, **31**, 669–95.

Freer, J. H. (1986). Membrane damage caused by bacterial toxins: recent advances and new challenges. In *Natural toxins* (ed. J. B. Harris), pp. 189–211, Clarendon Press, Oxford.

Habermann, E. (1980). Melittin, structure and activity. In *Natural toxins*. (ed. D. Eaker and T. Wadstrom), pp. 173–81, Pergamon Press, London

Hingaro, I., Pèrez-Payá, E., Pinilla, C., Appel, J. R., Houghten, R. A., and Blondelle, S. E. (1995). Activation of bee venom PLA_2 through a peptide-enzyme complex. *FEBS Lett.*, **372**, 131–4.

Kasimir, S., Schonfeld, W., Alouf, J. E. and Kijning, W. (1990). Effect of *Staphylococcus aureus δ-toxin* on granulocyte and PAF metabolism. *Infect. Immun.*, **58**, 1653–9.

Katsu, T., Kuroko, M., Morikawa, T., Sanchika, K, Fujita, Y., Yamamura, H., *et al.* (1989). Mechanism of membrane damage induced by peptides gramicidin S and melittin. *Biochim. Biophys. Acta.*, **983**, 135–41.

Kerr, I. D., Dufourcq, J., Rice, J. A., Fredkin, D. R. and Sansom, M. S. P. (1995). Ion channel formation by synthetic analogues of δ-toxin. *Biochim. Biophys. Acta*, **1236**, 219–27.

Lee, K. H., Fitton, J. E. and Wüthrich, K. (1987). NMR investigation of the conformation of δ-haemolysin bound to dodecyl phosphocholine. *Biochim. Biophys. Acta*, **911**, 144–53.

McKevitt, A. I., Bjomsonn, G. L., Mauracher, C.A., and Scheifele, D. W. (1990). Amino acid sequence of a delta like toxin from *Staphylococcus epidermidis. Infect. Immun.*, **58**, 1473–5.

Monette, M. and Lafleur, M. (1995). Modulation of melittin-induced lysis by surface charge density of membranes. *Biophys. J.*, **68**, 187–95.

Pott, T. and Dufourc, E. J. (1995). Action of Mel on DPPC-cholesterol liquid-ordered phases: a solid state 2H and 31P NHR study. *Biophys. J.*, **68**, 965–77.

Raghunathan, C., Seetharamulu, P., Brooks, B. R., and Guy, H. R. (1990). Models of δ-toxin membrane channels and crystal structures. *Proteins*, **8**, 213–25.
Sansom, M. S. P. (1991). The biophysics of peptide models if ion channels. *Prog. Biophys. Mol. Biol.*, **55**, 139–236.
Stankowski, S., Pawlak, M., Kaïfsheva, E., Robert, C. H., and Schwarz, G. (1991). A combined study of aggregation, membrane affinity and pore activity of natural and modified melittin. *Biochim. Biophys. Acta*, **1069**, 77–86.
Tappin, M. J., Pastore, A., Norton, R. S., Freer, J. H., and Campbell, I. D. (1988). High resolution ^{1}H NMR study of the structure of δ-hemolysin. *Biochemistry*, **27**, 1643–7.
Terwilliger, T. C. and Eisenberg, D. (1982). The structure of melittin. *J. Biol. Chem.*, **257**, 6016–22.
Thelestam, M. and Möllby, R. (1979). Classification of microbial, plant and animal cytolysins based on their membrane-damaging effects on human fibroblasts. *Biochim. Biophys. Acta*, **556**, 156–69.
Thiaudière, E., Siffert, O., Talbot, J. C., Bolard, J., Alouf, J. E. and Dufourcq, J. (1991). The amphipatic α-helix concept. Consequences for the structure of δ-toxin in solution and bound to lipids. *Eur. J. Biochem.*, **195**, 203–13.
Tosteson, M. T., Holmes, S. J., Razin, M., and Tosteson, D. C. (1985) Melittin lysis of red cells. *J. Membr. Biol.*, **87**, 35–44.
Van Veen, M. and Cherry, R. J. (1992). The effect of the presence of integral membrane protein in the membrane lytic properties of melittin in reconstituted systems. *FEMS Microbiol. Immun.*, **105**, 147–50.

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Aerolysin (*Aeromonas hydrophila*)

Aerolysin is a 48 kDa channel-forming protein secreted by species in the genus Aeromonas. It binds to a specific receptor on target cells and oligomerizes to form heptamers which can insert into the plasma membrane. This results in the production of well-defined channels that cause disruption of the permeability barrier and cell death.

Aerolysin is secreted as a 52 kDa protoxin by *Aeromonas* sp. (see van der Goot *et al.* 1994, for a recent review). The protein appears to be primarily responsible for the pathogenicity of *A. hydrophila* to mice (Chakraborty *et al.* 1987). The *aerA* structural genes from a number of different species have been sequenced, and the structure of the protein from *A. hydrophila* has been solved (Parker *et al.* 1994). The protein is a dimer in the crystal as well as in solution and each polypeptide chain contains four domains. More than 70 per cent of the molecule is in β-sheet. The protoxin is converted to the active toxin by proteolytic removal of approximately 43 amino acids from the C-terminus (Howard and Buckley 1985; van der Goot *et al.* 1992). This can be accomplished by proteases secreted by the bacteria, as well as by mammalian proteases such as trypsin, chymotrypsin, and furin. Aerolysin binds to a receptor on the surface of target cells. In the case of rats and mice, which are very sensitive to the toxin, the receptor on erythrocytes is a 47 kDa glycoprotein with high affinity (Kd 10^{-9}–10^{-10} M) for aerolysin (Gruber *et al.* 1994). We have recently found that the rat erythrocyte receptor is a member of a family of GPI-anchored membrane proteins that includes the T-cell receptor RT6. On human erythrocytes, which are less sensitive to the toxin, the receptor is likely glycophorin (Garland and Buckley 1988). Binding concentrates aerolysin on the surface of the cell and this promotes oligomerization of the toxin which results in the formation of heptameric structures that can insert into the plasma membrane (Wilmsen *et al.* 1992). The heptamers likely form β-barrels, similar to those produced by bacterial and mitochondria porins. The heptameric channels have some of the properties of porin channels. They are approximately 1.5 nm in diameter (Howard and Buckley 1982), and they are voltage gated (Wilmsen *et al.* 1990).

Aerolysin is a prototype of toxins that likely generate β-barrels in order to form channels. Other examples are the alpha toxins of *Staphylococcus aureus* and *Clostridium septicum*, the oxygen-labile toxins of many Gram-negative species, and *Pseudomonas aeruginosa* hemolysin. These toxins each contain a stretch of amino acids that is similar to the sequence from 250 to 300 in aerolysin. *C. septicum* alpha toxin is homologous throughout its sequence to domains 2, 3, and 4 of aerolysin.

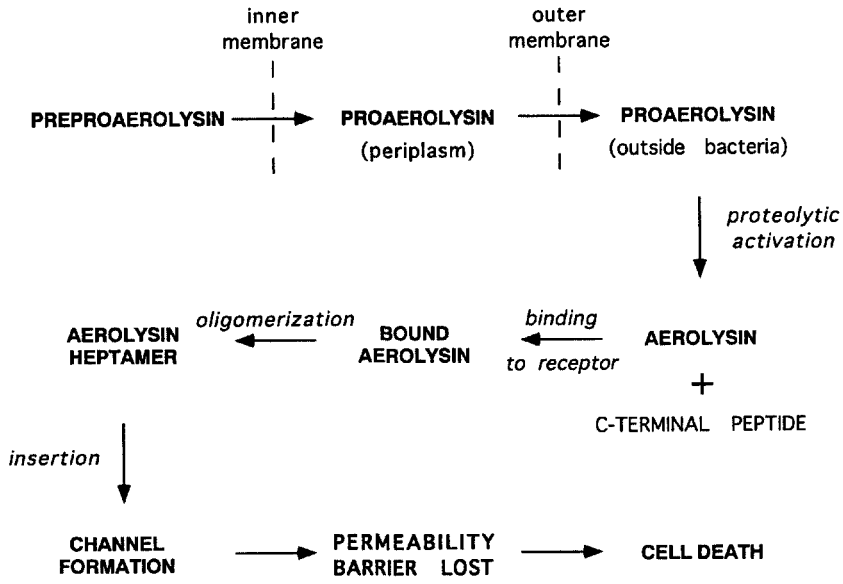

Figure 1. *Stages in the formation of a channel by aerolysin. During secretion the protein crosses the inner and outer membranes of the bacteria in separate steps. Proaerolysin can also bind to the receptor on the target cell.*

Purification and sources

Aerolysin has been isolated from culture supernatants of *Aeromonas hydrophila* (Buckley *et al.* 1981) and *Aeromonas sobria* (Chakraborty *et al.* 1984). Due to the presence of proteases, preparations are often a mixture of the protoxin and the active form and losses can be high, due to oligomerization and aggregation of the toxin. Large amounts of the *A. hydrophila* protoxin can be isolated from culture supernatants of a protease-deficient mutant of *A. salmonicida* containing the cloned *hydrophila* structural gene. Ammonium sulphate precipitation is used, followed by hydroxyapatite and DEAE cellulose chromatography. Proaerolysin can be converted to aerolysin by treatment with trypsin or with trypsin immobilized on agarose beads (Buckley 1990).

Toxicity

An LD_{50} for aerolysin has not been established for any species. In one experiment, intravenous injection of 100 ng of toxin killed all the mice tested within 24 hours (Buckley *et al.* 1981). Based on the *in vitro* experiments described above, aerolysin should be less toxic to humans.

Uses in cell biology

Aerolysin can be used to permeabilize cells in exactly the same way as *S. aureus* alpha toxin has been used, as well as for the other applications of alpha toxin that have recently been proposed (Krishnasastry *et al.* 1992). It has the advantage that it is more stable than alpha toxin, and that it can be converted to the active form on demand. Aerolysin has also been used for the isolation of intracellular parasites. This is based on the principle that although the host cells are disrupted by the toxin, parasites like trypanosomes are protected by their glycocalyx. Thus trypanosomes can be easily freed from erythrocytes as a first step in their purification (Pearson *et al.* 1982).

References

Buckley, J. T. (1990). Purification of cloned proaerolysin released by a low protease mutant of *Aeromonas salmonicida*. *Biochem. Cell. Biol.*, **68**, 221–4.

Buckley, J. T., Halasa, L. N., Lund, K. D., and MacIntyre, S. (1981). Purification and some properties of the hemolytic toxin aerolysin. *Can. J. Biochem.*, **59**, 430–6.

Chakraborty, T., Schmid, A., Notermans, S., and Benz, R. (1984). Aerolysin of *Aeromonas sobria*: evidence for formation of ion-permeable channels and comparison with alpha-toxin of *Staphylococcus aureus*. *Infect. Immun.*, **58**, 2127–32.

Chakraborty, T., Huhle, B., Hof., H., Bergbauer, H., and Goebel, W. (1987). Marker exchange mutagenesis of the aerolysin determinant in *Aeromonas hydrophila* demonstrates the role of aerolysin in *Aeromonas hydrophila* infections. *Infect. Immun.*, **55**, 2274–80.

Garland, W. J., and Buckley, J. T. (1988). The cytolytic toxin aerolysin must aggregate to disrupt erythrocytes and aggregation is stimulated by human glycoprotein. *Infect. Immun.*, **56**, 1249–54.

Gruber, H. J., Wilmsen, H. U., Cowell, S., Schindler, H., and Buckley, J. T. (1994). Partial purification of the rat erythrocyte receptor for the channel-forming toxin aerolysin and reconstitution into planar lipid bilayers. *Mol. Microbiol.*, **14**, 1093–11.

Howard, S. P., and Buckley, J. T. (1982). Membrane glycoprotein receptor and hole-forming properties of a cytolytic protein toxin. *Biochemistry*, **21**, 1662–7.
Howard, S. P., and Buckley, J. T. (1985). Activation of the hole-forming toxin aerolysin by extracellular processing. *J. Bacteriol.*, **163**, 336–40.
Krishnasastry, M., Walker, B., Zorn, L., Kasianowicz, J., and Bayley, H. (1992). In *Synthetic structures in biological research* (ed. J. M. Schnur and M. Peckerar), pp. 41–51, Plenum Press, New York.
Parker, M. W., Buckley, J. T., Postma, J. P. M., Tucker, A. D., Leonard, K., Pattus, F., *et al.* (1994). Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature*, **367**, 292–5.
Pearson, T. W., Saya, L. E., Howard, S. P., and Buckley, J. T. (1982). The use of aerolysin toxin as an aid for visualization of low numbers of African trypanosomes in whole blood. *Acta Tropica*, **39**, 73–7.
van der Goot, F. G., Lakey, J., Pattus, F., Kay, C. M., Sorokine, O., Van Dorsselaer, A., *et al.* (1992). Spectroscopic study of the activation and oligomerization of the channel-forming toxin aerolysin: identification of the site of proteolytic activation. *Biochemistry*, **31**, 8566–70.
van der Goot, F. G., Pattus, F., Parker, M. W., and Buckley, J. T. (1994). Aerolysin: from the soluble form to the transmembrane channel. *Toxicol.*, **87**, 19–28.
Wilmsen, H. U., Pattus, F., and Buckley, J. T. (1990). Aerolysin, a hemolysin from *Aeromonas hydrophila*, forms voltage-gated channels in planar lipid bilayers. *J. Membr. Biol.*, **115**, 71–81.
Wilmsen, H. U., Leonard, K., Tichelaar, W., Buckley, J. T., and Pattus, F. (1992). The aerolysin membrane channel is formed by heptamerization of the monomer. *EMBO J.*, **11**, 2457–63.

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Escherichia coli hemolysin

Hemolysin (Hly A) from Escherichia coli *is an extracellular, pore-forming toxin belonging to the RTX family of bacterial cytolysins. It is synthesized as an inactive protoxin of 110 kDa, which is activated within the* E. coli *cell by covalent fatty acid acylation. The secretion of the toxin into the culture medium is accomplished by a specific transport apparatus.* E. coli *hemolysin displays strong cytolytic and cytotoxic activity against a wide range of human and mammalian cells, including erythrocytes and leukocytes, and modulates the functions of several cell types at sublytic concentrations. The toxin represents an important virulence factor in the pathogenesis of extraintestinal* E. coli *infections.*

E. coli hemolysin (Hly A) is the most extensively studied member of the family of RTX toxins, which represents a group of structurally and functionally related cytolysins and cytotoxins produced by a variety of gram-negative bacteria. In addition to *E. coli* hemolysin, the RTX toxins include hemolysins from *Proteus vulgaris* and *Morganella morganii*, hemolysins, and cytotoxins from *Actinobacillus pleuropneumoniae* and *Actinobacillus suis*, leukotoxins from *Pasteurella haemolytica* and *Actinobacillus actinomycetemcomitans*, and the bifunctional adenylate cyclase-hemolysin (adenylate cyclase toxin) from *Bordetella pertussis* (Coote 1992). All RTX toxins are Ca^{2+}-dependent, pore-forming protein toxins. The most pronounced structural feature of these toxins is the presence of a characteristic repetitive domain, which is composed of a toxin-specific number of highly conserved glycine- and aspartate-rich repeat motifs of nine amino acids (XLXGGXGN/DD). The designation RTX (Repeats in ToXins) was deduced from this common structure.

The RTX toxins are synthesized as inactive proteins which have to be activated by an accessory protein prior to secretion from the bacterial cell. In the case of *E. coli* hemolysin, it has been shown that the 110 kDa hemolysin protein (proHlyA) is activated post-translationally by acyl carrier protein-dependent fatty acid acylation of two internal lysine residues, Lys-564 and Lys-690 (Issartel *et al.* 1991; Stanley *et al.* 1994). This covalent modification is mediated by the cytoplasmic protein HlyC (20 kDa) which is coexpressed together with HlyA, but the exact function of HlyC in the activation process is not completely understood.

The extracellular secretion of *E. coli* hemolysin and of the other RTX toxins is accomplished by specific, highly conserved transport systems that consist of at least three membrane-bound proteins. The transport of *E. coli* hemolysin, in particular, depends on two specific integral proteins of the cytoplasmic membrane, HlyB (80 kDa) and HlyD (55 kDa), and on the common *E. coli* outer membrane protein TolC (52 kDa) (Wagner *et al.* 1983; Wandersman and Delepelaire 1990; Wang *et al.* 1991). These proteins probably form a transenvelope complex that spans both membranes of *E. coli*, allowing the direct secretion of hemolysin into the medium without accumulation in the periplasmic space. Interestingly, HlyB is a member of the superfamily of ATP binding cassette (ABC) transporters and it has been shown that HlyB provides

energy for the translocation of HlyA by binding and hydrolyzing ATP (Koronakis *et al.* 1995).

Hemolysin is frequently produced by *E. coli* strains that cause extraintestinal infections in humans, especially those of the urinary tract. *In vivo* studies using several animal models indeed demonstrated that hemolysin significantly contributes to the virulence of these *E. coli* strains (Hacker *et al.* 1983; Cavalieri *et al.* 1984). However, the precise function of *E. coli* hemolysin in the pathogenesis of extraintestinal infections is unclear and probably multifactorial. Due to its cytotoxic activity against leukocytes and other nucleated cell types, it may impair or counteract the host immune defence system and cause tissue damage, thereby promoting the penetration of the bacteria into deeper tissue layers. In addition, the lysis of erythrocytes by hemolysin may stimulate bacterial growth in the host by increasing the level of available iron. Inflammatory mediators, which are released in large amounts from several types of human and mammalian cells upon contact with sublytic doses of *E. coli* hemolysin, may further impair the host and facilitate bacterial spreading.

Alternative names

α-Hemolysin, HlyA

Isolation

E. coli hemolysin was originally identified by its capacity to lyse erythrocytes. The genetic determinant coding for synthesis and secretion of active hemolysin was found to be located either on large plasmids or on the chromosome of hemolytic, wild-type *E. coli* strains. Cloning and sequencing of hemolysin determinants from different strains allowed the further characterization of the toxin.

Gene and sequence

The *E. coli* hemolysin determinant represents the prototype of an RTX toxin determinant. It consists of four structural genes which are arranged in an operon in the order *hly*C, *hly*A, *hly*B, and *hly*D (Felmlee *et al.* 1985; Hess *et al.* 1986) (GenBank accession numbers M10133, M12863, M14107, M81823, X02768, X07565). The gene *hly*A is the structural gene of the *E. coli* hemolysin and encodes the inactive protoxin (proHlyA, 110 kDa, 1024 amino acids); *hly*C encodes the cytoplasmic protein that is necessary for the conversion of proHlyA to the hemolytically active toxin; *hly*B and *hly*D encode the two inner membrane proteins which are required for the secretion of hemolysin (Goebel and Hedgpeth 1982; Wagner *et al.* 1983). The *hly*CABD operon is transcribed from a promoter region located upstream of *hly*C. TolC is encoded by a chromosomal gene not located in the *hly* gene cluster.

Protein

E. coli hemolysin is an extracellularly secreted, fatty acid-acylated protein of 110 kDa which is endowed with hemolytic and cytotoxic activity. The toxin is routinely isolated from the culture supernatant of exponentially growing *E. coli* strains containing the complete hemolysin determinant. Active hemolysin depleted of LPS can be purified from the culture supernatant by precipitation with polyethylene glycol followed by centrifugation in a glycerol density gradient (Jonas *et al.* 1993). Various monoclonal and polyclonal antibodies against *E. coli* hemolysin have been described (Pellett *et al.* 1990; Oropeza-Wekerle *et al.* 1991; Jarchau *et al.* 1994).

Several functional regions have been identified in the hemolysin protein (Fig. 1). The secretion signal necessary for the recognition of HlyA by the transport machinery is located within the C-terminal 50–60 amino acids of HlyA, but the exact structural features of this unprocessed signal are not well understood (Jarchau *et al.* 1994; Kenny *et al.* 1994). Binding of hemolysin to target cell membranes apparently requires the binding of Ca^{2+} to the repeat domain in the C-terminal half of HlyA as well as the HlyC-mediated fatty acid acylation of Lys-564 and Lys-690 in the region proximal to the repeat domain (Ludwig *et al.* 1988; Boehm *et al.* 1990). The transmembrane pore appears to be formed by a domain in the N-terminal half of HlyA which contains putative hydrophobic and amphipathic transmembrane sequences (Ludwig *et al.* 1991). Studies on artificial lipid bilayers have shown that the transmembrane pores generated by *E. coli* hemolysin are unstable, hydrophilic, and cation-specific. From the single channel conductance the pore diameter was estimated to be at least 1 nm (Benz *et al.* 1989).

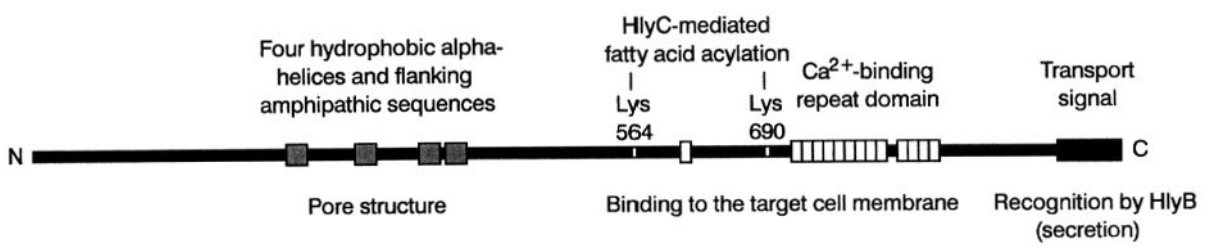

Figure 1. Model of *E. coli* hemolysin (HlyA) showing the location of functional domains.

Biological activities

E. coli hemolysin lyses erythrocytes from a wide range of species and it displays strong cytotoxic activity against a variety of nucleated cells including leukocytes, endothelial cells, and renal epithelial cells (Cavalieri *et al.* 1984; Jonas *et al.* 1993). Particularly, granulocytes, monocytes, and human T lymphocytes are killed by nanomolar or even subnanomolar concentrations of the toxin. The cytolytic and cytotoxic activity of *E. coli* hemolysin is most likely due to the formation of pores in the cytoplasmic membrane of the target cells, which cause a rapid and irreversible depletion of cellular ATP and eventually may lead to osmotic cell lysis. Very low, sublytic concentrations of *E. coli* hemolysin modulate normal functions of several types of host cells. They induce, for example, the production and release of inflammatory mediators from polymorphonuclear leukocytes, monocytes, and platelets, and cause a contraction of endothelial cells (Grimminger *et al.* 1991; König *et al.* 1994). Many of the host cell responses to very low concentrations of *E. coli* hemolysin are most likely mediated by a defined signal transduction cascade which is triggered by the formation of the transmembrane pores in the target cell membrane.

Biological regulation

Transcription of the *hly* gene cluster is positively regulated by sequences located upstream of the promoter region, which may act as binding sites for regulatory proteins. In some *E. coli* strains the expression of hemolysin was found to be negatively regulated by iron in the growth medium.

Mutagenesis studies

The significance of hemolysin as a virulence factor in extraintestinal *E. coli* infections has been established in several animal models. Experimental infections with hemolytic wild-type *E. coli* strains and nonhemolytic mutants as well as with genetically engineered, isogenic hemolysin-producing and nonproducing *E. coli* strains demonstrated that hemolysin is directly involved in the pathogenesis of infection (Hacker *et al.* 1983; Cavalieri *et al.* 1984).

Biological interactions

E. coli hemolysin interacts with a variety of eukaryotic cells. However, a specific receptor for hemolysin in target cell membranes has not been identified.

References

Benz, R., Schmid, A., Wagner, W., and Goebel, W., (1989). Pore formation by the *Escherichia coli* hemolysin: evidence for an association–dissociation equilibrium of the pore-forming aggregates. *Infect. Immun.*, **57**, 887–95.

Boehm, D. F., Welch, R. A., and Snyder, I. S. (1990). Domains of *Escherichia coli* hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. *Infect. Immun.*, **58**, 1959–64.

Cavalieri, S. J., Bohach, G. A., and Snyder, I. S. (1984). *Escherichia coli* α-hemolysin: characteristics and probable role in pathogenicity. *Microbiol. Rev.*, **48**, 326–43.

Coote, J. G. (1992). Structural and functional relationships among the RTX toxin determinants of Gram-negative bacteria. *FEMS Microbiol. Rev.*, **88**, 137–62.

Felmlee, T., Pellett, S., and Welch, R. A. (1985). Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J. Bacteriol.*, **163**, 94–105.

Goebel, W. and Hedgpeth, J. (1982). Cloning and functional characterization of the plasmid-encoded hemolysin determinant of *Escherichia coli*. *J. Bacteriol.*, **151**, 1290–8.

Grimminger, F., Scholz, C., Bhakdi, S., and Seeger, W. (1991). Subhemolytic doses of *Escherichia coli* hemolysin evoke large quantities of lipoxygenase products in human neutrophils. *J. Biol. Chem.*, **266**, 14262–9.

Hacker, J., Hughes, C., Hof, H., and Goebel, W. (1983). Cloned hemolysin genes from *Escherichia coli* that cause urinary tract infection determine different levels of toxicity in mice. *Infect. Immun.*, **42**, 57–63.

Hess, J., Wels, W., Vogel, M., and Goebel, W. (1986). Nucleotide sequence of a plasmid-encoded hemolysin determinant and its comparison with a corresponding chromosomal hemolysin sequence. *FEMS Microbiol. Lett.*, **34**, 1–11.

Issartel, J.-P., Koronakis, V., and Hughes, C. (1991). Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature*, **351**, 759–61.

Jarchau, T., Chakraborty, T., Garcia, F., and Goebel, W. (1994). Selection for transport competence of C-terminal polypeptides derived from *Escherichia coli* hemolysin: the shortest peptide capable of autonomous HlyB/HlyD-dependent secretion comprises the C-terminal 62 amino acids of HlyA. *Mol. Gen. Genet.*, **245**, 53–60.

Jonas, D., Schultheis, B., Klas, C., Krammer, P. H., and Bhakdi, S. (1993). Cytocidal effects of *Escherichia coli* hemolysin on human T lymphocytes. *Infect. Immun.*, **61**, 1715–21.

Kenny, B., Chervaux, C., and Holland, I. B. (1994). Evidence that residues –15 to –46 of the haemolysin secretion signal are involved in early steps in secretion, leading to recognition of the translocator. *Mol. Microbiol.*, **11**, 99–109.

König, B., Ludwig, A., Goebel, W., and König, W. (1994). Pore formation by the *Escherichia coli* alpha-hemolysin: role for mediator release from human inflammatory cells. *Infect. Immun.*, **62**, 4611–17.

Koronakis, E., Hughes, C., Milisav, I., and Koronakis, V. (1995). Protein exporter function and *in vitro* ATPase activity are correlated in ABC-domain mutants of HlyB. *Mol. Microbiol.*, **16**, 87–96.

Ludwig, A., Jarchau, T., Benz, R., and Goebel, W. (1988). The repeat domain of *Escherichia coli* haemolysin (HlyA) is responsible for its Ca^{2+}-dependent binding to erythrocytes. *Mol. Gen. Genet.*, **214**, 553–61.

Ludwig, A., Schmid, A., Benz, R., and Goebel, W. (1991). Mutations affecting pore formation by haemolysin from *Escherichia coli*. *Mol. Gen. Genet.*, **226**, 198–208.

Ludwig, A., Garcia, F., Bauer, S., Jarchau, T., Benz, R., Hoppe, J., and Goebel, W. (1996). Analysis of the in vivo activation of hemolysin (HlyA) from *Escherichia coli*. *J. Bacteriol.*, **178**, 5422–30.

Oropeza-Wekerle, R. L., Kern, P., Sun, D., Muller, S., Briand, J. P., and Goebel, W. (1991). Characterization of monoclonal antibodies against alpha-hemolysin of *Escherichia coli*. *Infect. Immun.*, **59**, 1846–52.

Pellett, S., Boehm, D. F., Snyder, I. S., Rowe, G., and Welch, R. A. (1990). Characterization of monoclonal antibodies against the *Escherichia coli* hemolysin. *Infect. Immun.*, **58**, 822–7.

Stanley, P., Packman, L. C., Koronakis, V., and Hughes, C. (1994). Fatty acylation of two internal lysine residues required for the toxic activity of *Escherichia coli* hemolysin. *Science*, **266**, 1992–6.

Wagner, W., Vogel, M., and Goebel, W. (1983). Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. *J. Bacteriol.*, **154**, 200–10.

Wandersman, C. and Delepelaire, P. (1990). TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proc. Natl. Acad. Sci. USA*, **87**, 4776–80.

Wang, R., Seror, S. J., Blight, M., Pratt, J. M., Broome-Smith, J. K., and Holland, I. B. (1991). Analysis of the membrane organization of an *Escherichia coli* protein translocator, HlyB, a member of a large family of prokaryote and eukaryote surface transport proteins. *J. Mol. Biol.*, **217**, 441–54.

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Toxins affecting signal transduction

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Introduction

Signal transduction

The ability to sense extracellular signals is essential for all living organisms. In animals, the presence of specialized tissues with different functions, that need to coordinate and regulate their activities, makes signal transduction even more essential. Signals are most often delivered to surface receptors by hormones, growth factors, cell to cell contacts, etc. Once triggered by the ligand, the receptor is activated and the signal is transduced across the cell membrane by receptor clustering and/or conformational changes, that results in biochemical changes on the cytosolic side of the membrane. These occur mainly by two mechanisms:

(1) tyrosine kinase receptor phosphorylation of specific sites of its carboxyterminal cytoplasmic domain, that recruits SH2-transducers to start a cascade of phosphorylation of intracellular targets;

(2) modification of a receptor-coupled G protein that transduces the signal to enzymes, releasing second messengers such as cyclic-AMP (cAMP), inositol-triphosphate (ITP), diacyglycerol (DG), to a cytoplasmic kinase etc.

So far, no toxins have been described that interfere with signal transduction mediated by receptors with a cytoplasmic kinase domain. In marked contrast, many toxins are known to interfere with G protein mediated signal transduction, or with one of the steps downstream from a G protein regulated process. As shown in Fig. 1, these are pertussis toxin, cholera and *Escherichia coli* LT toxins, exoenzyme C3 of *Clostridium botulinum*, the *Bordetella pertussis* adenylate cyclase-hemolysin, and the edema factor of *Bacillus antracis*, which are described in this chapter, and also exoenzyme S of *Pseudomonas aeruginosa* and the *C. botulinum* toxins A and B, which are described in other chapters of this manual.

G proteins

G proteins are a family of GTP-binding proteins composed of three subunits (α, β and γ), that are involved in signal transduction across the membrane in animals, plants, and fungi (Hepler and Gilman 1992; Linder and Gilman 1992). The α subunit is usually anchored to the cell membrane by a 14-carbon fatty acid (myristic acid) (Jones *et al.* 1990; Mumby *et al.* 1990; Spiegel *et al.* 1991).

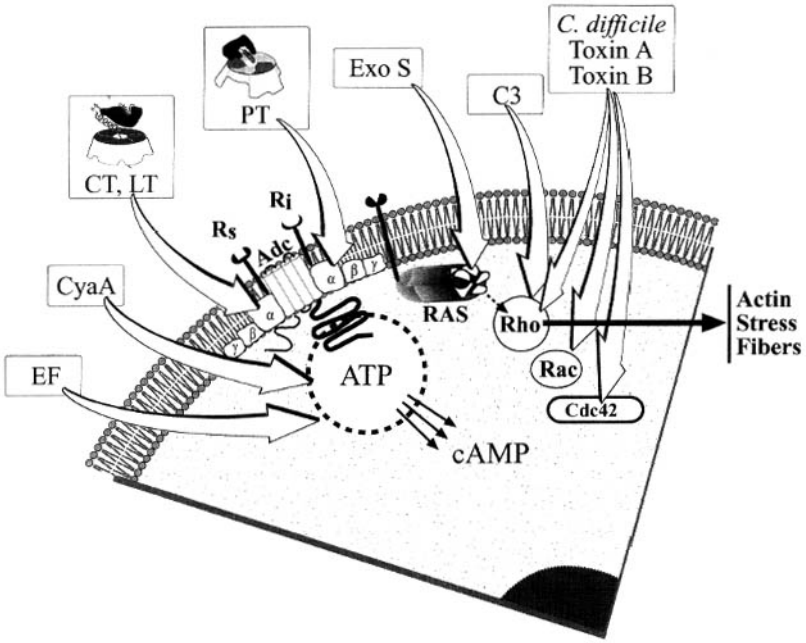

Figure 1. Schematic representation showing the bacterial toxins affecting signal transduction and their targets in an eukaryotic cell.

Typically, a signal that is sensed by a receptor on the surface of eukaryotic cells is received by the α subunit of the G protein, which consequently binds GTP, dissociates from the β and γ subunits, and transmits the signal to the enzymes that release second messengers such as adenylate cyclase, phospholipase C, and cyclic GMP phosphodiesterase. Adenylate cyclase, shown as an example in Fig. 1, is regulated by two classes of receptors that can transmit their signals to two different GTP-binding proteins: G_s and G_i. G_s receives signals from the stimulatory receptors and activates the adenylate cyclase, whereas G_i receives signals from the inhibitory receptors and inhibits the activity of adenylate cyclase. In addition to G_s and G_i, the family of G proteins contains several other proteins, including G_t, G_o, G_{olf} (Jones and Reed 1989; Hepler and Gilman 1992), and several other G proteins with a similar function, that have not yet been fully characterized. As shown in Fig. 2, cholera toxin ADP-ribosylates Arg-201 of

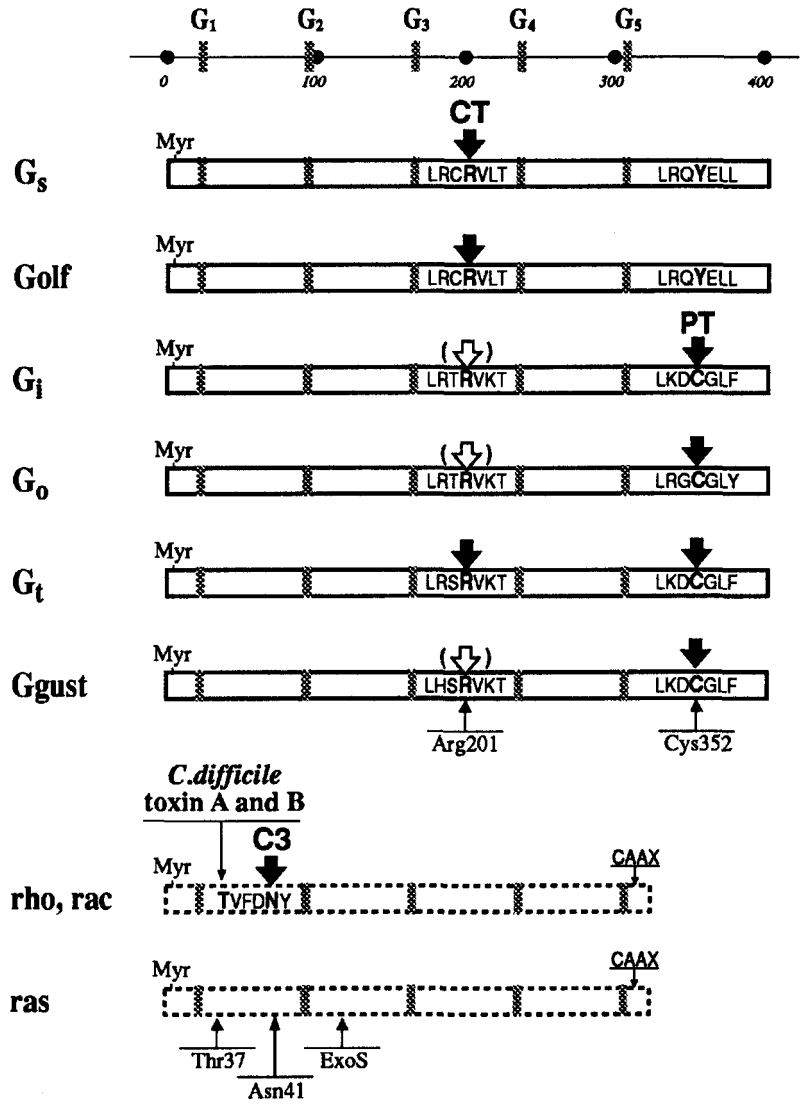

Figure 2. Schematic representation of the proteins involved in signal transduction that are modified by bacterial protein toxins. The top line shows the numbers of the amino acids. G_1 to G_5 represent the regions of homology within the target G proteins. Myr and CAAX indicate sites that are modified by the addition of myristic acid or a farnesyl residue, respectively. Amino acids are indicated in the one-letter code; those that are ADP-ribosylated by the toxins are in bold letters and indicated by large arrows. Small arrows indicate the glucosylation of Thr37 by *C. difficile* toxins A and B. G_{gust} stands for Gustducin. CT, PT, and C3 stand for cholera, pertussis, and *Clostridium botulinum* C3 toxins, respectively.

the α subunit of G_s, G_t and G_{olf} (Van Dop *et al.* 1984), whereas it ADP-ribosylates the corresponding Arg in G_i and G_o only when these are receptor-activated (Iiri *et al.* 1989). Pertussis toxin ADP-ribosylates Cys-352 residue in G_i, G_o, G_t, G_{gust}, and other G proteins, but is not able to modify G_s and G_{olf}, which have a tyrosine in position 352 (Katada and Ui 1982; Bokoch *et al.* 1983; West *et al.* 1985; Fong *et al.* 1988). ADP-ribosylation of G_s by cholera or LT toxins causes an irreversible block of the GTPase activity of the G_s protein, leading to the constitutive activation of adenylate cyclase, which results in intracellular accumulation of the second messenger cAMP. ADP-ribosylation of G_i by pertussis toxin uncouples this protein from the receptor in such a way that it is unable to transmit inhibitory signals to the adenylate cyclase.

Ras, Rho, Rac, CDC 42, and the small GTP-binding proteins

Ras, Rho, Rac, CDC 42, and other small GTP-binding proteins belong to a large family of regulatory proteins that are known to be involved in controlling diverse essential cellular functions, including growth, differentiation, cytoskeletal organization, intracellular vesicle transport, and secretion. They usually contain a carboxy terminal CAAX box that is modified by addition of a farnesyl lipid moiety, increasing the hydrophobicity and localizing them to the plasma membrane or to the membranes of intracellular vesicles (Hall 1990). The oncoprotein Ras, which is localized to the plasma membrane, controls cell growth and differentiation (Egan *et al.* 1993), and is a target of ADP-ribosylation by *Pseudomonas* exoenzyme S (Coburn *et al.* 1989). Following ADP-ribosylation, the GTPase activity of Ras is decreased (Tsai *et al.* 1985). Rho and Rac are structurally similar proteins involved in the organization of actin (Ridley and Hall 1992; Ridley *et al.* 1992), formation of focal adhesions, membrane ruffling, etc. They are ADP-ribosylated on Asn-41 by *C. botulinum* C3 (Aktories *et al.* 1989; Sekine *et al.* 1989), and glucosylated on Thr-37 by *C. difficile* toxins A and B (Aktories and Just 1995). Both modifications of Rho cause actin depolymerization and cell rounding.

Invasive adenylate cyclases

Some of the toxins are enzymes that bypass membrane signal transduction and interfere with signal transduction by generating directly second messengers, such as cAMP. Two of these enzymes, the adenylate cyclase-hemolysin of *B. pertussis* and the edema factor of *B. antracis*, are totally unrelated proteins that have evolved different ways to reach the intracellular space. However, once inside the cell they display the same catalytic activity: they use ATP to produce cAMP, thus altering the normal levels of this second messenger and producing the same effects that are indirectly induced by cholera and LT toxins.

References

Aktories, K. and Just, I. (1995). Monoglucosylation of low-molecular-mass GTP-binding Rho proteins by clostridial cytotoxins. *Trends Cell Biol.*, **5**, 441–3.

Aktories, K., Braun, U., Rosener, S., Just, I., and Hall, A. (1989). The rho gene product expressed in *E. coli* is a substrate of botulinum ADP-ribosyltransferase C3. *Biochem. Biophys. Res. Commun.*, **158**, 209–13.

Bokoch, G. M., Katada, T., Northup, J. K., Hewlett, E. L., and Gilman, A. G. (1983). Identification of the predominant substrate for ADP-ribosylation by islet activating protein. *J. Biol. Chem.*, **258**, 2072–5.

Coburn, J., Wyatt, R. T., Iglewski, B. H., and Gill, D. M. (1989). Several GTP-binding proteins, including p21c-H-ras, are preferred substrates of *Pseudomonas aeruginosa* exoenzyme S. *J. Biol. Chem.*, **264**, 9004–8.

Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993). Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature*, **363**, 45–51.

Fong, H. K., Yoshimoto, K. K., Eversole-Cire, P., and Simon, M. I. (1988). Identification of a GTP-binding protein by alpha subunit that lacks an apparent ADP-ribosylation site for pertussis toxin. *Proc. Natl. Acad. Sci. USA*, **85**, 3066–70.

Hall, A. (1990). The cellular functions of small GTP-binding proteins. *Science*, **249**, 635–40.

Hepler, J. R. and Gilman, A. G. (1992). G proteins. *Trends Biochem. Sci.*, **17**, 383–7.

Iiri, T., Tohkin, M., Morishima, N., Ohoko, Y., Ui, M., and Katada, T. (1989). Chemotactic peptide receptor-supported ADP-ribosylation of a pertussis toxin substrate GTP-binding protein by cholera toxin in neutrophil-type HL-60 cells. *J. Biol. Chem.*, **264**, 21394–400.

Jones, D. T. and Reed, R. R. (1989). G_{olf}: an olfactory neuron specific-G protein involved in odorant signal transduction. *Science*, **244**, 790–5.

Jones, T. L., Simonds, W. F., Merendino, J. J., Jr, Brann, M. R., and Spiegel, A. M. (1990). Myristoylation of an inhibitory GTP-binding protein alpha subunit is essential for its membrane attachment. *Proc. Natl. Acad. Sci. USA*, **87**, 568–72.

Katada, T. and Ui, M. (1982). ADP-ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. *J. Biol. Chem.*, **257**, 7210–16.

Linder, M. E. and Gilman, A. G. (1992). G proteins. *Sci. Am.*, **July**, 56–65.

Mumby, S. M., Heukeroth, R. O., Gordon, J. I., and Gilman, A. G. (1990). G-protein alpha-subunit expression, myristoylation, and membrane association in COS cells. *Proc. Natl. Acad. Sci. USA*, **87**, 728–32.

Ridley, A. J. and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*, **70**, 389–99.

Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*, **70**, 401–10.

Sekine, A., Fujiwara, M., and Narumuya, S. (1989). Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. *J. Biol. Chem.*, **264**, 8602–5.

Spiegel, A. M., Backlund, P. S., Jr., Butrynski, J. E., Jones, T. L., and Simonds, W. F. (1991). The G protein connection: molecular basis of membrane association. *Trends Biochem. Sci.*, **16**, 338–41.

Tsai, S. C., Adamik, R., Moss, J., Vaughan, M., Manne, V., and Kung, H.F. (1985). Effects of phospholipids and

ADP-ribosylation on GTP hydrolysis by *Escherichia coli*-synthesized Ha-ras-encoded p21. *Proc. Natl. Acad. Sci. USA*, **82**, 8310–14.
Van Dop, C., Tsubokawa, M., Bourne, H. R., and Ramachandran, J. (1984). Amino acid sequence of retinal transducin at the site ADP-ribosylated by cholera toxin. *J. Biol. Chem.*, **259**, 696–8.
West, R. E., Jr, Moss, J., Vaughan, M., Liu, T., and Liu, T. Y. (1985). Pertussis toxin-catalyzed ADP-ribosylation of transducin. Cysteine 347 is the ADP-ribose acceptor site. *J. Biol. Chem.*, **260**, 14428–30.

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Cholera toxin (*Vibrio cholerae*)

Cholera toxin (CT) is a protein of 82 kDa secreted by Vibrio cholerae, *that is responsible for the watery diarrhea typical of cholera, a severe disease causing 150 000 deaths each year. The toxicity is due to the ADP-ribosylating activity of the A subunit, an enzyme which modifies the α-subunit of G_s, a GTP binding protein involved in signal transduction that regulates the production of cAMP in the eukaryotic cells. This induces an alteration in the ion transport, with an increase in chloride secretion and an inhibition in sodium absorption, causing the diarrhea characteristic of the disease.*

CT is composed of two subunits, A and B, of 27 and 57.5 kDa respectively, held together by noncovalent interactions. The B subunit which contains the receptor binding site, is formed by five identical monomers, each of 11.5 kDa which assemble into a ring like oligomeric structure having a central hole. The CT receptor is the glycosfingolipid, GM1 ganglioside. The A subunit is composed of the enzymatically active A1 domain that is connected to the B subunit through the A2 domain, a long alpha helix inserted into the central hole of the B subunit (Fig. 1) (Rappuoli and Pizza 1991; Sixma *et al.* 1991*a*; Sixma *et al.* 1993; Merritt *et al.* 1994).

The genes coding for the two subunits are located in a 1.5 Kb DNA region (accession numbers: X58786 for El Tor and X58785 for Classical strains, respectively), and are transcribed from the same promoter that is regulated by ToxR (Mekalanos *et al.* 1983; Miller and Mekalanos 1984). The A and B subunits are synthesized as precursors with a signal peptide of 18 and 21 aminoacids, respectively, which are cleaved during the translocation to the cytoplasm. Holotoxin assembly takes place in the periplasm (Hirst *et al.* 1984; Hirst and Holmgren 1987). Only the fully assembled AB_5 toxin or the B_5 pentamer are secreted into the culture supernatant by a specialized secretory apparatus, that is present only in *Vibrio cholerae* strains (Sandkvist *et al.* 1993). Activation of the A subunit requires proteolitic cleavage of the loop which links the A1 to the A2 domains and reduction of a disulphide bridge that holds the two domains together (Lai *et al.* 1981). *V. cholerae* secretes a specific protease that cleaves the above loop (Booth *et al.* 1984). The A subunit binds NAD and transfers the ADP-ribose group to an Arg residue present in an... LRX**R**VXT motif located in the central part of all G proteins (Gill and Woolkalis 1991). However, only G_s, G_t, and G_{olf} are substrates for CT. G_i, G_o and G_{gust}, under physiological conditions, are not modified by CT. *In vitro*, the ADP-ribosylation activity of CT is enhanced by ADP-ribosylation factors (ARFs), which are a family of small GTP-binding proteins (Moss and Vaughan 1991).

Toxins with a similar A–B structure and with the same mechanism of action are diphtheria toxin (DT), exotoxin A of *Pseudomonas aeuriginosa* (PAETA), pertussis toxin (PT), and heat-labile enterotoxin (LT) (Domenighini *et al.* 1991, 1994). LT shares with CT an identical AB_5 structure and an 80% amino acid homology (Dallas and Falkow 1980; Domenighini *et al.* 1995).

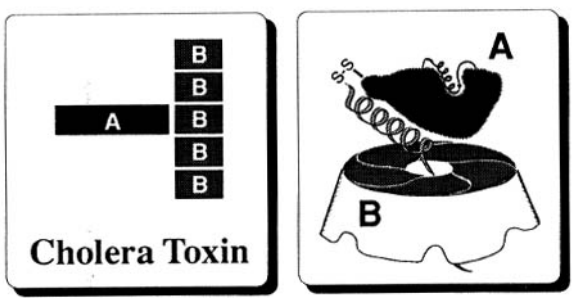

Figure 1. A schematic representation of the subunit composition (left panel), and of the 3D structure (right panel) of cholera toxin.

Structure and mutants

The 3D structure of LT has been solved (Sixma *et al.* 1991*b*, 1993). The A subunit shows a strong homology of the cavity containing the active site with the other ADP-ribosylating toxins. This cavity is formed by a β-strand bent over an α-helix, which form the floor and the ceiling of the cavity, respectively. Inside this cavity, many residues important for the enzymatic activity, have been identified (Domenighini *et al.* 1991, 1994). Based on this information, many non-toxic CT mutants have been generated by site-directed mutagenesis of residues located in the catalytic site (Hase *et al.* 1994; Fontana *et al.* 1995). The best characterized non toxic mutant of CT is CT-K63, containing the Ser $\rightarrow$ Lys substitution in position 63 (Fontana *et al.* 1995). Mutants in the B subunit with different GM1 binding affinity have been constructed. One of them, unable to bind the receptor, containing a Gly33 $\rightarrow$ Asp has been crystallized and the structure has been determined (Shoham *et al.* 1995).

Purification and sources

Cholera toxin can be purified from the supernatant of *V. cholerae* strain by precipitation with Sodium hexametaphosphate at pH 4.5 (Mekalanos 1988), and chromatography on CM-Sepharose column (Fontana *et al.* 1995). Cholera toxin can be purchased from Sigma Chemical Company, St. Louis, Mo., USA. The mutants in the A and the B subunits are available from the authors.

Toxicity

LD_{50} in NIH Swiss mice after intraperitoneal injection is 33.3 μg $\pm$ 7.3 μg (Dragunsky *et al.* 1992). However, a basic technique to study the toxic response to CT is the rabbit ileal loop. In this assay, 50 ng of CT are able to induce fluid accumulation in the intestine of rabbit. Studies of toxicity of CT in humans have shown that the ingestion of 25 μg of CT induces over 20 litres of diarrhea, while 5 μg of CT induces 1 to 6 litres of diarrhea (Levine *et al.* 1983).

Use in cell biology

CT has been widely used for the dissection of G_s-mediated signal transduction and the consequent regulation of adenylate cyclase activity in animal and even in plant cells (Beffa *et al.* 1995). In addition to this classical use, today it can be used to study tissue permeability to ions (Ussing chambers) (Field *et al.* 1969), and to study the structure–function of ARF proteins. *In vivo*, CT has been shown to be an immunogen of unusually high efficiency (Elson and Ealding 1984), and to have a potent adjuvant activity at the mucosal level (Holmgren *et al.* 1993). These properties may be used to dissect the mechanisms of antigen uptake and presentation *in vitro*. The presence of a KDEL sequence at the carboxy-terminal end of the A subunit suggests that this protein also has a mechanism of reaching the Golgi, and that the protein may be also used to study intracellular trafficking of endocytic vesicles and proteins (Lencer *et al.* 1995).

References

Beffa, R., Szell, M., Meuwly, P., Pay, A., Vögeli-Lange, R., Métraux, J.-P., *et al.* (1995). Cholera toxin elevates pathogen resistance and induces pathogenesis-related gene expression in tobacco. *EMBO J.*, **14(23)**, 5753–61.

Booth, B. A., Boesman-Finkelstein, M., and Finkelstein, R. A. (1984). *Vibrio cholerae* hemagglutinin/protease nicks cholera enterotoxin. *Infect. Immun.*, **45**, 558–60.

Dallas, W. S. and Falkow, S. (1980). Amino acid homology between cholera toxin and *Escherichia coli* heat labile toxin. *Nature*, **288**, 499–501.

Domenighini, M., Montecucco, C., Ripka, W. C., and Rappuoli, R. (1991). Computer modelling of the NAD binding site of ADP-ribosylating toxins: active-site structure and mechanism of NAD binding. *Mol. Microbiol.*, **5**, 23–31.

Domenighini, M., Magagnoli, C., Pizza, M., and Rappuoli, R. (1994). Common features of the NAD-binding and catalytic site of ADP-ribosylating toxins. *Mol. Microbiol.*, **14 (1)**, 41–50.

Domenighini, M., Pizza, M., Jobling, M. G., Holmes, R. K., and Rappuoli, R. (1995). Identification of errors among database sequence entries and comparison of correct amino acid sequences for the heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae*. *Mol. Microbiol.*, **15(6)**, 1165–67.

Dragunsky, E. M., Rivera, E., Aaronson, W., Dolgaya, T. M., Hochstein, H. D., Habig, W. H., *et al.* (1992). Experimental evaluation of antitoxic protective effect of new cholera vaccines in mice. *Vaccine*, **10**, 735–6.

Elson, C. O. and Ealding, W. (1984). Generalized systemic and mucosal immunity in mice after mucosal stimulation and cholera toxin. *J. Immunol.*, **132**, 2736–41.

Field, M., Fromm, D., Wallace, C. K., and Greenough III, W.B. (1969). Stimulation of active chloride secretion in small intestine by cholera exotoxin. *J. Clin. Invest.*, **48**, 24a.

Fontana, M. R., Manetti, R., Giannelli, V., Magagnoli, C., Marchini, A., Domenighini, M., *et al.* (1995). Construction of non toxic derivatives of cholera toxin and characterization of the immunological response against the A subunit. *Infect. Immun.*, **63**, 2356–60.

Gill, D. M . and Woolkalis, M. J. (1991). Cholera toxin-catalyzed [32P]ADP-ribosylation of proteins. *Methods Enzymol.*, **195**, 267–80.

Hase, C. C., Thai, L. S., Boesmanfinkelstein, M., Mar, V. L., Burnette, W. N., Kaslow, H. R., *et al.* (1994). Construction and characterization of recombinant *Vibrio cholerae* strains producing inactive cholera toxin analogs. *Infect. Immun.*, **62**, 3051–7.

Hirst, T. R. and Holmgren, J. (1987). Transient entry of enterotoxin subunits into the periplasm occurs during their secretion from *Vibrio cholerae*. *J. Bacteriol.*, **169**, 1037–45.

Hirst, T.R., Sanchez, J., Kaper, J.B., Hardy, S.J. and Holmgren, J. (1984). Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harboring plasmids that encode heat-labile enterotoxins of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, **81**, 7752–6.

Holmgren, J., Lycke, N. and Czerkinsky, C. (1993). Cholera Toxin and cholera-B subunit as oral mucosal adjuvant and antigen vector systems. *Vaccine*, **11**, 1179–84.

Lai, C., Cancedda, F., and Duffy, L. K. (1981). ADP-ribosyltransferase activity of cholera toxin polypeptide A1 and the effect of limited trypsinolysis. *Biochem. Biophys. Res. Commun.*,**102**, 1021–7.

Lencer, W. I., Constable, C., Moe, S., Jobling, M. G., Webb, H. M., Ruston, S., *et al.* (1995). Targeting of cholera toxin and *Escherichia coli* heat labile toxin polarized epithelia: role of COOH-terminal KDEL. *J. Cell Biol.*, **131(4)**, 951–62.

Levine, M. M., Kaper, J. B., Black, R. E. and Clements, M. L. (1983). New knowledge on pathogenesis of bacterial infections as applied to vaccine development. *Microbiol. Rev.*, **47**, 510–50.
Mekalanos, J. (1988). Production and purification of cholera toxin. *Methods Enzymol.*, **165**, 169–75.
Mekalanos, J. J., Swartz, D. J., Pearson, G. D., Harford, N., Groyne, F. and de Wilde, M. (1983). Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. *Nature*, **306**, 551–7.
Merritt, E. A., Sarfaty, S., Vandenakker, F., Lhoir, C., Martial, J. A., and Hol, W. G. J. (1994). Crystal structure of cholera toxin B-pentamer bound to receptor G(M1) pentasaccharide. *Protein Sci.*, **3**, 166–75.
Miller, M. and Mekalanos, J. J. (1984). Synthesis of cholera toxin is positively regulated at the transcriptional level by toxR. *Proc. Natl. Acad. Sci. USA*, **81**, 3471–5.
Moss, J. and Vaughan, M. (1991). Activation of cholera toxin and *Escherichia coli* heat-labile enterotoxins by ADP-ribosylation factors, a family of 20 kDa guanine nucleotide-binding proteins. *Mol. Microbiol.*, **5**, 2621–7.
Rappuoli, R. and Pizza, M. (1991). Structure and evolutionary aspects of ADP-ribosylating toxins. In *Sourcebook of bacterial protein toxins* (ed. J. Alouf and J. Freer), pp. 1–20, Academic Press, London and San Diego.
Sandkvist, M., Morales, V. and Bagdasarian, M. (1993). A protein required for secretion of cholera toxin through the outer membrane of *Vibrio cholerae*. *Gene*, **123**, 81–6.
Shoham, M., Scherf, T., Anglister, J., Levitt, M., Merritt, E. A., and Hol, W. G. J. (1995). Structural diversity in a conserved cholera toxin epitope involved in ganglioside binding. *Protein Sci.*, **4**, 841–8.
Sixma, T. K., Pronk, S. E., Kalk, K. H., Wartna, E. S., van Zanten, B. A., Witholt, B., *et al.* (1991*a*). Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E. coli* [see comments]. *Nature*, **351**, 371–7.
Sixma, T. K., Pronk, S. E., Kalk, K. H., Wartna, E. S., van Zanten, B. A., Witholt, B., *et al.* (1991*b*). Crystal structure of a cholera toxin-related heat-labile enterotoxin from E. coli. *Nature*, **351**, 371–7.
Sixma, T. K., Kalk, K. H., Vanzanten, B. A. M., Dauter, Z., Kingma, J., Witholt, B., *et al.* (1993). Refined Structure of *Escherichia coli* heat-labile enterotoxin, a close relative of cholera toxin. *J. Mol. Biol.*, **230**, 890–918.

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Heat-labile enterotoxins (*Escherichia coli*)

E. coli *heat-labile enterotoxins (LT) are classified into serogroups LT-I and LT-II. Each holotoxin structurally resembles cholera toxin (CT) and consists of one A polypeptide and five identical B polypeptides. The B pentamer binds to cell surface gangliosides and triggers uptake of holotoxin by receptor-mediated endocytosis. After proteolytic cleavage and reduction of polypeptide A, fragment A1 enters the cytoplasm, activates adenylate cyclase by ADP-ribosylation of the regulatory protein $G_s\alpha$, and initiates a cascade of signal transduction pathways that mediate the biologic effects of LT. In the small intestine, LT stimulates secretion of fluid and electrolytes into the lumen, resulting in watery diarrhea.*

The structure, function, and role in disease of the heat-labile enterotoxins (LT) of *Escherichia coli* have been recently reviewed (Hirst 1995; Hol *et al.* 1995; Holmes *et al.* 1995). Enterotoxigenic *E. coli* (ETEC) that cause acute diarrhea in humans and animals produce plasmid-encoded LT-I, heat-stable toxin (ST), or both. ETEC from pigs and humans produce different antigenic variants of LT-I, respectively called LTp-I and LTh-I. LT-II-producing ETEC are isolated from animals and food but rarely from humans, and they have not yet been shown to cause diarrheal disease. Two antigenic variants of LT-II, designated LT-IIa and LT-IIb have been characterized. A comparative analysis of database entries for the amino acid sequences of the A and B polypeptides of the recognized LT-I, LT-II, and CT variants, including correction of known errors in the published sequences, was recently reported (Domenighini *et al.* 1995). Original sources for the nucleotide sequences of genes that encode these toxins are cited in the same publication.

The structures of LTp-I, CT, the B pentamer of CT, and co-crystals of some of these proteins with galactose, lactose or the oligosaccharide from ganglioside GM1 have been reported (Sixma *et al.* 1993; Merritt *et al.* 1994*a*, *b*; Zhang *et al.* 1995*a*, *b*), and the structure of LT-IIb has recently been solved (van den Akker *et al.* 1996). The folds adopted by the homologous polypeptides of the type I and type II enterotoxins are very similar (Fig. 1), despite striking differences in the primary sequences among the B polypeptides. In each of these toxins the five B polypeptides associate to form a planar ring with a central pore that is penetrated by and interacts with the carboxy-terminal A2 domain of polypeptide A. The A1

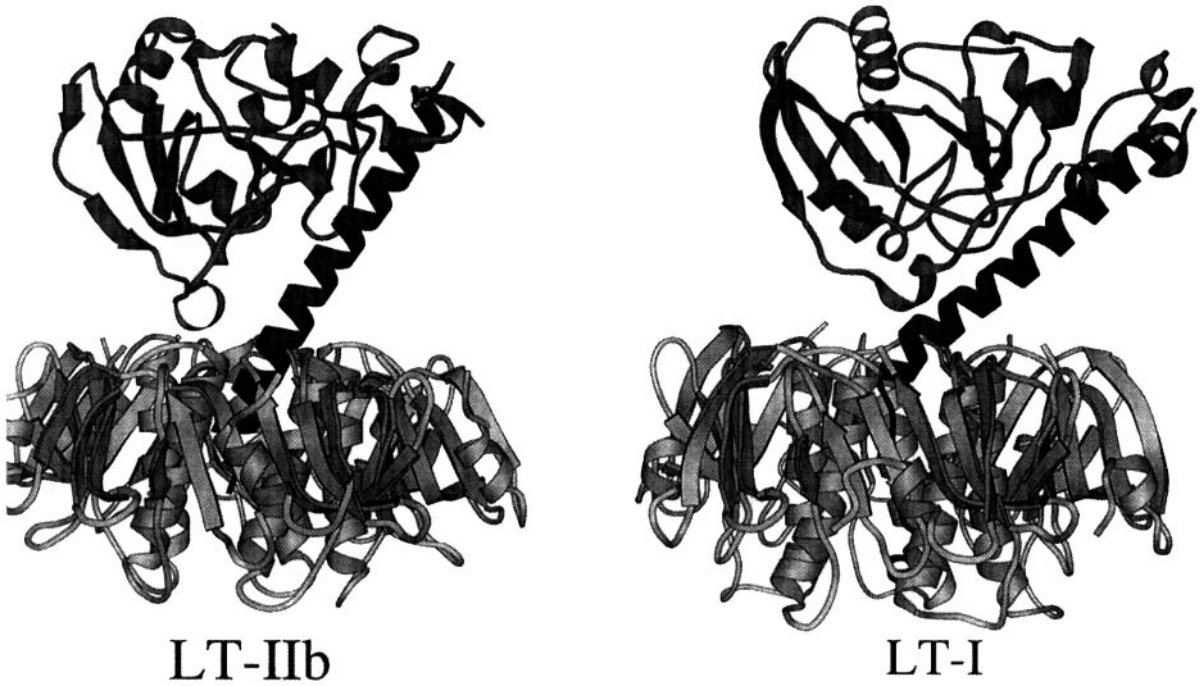

Figure 1. Structure of *Escherichia coli* heat-labile enterotoxins LT-IIb and LT-1. The structure of LT-IIb is remarkably similar to that of LT-I, both in the A subunit and in the B subunits. There is a 24° difference in the relative orientation between the A and B subunits in the two holotoxins, and in LT-IIb the A2 helix is longer and extends further into the pore of the B pentameter than in LT-I. Genetic data indicate that the locations of the ganglioside GD1a-binding sites in LT-IIb are similar to those demonstrated for the ganglioside GM1-binding sites in LT-1. (Figure kindly provided by Focco van den Akker and Wim G. J. Hol.)

domain, which corresponds to an inactive conformation of the ADP ribosyltransferase enzyme, extends outward from the plane of the B pentamer and is located on the opposite side from the ganglioside-binding domains of the B polypeptides. The B pentamers of LTp-I and CT have five binding sites for the GM1 oligosaccharide, and each GM1 oligosaccharide interacts predominantly with residues in a single B polypeptide.

The A and B polypeptides for representative heat-labile enterotoxins from *E. coli* and *V. cholerae* are compared in Table 1. The A polypeptides of type I and type II enterotoxins exhibit the greatest degree of sequence homology within their A1 domains (Pickett *et al.* 1987, 1989; Domenighini *et al.* 1995; Holmes *et al.* 1995). The A polypeptides of the type I enterotoxins CT, LTh-I, and LTp-I are related more closely to one another than to the type II enterotoxins LT-IIa and LT-IIb, and vice versa. Conserved features of the A polypeptides include: NAD:$G_s\alpha$ ADP-ribosyltransferase activity of fragment A1 that is stimulated by the ARF family of GTP-binding proteins; high susceptibility to trypsin of the surface-exposed, disulfide-linked oligopeptide loop between domains A1 and A2 that is selectively cleaved during generation of fragments A1 and A2 ; the ability of the A2 domain to interact with complementary B polypeptides during assembly of holotoxin; and the presence of KDEL

Table 1(a) Comparison of characteristics of A polypeptides of heat-labile enterotoxins

Toxin	Number of amino acid residues	Location of cysteine residues	Carboxyl terminal sequence	ADP ribosylation of		Stimulation by ARF	Percentage amino acid homology with		
				$G_s\alpha$	agmatine		CT	LTh-I	LT-IIa
CT	240	187, 199	KDEL	active	active	yes	–	81.3	50.4
LTh-I	240	187, 199	RNEL	active	active	yes	81.3	–	54.2
LTp-I	240	187, 199	RDEL	active	active	yes	81.3	99.2	54.2
LT-IIa	241	185, 197	KDEL	active	<1%	yes	50.4	54.2	–
LT-IIb	243	185, 197	KDEL	active	<1%	yes	51.7	53.4	75.5

or a related endoplasmic retention sequence at the carboxyl terminus of A2 that affects intracellular trafficking of the enterotoxins in target cells (Lee *et al.* 1991; Holmes *et al.* 1995; Lencer *et al.* 1995). A structural model for conversion of the inactive A1 domain of LTp-I to enzymatically active fragment A1 was recently proposed (van den Akker *et al.* 1995), and the structure of pseudomonas exotoxin A domain III complexed with AMP and nicotinamide (Li *et al.* 1995) has provided new insights into the probable mode of binding of NAD by other ADP ribosylating toxins, including the heat-labile enterotoxins. The B polypeptides of the type I enterotoxins are highly homologous, but they show little primary sequence identity with the B polypeptides of the type II enterotoxins (Domenighini *et al.* 1995; Holmes *et al.* 1995). The B polypeptides of LT-IIa and LT-IIb are homologous, but the extent of identity between them is less than that observed among the B polypeptides of the type I enterotoxins. These differences in amino acid sequence correlate with differences in ganglioside-binding activities among the members of the heat-labile enterotoxin family (Fukuta *et al.* 1988).

Purification and sources

The heat-labile enterotoxins are produced as periplasmic proteins by *E. coli*. Typically they are produced by overexpression of the cloned operons, and the recombinant enterotoxins are purified from lysed cells. CT and LT-I are easily purified in high yield by affinity chromatography on immobilized D-galactose, followed by elution with D-galactose (Uesaka *et al.* 1994). LT-IIa and LT-IIb are purified by combinations of chromatographic methods adapted for the characteristics of each toxin (Guth *et al.* 1986; Holmes *et al.* 1986).

Toxicity

The heat-labile enterotoxins alter the functions of target cells but do not kill them; they are cytotonic rather than cytotoxic. Treatment with trypsin of enterotoxins that have an intact ('un-nicked') A polypeptide increases their toxicity by 10- to 100-fold against cultured target cells that do not activate the enterotoxins via endogenous proteases. The most sensitive tissue culture systems (mouse Y1 adrenal tumor cells or Chinese hamster ovary cells) detect activated heat-labile enterotoxins with minimum detectable doses of approximately 1–25 picograms, and in Y1 adrenal cell culture systems the specific toxicity of LT-II is significantly greater than that of LT-I or CT (Guth *et al.* 1986; Holmes *et al.* 1986). The parenteral lethality of LT-I is assumed to be comparable with that of CT (about 250 μg i.v. for mice), since their activities are similar in several nonlethal assays, but smaller doses of LT-I or CT are lethal for susceptible animals by the enteral route (Gill 1982). A 25 μg oral dose of CT in volunteers caused diarrhea comparable to that of cholera (Levine *et al.* 1983). Unlike CT and LT-I, LT-II does not stimulate secretion in rabbit ileal segment assays, and data concerning lethality of LT-II in animals are not available (Guth *et al.* 1986; Holmes *et al.* 1986).

Uses in cell biology

CT and LT-I are widely used in cell biology as probes for cyclic AMP and G protein-dependent signal transduction pathways (Harnett, 1994). CT and LT-I are potent mucosal immunogens with a variety of immunomodulatory activities including musosal adjuvanticity (Xu-Amano *et al.* 1994). They are studied extensively as probes for the immune system and as tools for vaccine development . CT and LT-I enter target cells by receptor-mediated endocytosis and undergo both retrograde transport into Golgi cisternae and endoplasmic reticulum as well as transcytosis in polarized epithelial cell cultures (Lencer *et al.* 1995). They are under active investigation as probes for endocytotic and transcytotic pathways in eukaryotic cells. Uptake and retrograde transport of CT-B by neurons is widely used in neurobiology for mapping of neural pathways (Datiche *et al.* 1995). The recently characterized LT-II enterotoxins have not yet been used extensively in cell biology. Because they differ from CT and LT-I in ganglioside-binding specificity (Table 1(b)), they may be particularly useful in comparative studies with CT and LT-I to investigate the role of various gangliosides in endocytosis, transcytosis, and signal transduction pathways.

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Table 1(b) Comparison of characteristics of B polypeptides of heat-labile enterotoxins

Toxin	Number of amino acid residues	Location of cysteine residues	Relative binding activity for gangliosides	Percentage amino acid homology with		
				CT	LTh-I	LT-IIa
CT	103	9, 86	GM1 >GD1b	–	81.6	11.7
LTh-I	103	9, 86	GM1 >GD1b >GM2	81.6	–	10.7
LTp-I	103	9, 86	Not determined	80.6	97.1	10.7
LT-IIa	100	10, 81	GD1b >GD1a,GT1b,GQ1b GM1,GD2 >GM2,GM3	11.7	10.7	–
LT-IIb	99	10, 81	GD1a >GT1b >GM3	13.6	13.6	56.4

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References

Datiche, F., Luppi, P. H., and Cattarelli, M. (1995). Serotonergic and non-serotonergic projections from the raphe nuclei to the piriform cortex in the rat: a cholera toxin B subunit (CTb) and 5-HT immunohistochemical study. *Brain Res.*, **671**, 27–37.

Domenighini, M., Pizza, M., Jobling, M. G., Holmes, R. K., and Rappuoli, R. (1995). Identification of errors among database sequence entries and comparison of correct amino acid sequences for the heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae* [letter]. *Mol. Microbiology*, **15**, 1165–7.

Fukuta, S., Magnani, J. L., Twiddy, E. M., Holmes, R. K., and Ginsburg, V. (1988). Comparison of the carbohydrate-binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb. *Infect. Immun.*, **56**, 1748–53.

Gill, D. M. (1982). Bacterial toxins: a table of lethal amounts. *Microbiol. Rev.*, **46**, 86–94.

Guth, B. E., Twiddy, E. M., Trabulsi, L. R., and Holmes, R. K. (1986). Variation in chemical properties and antigenic determinants among type II heat-labile enterotoxins of *Escherichia coli*. *Infect. Immun.*, **54**, 529–36.

Harnett, M. M. (1994). Analysis of G-proteins regulating signal transduction pathways. *Meth. in Mol. Biol.*, **27**, 199–211.

Hirst, T. R. (1995). Biogenesis of cholera toxin and related oligomeric enterotoxins. In *Bacterial toxins and virulence factors in disease* (ed. J. Moss, B. Iglewski, M. Vaughan, and A. T. Tu), pp. 123–84, Marcel Dekker, New York.

Hol, W. G. J., Sixma, T. K., and Merritt, E. A. (1995). Structure and function of *E. coli* heat-labile enterotoxin and cholera toxin B pentamer. In *Bacterial toxins and virulence factors in disease* (ed. J. Moss, B. Iglewski, M. Vaughan, and A. T. Tu), pp. 185–223, Marcel Dekker, New York.

Holmes, R. K., Twiddy, E. M., and Pickett, C. L. (1986). Purification and characterization of type II heat-labile enterotoxin of *Escherichia coli*. *Infect. Immun.*, **53**, 464–73.

Holmes, R. K., Jobling, M. G., and Connell, T. D. (1995). Cholera toxin and related enterotoxins of gram negative bacteria. In *Bacterial toxins and virulence factors in disease* (ed. J. Moss, B. Iglewski, M. Vaughan, and A. T. Tu), pp. 225–55, Marcel Dekker, New York.

Lee, C. M., Chang, P. P., Tsai, S. C., Adamik, R., Price, S. R., Kunz, B. C., *et al.* (1991). Activation of *Escherichia coli heat*-labile enterotoxins by native and recombinant adenosine diphosphate-ribosylation factors, 20-kD guanine nucleotide-binding proteins. *J. Clin. Invest.*, **87**, 1780–6.

Lencer, W. I., Constable, C., Moe, S., Jobling, M. G., Webb, H. M., Ruston, S., *et al.* (1995). Targeting of cholera toxin and *Escherichia coli* heat labile toxin in polarized epithelia: role of COOH-terminal KDEL. *J. Cell. Biol.*, **131**, 951–62.

Levine, M. M., Kaper, J.B , Black, R. E., and Clements, M. L. (1983). New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. *Microbiol. Rev.*, **47**, 510–50.

Li, M., Dyda, F., Benhar, I., Pastan, I., and Davies, D. R. (1995). The crystal structure of *Pseudomonas aeruginosa* exotoxin domain III with nicotinamide and AMP: conformational differences with the intact exotoxin. *Proc. Natl. Acad. Sci. USA*, **92**, 9308–12.

Merritt, E. A., Sarfaty, S., van den Akker, F., L'hoir, C., Martial, J. A. and Hol, W. G. J. (1994*a*). Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein. Sci.*, **3**, 166–75.

Merritt, E. A., Sixma, T. K., Kalk, K. H., van Zanten, B. A. M., and Hol, W. G. J. (1994*b*). Galactose binding site in *E. coli* heat-labile enterotoxin (LT) and cholera toxin (CT). *Mol. Microbiol.*, **13**, 745–53.

Pickett, C. L., Weinstein, D. L., and Holmes, R. K. (1987). Genetics of type IIa heat-labile enterotoxin of *Escherichia coli*: operon fusions, nucleotide sequence, and hybridization studies. *J. Bacteriol.*, **169**, 5180–7.

Pickett, C. L., Twiddy, E. M., Coker, C., and Holmes, R. K. (1989). Cloning, nucleotide sequence, and hybridization studies of the type IIb heat-labile enterotoxin gene of *Escherichia coli*. *J. Bacteriol.*, **171**, 4945–52.

Sixma, T. K., Kalk, K. H., van Zanten, B. A., Dauter, Z., Kingma, J., Witholt, B. *et al.* (1993). Refined structure of *Escherichia coli* heat-labile enterotoxin, a close relative of cholera toxin. *J. Mol. Biol.*, **230**, 890–918.

Uesaka, Y., Otsuka, Y., Lin, Z., Yamasaki, S., Yamaoka, J., Kurazono, H., and Takeda, Y. (1994). Simple method of purification of *Escherichia coli* heat-labile enterotoxin and cholera toxin using immobilized galactose. *Microb. Pathog.*, **16**, 71–6.

van den Akker, F., Merritt, E.A., Pizza, M., Domenighini, M., Rappuoli, R. and Hol, W.G. (1995). The Arg7Lys mutant of heat-labile enterotoxin exhibits great flexibility of active site loop 47-56 of the A subunit. *Biochemistry*, **34**, 10996–1004.

van den Akker, F., Sarfaty, S., Twiddy, E. M., Connell, T. D., Holmes, R. K., and Hol, W. G. J. (1996). Crystal structure of a new heat-labile enterotoxin, LT-IIb. *Structure*, **4**, 665–78.

Xu-Amano, J., Jackson, R. J., Fujihashi, K., Kiyono, H., Staats, H. F., and McGhee, J. R. (1994). Helper Th1 and Th2 cell responses following mucosal or systemic immunization with cholera toxin. *Vaccine*, **12**, 903–11.

Zhang, R. G., Scott, D. L., Westbrook, M. L., Nance, S., Spangler, B. D., and Shipley, G. G. W., EM. (1995*a*). The three-dimensional crystal structure of cholera toxin. *J. Mol. Biol.*, **251**, 563–73.

Zhang, R. G., Westbrook, M. L., Westbrook, E. M., Scott, D. L., Otwinowski, Z., Maulik, P. R., *et al.* (1995*b*). The 2.4 A crystal structure of cholera toxin B subunit pentamer: choleragenoid. *J. Mol. Biol.*, **251**, 550–62.

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Pertussis toxin (*Bordetella pertussis*)

Pertussis toxin (PT), a bacterial protein toxin secreted in the culture supernatant by Bordetella pertussis, *intoxicates eukaryotic cells by ADP-ribosylating the alpha subunit of G proteins, thus interfering with receptor mediated signal transduction. PT which is a major virulence factor of* B. pertussis *and the main component of anti-pertussis vaccines, has been instrumental in the discovery of G proteins and the characterization of signal transduction in eukaryotic cells.*

PT (Sekura *et al.* 1985) is a protein of 105 000 daltons composed of five noncovalently linked subunits named S1 through S5, and organized into two functional domains called A and B (Tamura *et al.* 1982). The A domain, which is composed of the S1 subunit, is an enzyme that intoxicates eukaryotic cells by ADP-ribosylating their GTP-binding proteins (Rappuoli and Pizza 1991). The enzyme binds NAD and transfers the ADP-ribose group to a cysteine residue present in an ...XCGLX motif, located at the carboxy-terminal region of the alpha subunit of many G proteins such as G_i, G_o, G_t, G_{gust}, and others. G_s and G_{olf}, which in this position have a tyrosine instead of the cysteine, are not substrates for PT (Domenighini *et al.* 1995).

The B domain is a nontoxic oligomer formed by subunits S2, S3, S4, and S5 which are present in a 1:1:2:1 ratio. This domain binds the toxin receptor on the surface of eukaryotic cells and facilitates the translocation of the S1 subunit across the cellular membrane, so that it can reach the target proteins. The B oligomer binds to glycoproteins having a branched mannose core with *N*-acetyl glucosamine attached, such as fetuin (Sekura and Zhang 1985). The crystal structure of the protein is known (Stein *et al.* 1994). The genes coding for the five subunits of PT are located in 3.2 kilobases of DNA, and are organized in a bacterial operon, in the following order: S1, S2, S4, S5, S3. Each gene contains a sequence coding for a signal peptide (Locht and Keith 1986; Nicosia *et al.* 1986). The five subunits are cotranslationally secreted into the bacterial periplasm where the protein is assembled into the holotoxin. Secretion of the toxin occurs only after assembly of the holotoxin, or of the B domain (Pizza *et al.* 1990), and requires the product of the nine-gene *ptl* operon, which is located downstream from the PT operon and is regulated by the same promoter (Covacci and Rappuoli 1993; Weiss *et al.* 1993).

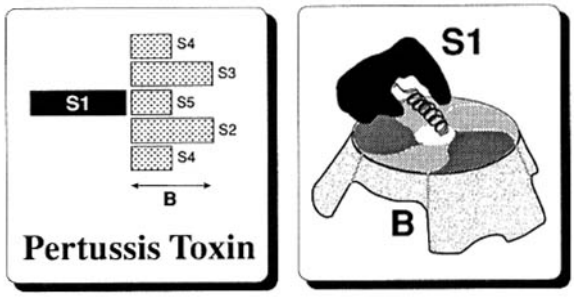

Figure 1. A schematic representation of the subunit composition (left panel), and of the 3D structure (right panel) of pertussis toxin.

Mutants

Many mutants containing single or multiple amino acid substitutions in the A or B subunits have been constructed by site-directed mutagenesis of the PT gene. The most popular one is PT-9K/129G, a nontoxic mutant, where Arg9 and Glu129 have been replaced by Lys and Gly respectively (Pizza *et al.* 1989). The mutation in these two amino acids which are located in the catalytic site make the S1 subunit enzymatically inactive and the mutant PT completely nontoxic. The nontoxic mutant is being used for vaccination against pertussis and therefore is produced in gram quantities (Rappuoli *et al.* 1995). Although not commercially available, it can be obtained from the authors of this entry.

Purification and sources

PT can be purified from the culture supernatant of *B. pertussis* by a two step procedure involving affi-gel blue chromatography, followed by affinity chromatography on a fetuin-containing column (Sekura *et al.* 1983). PT is also commercially available available from several companies, including Sigma Chemical Company, St. Louis, Mo., USA.

Toxicity

PT is lethal at 0.5 μg/mouse. However, PT induces a number of toxic effects at a very low concentration. It renders mice susceptible to death following histamine challenge at 0.5 ng/mouse, potentiates anaphylaxis at 9.5 ng/mouse, enhances insulin secretion at 2 ng/mouse, induces lymphocytosis at 8 ng/mouse, it enhances the IgE production at 0.1 ng/mouse (Munoz 1985), and it changes the permeability of intestinal tissue at a few nanograms per rat. Some of these effects can be measured in animals

a long time after the toxin administration. For instance, the change in intestinal permeability is still present eight months after toxin treatment (Kosecka *et al.* 1994).

Use in cell biology

PT was instrumental in the discovery of G_i protein and has been extensively used for the dissection of receptor mediated signal transduction (Katada and Ui 1982; Bokoch *et al.* 1983). Generally, receptors have been divided into 'pertussis toxin sensitive' and 'pertussis toxin insensitive', depending on whether PT interfered or not with signal transduction. The nontoxic mutants are the best controls to understand whether the observed effects are due to the modification of the G proteins mediated by the enzymatic activity of the toxin, or just to the binding of the toxin to its receptor.

References

Bokoch, G. M., Katada, T., Northup, J. K., Hewlett, E. L., and Gilman, A. G. (1983). Identification of the predominant substrate for ADP-ribosylation by islet activating protein. *J. Biol. Chem.*, **258**, 2072–5.

Covacci, A. and Rappuoli, R. (1993). Pertussis toxin export requires accessory genes located downstream from the pertussis toxin operon. *Mol. Microbiol.*, **8**, 429–34.

Domenighini, M., Pizza, M., and Rappuoli, R. (1995). Bacterial ADP-ribosyltransferases. In *Bacterial toxins and virulence factors in disease* (ed. J. Moss, B. Iglewski, M. Vaughan, and A. T. Tu), pp. 59–80, Marcel Dekker, New York.

Katada, T. and Ui, M. (1982). ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. *J. Biol. Chem.*, **257**, 7210–16.

Kosecka, U., Marshall, J. S., Crowe, S. E., Bienenstock, J., and Perdue, M. H. (1994). Pertussis toxin stimulates hypersensitivity and enhances nerve-mediated antigen uptake in rat intestine. *Am. J. Physiol-Gastrointest. L.*, **30**, G745–53.

Locht, C. and Keith, J. M. (1986). Pertussis toxin gene: nucleotide sequence and genetic organization. *Science*, **232**, 1258–64.

Munoz, J. J. (1985). Biological activity of pertussigen (pertussis toxin). In *pertussis toxin* (ed. R. D. Sekura, J. Moss, and M. Vaughan), pp. 1–18, Academic Press, Orlando, Florida.

Nicosia, A., Perugini, M., Franzini, C., Casagli, M. C., Borri, M. G., Antoni, G., *et al.* (1986). Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. *Proc. Natl. Acad. Sci. USA*, **83**, 4631–5.

Pizza, M., Covacci, A., Bartoloni, A., Perugini, M., Nencioni, L., de Magistris, M. T., *et al.* (1989). Mutants of pertussis toxin suitable for vaccine development. *Science*, **246**, 497–500.

Pizza, M., Covacci, A., Bugnoli, M., Manetti, R., and Rappuoli, R. (1990). The S1 subunit is important for pertussis toxin secretion. *J. Biol. Chem.*, **265**, 17759–63.

Rappuoli, R. and Pizza, M. (1991). Structure and evolutionary aspects of ADP-ribosylating toxins. In *Sourcebook of bacterial protein toxins* (ed. J. Alouf and J. Freer), pp. 1–20, Academic Press, London and San Diego.

Rappuoli, R., Douce, G., Dougan, G., and Pizza, M. (1995). Genetic detoxification of bacterial toxins: a new approach to vaccine development. *Int. Arch. Allergy Immunol.*, **108**, 327–33.

Sekura, R. D. and Zhang, Y. (1985). Pertussis toxin: Structural elements involved in the interaction with cells. In *Pertussis toxin* (ed. R. D. Sekura, J. Moss, and M. Vaughan), pp. 45–64, Academic Press, Orlando, Florida.

Sekura, R. D., Fish, F., Manclark, C. R., Meade, B., and Zhang, Y. L. (1983). Pertussis toxin. Affinity purification of a new ADP-ribosyltransferase. *J. Biol. Chem.*, **258**, 14647–51.

Sekura, R., Moss, J., and Vaughan, M. (1985). *Pertussis toxin*, Academic Press, Orlando, Florida; pp. V–255.

Stein, P. E., Boodhoo, A., Armstrong, G. D., Cockle, S. A., Klein, M. H., and Read, R. J. (1994). The crystal structure of pertussis toxin. *Structure*, **2**, 45–57.

Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., *et al.* (1982). Subunit structure of the islet-activating protein, pertussis toxin, in conformity with the A-B model. *Biochemistry*, **21**, 5516–22.

Weiss, A. A., Johnson, F. D., and Burns, D. L. (1993). Molecular characterization of an operon required for pertussis toxin secretion. *Proc. Natl. Acad. Sci. USA*, **90**, 2970–4.

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Exoenzyme C3 (*Clostridium botulinum*)

Exoenzyme C3 (C3) is an M_r *23.6 kDa protein produced by strains C and D of* C. botulinum. *C3 is an ADP-ribosyltransferase which inactivates selectively the small GTP-binding proteins Rho (A, B, and C) by modification of its asparagine 41. C3 is not a toxin and therefore cannot enter into cells. Upon introduction into cells, for instance by microinjection, C3 inactivation of Rho is followed by the collapse of actin stress fibres. C3 is used in cell biology to inactivate Rho* 'in vitro' *and* 'in vivo'.

ADP-ribosylating toxins have proved to be valuable tools for studying their target proteins. The targets generally suffer important changes in function upon modification, and they become labelled when radioactive NAD is used. In this respect C3 has proved to be extremely useful in understanding the physiological role of the small GTP-binding protein Rho (Narumiya and Morii 1993; Hall 1994).

C3 is an M_r 23.6 kDa ADP-ribosyl transferase which modifies selectively the small GTP-binding proteins Rho (Chardin *et al.* 1989; Aktories *et al.* 1989) on Asparagine 41 (Sekine *et al.* 1989), a residue in close proximity of the Rho effector domain. C3 is secreted by the C and D types of *C. botulinum* (Aktories *et al.* 1987; Rubin *et al.* 1988). Several C3 related exoenzymes have been isolated from other bacteria than *C. botulinum*, such as certain strains of *Staphylococcus aureus* (EDIN) (Inoue *et al.* 1991), *Clostridium limosum* (Just *et al.* 1992a), *Bacillus cereus* (Just *et al.* 1992*b*) and *Legionella pneumophila* (Belyi *et al.* 1991). However, C3 from *C. botulinum* is the most utilized Rho specific ADP-ribosyltransferase to inactivate and label this GTP-binding protein *in vitro* and *in vivo*. Other members of the Rho family of small GTP-binding proteins such as Rac and Cdc42 poor substrates for C3 (100-folds less for Rac, 400-folds less for Cdc42). C3 has apparently no role as a bacterial virulence factor and an extremely low toxicity upon injection into mice (about 400 $\mu g\ kg^{-1}$ ip).

C3 is composed of 211 amino acids (the preprotein has an M_r of 7.8 kDa and 251 amino acids) and is strongly positively charged (p*I* = 10.4). C3 is a very stable protein and renatures easily after heat- or detergent-denaturation. The C3 gene is borne on a mobile element by *C. botulinum* C and D phage DNAs, which also carry the neurotoxin C1 and D genes (Popoff *et al.* 1990; 1991; Hauser *et al.* 1993) (EMBL Data Library access numbers for C3 genes: X59039 for *C. botulinum* C and X59040 for *C. botulinum* D).

Purification and sources

The classical method to obtain C3 is to cultivate anaerobically, in TGY medium, *C. botulinum* D strain 1873 or C strain ATCC 17784 and to follow for purification the procedure described by Rubin *et al.* (1988) or Aktories *et al.* (1987). These methods have been made obsolete by the fact that recombinant C3 in *E. coli* can be obtained easily, in large quantities, and without C2 toxin contamination. Two main methods have been proposed to produce recombinant C3:

1. From the C3 gene cloned in an expression vector (Popoff *et al.* 1991), followed by a classical purification of C3 (Aktories *et al.* 1987; Rubin *et al.* 1988).
2. From a glutathione-*S*-transferase (GST) C3 fusion (Nobes and Hall 1995), and from a poly-Histidine tag C3 fusion (Lemichez *et al.* manuscript in preparation). For GST-C3 or His-C3 fusions, purification of recombinant C3 is obtained easily by affinity chromatography.

C3 purified from *C. botulinum* can be purchased from LIST Biological Laboratories, 501-B Vandell Way, Cambell, CA, 95008-6967, USA (fax: (408) 866-6364) or from BIOMOL Research Laboratories (ref catalog G-130), 5166 Campus Drive, Plymouth Meeting, PA, 19462, USA (fax: (610) 941 9252). Rabbit polyclonal antibodies against C3 are obtained easily upon immunization with a classical procedure. These antibodies can be used to immunoblot or neutralize C3.

Use in cell biology

C3 is used to selectively inactivate the small GTP-binding protein Rho. Although C3 can be used with [32P]-NAD to label *in vitro* or inactivate Rho proteins from cell lysates or in reconstituted systems (at a concentration of 1ng μl^{-1} in 20 mM HEPES buffer pH 8.00, containing 5 μM [32P]-NAD; presence of high salt concentrations and EDTA decrease the C3 enzymatic activity), C3 does not penetrate into cells. Several techniques have thus been proposed to inactivate Rho by C3 in cultured cells. The first one is to incubate cells with high concentrations of C3 (3–40 μg/ml depending on cell types). This method, however, remains expensive in term of C3 amounts. Microinjection of C3 is a classical method which is performed by injecting C3 into the cell, usually at a concentration of 100 $\mu g\ ml^{-1}$ in the injecting pipette (Nobes and Hall 1995). A third method is to use a recombinant chimeric protein made by the fusion of the C3 gene with the B portion of diphtheria toxin (DT) DNA. This chimeric toxin named C3B can be used on cells sensitive to DT

(mouse and rat cells are resistant to DT) at concentrations of about 10^{-8} M (Aullo *et al.* 1993). A recently published method is to transfect cells with a eukaryotic vector, expressing the C3 gene (N-terminally C-myc epitope tagged C3 transferase) (Hill *et al.* 1995). Introduction of C3 into cells is followed more or less rapidly (a few minutes by microinjection, hours by the chimeric C3B toxin) by morphological changes due to inactivation of the Rho GTP-binding proteins. The first sign of intoxication is a rounding of the cell body with formation of arborescent extensions. Staining of the F-actin cell structures with FITC-phalloidin, shows that C3 induces a total disruption of actin stress fibres indicating that Rho is a major player in the regulation of focal adhesion points (Chardin *et al.* 1988; Ridley and Hall 1992). Recently, Rho has been shown to regulate a phosphatidyl inositol 4-phosphate 5-kinase (Chong *et al.* 1994) producing 4,5-PIP2 (a phosphorylated inositol lipid required in the activation of proteins such as the actin-binding protein profilin or, involved in the intracellular traffic such as phospholipase D). Rho also plays a role in the signalling cascade triggered by growth factors such as lysophosphatidic acid (LPA) leading to transcriptional activation of SRE by SRF (Hill *et al.* 1995). In all these cases, C3 has been used to demonstrate the role of Rho in these reactions.

References

Aktories, K., Weller, U., and Chaatwal, G. S. (1987). *Clostridium botulinum* type C produces a novel ADP-ribosyl transferase distinct from botulinum C2 toxin. *FEBS Lett.*, **212**, 109–13.

Aktories, K., Braun, U., Rösener, S., Just, I. and Hall, A. (1989). The *rho* gene product expressed in *E. coli* is a substrate of botulinum ADP-ribosyl transferase C3. Biochem. *Biophys. Res.Comm.*, **158**, 209–13.

Aullo, P., Giry, M., Olsnes, S., Popoff, M. R., Kocks, C., and Boquet, P. (1993). A chimeric toxin to study the role of the p21 GTP-binding protein Rho in the control of actin microfilament assembly. *EMBO J.*, **12**, 921–31.

Belyi, Y. F., Tatakovskii, I. S., Vertief, Y. V., and Prosorovskii, S. V. (1991). Partial purification and characterization of an ADPribosyltransferase produced by *Legionella pneumophila*. *Biomed. Sci.*, **2**, 169–74.

Chardin, P., Boquet, P., Madaule, P., Popoff, M. R., Rubin, E. J., and Gill, D. M. (1989). The mammalian G-protein Rho C is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO J.*, **8**, 1087–92.

Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994). The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell*, **79**, 507–513.

Hall, A. (1994). GTPases and the actin cytoskeleton. *Ann. Rev. Cell Biol.*, **10**, 31–54.

Hauser, D., Gibert, M., Eklund, M. W., Boquet, P., and Popoff, M. R. (1993). Comparative analysis of C3 and botulinal neurotoxin genes and their environment in *Clostridium botulinum* types C and D. *J. Bacteriol.*, **175**, 7260–8.

Hill, C. S., Wynne, J. and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and Cdc42Hs regulate transcriptional activation by SRF. *Cell*, **81**, 1159–70.

Inoue, S., Sugai, M., Muruooka, Y., Paik, S. Y., Hong, Y. M. Ohgai, H., *et al.* (1991). Molecular cloning and sequencing of the epidermal cell differentiation inhibitor gene from *Staphylocccus aureus. Biochem. Biophys. Res. Comm.*, **174**, 459–64.

Just, I., Mohrt, C., Schallehn, G., Menard, L., Didsbury, J. R., Vandekerckove, J., *et al.* (1992a). Purification and characterization of an ADP-ribosyltransferase produced by *Clostridium limosum. J. Biol. Chem.*, **267**, 10274–80.

Just, I., Schallenhn, G. and Aktories, K. (1992*b*). ADP-ribosylation of small GTP-binding proteins by *Bacillus cereus. Biochem. Biophys. Res. Comm.*, **183**, 931–6.

Narumiya, S. and Morii, N. (1993). *rho* gene products, botulinum C3 exoenzyme and cell adhesion. *Cellular Signalling*, **5**, 9–19.

Nobes, C. D. and Hall, A. (1995). Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*, **81**, 53–62.

Popoff, M. R., Boquet, P., Gill, D. M., and Eklund, M. W. (1990). DNA sequence of exoenzyme C3, an ADP-ribosyltransferase encoded by *Clostridium botulinum* C and D phages. *Nucleic Acid Res.*, **18**, 1291.

Popoff, M. R., Hauser, D., Boquet, P., Eklund, M. W., and Gill, D. M. (1991). Characterization of the C3 gene of *Clostridium botulinum* types C and D and its expression in *E. coli. Infect. Immun.*, **59**, 3673–9.

Ridley, A. J. and Hall, A. (1992). The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*, **70**, 389–99.

Rubin, E. J., Gill, D. M., Boquet, P., and Popoff, M. R. (1988). Functional modification of a 21 kiloDalton G-protein when ADP-ribosylated by exoenzyme C3 from *Clostridium botulinum. Mol. Cell Biol.*, **8**, 418–26.

Sekine, A., Fujiwara, M., and Narumiya, S. (1989). Asparagine residue in the *rho* gene product is the modification site for botulinum ADP-ribosyl transferase. *J. Biol. Chem.*, **264**, 8602–5.

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Adenylate cyclase toxin (*Bordetella* sp.)

Adenylate cyclase toxin is a 177.7 kDa RTX protein secreted by pathogenic strains of Bordetella. *Upon post-translational fatty-acylation of an internal lysine, the toxin gains the capacity to insert into the plasma membrane of a wide variety of eukaryotic cells, to deliver its amino-terminal adenylate cyclase (AC) domain into their cytosol and to form small cation selective membrane channels. The AC domain binds intracellular calmodulin and converts ATP to cAMP, causing steep increase of intracellular cAMP. This results in disruption of cellular signalling and microbicidal capacities of immune effector cells.*

Adenylate cyclase toxin (ACT or CyaA) is a key virulence factor produced by the human whooping cough agents, *Bordetella pertussis* and *parapertussis* and by the related animal pathogens *B. bronchiseptica* and *B. avium* (Weiss and Hewlett 1986; Hanski and Coote 1991; Mock and Ullmann 1993). The toxin is essential in the early stages of bacterial colonization of the respiratory tract (Goodwin and Weiss 1990) and can induce apoptosis of lung alveolar macrophages (Khelef *et al.* 1993). ACT is produced as a single 177.7 kDa polypeptide chain protoxin of 1706 residues (Glaser *et al.* 1988) (EMBL accession number: Y00545), which is activated by a post-translational palmitoylation of the internal lysine residue 983 (Hackett *et al.* 1994), requiring an accessory protein, CyaC (Barry *et al.* 1991). As shown in Fig. 1, the toxin is a bifunctional protein consisting of a cell-invasive and calmodulin-dependent adenylate cyclase (AC) catalytic domain (400 amino-terminal residues), fused to a pore-forming hemolysin consisting of 1306 residues. The AC enzyme penetrates into target cells, where it binds calmodulin and catalyses high-level synthesis of the key signal molecule, cAMP, thereby disrupting cellular functions (Confer and Eaton 1982). The hemolysin portion of ACT accounts for membrane insertion of ACT and AC-delivery into cells and can itself form small cation selective membrane channels permeable for calcium (Benz *et al.* 1994; Iwaki *et al.* 1995). These cause colloido-osmotic cell-lysis and account for the weak hemolytic activity of ACT that is independent of the presence and activity of the AC domain (Sakamoto *et al.* 1992) The hemolysin is a typical RTX (**R**epeat in To**X**in) protein consisting of a hydrophobic pore-forming domain, fatty-acylation domain, 38 calcium-binding glycine and aspartate-rich nonapeptide repeat domain, and a carboxy-terminal secretion signal (Welch 1991; Sebo and Ladant 1993).

The toxin contains about 40 calcium binding sites of various affinities located in the repeats and its activity is calcium dependent (Hanski and Farfel 1985; Hewlett *et al.* 1991; Rose *et al.* 1995). The target cell(s) for ACT *in vivo* was not defined and is popularly believed to be alveolar macrophages and leukocytes. There is indirect evidence that delivery of AC domain into cells can be accomplished by toxin monomers (Fig. 2), while oligomerization of ACT is involved in formation of membrane channels (Sakamoto *et al.* 1992; Betsou *et al.* 1993; Szabo *et al.* 1994).

Purification and sources

ACT is extracted from the outer membrane of exponentially growing Bordetella organisms with buffered 4 M urea. Alternatively, recombinant ACT is extracted with buffered 8 M urea from cell debris of *Escherichia coli* strains expressing *cyaA* and *cyaC* genes (Sebo *et al.* 1991; Betsou *et al.* 1993). Nearly homogeneous toxin preparations are obtained by various combinations of sucrose density gradient centrifugation and chromatographies on

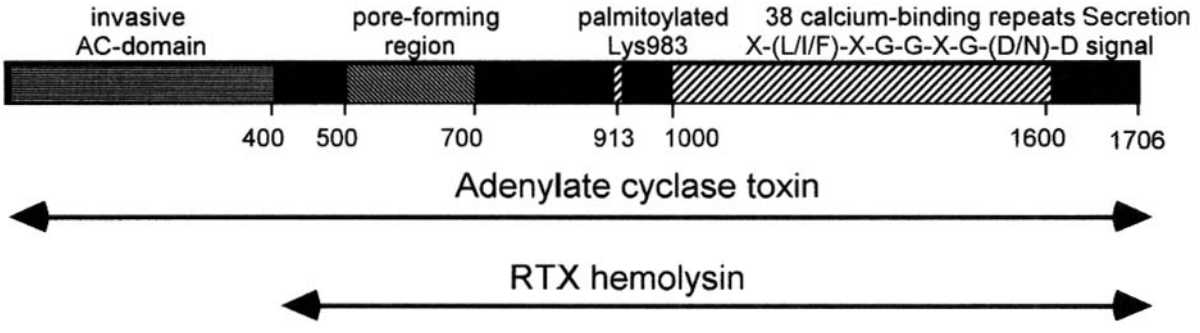

Figure 1. Schematic representation of ACT structure.

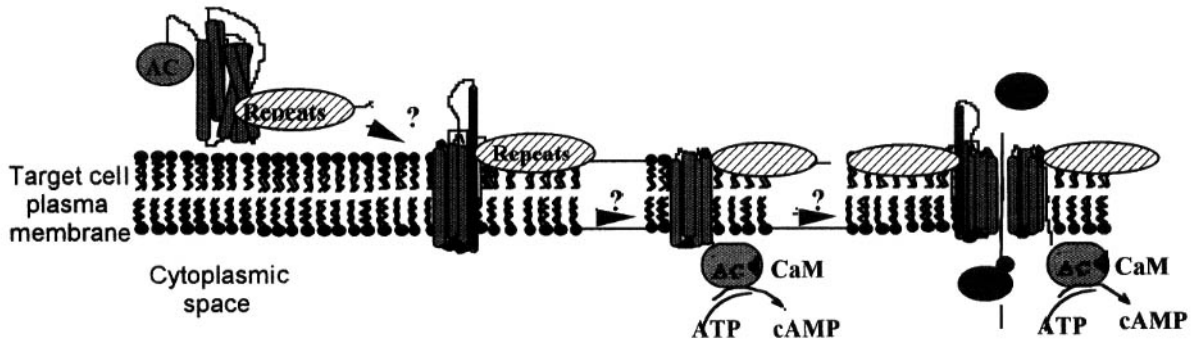

Figure 2. Schematic representation of ACT action.

hydrophobic, ion-exchange, and Calmodulin–Agarose affinity resins (Hewlett *et al.* 1989; Rogel *et al.* 1989; Gentile *et al.* 1990; Sakamoto *et al.* 1992). Purified toxin is diluted and stored frozen in 8 M urea and directly applied into urea free assay buffers containing target cells and millimolar calcium. Upon dialysis, the purified toxin rapidly loses activity. Intact toxin migrates abberantly on SDS-PAGE as a 220 kDa band. ACT cannot yet be purchased from commercial suppliers and submilligram amounts of toxin purified from *B. pertussis* can be provided by Drs E. Hanski (Hebrew University–Hadassah Medical School, Jerusalem), E. L. Hewlett (University of Virginia School of Medicine, Charlottesville), and J. G. Coote (Glasgow University, Scotland). Larger amounts of recombinant toxin can be obtained from Drs Agnes Ullmann (Institut Pasteur, Paris) and P. Sebo (Institute of Microbiology, Czech Acad. Sci., Prague).

Toxicity

Toxicity has not been evaluated. Mice tolerate intraperitoneal injections of 2.5 mg/kg without observable difficulties (C. Leclerc, personal communication). No problems to operators handling hundreds of milligrams of active toxin were observed. In addition, vaccination with active ACT protects mice against colonization by *B. pertussis* (Betsou *et al.* 1993).

Use in cell biology

Translocation of ACT into cells is strictly calcium dependent, with half maximal activity at 0.6 mM calcium concentration (Hanski and Farfel 1985; Hewlett *et al.* 1991). The toxin has the capacity to insert into the plasma membrane of a broad spectrum of eukaryotic cell types, including immune effector cells, and to deliver its AC domain into the cell interior by an unknown mechanism, directly across the plasma membrane and without the need for endocytosis (Gordon *et al.* 1989). ACT action does not appear to involve interaction with any specific proteinaceous receptor on the cell surface, and membrane insertion of ACT is apparently preceded by a rather unspecific adsorption to cell surface (Iwaki *et al.* 1995). Translocation of AC across membrane is stimulated by negative transmembrane potential (Otero *et al.* 1995). Inside nucleated cells, the AC enzyme is rapidly inactivated by an ATP-dependent mechanism (Gilboa-Ron *et al.* 1989). Because of its cell-invasive activity, recombinant ACT with inserted heterologous epitopes was used for delivery of viral epitopes into MHC class I, restricted antigen presentation pathway and induction of specific $CD8^+$ cytotoxic T lymphocytes in mice (Fayolle *et al.* 1996; Sebo *et al.* 1995).

The capacity of ACT to cause steep and transient increase of intracellular cAMP was used for reversibly maintaining meiotic arrest of rat oocytes (Aberdam *et al.* 1987).

References

Aberdam, E., Hanski, E., and Dekel, N. (1987). Maintenance of meiotic arrest in isolated rat oocytes by the invasive adenylate cyclase of *Bordetella pertussis*. *Biol. Reprod.*, **36**, 530–5.

Barry, E. M., Weiss, A. A., Ehrmann, I. E., Gray, M. C., Hewlett, E. L., and Goodwin, M. S. (1991). *Bordetella pertussis* adenylate cyclase toxin and hemolytic activities require a second gene, *cyaC*, for activation. *J. Bacteriol.*, **173**, 720–6.

Benz, R., Maier, E., Ladant, D., Ullmann, A., and Sebo, P. (1994). Adenylate cyclase toxin of *Bordetella pertussis* evidence for the formation of small ion-permeable channels and comparison with HlyA of *Escherichia coli*. *J. Biol. Chem.*, **269**, 27231–9.

Betsou, F., Sebo, P., and Guiso, N. (1993). CyaC-mediated activation is important not only for toxic but also for protective activities of *Bordetella pertussis* adenylate cyclase-hemolysin. *Infect. Immun.*, **61**, 3583–9.

Confer, D. L. and Eaton, J. W. (1982). Phagocyte impotence caused by an invasive bacterial adenylate cyclase. *Science*, **217**, 948–50.

Fayolle, C., Sebo, P., Ladant, D., Ullmann, A., and Leclerc, C. (1996). *In vivo* induction of CTL responses by recombinant adenylate cyclase of *Bordetella pertussis* carrying viral CD8+ T-cell epitope. *J. Immunol.*, **156**, 4697–706.

Gentile, F., Knipling, L. G., Sackett, D. L., and Wolff, J. (1990). Invasive adenylyl cyclase of *Bordetella pertussis*. *J. Biol. Chem.*, **265**, 10686–92.

Gilboa-Ron, A., Rogel, A., and Hanski, E. (1989). *Bordetella pertussis* adenylate cyclase inactivation by the host cell. *Biochem. J.*, **262**, 25–31.

Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullmann, A., and Danchin, A. (1988). The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression in *Escherichia coli*. *Mol. Microbiol.*, **2**, 19–30.

Goodwin, M. S. and Weiss, A. A. (1990). Adenylate cyclase toxin is critical for colonization and pertussis toxin is critical for lethal infection by *Bordetella pertussis* in infant mice. *Infect. Immun.*, **58**, 3445–7.

Gordon, V. M., Young Jr, W. W., Lechler, S. M., Gray, M. C., Leppla, S. H., and Hewlett, E. L. (1989). Adenylate cyclase toxins from *Bacillus anthracis* and *Bordetella pertussis*. Different processes for interaction with and entry into target cells. *J. Biol. Chem.*, **264**, 14792–6.

Hackett, M., Guo, L., Shabanowitz, J., Hunt, D. F., and Hewlett, E. L. (1994). Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis*. *Science*, **266**, 433–5.

Hanski, E. and Coote, J. G. (1991) *Bordetella pertussis* adenylate cyclase toxin. In *Sourcebook of bacterial toxins* (ed. J. E. Alouf and J. H. Freer), pp. 349–366, Academic Press, New York.

Hanski, E. and Farfel, Z. (1985). *Bordetella pertussis* invasive adenylate cyclase: partial resolution and properties of its cellular penetration. *J. Biol. Chem.*, **260**, 5526–32.

Hewlett, E. L., Gordon, V. M., McCaffery, J. D., Sutherland, W. M., and Gray, M. C. (1989). Adenylate cyclase toxin from *Bordetella pertussis*. Identification and purification of the holotoxin molecule. *J. Biol. Chem.*, **264**, 19379–84.

Hewlett, E. L., Gray, L., Allietta, M., Ehrmann, I. E., Gordon, V. M., and Gray, M. C. (1991). Adenylate cyclase toxin from *Bordetella pertussis*. Conformational change associated with toxin activity. *J. Biol. Chem.*, **266**, 17503–8.

Iwaki, M., Ullmann, A., and Sebo, P. (1995). Identification by *in vitro* complementation of regions required for cell-invasive activity of *Bordetella pertussis* adenylate cyclase toxin. *Mol. Microbiol.*, **17**, 1015–24.

Khelef, N., Zychlinsky, A., and Guiso, N. (1993). *Bordetella pertussis* induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. *Infect. Immun.*, **61**, 4064–70.

Mock, M. and Ullmann, A. (1993) Calmodulin-activated bacterial adenylate cyclases as virulence factors. *Trends Microbiol.*, **1**, 187–92.

Otero, A. S., Yi, X., Gray, M. C., Szabo, G., and Hewlett, E. L. (1995). Membrane depolarization prevents cell invasion by *Bordetella pertussis* adenylate cyclase toxin. *J. Biol. Chem.*, **270**, 9695–7.

Rogel, A., Schultz, J., Brownlie, R. M., Coote, J. G., Parton, R., and Hanski, E. (1989). *Bordetella pertussis* adenylate cyclase: purification and characterization of the toxic form of the enzyme. *EMBO J.*, **8**, 2755–60.

Rose, T., Sebo, P., Bellalou, J., and Ladant, D. (1995). Interaction of calcium with *Bordetella pertussis* adenylate cyclase toxin: Characterization of multiple calcium-binding sites and calcium induced conformational changes. *J. Biol. Chem.*, **270**, 26370–6.

Sakamoto, H., Bellalou, J., Sebo, P. and Ladant, D. (1992). *Bordetella pertussis* adenylate cyclase toxin: structural and functional independence of the catalytic and hemolytic activities. *J. Biol. Chem.*, **267**, 13598–602.

Sebo, P. and Ladant, D. (1993). Repeat sequences in the *Bordetella pertussis* adenylate cyclase toxin can be recognized as alternative carboxy-proximal secretion signals by the *Escherichia coli* a-haemolysin translocator. *Mol. Microbiol.*, **9**, 999–1009.

Sebo, P., Glaser, P., Sakamoto, H., and Ullmann, A. (1991). High-level synthesis of active adenylate cyclase toxin of *Bordetella pertussis* in a reconstructed *Escherichia coli* system. *Gene*, **104**, 19–24.

Sebo, P., Fayolle, P., d'Andria, O., Ladant, D., Leclerc, C., and Ullmann, A. (1995). Cell-invasive activity of the epitope-tagged adenylate cyclase of *Bordetella pertussis* allows *in vitro* delivery of foreign epitope into MHC class-I-restricted antigen processing pathway. *Infect Immun.*, **63**, 3851–7.

Szabo, G., Gray, M. C., and Hewlett, E. L. (1994). Adenylate cyclase toxin from *Bordetella pertussis* produces ion conductance across artificial lipid bilayers in a calcium and polarity-dependent manner. *J. Biol. Chem.*, **269**, 22496–9.

Weiss, A. A. and Hewlett, E. L. (1986) Virulence factors of *Bordetella pertussis*. *Ann. Rev. Microbiol.*, **40**, 661–86.

Welch, R. A. (1991). Pore-forming cytolysins of gram-negative bacteria. *Mol. Microbiol.*, **5**, 521–8.

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Anthrax edema factor (*Bacillus anthracis*)

Anthrax toxin edema factor is an 'invasive adenylyl cyclase' which is internalized into nearly all types of cells by interaction with the protective antigen toxin component. Translocation to the cytosol leads to unregulated conversion of ATP to cAMP, which activates cAMP-dependent protein kinase, leading to serious perturbations of regulatory mechanisms.

Anthrax edema factor (EF, originally designated Factor I) is a calmodulin-dependent adenylyl cyclase (Leppla 1982) which is one of the three proteins of the anthrax toxin complex (Smith *et al.* 1955; Leppla 1991a; 1995). The EF gene (Escuyer *et al.* 1988; Mock *et al.* 1988; Robertson *et al.* 1988; Tippetts and Robertson 1988) (GenBank accessions M23179 and M24074), located on the large pXO1 plasmid, encodes a precursor of 800 amino acids. Cleavage of the 33-amino acid signal peptide produces the 767 residue mature protein (PIR Protein database accession JS0029) having a mass of 88.8 kDa. The other proteins of the toxin complex are protective antigen (PA, GenBank M22589) and lethal factor (LF, GenBank M29081 and M30210; see separate entry in this book p. 00). None of the three proteins contain cysteine. As shown in Fig. 1, EF enters cells by binding to proteolytically activated, receptor-bound PA (Klimpel *et al.* 1992). EF (or LF) is then endocytosed and translocated from endosomes directly to the cytosol of cells, where it produces unregulated high concentrations of cAMP (Leppla 1982; Gordon *et al.* 1988). Entry of EF and LF is totally dependent on PA. The receptor for PA is unknown, but is present on nearly all types of cells (Escuyer and Collier 1991; Leppla 1991*a*).

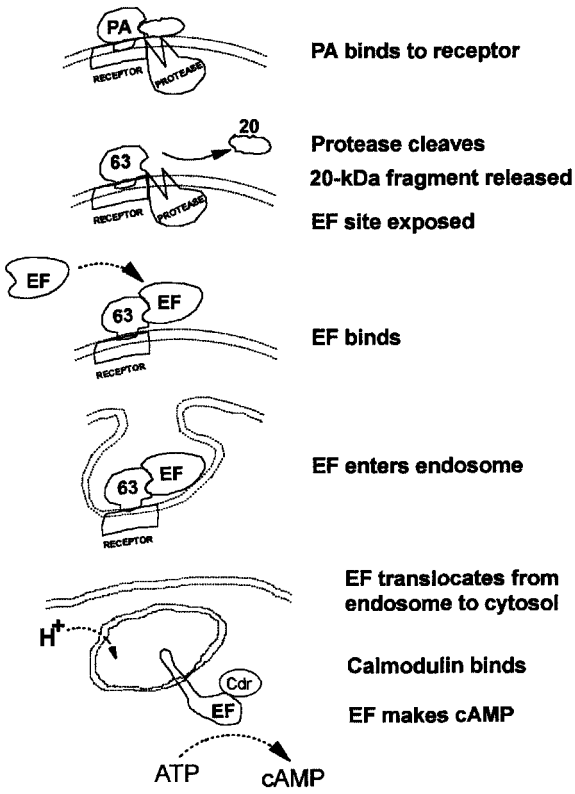

Figure 1. Schematic representation of the edema factor intoxication of eukaryotic cells.

EF has two recognized domains, an N-terminal region that causes binding to PA and internalization, and the C-terminal catalytic domain (Escuyer *et al.* 1988; Robertson 1988). Residues 1–250 constitute the PA-binding domain; this has strong sequence homology to LF. Fusion of residues 1–254 of LF or 1–260 of EF to the catalytic domains of other toxins (e.g. diphtheria, tetanus) produces potent cytotoxins, due to PA-dependent delivery to the cytosol (Arora and Leppla 1993). Residues 265–570 of EF have sequence homology to the other known 'invasive adenylate cyclase', that of *Bordetella pertussis* (Escuyer *et al.* 1988; Robertson 1988). Expression of residues 262–767 of EF in *Escherichia coli* yields a fully active adenylyl cyclase (Labruyere *et al.* 1991).

Several regions of EF have been shown to be involved in catalysis. Residues 314–321, GLNEHGKS, fit the consensus GxxxxGKS sequence of ATP binding sites; substitution of K320 destroys enzyme activity (Xia and Storm 1990). Identification of the EF sequences comprising the site to which calmodulin binds has been difficult. A synthetic peptide corresponding to EF residues 499–532 binds tightly to calmodulin (Munier *et al.* 1993), suggesting this region is part of the calmodulin-binding site.

Purification and sources

EF (and also PA and LF) are secreted by strains of *B. anthracis* containing plasmid pXO1. Mutant strains have been made which produce only EF, PA, or LF (Pezard *et al.* 1993). The three proteins collectively constitute more than half of the protein in a culture supernatant. Purification has been accomplished by ammonium sulfate

precipitation, followed by chromatography on hydroxyapatite, DEAE, and other anion exchange resins (Quinn *et al.* 1988; Leppla 1991*b*). Hydroxyapatite is an effective step because EF eluted last, well separated from the other components. EF is susceptible to proteolysis and is the most difficult of the three components to produce. Fermentor cultures yield 10 mg PA, 2 mg LF, and 0.5 mg EF per litre (Leppla 1991*b*). Purified PA should be examined by electrophoresis to screen for cleavage at residue 313, which destroys activity (Leppla 1991*a*). The proteins are not available commercially. Certain toxin components may be available from Stephen Leppla (NIDR, NIH, Bethesda, MD), Michelle Mock (Pasteur Institute, Paris), Peter Turnbull (CAMR, Porton Down, UK), or Joseph Farchaus (USAMRIID, Frederick, MD; PA only).

Toxicity

The edema toxin (PA + EF) was originally detected using vascular permeability assays in guinea pig skin (Smith *et al.* 1955). More convenient is measurement of elevated cytosolic cAMP concentrations in cultured cells (Leppla 1982; Gordon *et al.* 1988). Nearly all types of cells respond, although intracellular cAMP concentrations produced by edema toxin differ widely among cultured cells, probably due to differences in PA receptor number, cytosolic stability of EF, concentrations of Ca^{2+}-activated calmodulin, or activity of cAMP phosphodiesterase. Some cell types demonstrate 1000-fold increases, reaching 2000 μmol per mg cell protein (Leppla 1982; Gordon *et al.* 1989), which may represent conversion of 20–50 per cent of the cellular ATP. Elevated cAMP impairs phagocytic cells and thereby contributes to establishment of *B. anthracis* infections. Because all the effects of cAMP appear to be mediated by cAMP-dependent protein kinase, and this is fully activated by small increases in intracellular cAMP, no additional sequelae are expected from generation of very high concentrations of cAMP, except those caused by depletion of ATP. Elevated cAMP inhibits cell growth but is not lethal to most types of cells, and cultured cells generally will resume growth after toxin is removed. The anthrax toxin components are individually nontoxic and therefore present little hazard to users.

Catalytic properties

EF is a very efficient adenylyl cyclase (Labruyere *et al.* 1991; Leppla 1991*a*), having K_m for Mg^{+2}-ATP = 0.16 mM, and V_{max} = 1.2 mmol cAMP/min/mg enzyme. The catalytic activity is absolutely dependent on calmodulin and highly dependent on calcium. The truncated recombinant protein containing only residues 262–767 (Labruyere *et al.* 1991) has been useful in studying catalytic properties. This 62-kDa product has kinetic activities like those of the full-size protein, with a K_m = 0.25 mM for ATP, and an absolute requirement for calmodulin. The K_d for calmodulin activation is 23 nM.

Use in cell biology

Edema toxin (PA + EF) provides a convenient reagent for elevating cAMP concentrations in cultured cells. The effect of the toxin is qualitatively the same as that produced by cholera toxin, but the response occurs in a wider variety of cell types and higher concentrations of cAMP are typically produced. A further advantage as compared to cholera toxin is that cAMP concentrations decrease rapidly upon toxin removal, apparently because the toxin is inactivated within cells. It was estimated that the half-life of EF in CHO cells is less than 2 hours (Leppla 1982).

The high catalytic activity of EF suggests it could be used to synthesize cAMP analogues. If the enzyme can be shown to accept ATP analogues as substrates, then certain cAMP analogues could be prepared that might have useful pharmacological properties.

References

Arora, N. and Leppla, S. H. (1993). Residues 1–254 of anthrax toxin lethal factor are sufficient to cause cellular uptake of fused polypeptides. *J. Biol. Chem.*, **268**, 3334–41.

Escuyer, V. and Collier, R. J. (1991). Anthrax protective antigen interacts with a specific receptor on the surface of CHO-K1 cells. *Infect. Immun.*, **59**, 3381–6.

Escuyer, V., Duflot, E., Sezer, O., Danchin, A., and Mock, M. (1988). Structural homology between virulence-associated bacterial adenylate cyclases. *Gene*, **71**, 293–8.

Gordon, V. M., Leppla, S. H., and Hewlett, E. L. (1988). Inhibitors of receptor-mediated endocytosis block the entry of *Bacillus anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. *Infect. Immun.*, **56**, 1066–9.

Gordon, V. M., Young, W. W., Jr, Lechler, S. M., Gray, M. C., Leppla, S. H., and Hewlett, E. L. (1989). Adenylate cyclase toxins from *Bacillus anthracis* and *Bordetella pertussis*. Different processes for interaction with and entry into target cells. *J. Biol. Chem.*, **264**, 14792–6.

Klimpel, K. R., Molloy, S. S., Thomas, G., and Leppla, S. H. (1992). Anthrax toxin protective antigen is activated by a cell-surface protease with the sequence specificity and catalytic properties of furin. *Proc. Natl. Acad. Sci. USA*, **89**, 10277–81.

Labruyere, E., Mock, M., Surewicz, W. K., Mantsch, H. H., Rose, T., Munier, H., *et al.* (1991). Structural and ligand-binding properties of a truncated form of *Bacillus anthracis* adenylate cyclase and of a catalytically inactive variant in which glutamine substitutes for lysine-346. *Biochemistry*, **30**, 2619–24.

Leppla, S. H. (1982). Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc. Natl. Acad. Sci. USA*, **79**, 3162–6.

Leppla, S. H. (1991*a*). The anthrax toxin complex. In *Sourcebook of bacterial protein toxins* (ed. J. E. Alouf and J. H. Freer), pp. 277–302, Academic Press, London.

Leppla, S. H. (1991*b*). Purification and characterization of adenylyl cyclase from *Bacillus anthracis*. *Methods Enzymol.*, **195**, 153–68.

Leppla, S. (1995). Anthrax toxins. In *Bacterial toxins and virulence factors in disease. Handbook of natural toxins* Vol. 8 (ed. J. Moss, B. Iglewski, M. Vaughan, and A. Tu), pp. 543–72, Marcel Dekker, New York.

Mock, M., Labruyere, E., Glaser, P., Danchin, A., and Ullmann, A. (1988). Cloning and expression of the calmodulin-sensitive

Bacillus anthracis adenylate cyclase in *Escherichia coli*. *Gene*, **64**, 277–84.

Munier, H., Blanco, F. J., Precheur, B., Diesis, E., Nieto, J. L., Craescu, C. T., *et al.* (1993). Characterization of a synthetic calmodulin-binding peptide derived from *Bacillus anthracis* adenylate cyclase. *J. Biol. Chem.*, **268**, 1695–701.

Pezard, C., Duflot, E., and Mock, M. (1993). Construction of *Bacillus anthracis* mutant strains producing a single toxin component. *J. Gen. Microbiol.*, **139**, 2459–63.

Quinn, C. P., Shone, C. C., Turnbull, P. C., and Melling, J. (1988). Purification of anthrax-toxin components by high-performance anion-exchange, gel-filtration and hydrophobic-interaction chromatography. *Biochem. J.*, **252**, 753–8.

Robertson, D. L. (1988). Relationships between the calmodulin-dependent adenylate cyclases produced by *Bacillus anthracis* and *Bordetella pertussis*. *Biochem. Biophys. Res. Commun.*, **157**, 1027–32.

Robertson, D. L., Tippetts, M. T., and Leppla, S. H. (1988). Nucleotide sequence of the *Bacillus anthracis* edema factor gene (cya): a calmodulin-dependent adenylate cyclase. *Gene*, **73**, 363–71.

Smith, H., Keppie, J., and Stanley, J. L. (1955). The chemical basis of the virulence of *Bacillus anthracis*. V. the specific toxin produced by *B. anthracis in vivo*. *Brit. J. Exp. Path.*, **36**, 460–72.

Tippetts, M. T. and Robertson, D. L. (1988). Molecular cloning and expression of the *Bacillus anthracis* edema factor toxin gene: a calmodulin-dependent adenylate cyclase. *J. Bacteriol.*, **170**, 2263–6.

Xia, Z. G. and Storm, D. R. (1990). A-type ATP binding consensus sequences are critical for the catalytic activity of the calmodulin-sensitive adenylyl cyclase from *Bacillus anthracis*. *J. Biol. Chem.*, **265**, 6517–20.

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3

Toxins affecting protein synthesis

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Introduction

During the past decade there has been an enormous increase in our understanding of the structure–function relationships and molecular mechanism of action of many of the toxins that affect protein synthesis. The toxins considered in this section inhibit eukaryotic cellular protein synthesis either by the NAD^+ dependent adenosine diphosphate ribosylation of elongation factor 2 or function as an *N*-glycosidase to remove adenine 4324 (A^{4324}) from 28S rRNA which leads to an impaired ability of the ribosome to bind elongation factors. The X-ray crystal structure of ricin (Montfort *et al.* 1987; Rutenber *et al.* 1991), *Pseudomonas* exotoxin A (Allured *et al.* 1986), diphtheria toxin (Choe *et al.* 1992; Bennett *et al.* 1994), and Shiga toxin (Fraser *et al.* 1994) have been solved. Both crystallographic and biochemical genetic analysis have led to a detailed understanding of the adenosine diphosphate ribosyltransferase activity of diphtheria toxin and *Pseudomonas* exotoxin A (Collier 1990; Wick and Iglewski 1990) and the *N*-glycosidase activity of ricin, Shiga toxin, and the plant derived ribosome-inactivating proteins (Jimenez and Vasquez 1985; Stirpe and Barbieri 1986). Most all of these toxins have been highly purified, and their respective genes have been cloned and sequenced. In addition, many of their respective enzymatically active fragments or chains have been employed in the assembly of immunotoxins by the chemical crosslinking of the catalytic domains of either a bacterial or plant toxin to a monoclonal antibody (Ghetie and Vitetta 1994). More recently, recombinant DNA and protein engineering methods have been used to create chimeric genes in which the native receptor binding domain of either diphtheria toxin or *Pseudomonas* exotoxin A have been genetically substituted with one of a variety of genes encoding a polypeptide ligand that is directed toward a specific cell surface receptor or antigenic determinant on the target cell surface (for reviews see Kreitman and Pastan 1994; Murphy *et al.* 1995). The immunotoxins and the protein fusion toxins represent novel reagents for the study of cell biology and for the experimental treatment of human disease. Indeed, the diphtheria toxin-related IL-2 fusion toxin DAB_{389} IL-2 is currently being evaluated in Phase III clinical trials for the treatment of cutaneous T cell lymphoma.

References

Allured, V. S., Collier, R. J., Carroll, S. F., and McKay, D. B. (1986). Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-Angstorm resolution. *Proc. Natl. Acad. Sci. USA*, **83**, 1320–4.

Bennett, M. J., Choe, S., and Eisenberg, D. (1994). Domain swapping entangling alliances between proteins. *Proc. Natl. Acad. Sci. USA*, **91**, 3127–31.

Choe, S., Bennett, M. J., Fujii, G., Curmi, P. M. G., Kantardjieff, K. A., Collier, R. J. *et al.* (1992). The crystal structure of diphtheria toxin. *Nature*, **357**, 216–22.

Collier, R. J. (1990). In ADP-ribosylating toxins and g proteins (ed. J. Moss and M. Vaughan) pp. 3–19, American Society for Microbiology, Washington, DC.

Fraser, M. E., Chernaia, M. M., Kozlov, Y. V., and James, M. N. G. (1994). Crystal structure of the holotoxin from Shigella dysenteriae at 2. 5. resolution. *Nature Structural Biol.*, **1**, 59–64.

Ghetie, V. and Vitetta, E. (1994). Immunotoxins in the therapy of cancer: from bench to clinic. *Pharmacology & Therapeutics*, **63**, 209–34.

Jimenez, A. and Vazquez, D. (1985). Plant and fungal protein glycoprotein toxins inhibiting eukaryote protein synthesis. *(Annu.) Rev. Microbiol.*, **39**, 649–72.

Kreitman, R. J. and Pastan, I. (1994). Recombinant single-chain immunotoxins against T and B cell leukemias. *Leukemia & Lymphoma*, **13**, 1–10.

Montfort, W., Villafranca, J. E., Monzingo, A. F., Ernst, S., Katzin, B., Rutenber, E., *et al.* (1987). The three-dimensional structure of ricin at 2. 8 A. *J. Biol. Chem.*, **262**, 5389–403.

Murphy, J. R., vanderSpek, J. C., Lemichez, E., and Boquet P. (1995). In *Bacterial toxins, virulence factors and disease*, vol. 8 (ed. J. Moss, M. Vaughan, B. Iglweski, and A, Tu), pp. 23–45, Marcel Dekker, New York.

Rutenber, E., Katzin, B. J., Collins, E. J., Mlsna, D., Ernst, S. E., Ready, M. P., *et al.* (1991). Crytallographic refinement of ricin to 2. 5 A. *Proteins*, **10**, 240–50.

Stirpe, F. and Barbieri, L. (1986). Ribosome-inactivating proteins up to date. *FEBS Lett.*, **195**, 1–8.

Wick, M. J., and Iglewski, B. H. (1990). In *ADP-ribosylating toxins and g proteins* (ed. J. Moss and M. Vaughan), pp. 31–43, American Society for Microbiology, Washington,DC.

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Diphtheria toxin (*Corynebacterium diphtheriae*)

Diphtheria toxin is a 535 amino acid protein that is produced and secreted into the growth medium by toxigenic strains of Corynebacterium diphtheriae. *The toxin is a three domain protein whose X-ray crystal structure has been recently described (Choe* et al. *1992) and refined (Bennett* et al. *1994). Diphtheria toxin binds to its eukaryotic cell surface receptor, a heparin binding epidermal growth factor-like precursor, and is internalized by receptor mediated endocytosis. Intact toxin is cleaved by the endoprotease furin in the 14 amino acid protease sensitive loop between the catalytic and transmembrane domains and following acidification of the endosome, the transmembrane domain of the toxin inserts into the vesicle membrane and facilitates the delivery of the catalytic domain to the cytosol. Once delivered to the cytosol the catalytic domain specifically ADP-ribosylates cellular elongation factor 2 which results in the inhibition of protein synthesis and death of the cell.*

Diphtheria toxin is the primary virulence factor of toxigenic *C. diphtheriae* the etiologic agent of clinical diphtheria (Pappenheimer 1977). The structural gene for diphtheria toxin, *tox*, is carried by a closely related family of corynebacteriophages of which the β-phage has been the best studied (Buck *et al.* 1985; Bishai and Murphy 1988). The regulation of *tox* gene expression is controlled by the *C. diphtheriae* determined iron-activated repressor DtxR (Tao *et al.* 1994). Diphtheria toxin is produced in maximal yield only during the decline phase of the bacterial growth cycle when iron becomes the growth rate limiting substrate. The toxin is synthesized in precursor form and is co-translationally secreted into the growth medium (Smith *et al.* 1980). As shown in Fig. 1, diphtheria toxin is a three domain protein and is composed of the catalytic (C), transmembrane (T), and receptor binding (R) domains. The R domain has been shown to specially bind to a heparin binding epidermal growth factor-like precursor on the surface of sensitive eukaryotic cells (Naglich *et al.* 1992).

Diphtheria toxin is readily cleaved into two polypeptide fragments following mild digestion with trypsin or other serine proteases (Drazin *et al.* 1971; Gill and Dinius 1971). After cleavage, the toxin may be separated under denaturing conditions in the presence of a thiol into two polypeptides. The N-terminal polypeptide (catalytic domain or fragment A; M_r 21 167) is an enzyme that catalyses the NAD^+-dependent ADP-ribosylation of elongation factor 2 (EF2) according to the following reaction:

$$\text{NAD}^+ + \text{EF2} \xrightarrow{\text{fragment A (catalytic domain)}} \text{ADPR-EF2} + \text{nicotinamide} + \text{H}^+$$

Once EF2 is ADP-ribosylated it can no longer participate in protein synthesis. The C-terminal polypeptide (transmembrane and receptor binding domains or fragment B; M_r 37 199) is required for binding the toxin to its cell surface receptor on sensitive cells and for facilitating the transport of fragment A into the cytosol. The nucleic acid sequences of the native diphtheria toxin structural genes from corynebacteriophage β and ω have been shown to be identical (Greenfield *et al.* 1983; Ratti *et al.* 1983).

Purification and sources

Diphtheria toxin may be partially purified from the culture supernatant fluids of toxigenic *C. diphtheriae* by ammonium sulfate precipitation (65 per cent saturation) and ion exchange chromatography on DE-52 (Whatman) (Pappenheimer *et al.* 1972). In addition, diphtheria toxin in either its 'intact' or 'nicked' (i.e. cleaved after Arg190, Arg192, and/or Arg193) forms may be obtained from a variety of commercial sources (e.g. [intact or un-nicked toxin] List Laboratories, Campbell, CA; [nicked toxin] CalBiochem, La Jolla, CA; Connaught Laboratories, Toronto, Canada). In general, these preparations of toxin contain relatively high levels of contaminating nuclease activity. Diphtheria toxin may be further purified by high performance liquid chromatography (HPLC) gel filtration using a TSK-3000 column (Bodley *et al.* 1990).

Toxicity

Diphtheria toxin is extraordinarily potent; in sensitive species (e.g. humans, monkeys, rabbits, guinea pigs) as little as 100 to 150 ng/kg of body weight is lethal (Gill 1985). The relative sensitivity of a given sensitive eukaryotic cell line to the toxin has been shown to correlate with the number of cell surface receptors (Middlebrook *et al.* 1978). Since neither mouse nor rat cells express the diphtheria toxin receptor, they are resistant to its action (Pappenheimer 1977).

Immunity to diphtheria involves an antibody response to diphtheria toxin following clinical disease or immunization with diphtheria toxoid. Subclinical infection is no longer a source of diphtheria toxin antigen exposure and, if not boosted, antitoxin immunity wanes. As a conse-

Figure 1. Ribbon diagram of the X-ray crystal structure of native diphtheria toxin (Choe *et al.* 1992) as modified by Bennett *et al.* (1994). The catalytic domain (*upper left*), transmembrane domain (*bottom*), and receptor binding domain (*right*) are shown. N, N-terminal end of the toxin; PSL, 14 amino acid protease sensitive loop which separates the catalytic from transmembrane and receptor binding domains; C, C-terminal end of the toxin. The ribbon diagram was generated using MOLESCRIPT (Kraulis 1991).

quence, a large percentage of the adults (30 to 60 per cent) currently have antitoxin levels that are below the protective level (0. 01 IU/ml) and are at risk. The adult population should be re-immunized with diphtheria toxoid every 10 years. Given the current diphtheria epidemic in Russia and other countries of the former Soviet Union, booster immunization with diphtheria–tetanus toxoids should be administered to all persons travelling to regions with high rates of endemic diphtheria (Central and South America, Africa, Asia, Russia, and Eastern Europe).

Use of diphtheria toxin-related fusion proteins in cell biology and clinical medicine

In recent years, the diphtheria toxin structural platform has been used in the genetic construction of a family of eukaryotic cell receptor-specific fusion proteins, or fusion toxins (Murphy *et al.* 1995). In each instance, the substitution of the native receptor binding domain with either a polypeptide hormone or growth factor (e. g. α-melanocyte stimulating hormone [α-MSH], epidermal growth factor [EGF], interleukin (IL)-2, IL-6, etc.) has resulted in the formation of a unique fusion toxin. These chimeric toxins, combine the receptor binding specificity of the cytokine with the cytotoxic potency of the transmembrane and catalytic domains of diphtheria toxin. In each instance, the fusion toxins have been shown to selectively intoxicate only those cells which bear the appropriate targeted receptor. Indeed, the first of these genetically engineered fusion toxins, DAB_{389} IL-2, is currently being evaluated in human clinical trials for the treatment of refractory lymphomas and autoimmune diseases where cells with high affinity IL-2 receptors play a major role in pathogenesis. Administration of DAB_{389} IL-2 has been shown to be safe, well tolerated, and capable

of inducing durable remission from disease in the absence of severe adverse effects. It is likely that the diphtheria toxin-based fusion toxins will be important new biological agents for the treatment of specific tumors or disorders in which specific cell surface receptors may be targeted.

References

Bennett, M. J., Choe, S., and Eisenberg, D. (1994). Domain swapping entangling alliances between proteins. *Proc. Natl. Acad. Sci. USA*, **91**, 3127–31.

Bishai, W. R. and Murphy, J. R. (1988). In *The bacteriophages* (ed. I. Calendar), pp. 683–724, Plenum Publishing Corp, New York.

Bodley, J. W., Johnson, V. G., Wilson, B. A., Blanke, S. R., Murphy, J. R., Pappenheimer, A. M., Jr., and Collier, R. J. (1990). Does diphtheria toxin have nuclease activity? *Science*, **250**, 832–8.

Buck, G. A., Cross, R. E., Wong, T. P., Lorea, J., and Groman, N. B. (1985). DNA relationships among some tox-bearing corynebacteriophages. *Infect. Immun.*, **49**, 679–84.

Choe, S., Bennett, M. J., Fujii, G., Curmi, P. M. G., Kantardjieff, K. A., Collier, R. J. *et al.* (1992). The crystal structure of diphtheria toxin. *Nature*, **357**, 216–22.

Drazin, R., Kandel, J., and Collier, R. J. (1971). Structure and activity of diphtheria toxin. II. Attack by trypsin at a specific site within the intact toxin molecule. *J. Biol. Chem.*, **246**, 1504–10.

Gill, D. M. (1985). Bacterial toxins: a table of lethal amounts. *Microbiol. Rev.*, **46**, 86–94.

Gill, D. M. and Dinius, L. L. (1971). Observations on the structure of diphtheria toxin *J. Biol. Chem.*, **246**, 1485–91.

Greenfield, L., Bjorn, M. J., Horn, G., Fong, D., Buck, G. A., Collier, R. J. *et al.* (1983). Nucleotide sequence of the structural gene for diphtheria toxin carried by corynebacteriophage beta. *Proc. Natl. Acad. Sci. USA*, **80**, 6853–7.

Kraulis, P. J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.*, **24**, 946–50.

Middlebrook, J. L., Dorland, R. B., and Leppla, S. H. (1978). Association of diphtheria toxin with Vero cells. Demonstration of a receptor. *J. Biol. Chem.*, **253**, 7325–30.

Murphy, J. R., vanderSpek, J. C., Lemichez, E., and Boquet, P. (1995). In *Bacterial toxins, virulence factors and disease* Vol. 8, (ed. J. Moss, M. Vaughan, B. Iglweski, and A. Tu), pp. 23–45, Marcel Dekker, New York.

Naglich, J. G., Metherall, J. E., Russell, D. W., and Eidels, L. (1992). Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell*, **69**, 1051–61.

Pappenheimer, A. M., Jr (1977). Diphtheria toxin. *Annu. Rev. Biochem.*, **46**, 69–94.

Pappenheimer, A. M., Jr, Uchida, T., and Harper, A. (1972). An immunological study of the diphtheria toxin molecule. *Immunochemistry*, **9**, 891–906.

Ratti, G., Rappuoli, R., and Giannini, G. (1983). The complete nucleotide sequence of the gene coding for diphtheria toxin in the corynephage ω(tox$^+$) genome. *Nucleic Acids Res.*, **11**, 6589–95.

Smith, W. P., Tai, P. C., Murphy, J. R. and Davis, B. D. (1980). Precursor in cotranslational secretion of diphtheria toxin. *J. Bacteriol.*, **141**, 184–9.

Tao, X., Schiering, N., Zeng, H.-Y., Ringe, D., and Murphy, J. R. (1994). Iron, DtxR, and the regulation of diphtheria toxin expression. *Mol. Microbiol.*, **14**, 191–7.

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Pseudomonas aeruginosa exotoxin A

Exotoxin A from Pseudomonas aeruginosa *is a 66 kDa single chain protein that enters the cytosol and ADP-ribosylates elongation factor 2. The toxin thereby blocks protein synthesis and kills the cell. The toxin is used to form targeted cytotoxic agents by linking it to ligands that bind to defined cell surface molecules.*

Pseudomonas aeruginosa exotoxin A (PEA, MW 66 kDa, 613 amino acids, sequence accession number: PSEETA K01397) consists of three major domains (Allured *et al.* 1986), as indicated in Fig. 1. The N-terminal domain Ia (amino acids 1–252) binds to the α_2-macroglobulin receptor at the cell surface (Kounnas *et al.* 1992), domain II (amino acids 253–364) mediates translocation of itself and domain III to the cytosol, and the C-terminal domain III (amino acids 405–613) catalyses the transfer of ADP-ribose from NAD to the diphthamide residue of elongation factor 2, which is thereby inactivated (Iglewski and Kabat 1975; Carroll and Collier 1987; Wick *et al.* 1990*a*, *b*). Intoxication is initiated by binding of the toxin to its receptor and endocytotic uptake of the complex (Morris and Saelilnger 1986). Then follows proteolytic cleavage at a furin sensitive site between Arg^{279} and Gly^{280} and reduction of the disulfide between Cys^{265} and Cys^{287} (Ogata *et al.* 1990, 1992). The 37 kDa fragment thus generated is translocated to the cytosol. The translocation may occur from the lumen of the endoplasmic reticulum as the toxin contains a C-terminal REDLK sequence which, after removal of the C-terminal lysine residue, may function as an endoplasmic reticulum retention signal (Chaudhary *et al.* 1990). Removal of this signal strongly reduces the toxicity. Toxicity is also strongly reduced when His^{426} or Glu^{553} in domain III are

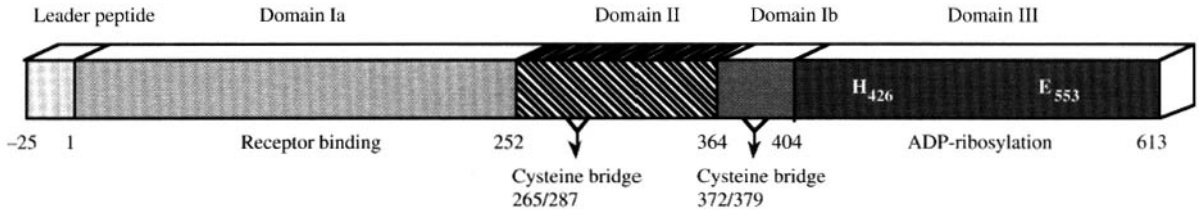

Figure 1. Schematic representation of *P. aeruginosa* exotoxin A.

mutated. In this case the toxin loses its enzymatic activity (Lukač and Collier 1988).

Domain Ia can be replaced by the variable domain of antibodies, or by lymphokines, growth factors and other molecules binding to defined cell surface molecules to make immunotoxins or related constructs for targeted cytotoxic therapy (Pastan and FitzGerald 1989).

Purification and sources

The toxin can be purified from the cell-free culture fluid of *Pseudomonas aeruginosa* (strain PA103, which produces little extracellular protease) as described (Iglewski and Sadoff 1979; Lory and Collier 1980). Wild-type and mutated toxin can also be purified from *E. coli* after transfection with the appropriate constructs (Gray *et al.* 1984; Benhar *et al.* 1994).

Toxicity

Toxicity can be assayed in a mouse lethality bioassay or in a cell toxicity assay (Iglewski and Sadoff 1979). The latter is fast and cheap and is most used. LD_{50} in mice weighing 17–20 g. is 0.1–0.3 μg. Intoxicated mice die within 2–6 days. The enzymatic activity can be assayed in an ADP-ribosylation assay (Iglewski and Sadoff 1979).

The toxin should be handled with care and one must avoid contact with the eyes or with open wounds. It is preferable to work with toxin solutions of 1 mg/ml or less. Lyophilized toxin should be handled with particular care to avoid inhalation.

Use in cell biology

Uncleaved PEA is not toxic to cells unless the cells are able to cleave the toxin, which occurs in the cells by furin (Moehring *et al.* 1993). PEA has been successfully used to select mutant cell lines lacking furin. The cells recover their toxin sensitivity upon transfection with furin or with the related Kex2 protease from *Saccharomyces cerevisiae*.

Use in targeted cytotoxic therapy

Due to its high toxic activity PEA has been extensively used in formation of immunotoxins and related constructs. The most common approach is to remove domain Ia and replace it with a ligand binding to cell surface molecules (Kondo *et al.* 1988). This may be the variable domain of immunoglobulins or various growth factors, hormones, and lymphokines. The toxicity of the constructs vary extensively. Endocytic uptake and transport to a deep membrane bounded compartment appears to be a prerequisite for translocation to the cytosol. The presence of a sequence resembling the retention signal for the endoplasmic reticulum (KDEL) suggests that transport of the toxin back to this location does take place. The finding that removal of the signal strongly reduces the toxicity indicates that this transport is required for translocation (Chaudhary *et al.* 1990).

References

Allured, V. S., Collier, R. J., Carrol, S. F., and McKay, D. B. (1986). Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-Angstrom resolution. *Proc. Natl. Acad. Sci. USA*, **83**, 1320–4.

Benhar, I., Wang, Q.-c., FitzGerald, D., and Pastan, I. (1994). Pseudomonas exotoxin A mutants. Replacement of surface-exposed residues in domain III with cysteine residues that can be modified with polyethylene glycol in a site-specific manner. *J. Biol. Chem.*, **269**, 13398–404.

Carroll, S. F. and Collier, R. J. (1987). Active site of *Pseudomonas aeruginosa* exotoxin A. Glutamic acid 553 is photolabeled by NAD and shows functional homology with glutamic acid 148 of diphtheria toxin. *J. Biol. Chem.*, **262**, 8707–11.

Chaudhary, V. K., Jinno, Y., FitzGerald, D. J., and Pastan, I. (1990). Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc. Natl. Acad. Sci. USA*, **87**, 308–12.

Gray, G. L., Smith, D. H., Baldridge, J. S., Harkins, R. N., Vasil, M. L., Chen, E. Y. *et al.* (1984). Cloning, nucleotide sequence, and expression in *Escherichia coli* of the exotoxin A strucutral gene of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, **81**, 2645–9.

Iglewski, B. H. and Kabat, D. (1975). NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. *Proc. Natl. Acad. Sci. USA*, **72**, 2284–8.

Iglewski, B. H. and Sadoff, J. C. (1979). Toxin inhibitors of protein synthesis: production, purification, and assay of *Pseudomonas aeruginosa* toxin A. *Methods Enzymol.*, **60**, 780–93.
Kondo, T., FitzGerald, D., Chaudhary, V. K., Adhya, S., and Pastan, I. (1988). Activity of immunotoxins constructed with modified Pseudomonas exotoxin A lacking the cell recognition domain. *J. Biol. Chem.*, **263**, 9470–5.
Kounnas, M. Z., Morris, R. E., Thompson, M. R., FitzGerald, D. J., Strickland, D. K., and Saelinger, C. B. (1992). The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J. Biol. Chem.*, **267**, 12420–3.
Lory, S. and Collier, R. J. (1980). Expression of enzymic activity by exotoxin A from *Pseudomonas aeruginosa*. *Infect. Immun.*, **28**, 494–501.
Lukač, M. and Collier, R. J. (1988). Restoration of enzymic activity and cytotoxicity of mutant, E553C, *Pseudomonas aeruginosa* exotoxin A by reaction with iodoacetic acid. *J. Biol. Chem.*, **263**, 6146–9.
Moehring, J. M., Inocencio, N. M., Robertson, B. J., and Moehring, T. J. (1993). Expression of mouse furin in a Chinese hamster cell resistant to Pseudomonas exotoxin A and viruses complements the genetic lesion. *J. Biol. Chem.*, **268**, 2590–4.
Morris, R. E. and Saelinger, C. B. (1986). Reduced temperature alters Pseudomonas exotoxin A entry into the mouse LM cell. *Infect. Immun.*, **52**, 445–53.
Ogata, M., Chaudhary, V. K., Pastan, I., and FitzGerald, D. J. (1990). Processing of Pseudomonas exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. *J. Biol. Chem.*, **265**, 20678–85.
Ogata, M., Fryling, C. M., Pastan, I., and FitzGerald, D. (1992). Cell-mediated cleavage of Pseudomonas exotoxin between Arg279 and Gly280 generates the enzymatically active fragment which translocates to the cytosol. *J. Biol. Chem.*, **267**, 25396–401.
Pastan, I. and FitzGerald, D. (1989). Pseudomonas exotoxin: chimeric toxins. *J. Biol. Chem.*, **264**, 15157–60.
Wick, M. J., Hamond, A. N., and Iglewski, B. H. (1990*a*). Analysis of the structure–function relationship of *Pseudomonas aeruginosa* exotoxin A. *Mol. Microbiol.*, **4**, 527–35.
Wick, M. J., Frank, D. W., Storey, D. G., and Iglewski, B. H. (1990*b*). Structure, function, and regulation of *Pseudomonas aeruginosa* exotoxin A. *Annu. Rev. Microbiol.*, **44**, 335–63.

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Shiga toxins (*Shigella dysenteriae* serotype I, *Escherichia coli*)

Shiga toxins are potent bacterial protein toxins that inhibit protein synthesis in target eukaryotic cells by cleaving an adenine residue from cytoplasmic ribosomes such that the ribosome no longer interacts with elongation factors eEF-1 and eEf-2.

The Shiga toxins (also known as verotoxins) comprise a family of toxins that inhibit protein synthesis in intoxicated cells (for reviews see O'Brien and Holmes 1987; O'Brien *et al.* 1992; Obrig 1994). The holotoxin consists of an A polypeptide (M_r 32 000) that has *N*-glycosidase activity which removes a single adenine residue from the 28 S rRNA (the same mode of action is found in the plant toxin ricin), and a pentamer of B polypeptides (M_r 7700 each) that confers specificity for the eukaryotic cell receptor, globotriaosylceramide or Gb_3 (Lingwood 1993). Toxin entry into the eukaryotic cell occurs via receptor-mediated endocytosis with subsequent routing of the toxin from the transgolgi through the endoplasmic reticulum to the cytosol (Sandvig and Deurs 1994). The A subunit contains a trypsin cleavage site near the C-terminus, but the importance of this nicking site for cytotoxicity is still under investigation. The cleaved A subunit is held together by a disulfide bridge that may get reduced after the toxin has been internalized. The crystal structure of Shiga toxin has been solved to 2.5 Å (Stein *et al.* 1992; Fraser *et al.* 1994). It is organized in two different domains: a pentameric oligosaccharide binding domain and a catalytic domain. The active site of the toxin appeared to be blocked by a portion of the A subunit, a finding which indicates that a conformational change may be required for full toxin activity. The active site of Shiga toxin appears to be the glutamic acid residue at position 167 in the mature A subunit, although neighbouring amino acids also appear to play a role (O'Brien *et al.* 1992).

The Shiga toxin family consists of Shiga toxin from *S. dysenteriae* and Shiga toxin types 1 and 2 found in *E. coli*. Shiga toxin and Stx1 are essentially identical,

with only one amino acid difference in the mature A subunit. Shiga toxin is about 60 per cent homologous to Stx2 at the amino acid level but polyclonal antisera to one toxin type does not cross react with the heterologous toxin. Variants of Shiga toxin type 2 are recognized based on biological differences or divergence in immunological reactivity or receptor binding. Based on these criteria, two variants of Stx2 have been identified: Stx2c (VT2c), which can be distinguished antigenically from Stx2 (Schmitt *et al.* 1991), and the edema disease toxin, Stx2e (VT2e, formerly called Stx2v or VT2v), which preferentially uses globotetraosylceramide (Gb_4) as a functional receptor rather than Gb_3 (Lingwood 1993).

Shiga toxin is chromosomally encoded (GenEMBL accession number M19437). Stxs are also found on the chromosome (Stx2e, some Stx1) or on bacteriophages (Stx1 and Stx2 GenEMBL accession numbers are M16625 and X07865, respectively). However, the toxins do share a conserved genetic structure in which the A and B subunit are encoded in an operon (O'Brien *et al.* 1992). The expression of Shiga toxin and Stx1, but not Stx2, is environmentally regulated such that high levels are made under iron-restricted conditions (O'Brien and Holmes 1987; O'Brien *et al.* 1992).

Shiga toxin producing organisms cause dysentery (*S. dysenteriae*), hemorrhagic colitis (*E. coli*), and the hemolytic uremic syndrome (*S. dysenteriae*, *E. coli*). One model for the role of these toxins in pathogenesis of disease is that the toxin binds to mature columnar epithelial cells in the intestine of the infected human or animal, and the toxin then enters those cells and halts protein synthesis. This inhibition of protein synthesis, in turn, leads to cell death and a subsequent loss of intestinal absorptive capacity (O'Brien and Holmes 1987). Recent hypotheses for how more serious Shiga toxin mediated sequelae may arise involve systemic delivery of toxin from the intestine followed by binding of the toxin to receptors on vascular endothelial cells of the central nervous system (edema disease), colon (hemorrhagic colitis), and kidney (HUS). Toxin bound to glomerular endothelia may cause direct damage to those cells or may act in concert with cytokines to mediate further damage to the endothelium (Tesh and O'Brien 1991).

Purification and sources

Shiga toxin can be isolated from shigellae grown in low iron medium or from K-12 strains carrying toxin clones or toxin-encoding phage. These toxin-producing bacteria are lysed, and toxin is purified by a series of step that include ammonium sulfate precipitation, anion exchange chromatography, chromatofocusing, and finally antitoxin affinity chromatography (O'Brien and Holmes 1987; O'Brien *et al.* 1992; Lindgren *et al.* 1994).

Toxicity

The intravenous or intraperitoneal LD_{50} for Shiga and Shiga toxin type 1 in mice has been reported to be from 28 ng/mouse (Yutsudo *et al.* 1986) to 400 ng/mouse (0.02 mg/kg; Tesh *et al.* 1993) to 0.450 mg/kg, (Eiklid and Olsnes 1983). For Shiga toxin type 2, the mouse LD_{50} is approximately 1 ng/mouse (5×10^{-5} mg/kg; Tesh *et al.* 1993; Lindgren *et al.* 1994). The intravenous LD_{50} of Shiga toxin for the rabbit is about 0.2 μg/kg (Richardson *et al.* 1992).

Use in cell biology

Shiga toxin has been used to study the endocytic pathway. In cells treated with butyric acid, Sandvig *et al.* (1992) have demonstrated that this toxin enters endosomal compartments, wherefrom it reaches the Golgi apparatus and the endoplasmic reticulum. Thus, the study of the membrane trafficking of this toxin revealed a pathway from the plasma membrane up to endosplasmic reticulum, that may be followed by more physiological ligands. It is expected that Shiga toxin will be a very useful marker, particular in sudies involving polarized and neuronal cells.

References

Eiklid, K. and Olsnes, S. (1983). Animal toxicity of *Shigella dysenteriae* cytotoxin: Evidence that the neurotoxic, enterotoxic, and cytotoxic activities are due to one toxin. *J. Immunol.*,**130**, 380–4.

Fraser, M., Chernaia, M., Kozlov, Y., and James, M. (1994). Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 Å resolution. *Struct. Biol.*, **1**, 59–64.

Lindgren, S., Samuel, J., Schmitt, C., and O'Brien, A. (1994). The specific activities of Shiga-like toxin type II (SLT-II) and SLT-II-related toxins of enterohemorrhagic *Escherichia coli* differ when measured by vero cell cytotoxicity but not by mouse lethality. *Infect. Immun.*, **62**, 623–31.

Lingwood, C. (1993). Verotoxins and their glycolipid receptors. *Adv. Lipid Res.*, **25**, 189–211.

O'Brien, A. D. and Holmes, R. K. (1987). Shiga and Shiga-like toxins. *Microbiol. Rev.*, **51**, 206–20.

O'Brien, A., Tesh, V., Donohue-Rolfe, A., Jackson, M., Olsnes, S., Sandvig, K., *et al.* (1992). Shiga toxin: Biochemistry, genetics, mode of action, and role in pathogenesis. *Curr. Top. Microbiol. Immunol.*, **180**, 65–94.

Obrig, T. (1994). Toxins that inhibit host protein synthesis. *Methods Enzymol.*, **235**, 647–56.

Richardson, S., Rotman, T., Jay, V., Smith, C., Becker, L., Petric, M., *et al.* (1992). Experimental verocytotoxemia in rabbits. *Infect. Immun.*, **60**, 4154–67.

Sandvig, K. and Deurs, B. (1994). Endocytosis and intracellular sorting of ricin and Shiga toxin. *FEBS Lett.*, **346**, 99–102.

Sandvig, K., Garred, O., Prydz, J, Kozlov, J. V., Hansen, H. and van Deurs, B. (1992). Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature*, **358**, 510–2.

Schmitt, C., McKee, M., and O'Brien, A. (1991). Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for

the antigenic heterogeneity of the O157:H_ strain E32511. *Infect. Immun.*, **59**, 1065–73.
Stein, P. E., Boodhoo, A., Tyrrell, G. J., Brunton, J. L., and Read, R. J. (1992). Crystal strucutre of the cell-binding B oligomer of verotoxin-1 from *E. coli*. *Nature*, **355**, 748–50.
Tesh, V. and O'Brien, A. (1991). The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. *Mol. Microbiol.*, **5**, 1817–22.
Tesh, V., Burris, J., Owens, J., Gordon, V., Wadolkowski, E., O'Brien, A. *et al.* (1993). Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect. Immun.*, **61**, 3392–402.
Yutsudo, T., Honda, T., Miwatani, T., and Takeda, Y. (1986). Characterization of purified Shiga toxin from *Shigella dysenteriae* 1. *Microbiol. Immunol.*, **30**, 1115–27.

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Ricin (*Ricinus communis*)

Ricin is a protein toxin found in the seeds of Ricinus communis. *The various isoforms of ricin have molecular weights of 63–66 kDa, and the toxin consists of two polypeptide chains connected by a disulfide bond. One of the chains, the B-chain binds to cell-surface glycoproteins and glycolipids with terminal galactose, whereas the other chain, the A-chain, enters the cytosol and blocks protein synthesis enzymatically by removal of one adenine from the 28 S RNA of the 60 S ribosomal subunit. Endocytosis and intracellular transport of ricin to the Golgi apparatus and possibly to the ER seem to be required for translocation of the A-chain to the cytosol.*

Ricin is synthesized in the endosperm cells of *Ricinus communis* seeds, and there are various isoforms with molecular weights of 63–66 kDa. The molecule is glycosylated (for review, see Barbieri *et al.* 1993; Lord *et al.* 1994). The ricin gene codes for a preprotoxin molecule which is processed in the plant (preproricin D: 576 amino acids, GenBank accession no. X52908). The leader sequence of ricin D has 35 amino acid residues, and the interchain linker between the two chains has 12 amino acids (Lord *et al.* 1994). The mature form of ricin D (Fig. 1) has an A-chain with 267 amino acids and a B-chain with 262 residues.

The intoxication consists of the following steps: The B-chain binds to cell surface receptors with terminal galactose and the toxin is endocytosed and transported to the organelle from where translocation of the A-chain to the cytosol takes place. Transport to the Golgi apparatus and possibly also retrograde transport to the endoplasmic reticulum (ER) may be involved in the internalization process (Pelham *et al.* 1992; Wales *et al.* 1992; Sandvig and van Deurs 1994*a*) (Fig. 2). After translocation to the cytosol, the A-chain inhibits the protein synthesis. Ricin A-chain is a *N*-glycosidase which removes one adenine from the 28 S RNA of the 60 S subunit of the ribosome (Endo *et al.* 1987). The toxin has no effect on prokaryotic ribosomes (Lord *et al.* 1994).

Ricin or parts of the toxin molecule, for instance the A-chain, is being used to construct immunotoxins or other conjugates where the aim is to selectively kill a subgroup of cells, for instance cancer cells (Olsnes *et al.* 1989; Barbieri *et al.* 1993; Lord *et al.* 1994).

Toxins with a similar two-chain structure as ricin are the plant proteins abrin, modeccin, volkensin, and viscumin which come from different plants, but which all inhibit protein synthesis in the same way as ricin. In spite of the similarities, the entry mechanisms of these toxins may differ (for review, see Olsnes *et al.* 1990).

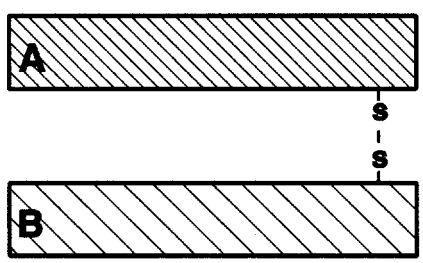

Figure 1. Schematic structure of ricin.

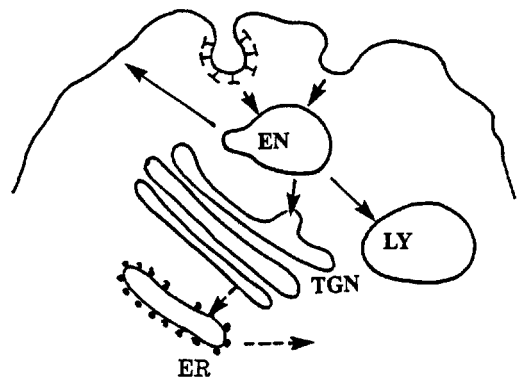

Figure 2. Intracellular pathways followed by ricin. Known pathways, arrows with intact lines; hypothetical pathways, arrows with broken lines. The toxin is endocytosed from clathrin-coated pits and also by a clathrin-independent mechanism, and it is transported to endosomes (EN), from where it may be recycled. The toxin is also transported to lysosomes (LY), to the trans-Golgi network (TGN), and possibly retrogradely through the Golgi to the endoplasmic reticulum (ER) from where it may be translocated to the cytosol.

Purification and sources

Ricin can be purified from the seeds of *Ricinus communis* (Olsnes 1978). The seeds are extracted with acetic acid, and ricin is purified by chromatography on CM-cellulose and Sepharose 4B. Ricin can also be purchased from Sigma Chemical Company, St. Louis, Mo., USA.

Toxicity

For humans the maximum tolerated dose has been estimated to be 23 $\mu g/m^2$ (about 40 μg for a person of 60 kg)

(Fodstad *et al.* 1984). LD_{50} for mice after parenteral injection is 2. 6 μg/kg (Barbieri *et al.* 1993), and the minimum lethal dose for dogs is 1. 75 μg/kg (Fodstad *et al.* 1984). The lethal dose of castor oil seed, the source of ricin, has been measured on a number of different animals (Balint 1974). The horse was found to be very sensitive, the lethal dose was 0. 1 g/kg, whereas the lethal dose for a hen is 14 g/kg. For safety, gloves should be used when working with ricin. If possible, avoid freeze-drying the toxin since this will increase the risk of inhaling the toxin.

Use in cell biology

Ricin has proven valuable in studies of endocytosis and intracellular trafficking (van Deurs *et al.* 1993; Eker *et al.* 1994; Sandvig and van Deurs 1994*a*). Since the toxin binds to a large number of different glycolipids and glycoproteins, ricin can be used as a membrane marker. Experiments with ricin have provided evidence for the existence of more than one endocytic pathway (Sandvig and van Deurs 1994*b*). The toxin is endocytosed even when the formation of coated vesicles from coated pits is blocked by acidification of the cytosol, and also when clathrin-coated pits are removed by potassium depletion of cells. Furthermore, ricin is transported to the lysosomes and to the Golgi apparatus, it is transcytosed across epithelial cell layers and it is recycled, and the toxin can therefore be used to study all these pathways. The mechanism for ricin translocation to the cytosol is of importance also in connection with the construction of immunotoxins, which often contain parts of the native ricin molecule (Olsnes *et al.* 1989; Barbieri *et al.* 1993; Lord *et al.* 1994). The efficiency of hybrid molecules like immunotoxins could be dependent on routing to the correct intracellular compartment for translocation to the cytosol. There is evidence that ricin has to be transported to the Golgi apparatus before translocation to the cytosol (for review, see Sandvig and van Deurs 1994*a*), and retrograde transport to the ER may be required for translocation to occur (Pelham *et al.* 1992; Wales *et al.* 1992; Sandvig and van Deurs 1994*a*). Ricin does not contain the ER retrieval signal KDEL, and more knowledge about the possible retrograde transport of this molecule should therefore provide important new information about trafficking of proteins in the Golgi/ER system. Some of the known and hypothetical pathways followed by ricin are shown in Fig. 2.

References

Balint, G. A. (1974). Ricin: The toxic protein of castor oil seeds. *Toxicology*, **2**, 77–102.

Barbieri, L., Battelli, M. G., and Stirpe, F. (1993). Ribosome-inactivating proteins from plants. *Biochim. Biophys. Acta*, **1154**, 237–82.

Eker, P., Holm, P. K., van Deurs, B., and Sandvig, K. (1994). Selective regulation of apical endocytosis in polarized MDCK cells by mastoparan and cAMP. *J. Biol. Chem.*, **269**, 18607–15.

Endo, Y., Mitsui, K., Motizuki, M., and Tsurugi, K. (1987). The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in the 28 S ribosomal RNA caused by the toxins. *J. Biol. Chem.*, **262**, 5908–12.

Fodstad, Ø., Kvalheim, G., Godal, A., Lotsberg, J., Aamdal, S., Høst, H., *et al.* (1984). Phase I study of the plant protein ricin. *Cancer Res.*, **44**, 862–5.

Lord, J. M., Roberts, L. M. and Robertus, J. D. (1994). Ricin: structure, mode of action, and some current applications. *FASEB J.*, **8**, 201–8.

Olsnes, S. (1978). The toxic lectins ricin and ricinus agglutinin. *Methods Enzymol.*, **50**, 330–5.

Olsnes, S., Sandvig, K., Petersen, O. W., and van Deurs, B. (1989). Immunotoxins–entry into cells and mechanism of action. *Immunol. Today*, **10**, 291–5.

Olsnes, S., Stenmark, H., Øivind Moskaug, J., McGill, S., Madshus, I. H. and Sandvig, K. (1990). Protein toxins with intracellular targets. *Microb. Pathog.*, **8**, 163–8.

Pelham, H. R. B., Roberts, L. M., and Lord, J. M. (1992). Toxin entry: how reversible is the secretory pathway? *Trends Cell Biol.*, **2**, 183–5.

Sandvig, K. and van Deurs, B. (1994*a*). Endocytosis and intracellular sorting of ricin and Shiga toxin. *FEBS Lett.*, **346**, 99–102.

Sandvig, K. and van Deurs, B. (1994*b*). Endocytosis without clathrin. *Trends Cell. Biol.*, **4**, 275–7.

van Deurs, B., Hansen, S. H., Olsnes, S., and Sandvig, K. (1993). Protein uptake and cytoplasmic access in animal cells. In *Biological barriers to protein delivery* (ed. K. L. Audus and T. J. Raub), pp. 71–104, Plenum Press, New York.

Wales, R., Chaddock, J. A., Roberts, L. M., and Lord, J. M. (1992). Addition of an ER retention signal to the ricin A chain increases the cytotoxicity of the holotoxin. *Exp. Cell Res.*, **203**, 1–4.

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Ribosome-inactivating proteins

Single-chain (type 1, as opposed to type 2, two-chain proteins) ribosome inactivating proteins are plant proteins possessing N-glycosidase activity which release adenine from rRNA, thus rendering ribosomes unable to perform proteins synthesis. They have antiviral properties and can be linked to antibodies from immunotoxins, selectively toxic to immunotoxins, selectively toxic to the cells recognized by the antibody.

Ribosome-inactivating proteins (RIPs, review by Barbieri *et al.* 1993) are a class of proteins present in various tissues of several plants which inactivate mammalian ribosomes and, with less activity and to variable extent, plant, fungal, and bacterial ribosomes. They are enzymes, *N*-glycosidases, which release adenine from rRNA. They are divided into two groups: type 1, consisting of a single peptide chain, and type 2, in which an A chain with enzymatic activity is linked to a slightly larger B chain with the properties of a galactosyl-specific lectin. The B chain binds to galactosyl-terminated receptors on the membrane of most animal cells, allowing the molecule to enter the cytoplasm. Thus most type 2 RIPs are potent toxins, ricin being the best known, although recently some nontoxic lectins with the structure of type 2 RIPs have been described. Type 1 RIPs do not enter easily into cells, and consequently are much less toxic than type 2 RIPs.

Sources, purification, and properties

Type 1 RIPs are widely distributed in the plant kingdom (Barbieri *et al.* 1993) and can be found in several tissues (roots, stems, leaves, seeds, latex) of many plants. Their concentration in plant tissues is highly variable, being particularly high in plants belonging to some families (Caryophyllaceae, Phytolaccaceae, Euphorbiaceae, Cucurbitaceae).

They can be purified by ion-exchange chromatography (Barbieri *et al.* 1987), taking advantage of their high isoelectric point. In some cases, additional chromatography on Blue Sepharose or gel filtration may be required.

Type 1 RIPs are 30 kDa proteins. Most of them are glycoproteins, and have a p*I* in a strongly basic region, usually > 9.5. A number of them have been sequenced and cloned.

Mechanism of action

Ribosome-inactivating proteins are *N*-glycosidases, and damage eukaryotic ribosomes by breaking the N-glycosidic bond of adenine in a precise position of rRNA (A_{4324} in the case of rat liver ribosomes), in a GAGA sequence in a loop at the top of a stem (review by Endo 1988). This renders ribosomes unable to bind elongation factor 2 (in same cases also elongation factor 1), thus arresting protein synthesis. RIPs act also on bacterial ribosomes and on purified rRNA, although at concentrations much higher than those effective on mammalian ribosomes. Some RIPs remove more than one adenine from all types of RNA tested, as well as from poly(A) and from DNA (Barbieri *et al.* 1994).

Toxicity and cytotoxicity

Type 1 RIPs do not have the B chain with lectin properties of type 2 RIPs, and consequently do not bind to cells and enter less easily into them. Thus they are much less toxic to cells and animals than type 2 RIPs: their LD_{50} for mice is of the order of mg/kg of body weight. They are very potent inhibitors of cell-free protein synthesis, with IC_{50}s (concentration causing 50 per cent inhibition of protein synthesis by HeLa cells) in the range 0.2–9.2 μM. Some cells (macrophages, trophoblasts, some malignant cell lines) are more sensitive to RIPs.

Biological properties

Single-chain ribosome-inactivating proteins have antiviral activity against both plant and animal viruses. It is commonly accepted that they act by entering more easily into cells infected by viruses, thus inactivating ribosomes and killing the cells, with consequent arrest of viral proliferation. They have also immunosuppressive activity, possibly due to killing of macrophages, for which they have a higher toxicity.

Possible uses

Ribosome-inactivating proteins can be linked to antibodies or other appropriate carriers (growth factors, hormones) to make immunotoxins or other conjugates selectively toxic to the cells recognized by the carrier (Frankel 1988; Magerstädt 1991). Immunotoxins can be used to selectively eliminate a given type of cells *in vitro* and *in vivo*, and are being experimented

(1) to remove contaminant cells from cultures;

(2) to remove malignant or immunocompetent cells *ex vivo* from bone marrow to be transplanted; and

(3) in clinical trials to treat patients with malignant tumours (Vallera 1994; Siegall *et al.* 1995).

Immunotoxins and other conjugates are used also to cause selective lesions in the central nervous system (Wiley and Lappi 1994).

Plants have been transfected with RIP genes to confer them with resistance to viruses (Lodge *et al.* 1993) or fungi (Logemann *et al.* 1992).

References

Barbieri, L., Stoppa, C., and Bolognesi, A. (1987). Large scale chromatographic purification of ribosome-inactivating proteins. *J. Chromatogr.*, **408**, 235–43.

Barbieri, L., Battelli, M. G. and Stirpe, F. (1993). Ribosome-inactivating proteins from plants. *Biochim. Biophys. Acta*, **1154**, 237–82.

Barbieri, L., Gorini, P., Valbonesi, P., Castiglioni, P., and Stirpe, F. (1994). Unexpected activity of saporins. *Nature*, **372**, 624.

Endo,Y. (1988). Mechanism of action of ricin and related toxins on the inactivation of eukaryotic ribosomes. In: *Immunotoxins* (ed. A. E. Frankel), pp. 75–89, Kluwer Academic, Boston.

Frankel, A. E. (ed.) (1988). *Immunotoxins*, Kluwer Academic, Boston.

Lodge, J. K., Kaniewski, W. K., and Tumer, N. E. (1993). Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. *Proc. Natl. Acad. Sci. USA*, **90**, 7089–93.

Logemann, J., Jach, G., Tommerup, H., Mundy, J., and Schell, J. (1992). Expression of a barley ribosome-inactivating protein leads to increased fungal protection in transgenic tobacco plants. *BioITechnol.*, **10**, 305–8.

Magerstädt, M. (1991). Immunotoxins. In *Antibody conjugates and malignant disease*, pp. 151–93, CRC Press, Boca Raton, Florida.

Siegall, C. B., Wolff, E. A., Gawlak, S. L., Paul, L., Chace, D., and Mixan, B. (1995). Immunotoxins as cancer chemotherapeutic agents. *Drug Develop. Res.*, **34**, 210–19.

Vallera, D. A. (1994). Immunotoxins: will their clinical promise be filled? *Blood*, **83**, 309–17.

Wiley, R. G. and Lappi, D. A. (1994). *Suicide transport and immunolesioning*, R. G. Landes Company, Austin.

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α-Sarcin and related toxins (*Aspergillus*)

α-Sarcin is a nonglycosylated single polypeptide chain of 17 kDa secreted by the mould Aspergillus giganteus. *It binds cell membrane and enters the cytosol where it specifically cleaves a single phosphodiester bond of the 28S rRNA, that causes ribosome disruption and impairs protein biosynthesis. This protein belongs to a family of fungal cytotoxic ribonucleases composed of mitogillin (produced by* A. restrictus*), restrictocin (from* A. restrictus*) and Asp fl (from* A. fumigatus*).*

Aspergillins are a family of toxic extracellular fungal ribonucleases displaying a high amino acid sequence homology (Table 1). α-Sarcin, mitogillin, and restrictocin were discovered and characterized as antitumoral agents displaying *in vivo* cytotoxity (Olson and Goerner 1965; Goldin *et al.* 1966). However, their clinical use was hampered since they revealed to be too toxic (Roga *et al.* 1971). Asp fl was recently discovered as an IgE-binding protein involved in the pathogenesis of invasive aspergillosis (Arruda *et al.* 1990; Lamy *et al.* 1991).

Aspergillins are encoded by a single gene that is translated into a precursor with a 27 residue amino terminal leader sequence that is removed upon maturation (Oka *et al.* 1990; Lamy and Davies 1991; Moser *et al.* 1992). The mechanism by which host ribosomes are protected from the toxin catalysis has not been deciphered yet although

Table 1 Aspergillins

Aspergillin	Source	Amino acid residues	Swiss Prot. Data Bank accession number	Gen Bank accession number
α-Sarcin	*A. giganteus* MDH 18894	150	P00655	X53394 (*sar*)
Mitogillin	*A. restrictus* NRRL 3050	149	PO4389 P19792	M94249 (*mif*)
Restrictocin	*A. restrictus* ATCC 34475, NRLL 2869	149	PO4389 P19792	M65257 (*res*)
Asp fl	*A. fumigatus* ATCC 42202, CBS 143.89	149	PO4389 P19792	M83781 (*asp fla*)

several hypotheses have been proposed (Lamy and Davies 1991). The mature form consists of a highly polar single polypeptide chain of 149 amino acid residues (150 in α-sarcin) that contains two intramolecular disulfide bonds and is rich in basic residues (Martínez del Pozo *et al.* 1988; Mancheño *et al.* 1995). The protein folds into a single αβ domain that exhibits a highly specific ribonucleolytic activity and the ability to interact with acid phospholipids (Gasset *et al.* 1994). Active site residues have been identified based on sequence similarities with other nontoxic phylogenetically related ribonucleases (Martínez del Pozo *et al.* 1988; Mancheño *et al.* 1995). Positive-charged loops and a theoretically predicted hydrophobic antiparallel β-sheet structurally support the electrostatic and hydrophobic interactions with membranes (Mancheño *et al.* 1995).

Purification and sources

α-Sarcin, mitogillin, restrictocin, and Asp fl are isolated and purified to homogeneity from culture supernatants of the respective *Aspergillus* genus producing strains by two chromatographic steps (Arruda *et al.* 1990; Gasset *et al.* 1994 and references therein). Several expression systems with suitable purification protocols for recombinant wild type and mutant protein forms are also available for all members of the family (Lamy and Davies 1991; Better *et al.* 1992; Moser *et al.* 1992; Lacadena *et al.* 1994). Purity assessment by SDS-PAGE and activity determination are recommended. Specific ribonuclease activity is ascertained by the appearance of α-fragment from the 28S rRNA of eukaryotic ribosomes using cell-free rabbit reticulocyte lysates (Endo and Wool 1982).

Toxicity

α-Sarcin *in-vivo* toxicity is tested by intraperitoneal injection of different amounts of toxin in Balb/c mice (LD_{50} = 12 mg/kg) (Olmo and Lizarbe, in preparation). Mitogillin LD_{50} was determined following a 24 i.v. daily injection protocol (LD_{50} = 0.17 mg/kg/day; dog LD_{50} = 0.035 mg/kg/day; monkey LD_{50} = 0.06 mg/kg/day) (Roga *et al.* 1971). Concentrations below the LD_{50} successfully inhibit the growth of different animal tumours (Roga *et al.* 1971; Olmo and Lizarbe, in preparation). The use of these toxins does not present any problem for healthy operators. The recent incorporation of Asp f1 to the aspergillin family underlines their potential harmful behaviour as allergens (Arruda *et al.* 1990; Lamy *et al.* 1991).

Use in cell biology

The cytotoxic action exhibited by these proteins is due to their ability to enter cells and hydrolyse a single phosphodiester bond on the 3′side of G4325 in eukaryotic 28S RNA, that results in protein biosynthesis inhibition (Endo and Wool 1982; Wool 1984). This bond is located in a universal conserved region, known as the α-sarcin loop, involved in the EF-1-dependent binding of aminoacyl-tRNA and EF-2-catalysed GTP hydrolysis and consequently essential for ribosome function (Wool *et al.* 1992). This highly specific ribonuclease actvity has been very useful in determining ribosome structure and protein biosynthesis mechanism. The reason why these proteins are not sensitive to the action of intracellular ribonuclease inhibitors is still an open question.

Aspergillins were widely used to probe cell membrane permeability to macromolecules since no surface receptor was found (Alonso and Carrasco 1980; Fernández-Puentes and Carrasco 1980; Otero and Carrasco 1986, 1988). Notwithstanding, aspergillins enter mammalian transformed cells interfering the proliferation pattern, inhibiting protein biosynthesis, and leading to cell death (Moser *et al.* 1992; Olmo *et al.* 1993; Turnay *et al.* 1993). The IC_{50} for protein biosynthesis varies from 0.01–0.4 nM in cell-free systems to 0.3–10 μM in intact cells (Fando *et al.* 1985; Turnay *et al.* 1993). These serial events emphasize the potential use of aspergillins as apoptosis-inducers. Furthermore, incorporation of aspergillin encoding genes into suicide cassettes, under the control of highly regulated promoter, will be useful to establish the function of specific cells and tissues during development. The cytotoxic efficiency is dramatically enhanced when these proteins are covalently linked to anti-cell specific antibodies (Conde *et al.* 1989; Wawrzynczak *et al.* 1991; Better *et al.* 1992). Fluid phase endocytosis is probably the pathway into the cytosol (Olmo *et al.* 1993). Biophysical studies have shown that α-sarcin binds, inserts, and translocates across acid phospholipid-containing model membranes (Gasset *et al.* 1994).

References

Alonso M. A. and Carrasco, L. (1980). Permeabilization of mammalian cells to proteins by the ionophore nigericin. *FEBS Lett.*, **127**, 112–14.

Arruda, K. L., Platts-Mills, T. A. E., Fox, J. W., and Chapman, M. (1990). Aspergillus fumigatus allergen I, a major IgE-binding protein, is a member of the mitogillin family of cytotoxins. *Exp. Med.*, **172**, 1529–32.

Better, M., Bernhard, S. L., Lei, S.-P., Fishwild, D. M., and Carroll, S. F. (1992). Activity of the recombinant mitogillin and mitogillin immunoconjugates. *J. Biol. Chem.*, **267**, 16712–18.

Conde, F. P., Orlandi, R., Canevari, S., Mezzanzanica, D., Ripamonti, M., Muñoz, S. M., *et al.* (1989). The Aspergillus toxin restrictocin is a suitable cytotoxic agent for generation of immunoconjugates with monoclonal antibodies directed against human carcinoma cells. *Eur. J. Biochem.*, **178**, 795–802.

Endo, Y. and Wool, I. G. (1982). The site of action of α-sarcin on eukaryotic ribosomes. *J. Biol. Chem.*, **257**, 9054–60.

Fando, J. L., Alaba, I., Escarmis, C., Fernández-Luna, J. L., Mendez, E., and Salinas, M. (1985). The mode of action of restrictocin and mitogillin on eukaryotic ribosomes. Inhibition of brain protein synthesis, cleavage and sequence of the ribosomal RNA fragment. *Eur. J. Biochem.*, **149**, 29–34.

Fernández-Puentes, C. and Carrasco, L. (1980). Viral infection permeabilizes mammalian cells to protein toxins. *Cell*, **20**, 769–75.

Gasset, M., Mancheño, J. M., Lacadena, J., Turnay, J., Olmo, N., Lizarbe, M. A., *et al.* (1994). α-Sarcin, a ribosome-inactivating

protein that translocates across the membrane of phospholipid vesicles. *Curr. Topics Peptide Prot. Res* **1**, 99–104.
Goldin, A., Serpik, A., and Mantel, N. (1966). Experimental screening procedures and clinical predictability value. *Cancer Chem. Rep.*, **50**, 173–218.
Lacadena, J., Martínez del Pozo, A., Barbero, J. L., Mancheño, J. M., Gasset, M., Oñaderra, M., *et al.* (1994). Overproduction and purification of biologically active native fungal α-sarcin in *Escherichia coli. Gene,* **142**, 147–51.
Lamy, B. and Davies, J. (1991). Isolation and nucleotide sequence of the *Aspergillus restrictus* gene coding for the ribonucleolytic toxin restrictocin and its expression in *Aspergillus nidulans*: the leader sequence protects producing strains from suicide. *Nucleic Acid Res.,* **19**, 1001–6.
Lamy B., Moutaouakil, M., Latge, J.-P., and Davies, J. (1991). Secretion of a potential virulence factor, a fungal ribonucleotoxin, during human aspergillosis infections. *Mol. Microbiol.*, **5**, 1811–15.
Mancheño, J. M., Gasset, M., Lacadena, J., Martínez del Pozo, A., Oñaderra, M., and Gavilanes, J. G. (1995). Predictive study of the conformation of the cytotoxic protein α-sarcin. A structural model to explain α-sarcin–membrane interaction. *J. Theor. Biol.*, **172**, 259–67.
Martínez del Pozo, A., Gasset, M., Oñaderra M., and Gavilanes, J. G. (1988). Conformational study of the antitumour protein α-sarcin. *Biochim. Biophys. Acta,* **953**, 280–8.
Moser, M., Crameri, R., Menz, G., Schneider, T., Dudler, T., Virchow, C., *et al.* (1992). Cloning and expression of recombinant *Aspergillus fumigatus* allergen I/a (rAsp f I/a) with IgE binding and type I skin test activity. *J. Immunol.*, **149**, 454–60.
Oka, T., Natori, Y., Tanaka, S., Tsuguri, K., and Endo, Y. (1990). Complete nucleotide sequence of cDNA for the cytotoxin α-sarcin. *Nucleic Acid Res.*, **18**, 1897.
Olmo, N., Turnay, J., Lizarbe, M. A., and Gavilanes, J. G. (1993). Cytotoxic effect of α-sarcin, a ribosome inactivating protein, in culture Rugli cells. *S.T.P. Pharma Sci.*, **3**, 93–6.
Olson, B. H. and Goerner, G. L. (1965). α-Sarcin, a new antitumor agent. I. Isolation, purification, chemical composition and the identity of a new amino acid. *Appl. Microbiol.*, **13**, 314–21.
Otero, M. J. and Carrasco, L. (1986). External ATP permeabilizes transformed cells to macromolecules. *Biochem. Biophys. Res. Commun.*, **134**, 453–60.
Otero, M. J. and Carrasco, L. (1988). Exogenous phospholipase C permeabilizes mammalian cells to proteins. *Exp. Cell Res.*, **177**, 154–61.
Roga, V., Hedeman, L. P., and Olson, B. H. (1971). Evaluation of mitogillin (NSC-629529) in the treatment of naturally occurring canine neoplasms. *Cancer Chemother. Rep. Part 1,* **55**, 101–13.
Turnay, J., Olmo, N., Jiménez, A., Lizarbe, M. A., and Gavilanes, J. G. (1993). Kinetic study of the cytotoxic effect of α-sarcin, a ribosome inactivating protein from *Aspergillus giganteus* on tumour cell lines: protein biosynthesis inhibition and cell binding. *Mol. Cell. Biochem.*, **122**, 39–47.
Wawrzynczak, E. J., Henry, R. V., Cumber, A. J., Parnell, G. D., Derbyshire, E. J., and Ulbrich, N. (1991). Biochemical, cytotoxic and pharmacokinetic properties of an immunotoxin composed of a mouse monoclonal antibody Fib75 and the ribosome-inactivating protein α-sarcin from *Aspergillus giganteus. Eur. J. Biochem.*, **196**, 203–9.
Wool, I. G. (1984). The mechanism of action of the cytotoxic nuclease α-sarcin and its use to analyze ribosome structure. *Trends Biochem. Sci.*, **9**, 14–17.
Wool, I. G., Glück, A., and Endo, Y. (1992). Ribotoxin recognition of ribosomal RNA and a proposal for the mechanism of translocation. *Trends Biochem. Sci.*, **17**, 266–9.

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Cytoskeleton-affecting toxins

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Introduction

Cytoskeleton

Many cellular functions depend on the cytoskeleton. The cytoskeleton controls the shape and the spatial organization of the cell. It participates in all kinds of cellular movement and transport and is involved in processes like endo- and exocytosis, vesicle transport, cell–cell contact, and mitosis. The cytoskeleton consists of a fibre network that is formed of three major filament systems which are highly dynamic structures: the microfilaments, the microtubules, and the intermediate filaments. Rapid structural changes of these cytoskeletal proteins are based on their ability to polymerize and depolymerize. Much of what we know about functions of these proteins has been learned from the use of cytotoxins which have been applied as cell biological tools. The group of cytoskeleton-affecting toxins comprises agents which act directly on elements of the cytoskeleton but also toxins that affect regulatory components (e.g. low molecular mass GTP-binding proteins) which control the organization of the cytoskeleton. The toxins can be subdivided into protein toxins with enzymatic activity, protein toxins without apparent enzymic activity, and small non-proteinaceous compounds which bind to the cytoskeleton proteins. The bacterial protein toxins appear to act predominantly on the actin cytoskeleton.

Microfilaments and toxins

Microfilaments have a diameter of 7–9 nm and are built of polymerized actin. Actin is a 42 kDa protein of 375 amino acids which consists of four domains (I, II, III, IV), binds Mg^{2+} and ADP or ATP, and possesses ATPase activity (Kabsch and Vandekerckhove 1992). At least six actin isoforms are known in mammals, which are more than 95 per cent identical. For the cytoskeleton nonmuscle β/γ-actin appears to be most important.

Actin filaments are organized in linear bundles, two-dimensional networks, and three-dimensional gels. Actin is spread all over the cells but the highest concentration is submembraneously located forming the cell cortex. In cultured cells (e.g. fibroblasts), actin filaments are basically involved in the formation of finger-like spikes (filopodia) and broad membrane sheets (lamellipodia). These processes start from the leading edge and attach to the matrix by adhesion plaques. Moreover, independent from the matrix, actin filaments form ruffles. At these different sites, microfilaments are stabilized by a large family of actin-binding proteins. For example, at the adhesion plaques, actin is colocalized with vinculin, talin, tensin, and α-actinin (Burridge *et al.* 1988; Bretscher 1991).

Actin filaments are polar structures with a plus (fast-polymerizing) and a minus (slow-polymerizing) end. Deduced from the microscopic features of filaments after decoration with S1 subunits of myosin, actin filament ends are also known as barbed and pointed ends. At the plus end, actin tends to polymerize and at the pointed, it tends to depolymerize. Polymerization is largely affected by actin binding proteins like profilin and β-4-thymosine, which induce and inhibit ADP/ATP exchange, respectively, thereby stimulating or blocking polymerization (Nachmias 1993; Theriot and Mitchison 1993; Pollard *et al.* 1994). Moreover, various actin severing proteins have been identified which cut F-actin and regulate the length of actin filaments in a Ca^{2+} dependent or independent manner. Some of these proteins (e.g. gelsolin), keep bound to actin ends, thereby capping the filaments and blocking actin polymerization (Bershadsky and Vasiliev 1988; Vandekerckhove 1990).

Control of the actin cytoskeleton involves low molecular mass GTP-binding proteins of the Rho subtype family (Hall 1994; Machesky and Hall 1996; Narumiya 1996). Members of this protein family are RhoA,B,C, Rac1,2, Cdc42, RhoG, and TC10. Cdc42 appears to be involved in filopodia formation, Rac in membrane ruffling, Rho in formation of adhesion plaque and stress fibres. A regulatory cascade by these proteins has been suggested (Nobes and Hall 1995). The proteins are regulated by an inherent GTPase cycle (Bourne *et al.* 1990, 1991). In the active GTP-bound form the proteins interact with their effectors (e.g. protein kinases). The activated state is terminated by hydrolysis of GTP. Various regulatory proteins control the functions of Rho subtype proteins by blocking (GDI, guanine nucleotide dissociation inhibitor) or stimulating (GDS, guanine nucleotide dissociation stimulator) the nucleotide exchange or by activating the GTPase activity (GAP, GTPase activating proteins).

Actin is directly modified by *Clostridium botulinum* C2 toxin and the related iota-like toxins (*C. perfringens* iota toxin), which ADP-ribosylate the protein at Arg-177 thereby inhibiting actin polymerization and causing depolymerization of microfilaments (Aktories and Wegner 1992). These binary toxins are constructed according the A,B model and consist of a biologically active component (A) and a binding component (B), which is important for the binding at the cell surface and the translocation into the cell. In contrast to other A,B-toxins, the components of these binary toxins are separate proteins.

Other bacterial factors appear to act indirectly on the actin cytoskeleton. The so-called large clostridial cytotoxins (*C. difficile* toxins, *C. sordellii* toxins, and the α-toxin from *C. novyi*) and the C3-toxins modify small GTP-binding proteins (Rho proteins) which are involved

in the regulation of the actin cytoskeleton by glycosylation and ADP-ribosylation, respectively (Aktories *et al.* 1992; Just *et al.* 1995). The cytotoxic necrotizing toxins (CNF1 and CNF2) from *E. coli* induce actin polymerization apparently by modification of Rho proteins (Oswald *et al.* 1994). ActA, which is produced by the *Listeria monocytogenes* is a bacterial cell surface protein which induces F-actin formation and enables the bacteria to move intracellularly (Domann *et al.* 1992). The zonula occludens toxin (ZOT) from *Vibrio cholerae* is an enterotoxin which acts on epithelial tight junctions to increase permeability (Baudry *et al.* 1992).

The nonproteinaceous toxins, which act on the actin cytoskeleton are cytochalasins and phalloidin (Cooper 1991). Cytochalasins are a family of fungal products which reversibly bind – somewhat similarly to capping proteins – to the barbed ends of actin filaments to block fast-actin polymerization. Like capping proteins they can nucleate polymerization. Therefore, in general, a complete depolymerization of actin is not achieved with cytochalasins. Cytochalasin B additionally inhibits sugar transport. Phallotoxins are cyclic oligopeptides which are isolated from *Amanita phalloides* (Wieland 1977). They bind to actin and decrease the critical concentration for actin polymerization thereby inducing and stabilizing microfilaments. Phallotoxins are not able to enter most cells but are taken-up by hepatocytes. Tagged with a fluorescence marker, they are widely used to stain F-actin by fluorescence microscopy (Faulstich *et al.* 1988). Jasplakinolide, which is a macrocyclic peptide produced by a marine sponge, decreases the critical concentration for actin polymerization in a manner similar to phallotoxins (Bubb *et al.* 1994). This agent is able to enter cells. Swinholde A is another cytotoxic agent from a marine sponge. This cytotoxin binds to actin dimers and severs F-actin (Bubb *et al.* 1995).

Table 1 Microfilament-affecting toxins
(a) Protein toxins

Toxin	Origin	Structure	Mechanism	Effect
C2 toxin Iota toxin Spiroforme toxin	*C. botulinum* type C,D *C. perfringens* *C. spiroforme*	Binary protein toxins	ADP-ribosylation of actin	Inhibition of actin polymerization, depolymerization of F-actin
Toxin A Toxin B Lethal (LT) toxin	*C. difficile* *C. difficile* *C. sordellii*	Single chain protein toxins	Glucosylation of Rho, Rac, Cdc42 (LT also Ras, Rap, Ral, but not Rho)	Depolymerization of F-actin in cells
α-Toxin	*C. novyi*		*N*-acetylglucose-aminylation of Rho, Rac ,Cdc42	Depolymerization of F-actin in cells
C3 toxin Limosum toxin EDIN Cereus toxin	*C. botulinum* type C,D *C. limosum* *S. aureus* *B. cereus*	Single chain toxins, no binding component	ADP-ribosylation of Rho	Depolymerization of F-actin in cells
Cytotoxic necrotizing factor 1 and 2 (CNF1,2)	*E. coli*	Single chain protein toxins	Modification of Rho proteins (?)	Actin polymerization in cells
Zonula occludens toxin (ZOT)	*V. cholerae*	Enterotoxin	Unknown	Increase in permeability of tight junctions
ActA	*Listeria monocytogenes*	Bacterial cell surface protein	Unknown	Polymerization of actin in cells

(b) Nonproteinaceous toxins

Toxin	Origin	Structure	Mechanism	Effect
Cytochalasins (A,B,C,D,E,H)	*Aspergillus clavatus* and various other fungi	Macrolide-like isoindol derivatives	Actin binding, capping function	Inhibition of actin polymerization
Phallotoxins Virotoxins	*Amanita phalloides*	Bicyclic heptapeptides	actin binding, decrease in the critical concentration of actin	Polymerization of actin
Jasplakinolide	*Jaspis johnstoni*	Cyclic peptide	Actin binding, decrease in the critical concentration of actin	Polymerization of actin
Swinholde A	*Theonella swinhoi*	Macrolide	Severing of F-actin	Depolymerization of F-actin

Microtubules and toxins

Microtubules have a diameter of 25 nm and consist of tubulin α/β heterodimers, which are often organized in 13 linear protofilaments forming a cylindric structure (Bershadsky and Vasiliev 1988). Therefore microtubules are more rigid than actin filaments. In mammals several tubulin isoforms exist, they consist of ~450 amino acids showing ~40 per cent identity. Whereas α-tubulin binds nonexchangeable GTP, β-tubulin has bound exchangeable GTP which is hydrolysed during polymerization. Microtubules are polar structures and contain a plus (fast-growing) and a minus (slow-growing or depolymerizing) end. Microtubules form parallel and radial arrays which often start at the microtubule organization centre (MTOC, centrosome). Various microtubule associated proteins (MAPs) modify the dynamic properties of tubulin. Microtubules are the scaffold for kinesins and dyneins, which are motor proteins for the intracellular transport to the plus and minus end, respectively (Gelfand and Bershadsky 1991).

In contrast to actin, no protein toxins are known which specifically affect microtubules. However, microtubules are targets for various nonproteinaceous toxins. Colchicine an alkaloid from the plant *Colchicum autumnale*, the related colcemid, nocadazole, podophyllotoxin, and vinca alkaloids inhibit polymerization or induce depolymerization of microtubules (Bergen and Borisy 1983; Goddette and Frieden 1986; Jordan *et al.* 1986). In contrast, taxol, a complex diterpene, isolated from certain types of the yew tree (*Taxus brevifolia*) induces tubulin polymerization even in the absence of GTP and microtubule-associated proteins, which are usually necessary for microtubule formation (Horwitz 1992).

Intermediate filaments

Intermediate filaments have a diameter of 10 nm and are built by a large spectrum of different protein classes (acidic and basic cytokeratins, vimentin/desmin, neurofilaments, and lamins), which form α-helical structures which assemble to rope-like fibres. Intermediate filaments are most important for the mechanical support of the cell and plays major role in cell–cell and cell–matrix contact. No evidence exists that intermediate filaments participate in motile functions (Bershadsky and Vasiliev 1988; Fuchs and Weber 1994).

So far, no toxins were shown to inhibit the functions of intermediate filaments. However, secondary to microfilament disruption by bacterial toxin, intermediate filaments are redistributed (Wiegers *et al.* 1991). The intermediate filament vimentin was identified to be an *in vitro* substrate for ADP-ribosylation by *Pseudomonas* exoenzyme S, which also modifies several different small GTP-binding proteins (Coburn *et al.* 1989).

References

Aktories, K. and Wegner, A. (1992). Mechanisms of the cytopathic action of actin-ADP-ribosylating toxins. *Mol. Microbiol.*, **6**, 2905–8.

Aktories, K., Mohr, C., and Koch, G. (1992). Clostridium botulinum C3 ADP-ribosyltransferase. *Curr. Top. Microbiol. Immunol.*, **175**, 115–31.

Baudry, B., Fasano, A., Ketley, J., and Kaper, J. B. (1992). Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. *Infect. Immun.*, **60**, No.2, 428–34.

Bergen, L. G. and Borisy G. G. (1983). Tubulin–colchicine complex inhibits microtubule elongation at both plus and minus ends. *J. Biol. Chem.*, **258**, 4190–4.

Bershadsky, A. D. and Vasiliev, J. M. (1988). *Cytoskeleton*, Plenum Press, New York.

Bourne, H. R., Sanders, D. A., and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. *Nature*, **348**, 125–32.

Bourne, H. R., Sanders, D. A., and McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature*, **349**, 117–27.

Bretscher, A. (1991). Microfilament structure and function in the cortical cytoskeleton. *Annu. Rev. Cell Biol.*, **7**, 337–74.

Bubb, M. R., Senderowicz, A. M., Sausville, E. A., Duncan, K. L. K., and Korn, E. D. (1994). Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competively inhibits binding of phalloidin to F-actin. *J. Biol. Chem.*, **269**, 14869–71.

Bubb, M. R., Spector, I., Bershadsky, A. D., and Korn, E. D. (1995). Swinholide A is a microfilament disrupting marine toxin that stabilizes actin dimers and severs actin filaments. *J. Biol. Chem.*, **270**, 3463–6.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988). Focal adhesions: Transmembrane junctions between the

Table 2 Microtubule affecting toxins

Toxin	Origin	Structure	Mechanism	Effect
Taxol	*Taxus brevifolia*	Complex diterpene derivative	Tubulin binding	Polymerization of tubulin
Colchicine Colcemid	*Colchicum autumnale*	Tropolone derivative	Tubulin binding	Depolymerization of tubulin
Vinca alkaloids (e.g.Vinblastin)	*Catharanthus roseus*	Monoterpene-indole alkaloid compl.	Tubulin binding	Depolymerization of tubulin
Podophyllotoxins	*Podophyllum peltatum*	Phenylpropane derivative	Tubulin binding	Depolymerization of tubulin
Nocodazol		Benzimidazole derivative	Tubulin binding	Depolymerization of tubulin

extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.*, **4**, 487–525.
Coburn, J., Dillon, S. T., Iglewski, B. H., and Gill, D. M. (1989). Exoenzyme S of *Pseudomonas aeruginosa* specifically ADP-ribosylates the intermediate filament protein vimentin. *Infect. Immun.*, **57**, 996–8.
Cooper, J. M. (1991). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.*, **105**, 1473–8.
Domann, E., Wehland, J., Rohde, M., Pistor, S., Hartl, M., Goebel, W., *et al.* (1992). A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. *EMBO J.*, **11**, 1981–90.
Faulstich, H., Zobeley, S., Rinnerthaler, G., and Small, J. V. (1988). Fluorescent phallotoxins as probes for filamentous actin. *J. Muscle Res. Cell Motil.*, **9**, 370–83.
Fuchs, E. and Weber, K. (1994). Intermediate filaments: Structure, dynamics, function, and disease. *Annu. Rev. Biochem.*, **63**, 345–82.
Gelfand, V. I. and Bershadsky, A. D. (1991). Microtubule dynamics: mechanism, regulation, and function. *Annu. Rev. Cell Biol.*, **7**, 93–116.
Goddette, D. W. and Frieden, C. (1986). The kinetics of cytochalasin D binding to monomeric actin. *J. Biol. Chem.*, **261**, 15970–3.
Hall, A. (1994). Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annu. Rev. Cell Biol.*, **10**, 31–54.
Horwitz, S. B. (1992). Mechanism of action of taxol. *Trends Pharmacol. Sci.*, **13**, 134–6.
Jordan, M. A., Margolis, R. L., Himes, R. H., and Wilson, L. (1986). Identification of a distinct class of vinblastine binding sites on microtubules. *J. Mol. Biol.*, **187**, 61–73.
Just, I., Selzer, J., Wilm, M., Von Eichel-Streiber, C., Mann, M.. and Aktories, K. (1995). Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature*, **375**, 500–3.
Kabsch, W. and Vandekerckhove, J. (1992). Structure and function of actin. *Annu. Rev. Biophys. Biophys. Chem.*, **21**, 49–76.
Machesky, L. M. and Hall, A. (1996). Rho: a connection between membrane receptor signalling and the cytoskeleton. *Trends Cell Biol.*, **6**. 304–10.
Nachmias, V. T. (1993). Small actin-binding proteins: The b-thymosin family. *Curr. Opin. Cell Biol.*, **5**, 56–62.
Narumiya, S. (1996). The small GTPase Rho: cellular functions and signal transduction. *J. Biochem.*, **120**, 215–28.
Nobes, C. D. and Hall, A. (1995). Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*, **81**, 53–62.
Oswald, E., Sugai, M., Labigne, A., Wu, H. C., Fiorentini, C., Boquet, P., *et al.* (1994). Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. *Proc. Natl. Acad. Sci. USA*, **91**, 3814–18.
Pollard, T. D., Almo, S., Quirk, S., Vinson, V., and Lattman, E. E. (1994). Structure of actin binding proteins: Insights about function at atomic resolution. *Annu. Rev. Cell Biol.*, **10**, 207–49.
Theriot, J. A. and Mitchison, T. J. (1993). The three faces of profilin. *Cell*, **75**, 835–8.
Vandekerckhove, J. (1990). Actin-binding proteins. *Curr. Opin. Cell Biol.*, **2**, 41–50.
Wiegers, W., Just, I., Müller, H., Hellwig, A., Traub, P., and Aktories, K. (1991). Alteration of the cytoskeleton of mammalian cells cultured *in vitro* by *Clostridium botulinum* C2 toxin and C3 ADP-ribosyltransferase. *Eur. J. Cell Biol.*, **54**, 237–45.
Wieland, T. (1977). Modification of actins by phallotoxins. *Naturwissenschaften*, **64**, 303–9.

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C2 toxin (*Clostridium botulinum* type C and D)

Clostridium botulinum *C2 toxin is a member of a family of binary cytotoxins that ADP-ribosylate monomeric G actin in arginine-177. Actin modification results in inhibition of actin polymerization and destruction of the actin cytoskeleton.*

C2 toxin is binary in structure and consists of a binding component (C2II, 100 kDa) and an enzyme component (C2I, 50 kDa) (Aktories *et al.* 1992). Both components are separated proteins. The gene of the enzyme component has been cloned recently (Fujii *et al.* 1996; EMBL Data Library D63903). C2II has to be activated by trypsin treatment to release an 75 kDa active fragment. C2II binds to a membrane receptor (not identified), which is present on all cell types studied so far. Binding of C2II induces a binding site for C2I. The toxin is taken-up by receptor-mediated endocytosis (Simpson 1989) (see Fig. 1). In artificial membranes, C2II induces cation selective and voltage-dependent channels (Schmid *et al.* 1994). C2I is an ADP-ribosyltransferase ($K_{m,NAD}$ = 4 μM) (Aktories *et al.* 1986) that modifies monomeric G-actin but not polymerized F-actin. Protein substrates are non-muscle β/γ actin and smooth muscle γ-actin, but not α-actin isoforms. Modification occurs specifically in

arginine-177 (Vandekerckhove *et al.* 1988), which is located in an actin–actin contact site. ADP-ribosylation inhibits actin polymerization (Aktories *et al.* 1986) and blocks actin ATPase activity. ADP-ribosylated actin binds to the 'fast-polymerizing' (barbed) ends of actin filaments in a capping protein-like manner to block polymerization of nonmodified actin (Wegner and Aktories 1988). ADP-ribosylated actin does not interact with the 'slow-polymerizing' (pointed) ends of actin filaments allowing depolymerization of actin (Fig. 1). Moreover, ADP-ribosylation of actin inhibits the nucleation activity of the gelsolin-actin complex (Wille *et al.* 1992).

Related to C2 toxin are *Clostridium perfringens* iota toxin, *Clostridium spiroforme* toxin, and an ADP-ribosyltransferase produced by *Clostridium difficile* (Considine and Simpson 1991). All these toxins are binary in structure. The gene for iota toxin has been sequenced (Perelle *et al.* 1993) [EMBL Data Library X73562]. The membrane receptors for iota toxin and C2 toxin are different. The binding components of *Clostridium perfringens* iota toxin and of *Clostridium spiroforme* toxin are interchangeable but not with that of C2II. Iota toxin modifies all actin isoforms including α-isoforms in Arg-177.

Purification and sources

The purification procedure reported for C2 toxin from *Clostridium botulinum* type C strain 92-13 (produces no neurotoxin) is based on ammonium sulfate precipitation, DEAE-Sephadex, CM-Sephadex, and gel filtration (Ohishi *et al.* 1980). We isolate C2II by CM-sephadex and gel filtration. C2I is purified from the ammonium sulfate precipitate by isoelectric focusing (*pI* ~4.5) and gel filtration.

Toxicity

The single components of C2 toxin are almost nontoxic. The LD_{50} of the activated holotoxin (C2I plus C2II, ratio 1:2) is about 5 and 50 ng per mouse for i.v. and i.p. administration, respectively (Ohishi *et al.* 1980). In rats C2 toxin induces hypotension, hemorrhaging and fluid accumulation into the lungs (Simpson 1982). Major toxic effects may be explained by massive increase in vascular permeability. C2 toxin is not a clostridial neurotoxin and plays no role in botulism. In intact cells, C2 toxin induces rounding-up of cells, destruction of the microfilament network, and complete depolymerization of actin filaments (Reuner *et al.* 1987). Effects on actin cytoskeleton occur with a latency (toxin up-take) of about 30 min.

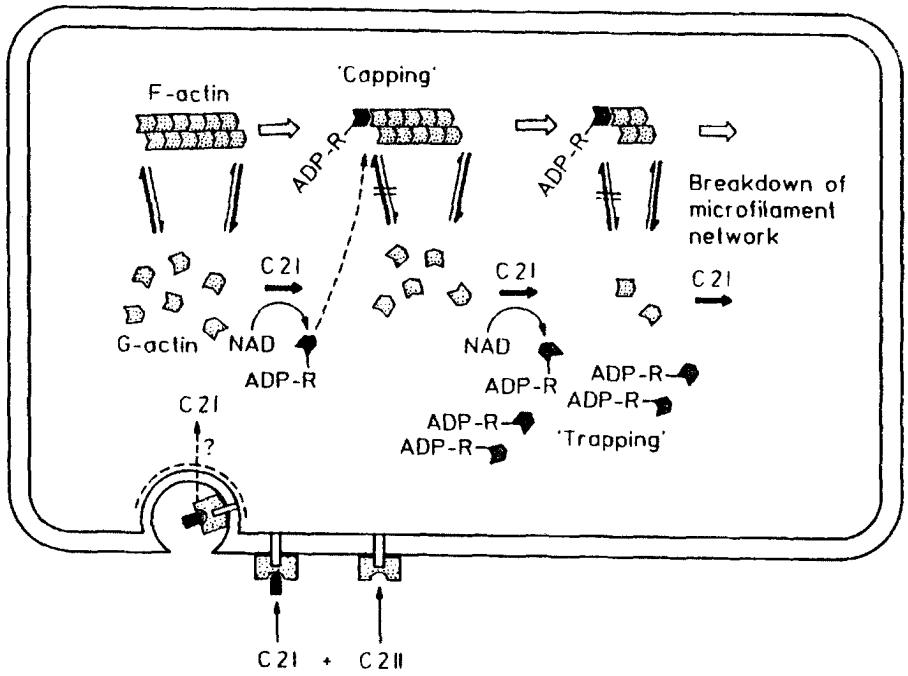

Figure 1. Model of the cytopathic effects of C2 toxin. The activated binding component (C2II) binds to the cell membrane receptor and induces the binding site for the enzyme component (C2I). After receptor-mediated endocytosis and translocation of C2I into the cytoplasma, actin is ADP-ribosylated. ADP-ribosylation inhibits actin polymerization. Furthermore, ADP-ribosylated actin acts like a capping protein to inhibit polymerization at the barbed ends of filaments. Depolymerization of F-actin occurs at the pointed ends of microfilaments (from Aktories *et al.* 1992).

Use in cell biology

C2 toxin is the most effective agent to depolymerize F-actin in intact cells (Aktories and Wegner 1992) and is applied to study the functional roles of the actin cytoskeleton (Aktories *et al.* 1992). In neutrophil leukocytes, C2 toxin inhibits migration and increases superoxide anion production and enzyme secretion induced by chemotactic agents (e.g. formyl-methionine-leucine-phenylalanine, FMLP) (Norgauer *et al.* 1988). FMLP-induced actin polymerization is inhibited and F-actin content is decreased by > 75 per cent. Endocytosis of the *N*-formyl peptide receptor is slowed down but still possible. C2 toxin augments ligand-induced phosphoinositide hydrolysis and increases diacylglycerol production several-fold and largely affects production of lipid mediators (Grimminger *et al.* 1991). The effects of C2 toxin were studied on catecholamine release in PC-12 cells (Matter *et al.* 1989), on steroid release in Y-1 cells (Considine *et al.* 1992), and on insulin secretion in pancreatic islets and HIT-T15 cells (Li *et al.* 1994). C2 toxin increases permeability and hydraulic conductivity in monolayers of endothelium cells (Suttorp *et al.* 1991). In isolated longitudinal muscle of guinea pig ileum, smooth muscle contraction is inhibited by C2 toxin (Mauss *et al.* 1989).

References

Aktories, K. and Wegner, A. (1992). Mechanisms of the cytopathic action of actin-ADP-ribosylating toxins. *Mol. Microbiol.*, **6**, 2905–8.

Aktories, K., Bärmann, M., Ohishi, I., Tsuyama, S., Jakobs, K. H., and Habermann, E. (1986). Botulinum C2 toxin ADP-ribosylates actin. *Nature*, **322**, 390–2.

Aktories, K., Wille, M., and Just, I. (1992). Clostridial actin-ADP-ribosylating toxins. *Curr. Top. Microbiol. Immunol.*, **175**, 97–113.

Considine, R. V. and Simpson, L. L. (1991). Cellular and molecular actions of binary toxins possessing ADP-ribosyltransferase activity. *Toxicon*, **29**, 913–36.

Considine, R. V., Simpson, L. L., and Sherwin, J. R. (1992). Botulinum C_2 toxin and steroid production in adrenal Y-1 cells: The role of microfilaments in the toxin-induced increase in steroid release. *J. Pharmacol. Exp. Ther.*, **260**, 859–64.

Fujii, N., Kubota, T., Shirakawa, S., Kimura, K., Ohishi, I., Moriishi, K., Isogai, E., and Isogai, H. (1996). Charaterization of component-I gene of botulinum C2 toxin and PCR detection of its gene in clostridial species. *Biochem. Biophys. Res. Commun.*, **220**, 353–9.

Grimminger, F., Sibelius, U., Aktories, K., Suttorp, N., and Seeger, W. (1991). Inhibition of cytoskeletal rearrangement by botulinum C2 toxin amplifies ligand-evoked lipid mediator generation in human neutrophils. *Mol. Pharmacol.*, **40**, 563–71.

Li, G., Rungger-Brändle, E., Just, I., Jonas, J.-C., Aktories, K., and Wollheim, C. B. (1994). Effect of disruption of actin filaments by *Clostridium botulinum* C2 toxin on insulin secretion in HIT-T15 cells and pancreatic islets. *Mol. Biol. Cell*, **5**, 1199–213.

Matter, K., Dreyer, F., and Aktories, K. (1989). Actin involvement in exocytosis from PC12 cells: studies on the influence of botulinum C2 toxin on stimulated noradrenaline release. *J. Neurochem.*, **52**, 370–6.

Mauss, S., Koch, G., Kreye, V. A. W., and Aktories, K. (1989). Inhibition of the contraction of the isolated longitudinal muscle of the guinea-pig ileum by botulinum C2 toxin: Evidence for a role of G/F-actin transition in smooth muscle contraction. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **340**, 345–51.

Norgauer, J., Kownatzki, E., Seifert, R., and Aktories, K. (1988). Botulinum C2 toxin ADP-ribosylates actin and enhances O^{2-} production and secretion but inhibits migration of activated human neutrophils. *J. Clin. Invest.*, **82**, 1376–82.

Ohishi, I., Iwasaki, M., and Sakaguchi, G. (1980). Purification and characterization of two components of botulinum C2 toxin. *Infect. Immun.*, **30**, 668–73.

Perelle, S., Gibert, M., Boquet, P., and Popoff, M. R. (1993). Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. *Infect. Immun.*, **61**, 5147–56.

Reuner, K. H., Presek, P., Boschek, C. B., and Aktories, K. (1987). Botulinum C2 toxin ADP-ribosylates actin and disorganizes the microfilament network in intact cells. *Eur. J. Cell Biol.*, **43**, 134–40.

Schmid, A., Benz, R., Just, I., and Aktories, K. (1994). Interaction of *Clostridium botulinum* C2 toxin with lipid bilayer membranes: formation of cation-selective channels and inhibition of channel function by chloroquine and peptides. *J. Biol. Chem.*, **269**, No.24, 16706–11.

Simpson, L. L. (1982). A comparison of the pharmacological properties of *Clostridium botulinum* type C1 and C2 toxins. *J. Pharmacol. Exp. Ther.*, **223**, 695–701.

Simpson, L. L. (1989). The binary toxin produced by *Clostridium botulinum* enters cells by receptor-mediated endocytosis to exert its pharmacologic effects. *J. Pharmacol. Exp. Ther.*, **251**, 1223–8.

Suttorp, N., Polley, M., Seybold, J., Schnittler, H., Seeger, W., Grimminger, F., *et al.* (1991). Adenosine diphosphate-ribosylation of G-actin by botulinum C2 toxin increases endothelial permeability *in vitro*. *J. Clin. Invest.*, **87**, 1575–84.

Vandekerckhove, J., Schering, B., Bärmann, M., and Aktories, K. (1988). Botulinum C2 toxin ADP-ribosylates cytoplasmic b/v-actin in arginine 177. *J. Biol. Chem.*, **263**, 696–700.

Wegner, A. and Aktories, K. (1988). ADP-ribosylated actin caps the barbed ends of actin filaments. *J. Biol. Chem.*, **263**, 13739–42.

Wille, M., Just, I., Wegner, A., and Aktories, K. (1992). ADP-ribosylation of the gelsolin–actin complex by clostridial toxins. *J. Biol. Chem.*, **267**, 50–5.

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Cytotoxic necrotizing factors *(Escherichia coli)*

Cytotoxic necrotizing factors (CNFs) are single-chain proteins of about 110 kDa elaborated by a number of pathogenic E. coli *strains. CNFs induce ruffling and stress fibre formation into cultured cells probably by activating the p21 Rho G-protein. Stimulation of actin assembly leads to the block of cytokinesis, resulting in multinucleation.*

Certain *Escherichia coli* strains belonging to the normal microflora present in the gastrointestinal tract, have been often associated with gastroenteritis, urogenital infections, septicemia, and bacteremia in both humans and animals. Among these pathogenic *E. coli,* a number of strains have been described as producing new putative virulence factors named cytotoxic necrotizing factors type 1 and 2 (CNF1 and CNF2). The first type of CNF was described as a cell-associated product of *E. coli* strains isolated from children with diarrhea causing necrosis of rabbit skin and multinucleation in cultured cells (Caprioli *et al.* 1983). Subsequently, a second type of CNF was detected in extracts of *E. coli* strains isolated from calves with enteritis (De Rycke *et al.* 1987). Purified CNF1 (Caprioli *et al.* 1984) and CNF2 (Oswald and De Rycke 1990) are immunologically related and similar in molecular weights (113.7 and 110 kDa, respectively). However, the toxins are distinguishable by:

(1) the morphology of multinucleated cells induced by each toxin in HeLa cell cytotoxic assays;

(2) their different responses in cross-neutralization assays; and

(3) the specific necrotic activity of CNF2 in the mouse footpads (De Rycke *et al.* 1990). Experimental infections of neonatal calves and pigs (Wray *et al.* 1993) have shown that orally inoculated CNF1- or CNF2-producing *E. coli* cause septicemia, enteritis, and histological changes characteristic of toxemic effects in the brain, heart, liver, and kidney. These lesions were similar to those observed after the intravenous inoculation of purified CNF1 in lambs (De Rycke and Plassiart 1990).

Purification

CNF1 and CNF2 are accumulated in the cytosol of producing bacteria during the exponential growth phase. CNFs-producing strains grown in LB medium, were harvested at the end of the exponential growth phase, centrifuged, and resuspended in 50 mM sodium phosphate buffer (pH 7.4). Bacteria were then disrupted by French press, centrifuged at high speed, and resuspended; the supernatant was precipitated with 50 per cent ammonium sulfate. The purification scheme for both toxins involves five main steps, as reported in Donelli *et al.* (1994). Briefly, after DEAE ion exchange, the fractions with toxin activity were pooled and purified by two consecutive gel filtrations, first on ACA54 and then on ACA44 columns. After a passage through a C6-Sepharose column, the fractions containing CNFs activity were pooled and purified by a monoQ FPLC column. CNFs purity was assessed by SDS-PAGE.

Molecular genetics

CNF1 is chromosomally encoded (Falbo *et al.* 1992), whereas the determinant for CNF2 is located on a large transmissible F-like plasmid called Vir (Oswald and De Rycke 1990). CNF1-producing strains isolated from diarrheal diseases and extraintestinal infections in humans, calves, pigs, cats, and dogs (Caprioli *et al.* 1983, 1987; De Rycke *et al.* 1987; Prada *et al.* 1987; Cherifi *et al.* 1990; Blanco *et al.* 1993) produce an alpha-hemolysin and induce mannose resistant hemagglutination. DNA hybridization with probes derived from a Pap adhesin operon and isolation of cosmids with a DNA fragment containing both *cnf1*, the gene encoding CNF1, and the alpha-hemolysin operon (Falbo *et al.* 1992) support the hypothesis that the genes of these three virulence factors (alpha-hemolysin, Pap-like adhesin, and CNF1) are closely associated on the chromosome. Most of the CNF2-producing strains are nonhemolytic, do not possess a Pap-like adhesin but carry virulence determinants for colonization of the calf and lamb intestine (Falbo *et al.* 1993). In fact, CNF2-producing strains are only isolated from the blood of diarrheal stools of domestic polygastric animals (De Rycke *et al.* 1987; Oswald *et al.* 1991; Blanco *et al.* 1993).

Recently, the genes encoding CNFI *(cnf1)* and CNF2 *(cnf2)* have been cloned (Falbo *et al.* 1993; Oswald *et al.* 1994) and the sequences of both genes are available (cnf1: accession no. X70670; *cnf2*: accession no. U01097). Both toxins are encoded by a single structural gene with a low GC content (35 per cent). No classical signal sequence was found in the N-terminal 50 residues of these toxins. When the deduced amino acid sequences of the two toxins were compared, 85 per cent identical and

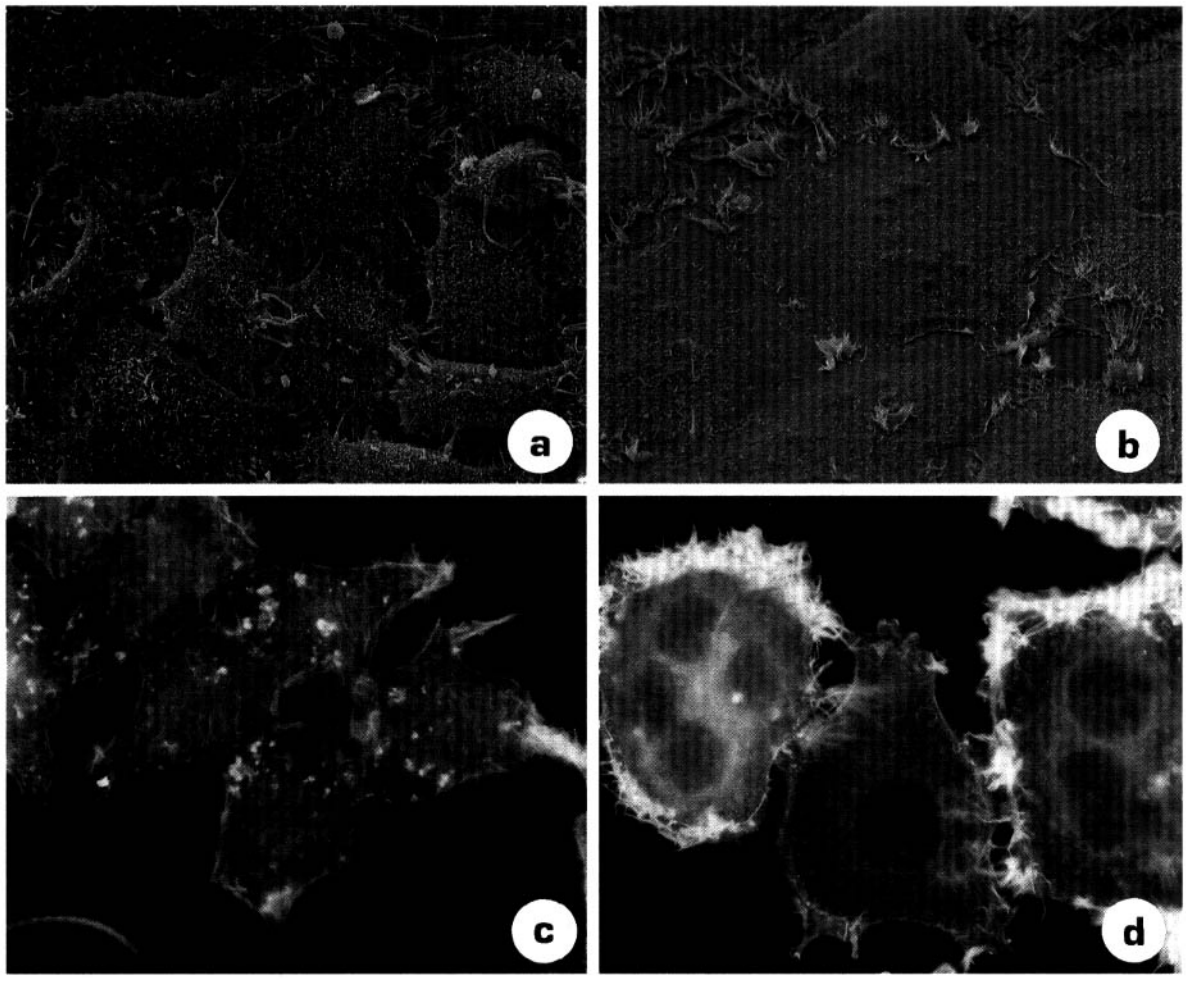

Figure 1. Scanning electron (a, b) and fluorescence (c, d) micrographs of HEp-2 cells treated with CNF1 (10^{-10}) M for 48 h. (Untreated (a, c) and CNF1-treated (b, d) cells.) Cells exposed to the toxin showed (b) the enlargement and flattening of the cell body, and (d) multinucleation accompanied by an intensive membrane ruffling positive for F-actin.

99 per cent conserved residues over 1014 amino acids were found. Nucleotide and protein data base searches showed significant homology between CNFs and the dermonecrotic toxin of *Pasteurella multocida* (Falbo *et al.* 1993; Oswald *et al.* 1994).

Use in cell biology

In cultured mammalian cells both CNFs act as classical bacterial toxins which have to be internalized by endocytosis in order to exert their effects (Falzano *et al.* 1993; Oswald *et al.* 1994). The two toxins are able to provoke a time- and dose-dependent increase in F-actin structures, inducing an intense and generalized ruffling activity and a remarkable increase in stress fibres (Fiorentini *et al.* 1988; Falzano *et al.* 1993; Oswald *et al.* 1994). Reorganization of the F-actin cytoskeleton by CNFs results in the inability of the cells to achieve correctly their cytokinesis and thus to divide normally, giving rise to extremely flat and large multinucleated cells.

Actin assembly/disassembly has been reported to be controlled by Rho proteins, a subgroup of small GTP-binding molecules belonging to the p21 Ras superfamily. Interestingly, CNFs are able to modify Rho by causing a shift of this protein towards a higher molecular weight (Oswald *et al.* 1994). A number of bacterial toxins, such as *Clostridium difficile* toxin B or *Clostridium botulinum* exoenzyme C3, are known to disrupt the actin cytoskeleton by inactivating the Rho protein (Donelli and Fiorentini 1994). However, in contrast to these toxins, CNF1 probably induces a covalent modification of Rho able to maintain this small GTP-binding protein in an

activated form, thereby promoting F-actin assembly in the cell (Fiorentini *et al.* 1995).

References

Blanco, J., Blanco, M., Wong, I., and Blanco, J. E. (1993). Haemolytic *Escherichia coli* strains isolated from stools of healthy cats produce cytotoxic necrotizing factor type 1 (CNF1). *Vet. Microbiol.*, **38**, 157–65.

Caprioli, A., Falbo, V., Roda, L. G., Ruggeri, F. M., and Zona, C. (1983). Partial purification and characterization of an *Escherichia coli* toxic factor that induces morphological cell alterations. *Infect. Immun.*, **39**, 1300–6.

Caprioli, A., Donelli, G., Falbo, V., Possenti, R., Roda, L. G., Roscetti, G., *et al.* (1984). A cell division-active protein from *E. coli*. *Biochem. Biophys. Res. Commun.*, **118**, 587–93.

Caprioli, A., Falbo, V., Ruggeri, F. M., Baldassarri, L., Bisicchia, R., Ippolito, G., *et al.* (1987). Cytotoxic necrotizing factor production by hemolytic strains of *Escherichia coli* causing extraintestine infections. *J. Clin. Microbiol.*, **25**, 146–9.

Cherifi, A. M., Contrepois, M., Picard, B., Goullet, P., De Rycke J., Fairbrother, J., *et al.* (1990). Factor and markers of virulence in *Escherichia coli* from human septicemia. *FEMS Microbiol. Lett.*, **70**, 279–84.

De Rycke J. and Plassiart, G. (1990). Toxic effects for lambs of cytotoxic necrotizing factor from *Escherichia coli*. *Res. Vet. Sci.*, **49**, 349–54.

De Rycke, J., Guillot, J. F., and Boivin, R. (1987). Cytotoxins in non-enterotoxigenic strains of *Escherichia coli* isolated from feces of diarrheic calves. *Vet. Microbiol.*, **15**, 137–57.

De Rycke, J., Gonzàlez, E. A., Blanco, J., Oswald, E., Blanco, M., and Boivin, R. (1990). Evidence of two types of cytotoxic necrotizing factors (CNF1 and CNF2) in human and animal clinical isolates of *Escherichia coli*. *J. Clin. Microbiol.*, **28**, 694–9.

Donelli, G., and Fiorentini, C. (1994). Bacterial protein toxins acting on the cell cytoskeleton. *Microbiologica*, **17**, 345–62.

Donelli, G., Fiorentini, C., Falzano, L., Pouchelet, M., Oswald, E., and Boquet, P. (1994). Effects induced by the cytotoxic necrotizing factor 1 (CNF1) from pathogenic *E. coli* on cultured epithelial cells. *Zbl. Bakt. Suppl.*, **24**, 60–71.

Falbo, V., Famiglietti, M., and Caprioli, A. (1992). Gene block encoding production of cytotoxic necrotizing factor 1 and hemolysin in *Escherichia coli* isolates from extraintestinal infections. *Infect. Immun.*, **60**, 2182–7.

Falbo, V., Pace, T., Picci, L., Pizzi, E., and Caprioli, A. (1993). Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of *Escherichia coli*. *Infect. Immun.*, **61**, 4909–14.

Falzano, L., Fiorentini, C., Donelli, G., Michel, E., Kocks, C., Cossart, P., *et al.* (1993). Induction of phagocytic behaviour in human epithelial cells by *E. coli* cytotoxic necrotizing factor type 1. *Mol. Microbiol.*, **9**, 1247–54.

Fiorentini, C., Arancia, G., Caprioli, A., Falbo, V., Ruggeri, F. M., and Donelli, G. (1988). Cytoskeletal changes induced in HEp-2 cells by the cytotoxic necrotizing factor of *Escherichia coli*. *Toxicon*, **26**, 1047–56.

Fiorentini, C., Donelli, G., Matarrese, P., Fabbri, A., Paradisi, S., and Boquet, P. (1995). *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1): evidence for induction of actin assembly by constitutive activation of the p21 Rho GTPase. *Infect. Immun*, **63**, 3936–44.

Oswald, E. and De Rycke, J. (1990). A single protein of 110 kDa is associated with the multinucleating and necrotizing activity coded by the Vir plasmid of *Escherichia coli*. *FEMS Microbiol. Lett.*, **68**, 279–84.

Oswald, E., De Rycke, J., Lintermans, P., Van Muylem, K., Mainil, J., Daube, G., *et al.* (1991). Virulence factors associated with cytotoxic necrotizing factor type two in bovine diarrheic and septicemic strains of *Escherichia coli*. *J. Clin. Microbiol.*, **29**, 2522–7.

Oswald, E., Motoyuki, S., Labigne, A., Wu, H. C., Fiorentini, C., Boquet, P., *et al.* (1994). Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. *Proc. Natl. Acad. Sci. USA*, **91**, 3814–18.

Prada, J., Baljer, G., De Rycke, J., Steinruck, H., Zimmerrnann, S., Stephan, R. *et al.* (1987). Characteristics of α-haemolyitc strains of *Escherichia coli* isolated from dogs with gastroenteritis. *Vet. Microbiol.*, **29**, 59–73.

Wray, C., Piercy, D. W. T., Carroll, P. J., and Cooley, W. A. (1993). Experimental infections of neonatal pigs with CNF toxin-producing strains of *Escherichia coli*. *Res. Vet. Sci.*, **54**, 290–8.

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Enterotoxin A and cytotoxin B (*Clostridium difficile*)

Clostridium difficile enterotoxin A (TcdA, 308 kDa) and cytotoxin B (TcdB, 270 kDa) belong to the group of large clostridial cytotoxins (LCT). The toxins are secreted into the culture supernatant of the growing bacteria, specifically bind to eukaryotic cells, and are then taken up by receptor mediated endocytosis. Intracellularly they monoglucosylate small GTP-binding proteins, mainly of the Rho subfamily, at their effector domain. The GTPases are thus functionally inactivated, the result is a breakdown of the cellular actin stress fibres, a block of cytokinesis, but not a loss of vitality of the cells.

The human pathogen

The enterotoxin A (TcdA) and the cytotoxin B (TcdB) of *Clostridium difficile* are the two virulence factors responsible for the induction of antibiotical associated diarrhea (AAD) or pseudomembranous colitis (PMC) (Lyerley *et al.* 1988; Knoop *et al.* 1993). Diagnosis of the disease is done by identifying the toxins in stool specimens.

Purification and sources

The high molecular weight cytotoxins are secreted by the bacterium into its culture supernatants from which they may be isolated. One high producer (Sullivan *et al.* 1982), *C. difficile* VPI10463 (see Table 1), is used in many laboratories. The culture technique first introduced by Sterne and Wentzel (1950) is recommended for isolation. The central and final purification step is an ion-exchange chromatography most effectively done on a high resolution column like MonoQ (Eichel-Streiber *et al.* 1987). The other large clostridial cytotoxins (LCT, see Table 1) may be purified by the same route. Starting from a 3 litre culture, good preparations yield a total of 6–18 mg TcdA (0.6–3 mg/ml) and 2–10 mg of TcdB (0.2–1.0 mg/ml). On SDS-PAGE gels the polypeptides migrate above the 200 kDa myosin standard protein. Major contaminating proteins are a 46 kDa glutamate dehydrogenase in TcdA (Lyerly *et al.* 1991) and a 150 kDa protein in TcdB (Pothoulakis *et al.* 1986). Special protocols have been described for TcdA purification using thyroglobulin affinity chromatography (Krivan and Wilkins 1987) and for TcdB purification using the addition of $CaCl_2$ as an additive during anion-exchange chromatography (Meador and Tweten 1988).

Antibodies

Polyclonal antisera against TcdA are almost exclusively directed against the C-terminal repetitive domain (Eichel-Streiber *et al.* 1989). Polyclonal antiserum against TcdB is difficult to raise. In many instances a polyclonal antiserum against the lethal toxin (TcsL) of *C. sordellii* proved to

Table 1 Properties of large clostridial cytotoxins

	Clostridium difficile			*C. sordellii*		*C. novyi*
Strain	VPI10463 (ATCC43255)		1470 (ATCC43598)	VPI9048 IP82		ATCC19402
Toxin (former abbreviation)	TcdA (ToxA)	TcdB (ToxB)	TcdB-1470 (TcdBF)	TcsH (HT)	TcsL (LT)	Tcnα (–)
Synonym	enterotoxin	cytotoxin	cytotoxin	hemorrhagic toxin	lethal toxin	alpha-toxin
M_r	308.000	270.000	269.000	300.000	250.000	258.000
PI	5.3	4.1	4.1	6.1	4.55	5.9
Cell culture cytotoxicity (ng/m)	10	1–50	10	15–500	1.6–16	0.1–10
Cytopathic effect	TcdB-like	TcdB	TcsL-like	TcsL-like	TcsL	TcdB-like
Mouse (lethal dose)	50–100 ng	50–100 ng	–	75–120 ng	3–5 ng	5–10 ng
Target GTPase	Rho, Rac Cdc42	Rho, Rac Cdc42	Rac, Rap	Rac, Ras, Rap, Ral	n. d.	Rho, Rac, Cdc42
mAbs	TTC8 PCG-4	2CV	2CV	TTC8 PCG-4	2CV	–
Gene	EMBL X51797	EMBL X53138	EMBL Z23277	–	EMBL X82638	EMBL Z48636

react effectively with TcdB (Eichel-Streiber *et al.* 1990). Although TcdA and TcdB are highly homologous to each other (Eichel-Streiber *et al.* 1992), properly diluted, neither polyclonal antiserum is crossreactive.

Two mAbs (TTC8 and PCG-4) against TcdA have been raised, they react with epitopes of the repeat structures (Lyerly *et al.* 1986; Sauerborn *et al.* 1994) and neutralize TcdA *in vivo*. TTC8 crossreacts with TcsH of *C. sordellii*. Only a few antibodies against TcdB exist (Muller *et al.* 1992*a*; Sauerborn *et al.* 1994). The best characterized is 2CV reacting with a sequence of the C-terminal repeat of TcdB (Sauerborn *et al.* 1994). The latter mAb does not neutralize the TcdB action. Again 2CV crossreacts with TcsL of *C. sordellii*.

Interaction with cells

The toxins are taken up by receptor mediated endocytosis (Eichel-Streiber *et al.* 1991). The repeat-domain of TcdA has been identified as the site of interaction with a carbohydrate (Galα1-3Galβ1-4GlcNAc) structure (Krivan and Wilkins 1987). A similar structure is present in thyroglobulin (Krivan *et al.* 1986) and also on a partially purified glycoprotein receptor molecule (Rolfe and Song 1993). Inactivation of TcdA with TTC8 is due to blocking the receptor–ligand interaction (Sauerborn and Eichel-Streiber 1995). No partner molecule for TcdB on the cell surface has yet been found. Due to their similar architecture an interaction of the TcdB repeat with another oligosaccharide is anticipated as the basis for its uptake.

During the passage of the toxins into the cell they are blocked by neutralization of the endosomal pH (Florin and Thelestam 1986; Henriques *et al.* 1986). Since both TcdA and TcdB are equally active when microinjected into the cell cytoplasm (Muller *et al.* 1992*b*), it was speculated that they do not need to be activated. The extreme amount of toxin needed for *in vitro* modification of the target proteins (Just *et al.* 1995), now indicates that such processing is indeed essential to deliver the full activity. Once inside the cell the toxins affect small GTP-binding proteins by an enzymatic mechanism described below (Just *et al.* 1995).

Genes and genetic variation

The toxin genes *tcd*A and *tcd*B together have been cloned and sequenced (Dove *et al.* 1990; Eichel-Streiber and Sauerborn 1990; Eichel-Streiber *et al.* 1990; Johnson *et al.* 1990). Together with three accessory genes (*tcd* C-E) they constitute the pathogenicity locus (PaLoc) of *C. difficile* (Braun *et al.* 1996). The single chain proteins are encoded by genes of 8130 and 7098 bp length (Eichel-Streiber 1993). Comparative analysis of a greater variety of strains proved that considerable genetic polymorphisms of the toxin genes exist (Rupnik *et al.* 1995). To date, for some isolates species-classification as *C. difficile* is disputed (Depitre *et al.* 1993). Since different LCTs modify a different spectrum of GTPases, dealing with '*C. difficile*' toxins does not necessary mean that the toxins react with identical targets. If LCTs are used as tools in molecular cell biology this has to be taken into account.

Characterization of the protein

The molecular sizes and isoelectric points of TcdA and TcdB are 308 kDa, p*I* 5.3 and 270 kDa, p*I* 4.1, respectively (Eichel-Streiber 1993). The polypeptides are not modified by mercaptoethanol addition. Protease digestion does not lead to production of defined fragments but rather totally breaks the toxins into pieces (Eichel-Streiber, unpublished data).

The protein sequences derived from the *tcd*A and *tcd*B genes share 63 per cent homologous amino acid positions (Eichel-Streiber *et al.* 1992). Structurally they are both composed of two parts, the N-terminal nonrepetitive two thirds, and the C-terminal third which is highly repetitive (Eichel-Streiber *et al.* 1992; Eichel-Streiber 1993). The repeats have been designated CROPs (clostridial repetitive oligopeptides) (Eichel-Streiber *et al.* 1992; Eichel-Streiber 1993; Hofmann *et al.* 1995). This part of the toxin shares sequences with glucosyltransferases of Streptococci (Eichel-Streiber *et al.* 1992).

Sequencing gave raise to a three domain structure of the toxins, with a C-terminal repetitive area recognized as the ligand part by neutralizing mAbs (Sauerborn and Eichel-Streiber 1995), a central hydrophobic part encompassing several transmembranal segments (Hofmann *et al.* 1995), probably functioning as a translocation unit, and an N-terminal part which obviously encodes for the catalytic domain (Eichel-Streiber *et al.* 1995).

Large clostridial cytotoxins

C. difficile toxins are A–B type bacterial toxins belonging to the group of large clostridial cytotoxins (see Table 1). The other three members of LCTs are *C. sordellii* hemorrhagic (TcsH) and the lethal (TcsL) toxins and *C. novyi* α-toxin (Tcnα). The *tcn*α (Hofmann *et al.* 1995) and *tcsL* (Grenn *et al.* 1995) genes are sequenced, again CROPs were identified and both toxins are homologous to the *C. difficile* toxins TcdA and TcdB. The latter homology supports the notion that the toxins of *C. sordellii* and *C. difficile* are immunologically related (Popoff 1987; Martinez and Wilkins 1988). One *C. difficile* strain produces a cytotoxin B with a modified mode of action (Eichel-Streiber *et al.* 1995). The cytotoxic response of treated cells is that of TcsL of *C. sordellii* rather than that of TcdB of *C. difficile* (Eichel-Streiber *et al.* 1995). This again points to the possibility that more cytotoxins exist which should be placed into the LCT-group and that the spectrum of GTPases modified might be greater than defined today.

Different from the diphtheria-type of toxins equimolarly composed of one A and one B subunit (Choe *et al.* 1992), and cholera-type toxins being composed of one A and five B subunits (Sixma *et al.* 1991), *C. difficile* toxins have a single chain containing several ligand domains. Thus they form a novel third class of ABI toxins (Eichel-Streiber *et al.* 1996).

Catalytic activity

TcdA and TcdB are enzymes which monoglucosylate small GTP-binding proteins of the Ras superfamily (Just *et al.*

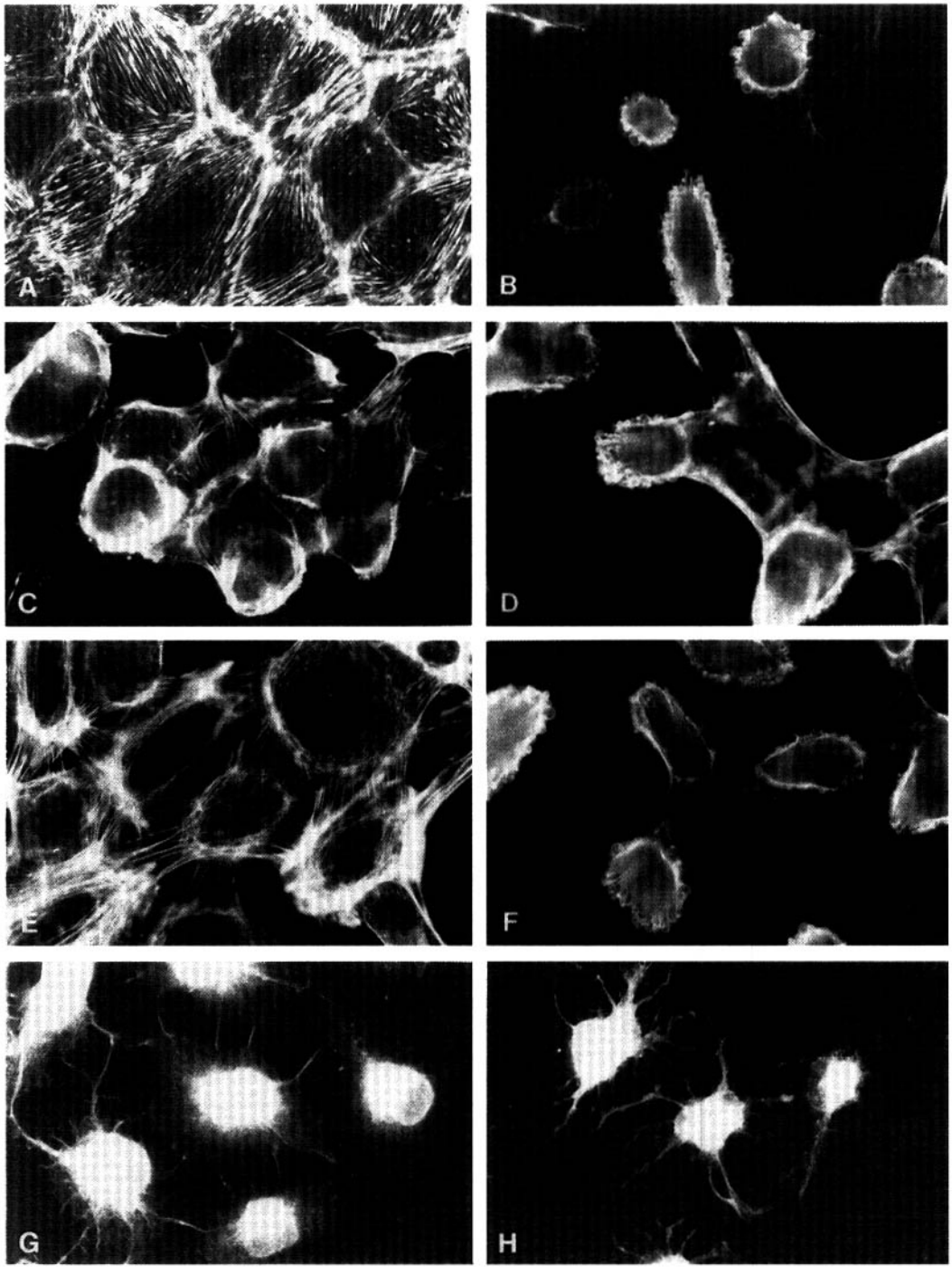

Figure 1. Distribution of actin filaments after intoxication with large clostridial cytotoxins. Endothelial cells from the pig pulmonary artery were taken into culture and incubated with different toxin concentrations for the times indicated. A, Untreated control; B–D, treatment with TcdB-1470: B, 1000 mg/ml 8 h; C, 200 ng/ml 4 h; D, 1000 ng/ml 4 h; E–F, treatment with *C. sordellii* TcsL: E, 200 ng/ml 4 h; F, 1000 ng/ml 4 h; G–H, treatment with TcdB of *C. difficile* VPI10463: G, 5 ng/ml 4 h; H, 200 ng/ml 4 h. Cells were stained with phalloidin-fluorescein to visualize F-actin.

1994, 1995). Three target proteins have been identified, Rho, Rac, and CDC42, all belonging to the Rho subfamily of GTPases. For Rho the site of modification is Thr-37 (Just *et al.* 1995). TcdB-glucosylated-Rho is no longer a substrate for the ADP-ribosyltransferase C3 of *C. botulinum* (Just *et al.* 1994). Transient overexpression of Rho mediates resistance of cells against *C. difficile* toxin A and B but not against *C. sordellii* lethal toxin (Giry *et al.* 1995).

The cofactor in the reaction is an activated glucose moiety (UDP-glucose) (Just *et al.* 1995). UDP-14C-glucose is solved in ethanol which has to be evaporated before the reaction is started. For Tcnα UDP-GlcNac is the cofactor (Selzer *et al.* 1995). The source of target GTPases is recombinant material or the pool of GTPases present in highly concentrated cell lysates. The reaction buffer is adapted to the intracellular medium. First GDP-loading of the GTPases is performed, thereafter toxin and cofactor are added and the labelling reaction is carried out. The reaction mixture is subjected to SDS-PAGE electrophoresis and radioactivity is measured in a phosphoimager.

The target proteins are GTPases of the Ras-superfamily of small GTP-binding proteins. The toxins analysed so far modify GTPases of the Rho- and Ras-subfamilies. Known targets of the individual toxins are listed in Table 1 (Eichel-Streiber *et al.* 1996; Hofmann *et al.* 1996; Just *et al.* 1995; Popoff *et al.* 1996; Selzer *et al.* 1996).

In vivo activities

A great variety of adherent cells is rounded after intoxication. Only after chemical mutagenesis could a cell resistent to TcdB be obtained (Florin 1991). The cell has a defect in the UDP-glucose metabolism which results in a decreased UDP-concentration in the cytosol (Chaves-Olarte *et al.* 1996). In cell cultures the active concentrations (Eichel-Streiber *et al.* 1987; Popoff 1987; Martinez and Wilkins 1988; Ball *et al.* 1993) of the five LCT toxins are between 0.001 and 10 ng/ml (see Table 1). When used *in vitro* as an enzyme on lysed cells much higher concentrations have to be applied (Just *et al.* 1995). This points to the fact that the toxins are further processed and toxified, once they have reached the cytosol.

Applied intraperitoneally into mice, a single dose of the toxin acts lethal (see Table 1). This lethal effect was a major obstacle to getting TcdB-antibody responses in test animals (Eichel-Streiber, unpublished data). When given perorally in hamsters TcdA induces the disease and the hamsters finally die (Lyerly *et al.* 1985). In humans TcdB seems to be more important (Riegler *et al.* 1995). Probably both toxins act synergistically and are both needed for the onset of the disease.

Use in cell biology

The target GTPases of TcdA and TcdB are of major importance for the cell. CDC42, Rac, and Rho have been described as being involved in a cascade of reactions (Chant and Stowers 1995; Nobes and Hall 1995) connected with lamellipodia (CDC42), membrane ruffling (Rac), and stress fibre formation (Rho). Rac is involved in oxidative burst of granulocytes (Ridley 1995). Rac and Rho play an important role in mast cell secretion (Price *et al.* 1995). Activated Rho induces PIP 5-kinase thus influencing the actin cytoskeleton and signal-transduction (Chong *et al.* 1994). Inactivation of Rho-GTPases by *C. difficile* toxins A and B leads to a decrease in phospholipase C and D activity due to a reduction in substrate supply (Schmidt *et al.* 1996*a*; Schmidt *et al.* 1996*b*). This is the first example for the use of *C. difficile* toxins in cell biology. These and the other LCTs will obviously soon become tools to dissect the interplay (signal transduction) between GTPases and the cell machinery.

References

Ball, D. W., van Tassell, R. L., Roberts, M. D., Hahn, P. E., Lyerly, D. M., and Wilkins, T. D. (1993). Purification and characterization of alpha-toxin produced by *Clostridium novyi* type A. *Infect. Immun.*, **61**, 2912–18.

Braun, V., Hundsberger, T., Leukel, P., Sauerborn M., and Eichel-Streiber, C. v (1996). Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. *Gene*, **181**, 29–38.

Chant, J. and Stowers, L. (1995). GTPase cascades choreographing cellular behaviour: movement, morphogenesis, and more. *Cell*, **81**, 1–4.

Chaves-Olarte E., Florin, I., Boquet, P., Popoff, M., Eichel-Streiber, C. v., and Thelestam, M. (1996). Low level of UDP-glucose in a mutant cell resistant to glucosyl-transferase toxins from *Clostridium difficile* and *C. sordellii*. *J. Biol. Chem.*, **271**, 6925–32.

Choe, S., Bennett, M. J., Fujii, G., Curmi, P. M. G., Kantardjieff, K. A., Collier, R. J., *et al.* (1992). The crystal structure of diphtheria toxin. *Nature*, **357**, 216–22.

Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994). The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell*, **79**, 507–13.

Depitre, C., Delmee, M., Avesani, V., L'Haridon, R., Roels, A., Popoff, M., *et al.* (1993). Serogroup F strains of *Clostridium difficile* produce toxin B but not toxin A. *J. Med. Microbiol.*, **38**, 434–41.

Dove, C. H., Wang, S. -Z., Price, S. B., Phelbs, C. J., Lyerly, D. M., Wilkins, T. D., *et al.* (1990). Molecular characterization of the *Clostridium difficile* toxin A gene. *Infect. Immun.*, **58**, 480–8.

Eichel-Streiber, C. v. (1993). Molecular biology of *Clostridium difficile* toxins. In *Genetics and molecular biology of anaerobic bacteria* (ed. M. Sebald), pp. 265–89, Springer Verlag, New York, Berlin.

Eichel-Streiber, C. v. and Sauerborn, M. (1990). *Clostridium difficile* toxin A carries a C-terminal repetitive structure homologous to the carbohydrate binding region of streptococcal glycosyltransferases. *Gene*, **96**, 107–13.

Eichel-Streiber, C. v., Harperath, U., Bosse, D., and Hadding, U. (1987). Purification of two high molecular weight toxins of *Clostridium difficile* which are antigenically related. *Microbial Pathogen.*, **2**, 307–18.

Eichel-Streiber, C. v., Suckau, D., Wachter, M., and Hadding, U. (1989). Cloning and characterization of overlapping DNA fragments of the toxin A gene of *Clostridium difficile*. *J. Gen. Microbiol.*, **135**, 55–64.

Eichel-Streiber, C. v., Laufenberg-Feldmann, R., Sartingen, S., Schulze, J., and Sauerborn, M. (1990). Cloning of *Clostridium difficile* toxin B gene and demonstration of high N-terminal homology between toxin A and B. *Med. Microbiol. Immunol.*, **179**, 271–9.

Eichel-Streiber, C. v., Warfolomeow, I., Knautz, D., Sauerborn, M., and Hadding, U. (1991). Morphological changes in adherent cells induced by *Clostridium difficile* toxins. *Biochem. Soc. Trans.*, **19**, 1154–60.

Eichel-Streiber, C. v., Sauerborn, M., and Kuramitsu, H. K. (1992). Evidence for a modular structure of the homologous repetitive C-terminal carbohydrate-binding sites of *Clostridium difficile* toxins and *Streptococcus mutans* glucosyltransferases. *J. Bacteriol.*, **174**, 6707–10.

Eichel Streiber, C. v., Laufenberg-Feldmann, R., Sartingen, S., Schulze, J., and Sauerborn, M. (1992). Comparative sequence analysis of the *Clostridium difficile* toxins A and B. *Mol. Gen. Genet.*, **233**, 260–8.

Eichel-Streiber, C. v., Meyer zu Heringdorf, D., Habermann, E., and Sartingen, S. (1995). Closing in on the toxic domain through analysis of a variant *Clostridium difficile* cytotoxin. *Mol. Microbiol.*, **17**, 313–21.

Eichel-Streiber, C. v., Boquet, P., Sauerborn M., and Thelestam, M. (1996). Large clostridial cytotoxins – a family of glycosyltransferases modifying small GTP-binding proteins. *Trends in Microbiology*, **14**, 375–82.

Florin, I. (1991). Isolation of a fibroblast mutant resistant to *Clostridium difficile* toxins A and B. *Microb. Pathogen.*, **11**, 337–46.

Florin I., and Thelestam, M. (1986). Lysosomal involvement in cellular intoxication with *Clostridium difficile* toxin B. *Microb. Pathogen.*, **1**, 373–385.

Giry M., Arpin, M., Eichel-Streiber, C. v., Popoff, M. and Boquet, P. (1995). Transient expression of Rho A, B, C GTPases into Hela cells potentiates their resistance to *Clostridium difficile* A and B but not to *Clostridium sordellii* lethal toxin. *Infect. Immun*, **63**, 4063–71.

Green G. A., Schue V., and Monteil, H. (1995). Cloning and characterization of the cytotoxin L-encoding gene of *Clostridium sordelli*: homology with *Clostridium difficile* cytotoxin B. *Gene*, **161**, 57–61.

Henriques B., Florin, I., and Thelestam, M. (1986). Cellular internalization of *Clostridium difficile* toxin A. *Microb Pathogen.*, **1**, 455–463.

Hofmann, F., Hermann, A., Habermann, E., and von Eichel-Streiber, C. (1995). Sequencing and analysis of *Clostridium novyi's* α-toxin gene supports definition of a subgroup of clostridial toxins. *Mol. Gen. Genet.*, **247**, 904–13.

Hofmann, F., Rex, G., Aktories, K., and Just, J. (1996). The Ras related protein Ral is monoglucosylated by *Clostridium sordellii* lethal toxin. *Biochem. Biophys. Res. Commun.*, **227**, 77–81.

Johnson, J. L., Phelps, C., Barroso, L., Roberts, M. D., Lyerly, D. M., and Wilkins, T. D. (1990). Cloning and expression of the toxin B gene of *Clostridium difficile*. *Curr. Microbiol.*, **20**, 397–401.

Just, I., Fritz, G., Aktories, K., Giry, M., Popoff, M. R., Boquet, P., *et al.* (1994). *Clostridium difficile* toxin B acts on the GTP-binding Rho. *J. Biol. Chem.*, **269**, 10706–12.

Just, I., Selzer, J., Wilm, M., Eichel-Streiber, C. v., Mann, M., and Aktories, K. (1995). Glycosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature*, **375**, 500–3.

Knoop, F. C., Owens, M., and Crocker, I. C. (1993). *Clostridum difficile*: clinical disease and diagnosis. *Clin. Microbiol. Rev.*, **6**, 251–65.

Krivan, H. C. and Wilkins, T. D. (1987). Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilized thyroglobulin. *Infect. Immun.*, **55**, 1873–7.

Krivan, H. C., Clark, G. F., Smith, D. F., and Wilkins, T. D. (1986). Cell surface binding site for Clostridium enterotoxin: evidence for a glycoconjugate containing the sequence Gal alpha 1-3Galbeta 1-4GlcNAc. *Infect. Immun.*, **53**, 573–81.

Lyerly, D. M., Saum, K. E., MacDonald, D. K., and Wilkins, T. D. (1985). Effects of *Clostridium difficile* toxins given intragastrically to animals. *Infect. Immun.*, **47**, 349–52.

Lyerly, D. M., Phelps, C. J., Toth, J., and Wilkins, T. D. (1986). Characterization of toxins A and B of *Clostridium difficile* with monoclonal antibodies. *Infect. Immun.*, **54**, 70–6.

Lyerly, D. M., Krivan, H. C., and Wilkins, T. D. (1988). *Clostridium difficile:* its disease and toxins. *Clin. Microbiol. Rev.*, **1**, 1–18.

Lyerly, D. M., Barroso, L. A., and Wilkins, T. D. (1991). Identification of the latex test-reactive protein of *Clostridium difficile* as glutamate dehydrogenase [see comments]. *J. Clin. Microbiol.*, **29**, 2639–42.

Martinez, R. D. and Wilkins, T. D. (1988). Purification and characterization of *Clostridium sordellii* hemorrhagic toxin and cross-reactivity with *Clostridium difficile* toxin A (enterotoxin). *Infect. Immun.*, **56**, 1215–21.

Meador III, J. and Tweten, R. K. (1988). Purification and characterization of toxin B from *Clostridium difficile*. *Infect. Immun.*, **56**, 1708–14.

Müller, F., Stiegler, C., and Hadding, U. (1992*a*). Monoclonal antibodies specific for *Clostridium difficile* toxin B and their use in immunoassays. *J. Clin. Microbiol.*, **30**, 1544–50.

Müller, H., Eichel Streiber, C. v., and Habermann, E. (1992*b*). Morphological changes of cultured endothelial cells after microinjection of toxins that act on the cytoskeletons. *Infect. Immun.*, **60**, 3007–10.

Nobes, C. D. and Hall, A. (1995). Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamillipodia, and filopodia. *Cell*, **81**, 53–62.

Popoff, M. R. (1987). Purification and characterization of *Clostridium sordellii* lethal toxin and cross-reactivity with *Clostridium difficile* cytotoxin. *Infect. Immun.*, **55**, 35–43.

Popoff M.R., Chaves-Olarte, E., Lemichez, E., Eichel-Streiber, C. v., Thelestam. M., Chardin, P., Cussac, D., Antonny, B., Chavier, P., Flatau, G., Giry, M. and Boquet, P. (1996). Ras, Rap and Rac are the target GTP-binding proteins of *C. sordellii* lethal toxin glucosylation. *J. Biol. Chem.*, **271**, 10217–24.

Pothoulakis, C., Barone, L. M., Ely, R., Faris, B., Clark, M. E., Franzblau, C., *et al.* (1986). Purification and properties of *Clostridium difficile* cytotoxin B. *J. Biol. Chem.*, **261**, 1316–21.

Price, L. S., Norman, J. C., Ridley, A. J., and Koffer, A. (1995). The small GTPases Rac and Rho as regulators of secretion in mast cells. *Curr. Biol.*, **5**, 68–73.

Ridley, A. J. (1996). Rho: theme and variations. *Curr. Biol.*, **6**, 1256–64.

Riegler, M., Sedivy, R., Pothoulakis, C., Hamilton, G., Zacherl, J., Bischof, G., *et al.* (1995). *Clostridium difficile* toxin B is more potent than toxin A in damaging human clonic epithelium *in vitro*. *J. Clin. Invest.*, **95**, 2004–11.

Rolfe, R. D. and Song, W. (1993). Purification of a functional receptor for *Clostridium difficile* toxin A from intestinal brush border membranes of infant hamsters. *Clin. Infect. Dis.*, **16**, (Suppl. 4), S219–27.

Rupnik, M., Braun, V., Soehn, F., Hofstetter, M., Janc, M., and Eichel-Streiber, C. v. (1996). Lack of detection of *Clostridium difficile* toxins due to genetic polymorphism of the toxic genes. *Zentralbl. Bakteriol. Suppl.* (accepted for publication).

Sauerborn, M. and Eichel-Streiber, C. v. (1996). Interaction of toxin A of *C. difficile* with its cell receptor. *Zentralblatt. Bakteriol. Suppl.* (accepted for publication).

Sauerborn, M., Hofstetter, M., Laufenberg-Feldmann, R., and Eichel-Streiber, C. v. (1991). The C-terminal repetitive domain of *Clostridium difficile* toxin A and its significance

for the induction disease. *Zentralblatt Bakteriol.*, Suppl. **23**, 144–5.
Sauerborn, M., Hegenbarth, S., Laufenberg-Feldmann, R., Leukel, P., and Eichel-Streiber, C. v. (1994). Monoclonal antibodies discriminating between *Clostridium difficile* toxin A and toxin B. *Zentralblatt. Bakteriol. Suppl.*, **24**, 510–11.
Schmidt, M., Rümenapp, U., Bienek, C. Keller, J., Eichel-Streiber, C. v., and Jakobs, K. H. (1996*a*). Inhibition of receptor signalling to phospholipase D by *Clostridium difficile* toxin B. *J. Biol. Chem.*, **271**, 2422–6.
Schmidt M., Bienek. C., Rümenapp, U., Zhang, C., Jakobs, K. H., Just, I., Aktories, K., Moos, M., and Eichel-Streiber, C. v. (1996*b*). Inhibition of receptor- and G protein-stimulated phospholipase C by *Clostridium difficile* toxin B. *Naunyn-Schmiedeberg's Archiv.* **354**, 87–94.
Selzer, J., Hofmann, F., Rex, G., Wilm, M., Mann, M., Just, I., and Aktories, K. (1996). *Clostridium novyi* alpha toxin catalyzed incorporation of GlcNAc into Rho subfamily proteins. *J. Biol. Chem.*, **271**, 25173–7.
Sixma, T. K., Pronk, S. E., Kalk, K. H., Wartna, E. S., van Zanten, B. A. M., Witholt, B., *et al.* (1991). Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E. coli* [see comments]. *Nature*, **351**, 371–7.
Sterne, M. and Wentzel, L. M. (1950). A new method for the large scale production of high-titered botulium formal-toxoid types C and D. *J. Immunol.*, **65**, 175–83.
Sullivan, N. M., Pellett, S., and Wilkins, T. D. (1982). Purification and characterization of toxins A and B of *Clostridium difficile*. *Infect. Immun.*, **35**, 1032–40.

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ActA (*Listeria monocytogenes*)

ActA is a 90 kDa protein expressed on the surface of the facultative intracellular bacterium Listeria monocytogenes. *It is necessary and sufficient to induce actin polymerization at one pole of the bacterium. Actin polymerization is thought to provide the propulsive force for intracellular movement and cell-to-cell spread. ActA is a major virulence factor of* L. monocytogenes.

ActA is a major surface protein of the Gram positive human bacterial pathogen *L. monocytogenes*. It is encoded by gene *actA* (Genebank accession number M82881 and EMBL Data library accession number X59723) whose sequence predicts a 639-amino-acid protein with an amino-terminal signal peptide and a C-terminal membrane anchor (Domann *et al.* 1992; Vazquez-Boland *et al.* 1992). The N-terminus is processed at the predicted site and the mature 610-amino-acid protein is expressed on the bacterial surface and anchored into the bacterial membrane via its hydrophobic C-terminal region (Domann *et al.* 1992; Kocks *et al.* 1992). ActA (predicted isoelectric point: 4.74) is rich in glutamic acid (12 per cent) and proline (9.2 per cent). The N-terminal domain of ActA is highly charged. Its central region contains a succession of proline/glutamic rich stretches. The C-terminal part is more hydrophobic. As shown by immunogold labelling, the two first parts of the protein protrude from the cell wall and are thus able to interact with cytoskeleton components. ActA displays a polar distribution on the bacterial body. It is highly expressed at one pole of the bacterium and its distribution gradually decreases towards the other (Kocks *et al.* 1993).

ActA is required for actin assembly once the bacterium has escaped from the phagosomal compartment and resides in the cytosol (Tilney and Tilney 1993; Cossart and Kocks 1994; Cossart 1995). Actin filaments are formed at the posterior end of the bacterium, at the site of high ActA expression. The actin filaments are subsequently released and crosslinked, generating an actin tail which is left behind by the moving bacterium. The exact function of ActA in the actin assembly process is unknown. Up to now, there is no evidence that ActA has the capacity to interact directly with actin. The only identified ligand of ActA is VASP, the vasodilator-stimulated phosphoprotein (Chakraborty *et al.* 1995), a focal adhesion and microfilament associated proline-rich protein, which has the capacity to interact with profilin, an actin monomer sequestering protein which can induce actin polymerization. Profilin and VASP have been localized at the base of the actin tail (Theriot *et al.* 1994). VASP binds to the proline rich region of ActA (Pistor *et al.* 1995). It is proposed that VASP would bind to ActA and bring profilin–actin complexes in the vicinity of the bacterium. These complexes would contribute to the polymerization process. However, transfection of mammalian cells with various parts of ActA or expression of various parts of ActA in *L. monocytogenes* have shown that the N-terminal part of ActA is critical for actin assembly while the proline-rich region only acts as a stimulator (Friederich *et al.* 1995; Lasa *et al.* 1995; Pistor *et al.* 1995). Consequently, VASP may not be absolutely required for movement. The present challenge is to identify the putative 'actin nucleator' protein binding to the N terminal of ActA or the ligand allowing the N-terminal to directly nucleate actin monomers.

Purification

ActA can be extracted from the bacterial surface by mild treatment with SDS, but this method may not be appropriate for subsequent functional characterization. Monoclonal antibodies have been produced against ActA eluted from gels and these have been used to affinity purify both ActA from cell surface extracts and a soluble form of ActA lacking the carboxy-terminal region (Niebuhr *et al.* 1993). However, degradation products with the same amino-terminal sequence as ActA copurify with it, probably following proteolysis of ActA by a listerial protease.

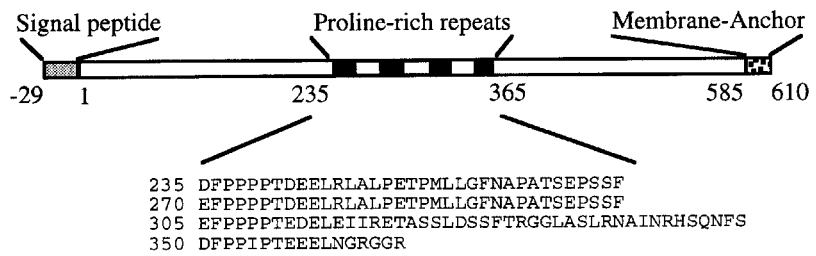

Figure 1. Schematic representation of ActA.

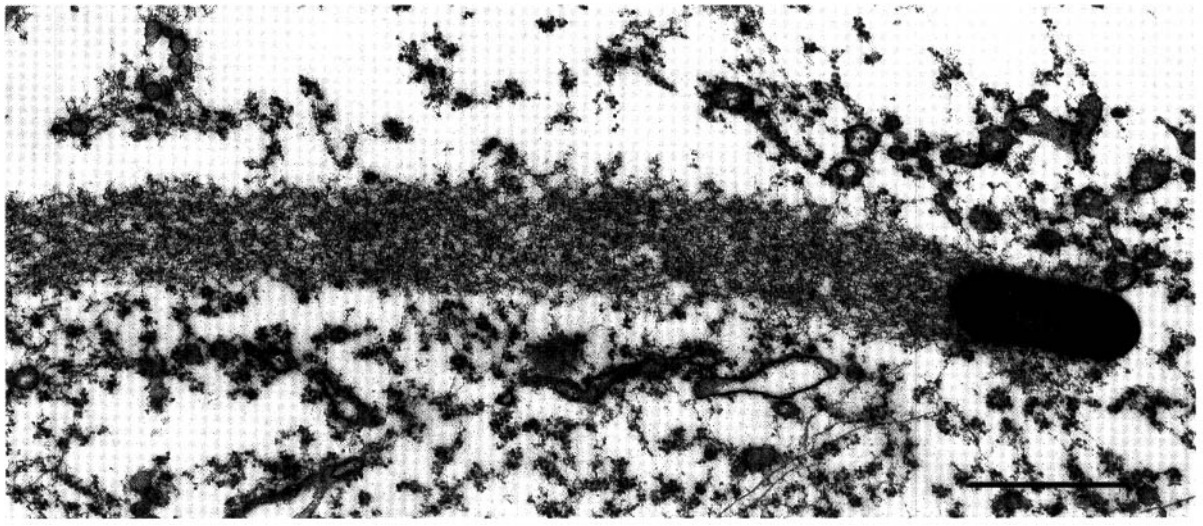

Figure 2. Ultra-thin section through an *L. monocytogenes* infected J774-macrophage at 4 h of infection. Actin filaments (electron dense filamentous material) assemble from the rear half of the bacterium into a tail thereby generating movement towards the opposite direction (Reproduced from Kocks *et al.* 1992 with permission from *Cell*). Bar = 1 μm.

Use in cell biology

ActA is the first bacterial protein known to induce by itself actin polymerization either in mammalian cells or in cytoplasmic extracts of *Xenopus Laevis* eggs (Pistor *et al.* 1995; Friederich *et al.* 1995; Kocks *et al.* 1995; Smith *et al.* 1995). To dissect ActA function, ActA has been mostly expressed from eukaryotic expression vectors in transfection experiments involving mammalian cell lines. When the complete ActA protein is expressed, ActA is targeted to the mitochondria where it induces actin assembly (Pistor *et al.* 1994, 1995). If the C-terminal membrane anchor is deleted, a soluble form of ActA is expressed in the cytosol where it induces actin assembly (Pistor *et al.* 1994; Friederich *et al.* 1995). If ActA is fused to a CAAX box, the ActA–CAAX protein is targeted to the inner face of the plasma membrane, due to isoprenylation of the C residue. At this location, ActA–CAAX induces actin polymerization, membrane extensions, and dramatic cell shape changes (Friederich *et al.* 1995). Thus there is a developing trend to use ActA as a tool to understand the correlation between actin polymerization and cell shape changes and/or cell motility. Moreover, immunologically cross reactive proteins have been detected in extracts of several organisms (Lasa *et al.* 1995; E. Friedrich and D. Louvard, personal communication; and C. Kocks and P. Cossart, unpublished results). The identification of eukaryotic ActA homologues should be very interesting.

References

Chakraborty, T., Ebel, F., Dommann, E., Niebuhr, K., Gerstel, B., Pistor, S., *et al.* (1995). A focal adhesion factor directly linking intracellularly motile *Listeria monocytogenes* and *Listeria ivanovii* to the actin-based cytoskeleton of mammalian cells. *EMBO J.*, **14**, 1314–21.

Cossart, P. (1995). Bacterial actin based motility. *Curr. Opin. Cell. Biol.*, **7**, 94–101.

Cossart, P. and Kocks, C. (1994). The actin based motility of the intracellular pathogen *Listeria monocytogenes*. *Mol. Microbiol.*, **13**, 395–402.

Domann, E., Wehland, J., Rohde, M., Pistor, S., Hartl, M., Goebel, W., *et al.* (1992). A novel bacterial gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. *EMBO J.*, **11**, 1981–90.

Friederich, E., Gouin, E., Hellio, R., Kocks, C., Cossart, P., and Louvard, D. (1995). Targeting of *Listeria monocytogenes* ActA protein to the plasma membrane as a tool to dissect both actin-based cell morphogenesis and ActA function. *EMBO J.*, **14**, 2731–44.

Kocks, C., Gouin, E., Tabouret, M., Berche, P., Ohayon, H., and Cossart, P. (1992). *Listeria monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. *Cell*, **68**, 521–31.

Kocks, C., Hellio, R., Gounon, P., Ohayon, H., and Cossart, P. (1993). Polarized distribution of *Listeria monocytogenes* surface protein ActA at the site of directional actin assembly. *J. Cell Sci.*, **105**, 699–710.

Kocks, C., Marchand, J. B., Gouin, E., d'Hauteville, H., Sansonetti, P. J., Carlier, M. F., and Cossart, P. (1995). The unrelated surface proteins ActA of *Listeria monocytogenes* and IcsA of *Shigella flexneri* are sufficient to confer actin-based motility to *L. innocua* and *E. coli* respectively. *Mol. Microbiol.*, **18**, 413–23.

Lasa, I., David, V., Gouin, E., Marchand, J., and Cossart, P. (1995). The amino-terminal part of ActA is critical for the actin based motility of *Listeria monocytogenes*; the central proline-rich region acts as a stimulator. *Mol. Microbiol.*, **18**, 425–36.

Niebuhr, K., Chakraborty, T., Rohde, M., Gazlig, T., Jansen, B., Kollner, P., *et al.* (1993). Localization of the ActA polypeptide of *Listeria monocytogenes* in infected tissue culture cell lines: ActA is not associated with actin comets. *Infect. Immun.*, **61**, 2793–802.

Pistor, S., Chakraborty, T., Niebuhr, K., Domann, E., and Wehland, J. (1994). The ActA protein of *L. monocytogenes* acts

as a nucleator inducing reorganization of the actin cytoskeleton. *EMBO J.*, **13**, 758–63.
Pistor, S., Chakraborty, T., Walter, U., and Wehland, J. (1995). The bacterial actin nucleator protein ActA of *Listeria monocytogenes* contains multiple binding sites for host microfilament proteins. *Curr. Biol.*, **5**, 517–25.
Smith, G. A., Portnoy, D. A., and Theriot, J. A. (1995). Asymmetric distribution of the *Listeria monocytogenes* ActA protein is required and sufficient to direct actin-based motility. *Mol. Microbiol.*, **17**, 945–51.
Theriot, J. A., Rosenblatt, J., Portnoy, D. A., Goldschmidt-Clermont, P. J., and Mitchison, T. J. (1994). Involvement of profilin in the actin-based motility of *L. monocytogenes* in cells and in cell-free extracts. *Cell*, **76**, 505–17.
Tilney, L. G. and Tilney, M. S. (1993). The wily ways of a parasite: induction of actin assembly by *Listeria*. *Trends Microbiol.*, **1**, 25–31.
Vazquez-Boland, J.-A., Kocks, C., Dramsi, S., Ohayon, H., Geoffroy, C., Mengaud, J., *et al.* (1992). Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. *Infect. Immun.*, **60**, 219–30.

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IcsA (*Shigella flexneri*)

IcsA is an outer membrane protein produced by virulent strains of Shigella flexneri. *It promotes the intraepithelial dissemination of the bacteria in infected cells by inducing polymerization of an actin comet at one pole of the microorganism.*

Shigella flexneri, the causative agent of bacillary dysentery, is a facultative intracellular pathogen that can enter and spread inside the host epithelial cells. This invasion process involves a sequence of molecular interactions between bacterial virulence factors and the host cell cytoskeleton (Sansonetti *et al.* 1994).

IcsA (intra/intercellular spread protein A) or VirG, a *S. flexneri* outer membrane protein, is essential for the intracellular movement and intercellular spread (Makino *et al.* 1986; Bernardini *et al.* 1989; Lett *et al.* 1989). Upon infection of Hela cell monolayers by *S. flexneri*, IcsA induces formation of an actin tail at one pole of the bacterium. Accumulation and polymerization of F-actin filaments provide a motive force which results in movement of the bacterium inside the cytoplasm. This event is followed by the formation of extracellular finger-like protusion, through which the bacteria penetrate adjacent cells. A strain of *S. flexneri* containing a mutation in *icsA* neither polymerizes actin on its surface nor spreads within the cytoplasm or to the adjacent cells. It rather forms localized microcolonies near the nucleus (Bernardini *et al.* 1989).

The IcsA protein is encoded by a 3.6 kb gene present on the virulence plasmid (Lett *et al.* 1989) (GenBank accession number M22802). Translocation and anchorage of IcsA to the membrane is achieved by a mechanism similar to that described for translocation of the IgA of *Neisseria gonorrhoeae* (Klauser *et al.* 1993; Suzuki *et al.* 1995). Accordingly, IcsA can be divided in three main portions: the signal peptide (L) (residues 1–53), the extramembrane domain B (residues 54–758), and the intramembrane (residues 758–1102). Within this IcsAα domain several glycine-rich repeats can be found.

Secretion of the IcsAα occurs after cleavage of the protein between two arginine residues at the positions 758 and 759 on an SSRRASS sequence (d'Hauteville *et al.* 1995; Fukada *et al.* 1995). This site is also a target for cyclic AMP dependent protein kinase (PKA) mediated phosphorylation in *in vitro* assays (d'Hauteville and Sansonetti 1992). Site-directed mutagenesis of this sequence results in lack of cleavage with subsequent abnormal localization of the protein on the bacterial surface leading to aberrant movement (d'Hauteville and Sansonetti 1995). It is still debated whether the secreted IcsAα is present within the comet (d'Hauteville *et al.* 1995).

By immunolabelling, IcsA is detected at the interface between the actin comet and the bacteria (Goldberg *et al.* 1993). The membrane-bound form of the protein is sufficent to elicit actin nucleation (Goldberg and Theriot 1995; Fukuda *et al.* 1995). Decoration of the actin filaments with the S1 subunit of myosin has shown that actin filaments display a unidirectional polarity, with the barbed ends always associated to the surface of the bacteria. Also incorporation of the actin monomers occurs at the comet– bacterium interface.

The interaction of IcsA with G-actin is weak and ATP-dependent. *In vitro* experiments have shown that IcsA binds and hydrolyses ATP (Goldberg *et al.* 1993). It has also been postulated that IcsA may not be itself the actin

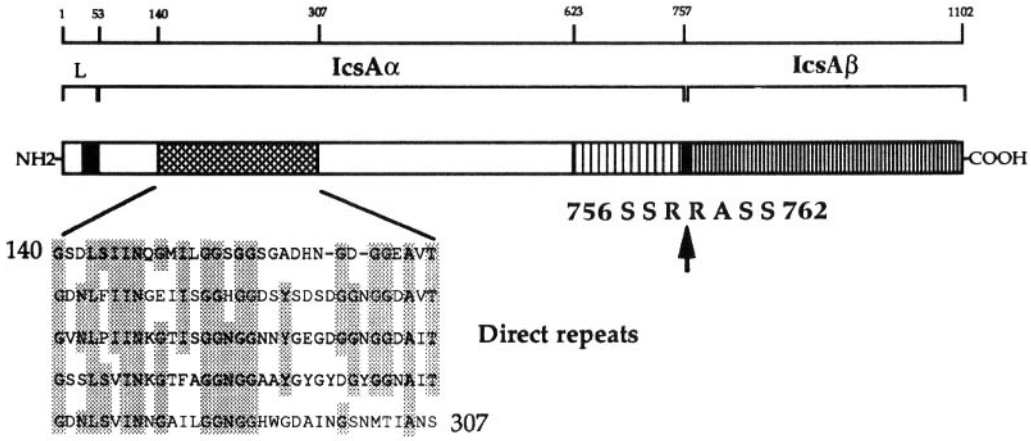

Figure 1. Schematic representation of the IcsA protein of *S. flexneri*.

nucleator and that it may recruit a cellular partner to promote actin nucleation.

Purification and sources

IcsA p. 120 has been overexpressed as in *E.coli* strain JM109. In this strain, *icsA* gene has been placed under the control of the inducible *tac* promoter in vector pKK223-3. Urea-denatured proteins have then been fractionated by an Ultrogel AcA22 column (Goldberg *et al.* 1993).

Recently, different portions of IcsAα have been expressed as GST fusion proteins, and purified by affinity chromatography (Suzuki *et al.* 1996).

Antibodies

Rabbit polyclonal and mouse monoclonal antibodies have been raised against the whole protein (Goldberg *et al.* 1993). These antibodies have been used in ELISA, Western-blotting, immunofluorescence, and immunoprecipitation assays (d'Hauteville *et al.* 1995).

Use in cell biology

Since regulation of actin dynamics and polymerization of actin filaments in intact moving cells are still poorly understood, the actin-based movement of *S. flexneri* can be used as a model to dissect the molecular basis of actin-based cell motility.

Currently, the exact mechanism of action of IcsA is under studies in our laboratory. Recent development of cell-free assays will facilitate this study (Theriot *et al.* 1994). In these assays, which use cytoplasmic extracts of Xenopus oocytes, actin polymerization and bacterial motility can be reproduced by using a strain of *E. coli* that expresses only the membrane-bound form of IcsA (Goldberg and Theriot 1995; Kocks *et al.* 1995).

References

Bernardini, M. L., Mounier, J., d'Hauteville, H., Coquis-Rondon, M., and Sansonetti, P. J. (1989). Identification of *icsA*, a plasmid locus of *Shigella flexneri* which governs bacterial intra- and intercellular spread through interaction with F-actin. *Proc. Natl. Acad. Sci. USA*, **86**, 3867–71.

d'Hauteville, H. and Sansonetti, P. J. (1992). Phosphorylation of IcsA by cAMP-dependent protein kinase and its effect on intercellular spread of *Shigella flexneri*. *Mol. Microbiol.*, **6**, 833–41.

d'Hauteville, H., Dufourcq Lagelouse, R., Nato, F., and Sansonetti, P. J., (1995). Lack of cleavage of IcsA in *Shigella flexneri* causes aberrant movement and allows demonstration of cross reactive eucaryotic protein. *Infect. Immun.*, **64**, 511–17.

Fukada, I., Suzuki, T., Munakata, H., Hayashi, N., Katayama, E., Yoshikawa, M., *et al.* (1995). Cleavage of *Shigella* surface protein VirG occurs at a specific site, but the secretion is not essential for intracellular spreading. *J. Bacteriol.*, **177**, 1719–26.

Goldberg, M. B. and Theriot, J. A. (1995). *Shigella flexneri* surface protein IcsA is sufficient to direct actin-based motility. *Proc. Natl. Acad. Sci. USA*, **92**,

Goldberg, M., Barzu, O., Parsot, C., and Sansonetti, P. J. (1993). Unipolar localization and ATPase activity of IcsA, a *Shigella flexneri* protein involved in intracellular movement. *J. Bacteriol.*, **175**, 2189–96.

Klauser, T., Pohlner, P., and Meyer, T. (1993). The secretion pathway of IgA protease-type proteins in gram negative bacteria. *Bioassays*, **15**, 799–805.

Kocks, C., Marchand, J. B., Gouin, E., d'Hauteville, H., Sansonetti, P. J., Carlier, M. F., *et al.* (1995). The unrelated proteins ActA of Listeria monocytogenes and IcsA of *Shigella flexneri* are sufficient to confer actin-based motility on *L. innocua* and *Escherichia coli* respectively. *Mol. Microbiol.* **18**, 413–23.

Lett, M. C., Sasakawa, C., Okada, N., Sakai, T., Makino, S., Yamada, M., *et al.* (1989). *virG*, a plasmid-coded virulence gene of *Shigella flexneri*: identification of the VirG protein and determination of the complete coding sequence. *J. Bacteriol.*, **171**, 353–9.

Makino, S., Sasakawa, C., Kamata, K., Kurata, T., and Yoshikawa, M. (1986). A virulence determinant required for continuous reinfection of adjacent cells on large plasmid in *Shigella flexneri* 2a. *Cell*, **46**, 551–5.

Sansonetti, P. J., Mounier J., Prévost M. C., and Mege R. M. (1994). Cadherin expression is required for the spread of *Shigella flexneri* between epithelial cells. *Cell*, **76**, 829–39.

Suzuki, T., Saga, S., and Sasakawa, C. (1996). Functional analysis of *Shigella* Vir G domains essential for interaction with vinculin and actin-based motility. *J. Biol. Chem.*, **271**, 21878–85.
Theriot, J. A., Rosenblatt, J., Portnoy, J. A., Goldschmidt-Clermont, P. J., and Mitchison, T. J. (1994). Involvement of profilin in actin-based motility of *L. monocytogenes* in cells and cell-free extracts. *Cell*, **76**, 505–17.

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Zonula occludens toxin (*Vibrio cholerae*)

Zonula occludens toxin is a 44. 8 kDa protein elaborated by Vibrio cholerae. *It binds to a specific receptor whose distribution within the intestine varies, being more represented in the jejunum and ileum, and decreasing along the villous-crypt axis. The toxin activates a complex cascade of intracellular events that regulate the tight junction permeability.*

Zonula occludens toxin (ZOT) is a single peptide chain of 44. 8 kDa (399 amino acid residues, predicted p*I* of 8. 5, GenBank accession number M83563) (Baudry *et al.* 1992). It contains a modified version of the purine NTP-binding motif with a drastic substitution of tyrosine for a conserved glycine (Koonin 1992). ZOT seems to be distantly but reliably related to the product gene I of filamentous bacteriophages, which is a putative ATPase containing the classic NTP-binding motif (Koonin 1992). ZOT hydropathy plots revealed one membrane-spanning segment located downstream from the putative NTPase domain.

Genetic analysis by Mac Vector Analysis Software revealed a conserved region of the gE gene of herpes virus that overlaps the region US9 gene of cytomegalovirus and has a remarkable similarity to the *zot* gene (Pereira *et al.* 1996). Both US9 glycoprotein and gE glycoprotein alter tight junctions and facilitate cell–cell spread of the viruses through the paracellular pathway (Pereira *et al.* 1995). These data suggest that ZOT is the archetype of a new family of enterotoxins that are responsible for a completely new mechanism of microbial pathogenesis.

Preliminary data suggest that ZOT binds to a glycoprotein receptor, whose surface expression among various cell lines and within the intestine varies (Fasano *et al.* 1995). Following this binding, the protein is internalized and activates an intracellular cascade of events leading to the regulation of tight junction permeability.

■ Purification

ZOT was originally prepared from culture supernatants of *Vibrio cholerae* (Fasano *et al.* 1991). In its native form the toxin is extremely unstable, since the biological effect disappears within a few hours of the preparation of the supernatant and is abrogated by freezing or lyophilization (Fasano *et al.* 1991). Moreover, the amount of ZOT that can be obtained either from supernatants or cell lysates of *Vibrio cholerae* cultures is very limited. To increase the amount of ZOT produced, *zot* gene has been fused with the maltose binding protein (MBP) gene and placed under the control of the inducible *tac* promoter. The fusion protein is expressed in *E. coli* and obtained by disrupting the cells, passing the cell lysate supernatant over an amylose column, and eluting the column with maltose. Purified ZOT is finally cleaved from the fusion protein using factor Xa (Uzzau and Fasano, unpublished data). Currently, ZOT is not commercially available (contact person: Alessio Fasano).

■ Activities

ZOT, either as a purified protein or MBP-fusion product, interacts with a membrane receptor present in the jejunum and ileum (particularly on the brush border of mature cells of the tip of the villi), but not in the colon. Once internalized, the toxin induces time- and dose-dependent rearrangement of the cytoskeleton (Fasano *et al.* 1995). This rearrangement is related to the PKCa-dependent polymerization of actin monomers into actin filaments and is mechanistically linked to the redistribution of the ZO-1 protein from the tight junctional complex (Fasano *et al.* 1995) (see Fig. 1). *In-vivo* animal studies showed that, as a consequence of the alteration of the paracellular pathway, the intestinal mucosa becomes more permeable, and water and electrolytes, under the force of hydrostatic pressure, leak into the lumen, resulting in diarrhea. The effect of ZOT on cytoskeleton rearrangement and tissue permeability is completely reversible. The MBP–ZOT fusion protein retains all the biological properties of ZOT.

■ Antibodies

Rabbit polyclonal antibodies showing very good sensitivity (titer 1:5000–1:10000) have been obtained, but are not yet commercially available.

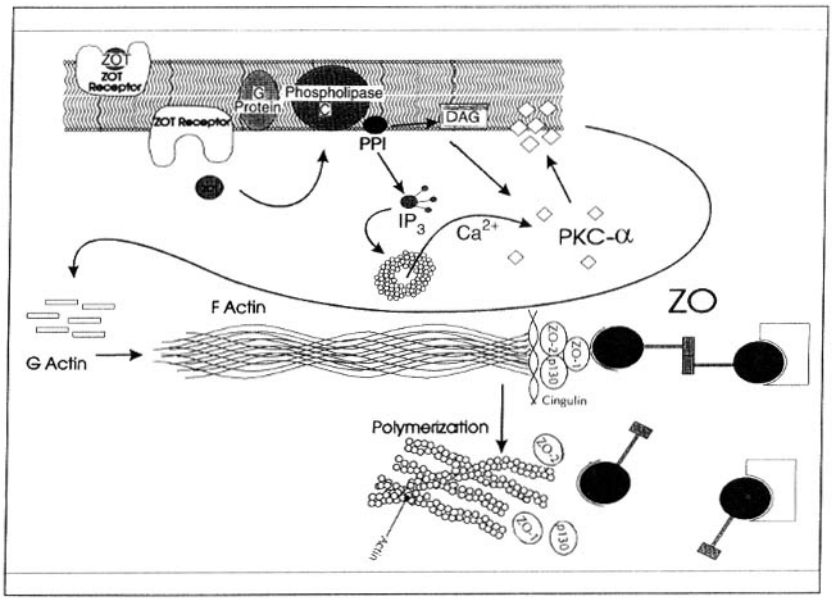

Figure 1. A cartoon depicting the possible mechanism of action of ZOT. Once the toxin is internalized, it activates phospholipase-C (PLC) that will catalyse the production of diacylglycerol (DAG) and inositol-triphosphate (IP_3) from phosphatidyl inositol (PPI). Consequently, Ca^{2+} is released from intracellular stores and activates PKC-α. The phosphorylation of a target protein(s), not yet identified, induces polymerization and rearrangement of actin filaments with subsequent displacement of ZO-1 protein from the junctional complex and opening of tight junctions.

Genes

zot gene is located on the *Vibrio cholerae* chromosome immediately adjacent to the cholera toxin genes (*ctx*) (Baudry *et al.* 1992), within a highly dynamic region of the chromosome defined as the 'virulence cassette' (Trucksis *et al.* 1993). The high concurrence of *zot* gene with *ctx* genes among *Vibrio cholerae* (Johnson *et al.* 1993; Chowdhury *et al.* 1994) suggest a possible synergistic role of ZOT in the causation of the acute dehydrating diarrhea typical of cholera.

Recent evidences seem to suggest that the virulence cassette genes upstream ctx belong to a filamentous phage (CTXø) (Waldor and Mekalanos 1996).

References

Baudry, B., Fasano, A., Ketley, J., and Kaper, J. B. (1992). Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. *Infect. Immun.*, **60**, 428–34.

Chowdhury, M. A. R., Hill, R. T., and Colwell, R. R. (1994). A gene for the enterotoxin zonula occludens toxin is present in *Vibrio mimicus* and *Vibrio cholerae* O139. *FEMS Microbiol. Lett.*, **119**, 377–80.

Fasano, A., Baudry, B., Pumplin, D. W., Wasserman, S. S., Tall, B. D., Ketley, J. M., *et al.* (1991). *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. *Proc. Natl. Acad. Sci. USA*, **88**, 5242–6.

Fasano, A., Fiorentini, C., Donelli, G., Uzzau, S., Kaper, J. B., Margaretten, K., *et al.* (1995) Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, *in vitro*. *J. Clin. Invest.*, **96**, 710–20.

Johnson, J. A., Morris, G. J. Jr, and Kaper, J. B. (1993). Gene encoding zonula occludens toxin (*zot*) does not occur independently from cholera enterotoxin genes (*ctx*) in *Vibrio cholerae*. *J. Clin. Microbiol.*, **31**, 732–3.

Koonin, E. V. (1992). The second cholera toxin, ZOT, and its plasmid-encoded and phage-encoded homologues constitute a group of putative ATPases with an altered purine NTP-binding motif. *FEBS*, **312**, 3–6.

Pereira, L., Maidjy, E., Tugizov, S., and Jones, T. (1995). Deletion mutants in human cytomegalovirus glycoprotein US9 are impaired in cell–cell transmission and in altering tight junctions of polarized human retinal pigment epithelial cells. *Scand. J. Infect. Dis.*, **99**, 82–87.

Trucksis, M., Galen, J. E., Michalski, J., Fasano, A., and Kaper, J. B. (1993). Accessory cholera enterotoxin (ACE), the third member of a *Vibrio cholerae* virulence cassette. *Proc. Natl. Acad. Sci. USA*, **90**, 5267–71.

Waldor, M. K. and Mekalanos J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science*, **272**, 1910–14.

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Toxins affecting the immune and inflammatory response

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Introduction

The study of the effects of bacterial toxins on immune system (IS) cells and functions, includng inflammatory cascade, was very limited until the mid 1980s as compared to the outstanding expansion of other areas of bacterial toxinology, particularly molecular genetics, structural biochemistery, and cell biology of these molecules. The slower pace of the research on IS–toxin interaction stems very likely from the high complexity of the IS network and for methodological reasons. However, the past few years have witnessed remarkable achievements in our knowledge of the molecular mechanisms and the physiological and pathological consequences of toxin–IS interaction. The discovery of the superantigenic properties of certain toxins (following entry p. 89) greatly contributed to this progress.

General features of toxin effects

Two different situations should be considered in this respect:

1. Bacterial toxins whose main targets are exclusively or mainly IS cellular and molecular effectors *per se,* for which the generic name *'immunocytotropic toxins'* could be designated, as proposed by the author.
2. Bacterial toxins which may affect or modulate certain functions of IS cells but which mainly act on cellular and molecular targets unrelated to the IS network. They could be qualified as *'Immunoactive toxins'.*

The modification of IS cell functions and homeostatis by both groups of toxins may lead to various pathophysiological disorders and diseases including host death, resulting from the production by target cells of potent pharmacological and toxic effectors (cytokines, inflammatory cascade products, biogenic amines, etc.).

Immunocytotropic toxins

These proteins released by certain Gram-positive and Gram-negative bacteria act directly on various IS cells particularly T lymphocytes, causing the modulation and disregulation of many cellular functions, T cell mitogenesis (polyclonal proliferation), cell activation *(de novo* expression of cell-surface proteins, which may behave as receptors for various biological ligands), cell apoptosis, anergy, suppression or impairment of humoral and (or) cell-mediated immune responses, in addition to the release of pharmacological effectors.

Two main classes of mitogenic immunocytotropic toxins could be distinguished:

1. Those members of the family of the so-called *superantigens* (see entry p. 89), namely *S. aureus* enterotoxins and TSST-1, *S. pyogenes,* pyrogenic (erythrogenic) exotoxins A and C, and newly discovered mitogens, *Y. pseudotuberculosis* and *Y. enterocolitica* mitogens and *C. perfringens* enterotoxin (Alouf *et al.* 1991; Bowness *et al.* 1992; Stuart and Woodward 1992; Geoffroy *et al.* 1994; Norrby-Teglund 1994; Yasuhiko *et al.* 1995).

   These toxins bind to MHC class IT molecules on antigen presenting cells (APC) and elicit in-parallel polyclonal proliferation of T lymphocytes expressing particular motifs on the V β chain of T cell receptor (Kotzin *et al.* 1993). Independently, they may react with other IS cells such as polymorphonuclear neutrophils leading to the release of inflammatory mediators and expression of heat shock proteins (Hensler *et al.* 1993).
2. Other mitogenic toxins such as pertussis toxin (Rappuoli and Pizza 1991) trigger potent lymphocyte proliferation by a pathway different from that elicited by the superantigens and also disregulate the IS by provoking the inhibition of the migration of peritoneal macrophages, lymphocytosis, immunopotentiation, and modulation of leukotriene generation (Munoz 1988; Hensler *et al.* 1989; Lobet *et al.* 1993).

Immunoactive toxins

A great number of pore-forming, ADP-ribosylating, and other toxins, acting on various eukaryotic cellular or intracellular targets, may also interact with IS cells leading to a variety of modifications, activation, or disregulation of the functions of target immunocytes. This is the case for *E. coli* hemolysin which is a potent stimulus for superoxide generation; the release of many effectors of the inflammatory cascade from human PMN, platelets, or lymphocyte/monocyte/basophil cells; and down-regulation of the release of certain proinflammatory cytokines (Bhakdi and Martin 1991; Grimminger *et al.* 1991; König *et al.* 1994). The sulfydryl-activated toxins (Bremm *et al.* 1987), staphyloccocal alpha toxin (Bhakdi and Tranum-Jensen 1991), and delta toxin (Kasimir *et al.* 1990) also provoke similar effects and complement activation and immunoglobulins Fc binding property (Mitchell *et al.* 1991).

Cholera toxin is also a highly active toxin on the IS network. It exhibits strong systemic and mucosal adjuvant

enhancing properties on IgG and IgA response, through IL-1 production and potentiation of APC activity and prevention of the induction of oral tolerance against bystander antigens (Brommander *et al.* 1991; Pierre *et al.* 1992). *Pseudomonas aeruginosa* exotoxin A elicited immunosuppression of IgG and IgM production by B-cell blasts (Vidal *et al.* 1993).

These few examples illustrate the great theoretical, clinical, and biotechnological interest of investigating toxin interaction with the IS.

References

Alouf, J. E., Knöll, H., and Köhler, W. (1991). The family of mitogenic, shock-inducing and superantigenic toxins from staphylococci and streptococci. In *Sourcebook of bacterial protein toxins* (ed J. E. Alouf and J. H. Freer), pp. 367–414, Academic Press, London.

Bhakdi, S. and Martin, E. (1991). Superoxide generation by human neutrophils induced by low doses of *Escherichia coli* hemolysin. *Infect. Immun.*, **59**, 2955–62.

Bhakdi, S. and Tranum-Jensen, J. (1991). Alpha-toxin of *Staphylococcus aureus*. *Microbiol. Rev.*, **55**, 733–51.

Bowness, P., Moss, A. H., Tranter, H., Bell, I. I., and McMichael, J. (1992). *Clostridium perfringens* enterotoxin is a superantigen reactive with human T cell receptors VB6.9 and VB22. *J. Exp. Med.*, **176**, 893–6.

Bremm, K. D., König, W., Thelestom, M., and Alouf, J. E. (1987). Modulation of granulocyte function by bacterial exotoxins and endotoxins. *Immunology*, **62**, 363–71.

Brommander, A., Holmgren, J., and Lycke, N. (1991). Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages *in vitro*. *J. Immunol.*, **146**, 2908–14.

Geoffroy, C., Müller-Alouf, H., Champagne, E., Cavaillon, J. M., and Alouf, J. E. (1994). Identification of a new extracellular mitogen from group A streptococci. *Zentralbl. Bakteriol. Suppl.*, **24**, 90–1.

Grimminger, F., Scholz, C., Bhakdi, S., and Seeger, W. (1991). Subhemolytic doses of *Escherichia coli* hemolysin evoke large quantities of lipoxygenase products in human neutrophils. *J. Biol. Chem.*, **266**, 14262–9.

Hensler, T., Raulf, M., Megret, F., Alouf, J. E., and König, W. (1989). Modulation of leukotriene generation by pertussis toxin. *Infect. Immun.*, **57**, 1163–71.

Hensler, T., Köller, M., Geoffroy, C., Alouf, J. E., and König, W. (1993). *Staphylococcus aureus* toxic shock syndrome, toxin-1 and *Streptococcus pyogenes* erythrogenic toxin A modulate inflammatory mediator release from human neutrophils. *Infect. Immun.*, **61**, 1055–61.

Kasimir, S., Schonfeld, W., Alouf, J. E., and König, W. (1990). Effect of *Staphylococcus aureus* delta-toxin on human granulocyte functions and platelet-activating factor metabolism. *Infect. Immun.*, **58**, 1653–9.

König, B., Ludwig, A., Goebel, W., and König, W. (1994). Pore formation by the *Escherichia coli* alpha-hemolysin: role for mediator release from human inflammatory cells. *Infect. Immun.*, **62**, 4611–17.

Kotzin, B. L., Leung, D. Y., Kappler, J., and Mamck, P. (1993). Superantigens and their potential role in human disease. *Adv. Immunol.*, **54**, 99–166.

Lobet, Y., Feron, C., Dequesne, O., Simoen, E., Hauser, P., and Locht, C. (1993). Site-specific alterations in the B oligomer that affect receptor-binding activities and mitogenicity of pertussis toxin. *J. Exp. Med.*, **177**, 79–87.

Mitchell, T. J., Andrew, P. W., Saunders, F. K., Smith, A. N., and Boulnois, G. J. (1991). Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. *Mol. Microbiol.*, **5**, 1883–8.

Munoz, S. S. (1988). Actions of pertussigen (pertussis toxin) on the host immune system. In *Pathogenesis and immunity in pertussis* (ed. A. C. Wardlaw and R. Parton), pp. 173–92, Chichester.

Norrby-Teglund, A., Norgren, M., Holm, S. E., Andersson, U., and Andersson, J. (1994). Similar cytokine induction profiles of a novel streptococcal exotoxin, MF and pyrogenic exotoxins A and B. *Infect. Immun.*, **62**, 3731–8.

Pierre, P., Denis, O., Bazin, H., Mbella, E. M., and Vaerman, J. P. (1992). Modulation of oral tolerance to ovalbumin by cholera toxin and its B-subunit. *Eur. J. Immun.*, **22**, 3179–82.

Rappuoli, R. and Pizza, M. (1991). Structure and evolutionary aspects of ADP-ribosylating toxins. In *Sourcebook of bacterial proteins toxins* (ed. J. E. Alouf and J. H. Freer), pp. 1–21, Academic Press, London.

Stuart, P. M. and Woodward, J. G. (1992). *Yersinia enterocolitica* produces superantigenic activity. *J. Immunol.*, **148**, 225–33.

Vidal, D. R., Garrone, P., and Banchereau, J. (1993). Immunosuppressive effects of *Pseudomonas aeruginosa* exotoxin A on human B-lymphocytes. *Toxicon*, **31**, 27–34.

Yasuhiko, I., June, A., Ken-ichi, Y., Tae, T., and Takao, K. (1995). Sequence analysis of the gene for a novel superantigen produced by *Yersinia pseudotuberculosis* and expression of the recombinant protein. *J. Immunol.*, **154**, 5896–906.

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Pyrogenic exotoxins (superantigens) (*Staphylococcus aureus* and *Streptococcus pyogenes*)

The pyrogenic exotoxins produced by two genera of Gram-positive cocci comprise a polymorphic family of genetically related toxins consisting of the the staphylococcal enterotoxins (SE) and the toxic shock syndrome toxin-1 (TSST-1) produced by S. aureus *and the streptococcal pyrogenic exotoxins (SPE) produced by* S. pyogenes. *These molecules of 22 to 29 kDa share a special mechanism of stimulation of T lymphocytes of several species: they crosslink antigen receptor molecules of T cells with major histocompatibility complex (MHC) class II molecules on antigen-presenting cells. This leads to the activation of a large fraction of all T cells to cytokine secretion and proliferation* in vitro *and* in vivo. *The consequences* in vivo *are shock and immunosuppression.*

The members of the pyrogenic exotoxin family are listed in Table 1 (reviewed in Fleischer 1995). The staphylococcal enterotoxins SEA to SEE have been divided into serotypes by antisera, there are three subtypes of SEC (SEC1, SEC2 and SEC3). Recently, SEG and SEH have been cloned. Sequencing of genes of different isolates has shown a marked microheterogeneity due to mutations. Other members of the family are the streptococcal toxins SPEA and SPEC (also designated as erythrogenic toxins) and the streptococcal superantigen SSA. These molecules consist of a single chain of approximately 230 AA, with one central disulphide bond, they have more or less extensive homologies within the family (Betley *et al.* 1992). Many of them are encoded on mobile genetic elements. The structure of TSST-1 (originally designated SEF) differs, it has 194 AA and no cysteins. It has only a very low homology to the other toxins but its 3D-structure is very similar (Kim *et al.* 1994). All toxin genes have been cloned, their molecular biology is reviewed in Betley *et al.* 1992). Toxins of ovine and bovine *S. aureus* strains show slight sequence differences. Gen Bank accession numbers of isolates from human strains are listed in Table 1.

Table 1 Members of the staphylococcal enterotoxin family

Toxin	Producer	M_r	Gen Bank Accession No.
SEA	*S. aureus*	27 100	M18970
SEB		28 366	M11118
SEC1		27 531	X05815
SEC2		27 589	not available
SEC3		27 563	M28364
SED		26 360	M94872
SEE		26 425	M21319
SEG		27 107	not available
SEH		25 140	U11702
TSST-1		22 049	J02615
SPEA	*S. pyogenes*	25 787	M19350
SPEC		24 354	M35514
SSA		26 892	L29565

Alternative names

Staphylococcal enterotoxins (SE) = staphylococcal pyrogenic exotoxins. Streptococcal pyrogenic exotoxins (SPE) = erythrogenic toxins.

Molecular mechanism of action

The toxins are bivalent molecules with two distinct interaction sites (Fig. 1). One is for MHC class II molecules.

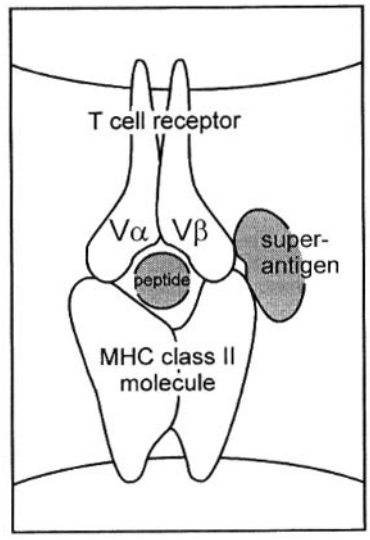

Figure 1. Schematic structure of pyrogenic exotoxins.

Binding affinities vary from 1.11×10^{-5} (SEE) to 8.22×10^{-8} (SEA). Different mechanisms of binding are used: SEA, SED, and SEE bind via a Zn^{2+} bridge involving three histidine residues in the toxin and one histidine residue in the β-chain of the class II molecule. Other toxins such as SEB, SPEA, or TSST-1 do not require Zn^{2+} and bind to different sites on the class II (Jardetzky *et al.* 1994; Kim *et al.* 1994).

With a second interaction site the toxins interact with variable parts of the T cell antigen receptor. Interaction is of very low affinity, physical binding is not detectable. On the $\alpha\beta$-T cell receptor the toxin binding site is the variable part of the β-chain (Vβ), on the $\gamma\delta$-T cell receptor it is the Vγ.

Other bacterial superantigens

Note that there are several bacterial proteins that have been proposed to be superantigens. A novel superantigen is the mitogen of *Y. pseudotuberculosis*, a recently described 14 kDa secreted protein without homology to the staphylococcal enterotoxin family (Mioshi-Akiyama *et al.* 1995). This protein stimulates human Vβ3$^+$, Vβ9$^+$, and Vβ13$^+$ T cells in native and recombinant form. Many other candidates probably have no superantigen activity. Examples are the epidermolytic (exfoliative) toxins of *S. aureus*, the M-proteins and the streptococcal pyrogenic exotoxin B (a protease) of *S. pyogenes*, and the enterotoxin of *C. perfringens* (see Fleischer *et al.* 1995 for review). These molecules are inactive in recombinant form.

Purification and sources

Approximately 40 per cent of *S. aureus* strains secrete one or two (or rarely three) toxins into the culture medium. Concentrations vary between a few ng/ml and more than 100 μg/ml. The situation is similar with *S. pyogenes* strains, although usually lower amounts of toxins are produced. Several protocols have been developed to purify the toxins from the culture supernatant, using ion exchange chromatography or dye matrix absorption chromatography (Alouf *et al.* 1991). Details of these procedures are different for each toxin. Many SE and SPE are commercially available (most of them from Toxin Technology, Sarasate, Florida, and Serva, Heidelberg, Germany, some from Sigma and Calbiochem). Some of these preparations are contaminated with traces of other SE or SPE. Due to the extremely potent activity of superantigens such contaminations are difficult to detect by biochemical methods and can cause problems (Fleischer *et al.* 1995). The toxins can be easily expressed in *E. coli* as native proteins in the cytoplasm or in *S. aureus*.

Toxicity

Enterotoxicity (vomiting and diarrhea) is induced in man and primates by oral ingestion of a few micrograms. Systemic introduction by infection with toxinogenic bacteria leads to toxic-shock syndrome (Schlievert *et al.* 1981; Chatila *et al.* 1991). The use of the toxins does not present a hazard to operators if oral uptake is strictly avoided.

In vivo effects in mice on the immune system are found after injection of less than 1 µg/mouse (iv, ip, or sc). Effects start within 1 to 2 hours (Bette *et al.* 1993). The toxicity, however, is low in mice due to low sensitivity to the effects of liberated cytokines, but lethal shock results if mice are pretreated with D-galactosamin (Miethke *et al.* 1992).

Rabbits are quite sensitive to the effects of the toxins. Pyrogenicity (fever induction) is found after injection of only 0.1 to 1 μg/kg, depending on the toxin (Alouf *et al.* 1991). There is a strong synergy with lipopolysaccharide, sensitivity to lipopolysaccharide is enhanced 50 000-fold, leading to lethal shock.

Use in cell biology

Because the mechanism of T cell stimulation by superantigens has many similarities to stimulation by recognition of MHC/antigen (but there are some differences) superantigens have been widely used as a substitute for specific antigen in immunology. Major progress in the understanding of tolerance, thymic selection, and anergy have been obtained using bacterial and viral superantigens (Herman *et al.* 1991). *In vitro*, superantigens are a much more effective 'physiological' stimulus than the commonly used lectins or anti-CD3 antibodies, especially if used in low ('physiological') concentrations of 1 to 100 ng/ml. T cells then use the same adhesion and signalling molecules when responding to superantigen (with the apparent exception of CD4 and CD8) that are required for responding to specific antigens. Superantigens are involved in certain diseases (although there is disagreement in many cases) and they have been valuable for the study of the pathophysiology of shock.

References

Alouf, J. E., Knöll, H., and Köhler, W. (1991). The family of mitogenic, shock-inducing and superantigenic toxins from staphylococci and streptococci. In *Sourcebook of bacterial protein toxins*. (ed. J. E. Alouf and J. H. Freer) pp. 367–414, Academic Press, New York.

Betley, M. J., Borst, D. W., and Regassa, L. B. (1992). Staphylococcal enterotoxins, toxic shock syndrome toxin and streptococcal pyrogenic exotoxins: a comparative study of their molecular biology. *Chem. Immunol.*, **55**, 1–35.

Bette, M., Schäfer, M. K., van Rooijen, N., Weihe, E., and Fleischer, B. (1993). Distribution and kinetics of superantigen-induced cytokine gene expression in mouse spleen. *J. Exp. Med.*, **178**, 1531–9.

Chatila, T., Scholl, P., Spertini, F., Ramesh, N., Trede, N., Fuleihan, R., *et al.* (1991). Toxic shock syndrome toxin-1, toxic shock and the immune system. *Curr. Top. Microbiol. Immunol.*, **174**, 63–80.

Fleischer, B. (1995). Bacterial superantigens. *Rev. Med. Microbiol.*, **6**, 49–57.

Fleischer, B., Gerlach, D., Fuhrmann, A., and Schmidt, K. H. (1995). Superantigens and pseudosuperantigens of Gram-positive cocci. *Med. Microbiol. Immunol.*, **184**, 1–8.
Herman, A., Kappler, J. W., Marrack, P., and Pullen, A.M. (1991). Superantigens: mechanism of T cell stimulation and role in immune responses. *Ann. Rev. Immunol.*, **9**, 745–72.
Jardetzky, T. S., Brown, J. H., Gorga, J. C., Stern, L. J., Urban, R. G., Chi, Y. I., *et al.* (1994). Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. *Nature*, **368**, 711–18.
Kim, J., Urban, R. G., Strominger, J. L., and Wiley, D. C. (1994). Toxic shock syndrome toxin-1 complexed with a class II major histocompatibility molecule HLA-DR1. *Science*, **266**, 1870–4.
Miethke, T., Wahl, C., Heeg, K., Echtenacher, B., Krammer, P. H., and Wagner, H. (1992). T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J. Exp. Med.*, **175**, 91–8.
Mioshi-Akiyama, T., Abe, A., Kato, H., Kawahara, K., Narimatsu, H., and Uchiyama, T. (1995). DNA sequencing of the gene encoding a bacterial superantigen, Yersinia pseudotuberculosis-derived mitogen (YPM), and characterization of the gene product, cloned YPM. *J. Immunol.*, **154**, 5228–34.
Schlievert, P. M., Shands, K. N., Dan, B. B., Schmid, G. P., and Nishimura, R. D. (1981). Identification and characterization of an exotoxin from *Staphylococcus aureus* associated with toxic-shock syndrome. *J. Infect. Dis.*, **143**, 509–16.

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Anthrax lethal toxin (*Bacillus anthracis*)

Anthrax lethal toxin (LeTx) causes the shock-like symptoms observed in systemic anthrax infections by inducing macrophages to overexpress proinflammatory cytokines. LeTx is comprised of two proteins, both of which are required for toxicity. The protective antigen (PA) binds to cellular receptors and is responsible for translocation of the lethal factor (LF), the catalytic moiety, across the plasma membrane into the cytosol. Sequence analysis suggests that LF may be a metalloprotease whose substrate remains unidentified.

Anthrax lethal toxin (LeTx) induces shock and sudden death in test animals, mimicking the symptoms of systemic anthrax infections (Leppla 1991; Hanna *et al.* 1993). Bacterial strains lacking either of the two LeTx components are highly attenuated, and immunization against the toxin protects animals from *B. anthracis* challenge (Cataldi *et al.* 1990; Pezard *et al.* 1991; Turnbull 1991). LeTx is composed of two proteins (Fig. 1): protective antigen (mature PA, 82.7 kDa, 735 amino acids, Swiss Prot databank: PAG-BACAN) and lethal factor

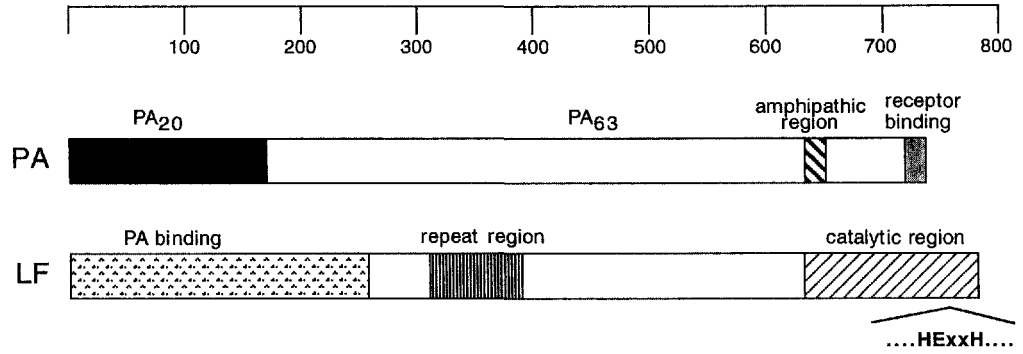

Figure 1. Schematic representations of the two mature anthrax lethal toxin proteins, protective antigen (PA) and lethal factor (LF). Both are required for toxicity. PA is responsible for binding to receptors on mammalian cells and for membrane translocation of LF into the cytosol. LF is believed to serve a catalytic function, perhaps specific proteolysis of an intracellular target protein (see text).

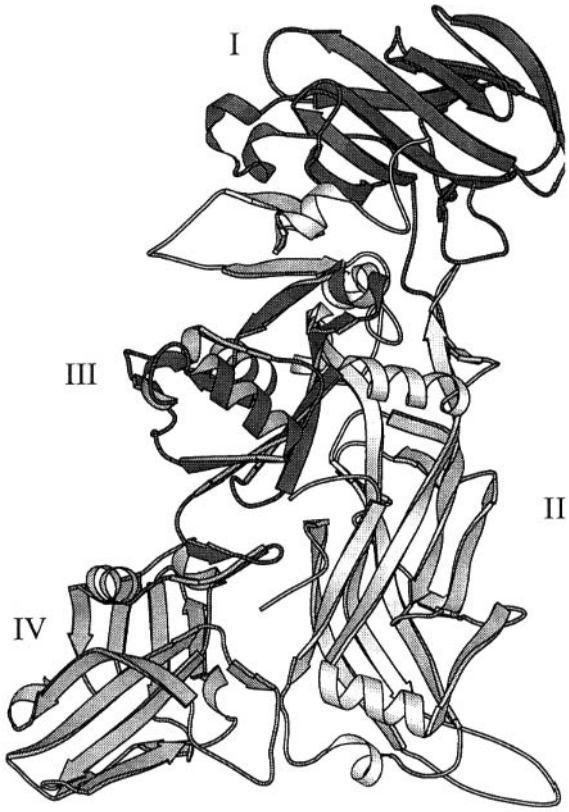

Figure 2. Ribbon diagram of the crystallographic structure of protective antigen (PA), showing the four folding domains (unpublished results, C. Petosa, R. Liddington). Note that the protein consists largely of β structure. Proteolytic activation results in removal of domain I, leaving PA_{63}, corresponding to domains II–IV, which is capable of binding LF and oligomerizing to form heptameric, ring-shaped structures (see Fig. 3). Ribbon diagram was generated using MOLSCRIPT (Kraulis 1991).

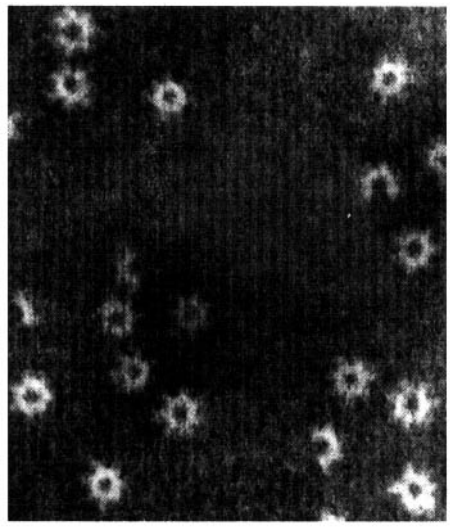

Figure 3. Electron micrograph of negatively stained heptameric rings of PA_{63}. Taken from Milne *et al.* (1994).

(mature LF, 90.2 kDa, 776 amino acids, Swiss Prot databank: LEF-BACAN). The crystallographic structure of PA has recently been determined (Fig. 2) (unpublished results, courtesy of C. Petosa, R. Liddington). Notably, there are no cysteine residues in the entire LeTx complex (Bragg and Robertson 1989). After binding to its receptor on cells, PA is cleaved by a cellular protease with the specificity of furin, and the PA C-terminal 63 kDa peptide (PA_{63}) remains associated with the membrane (Klimpel *et al.* 1992). PA_{63} binds LF avidly. During normal receptor-mediated endocytosis and acidification of the endosome, PA_{63} undergoes conformational changes, oligomerizing into rings with seven-fold symmetry and forming pores in artificial lipid-bilayers and membranes (Fig. 3). These conformational changes are believed to be relevant to the the role of PA_{63} in translocation of the catalytic moiety, LF, into the cytosol (Koehler and Collier 1991; Milne and Collier 1993; Milne *et al.* 1994). No intracellular target for LF has been identified, but LF contains the consensus sequence —HExxH— that is found in the active sites of Zn^{2+}-metalloproteases such as thermolysin and the clostridial neurotoxins (Klimpel *et al.* 1994). Independent reports cite evidence that LF binds to either one (Klimpel *et al.* 1994) or three (Kochi *et al.* 1994) Zn^{2+} ions.

Although LeTx is believed to enter most types of cells, only macrophages have been reported to respond (Freidlander 1986). At high LeTx concentrations, a hyper-stimulation of the oxidative burst results in reactive oxygen-mediated lysis *in vitro* within 60–90 minutes (Hanna *et al.* 1994). At sublytic toxin concentrations, macrophages are induced to express proinflammatory mediators such as IL-1β and TNFα, and it the release of high levels of these mediators that are believed to be responsible for the symptoms and lethal shock associated with anthrax (Hanna *et al.* 1993).

Purification and sources

PA and LF, both of which are plasmid-encoded, are expressed from capsule-minus (attenuated) strains of *B. anthracis* cultures grown in defined RM medium in the presence of physiological concentrations of the transcriptional inducer, CO_2 (in the form of $NaHCO_3$) (Leppla 1988). Virtually pure toxin species are obtained after ammonium sulfate precipitation and anion exchange FPLC. Additionally, recombinant forms of PA and LF, as well as toxin fragments and chimeric fusions, can be obtained from *E. coli* (Arora and Leppla 1994; Milne *et al.* 1995). LeTx is not commercially available.

Toxicity

Anthrax toxin is lethal to most animal species, the most sensitive being the Fischer 334 rat (Lincoln and Fish 1970). The rat model was used in the original derivation of units of lethal toxin activity. Toxicity varies greatly among species, with an interesting inverse correlation between sensitivity to LeTx and sensitivity to *B. anthracis* spores (Lincoln and Fish 1970). This inverse correlation holds between different strains of inbred mice (Welkos *et al.* 1986). Use of LeTx does not present hazards to researchers if normal safety precautions are heeded, but as a precaution, PA and LF should be handled separately whenever possible. Vaccines are available but not required under most laboratory circumstances.

Use in cell biology

Use of LeTx as a tool for studying cellular processes has centered primarily on the basic biological problem of delivery of large hydrophilic proteins across the plasma membrane (e.g. receptor-binding, proteolytic activation, oligomerization, membrane insertion, translocation) (Leppla *et al.* 1988; Koehler and Collier 1991; Leppla 1991; Klimpel *et al.* 1992; Milne *et al.* 1994; Milne and Collier 1993). The toxin structures and domain motifs involved in these processes are currently being described (Fig. 2), and molecular models of translocation have been proposed (Freidlander 1990; Milne and Collier 1993; Milne *et al.* 1994). LeTx has also been used as a specific stimulator of acute macrophage immune responses that are both metabolically and genetically regulated (e.g. oxidative burst, cytokine expression) (Hanna *et al.* 1993, 1994). It is believed that both the cytotoxic and lethal clinical manifestations of LeTx are mediated directly by hyper-stimulation of normal macrophage immune activities (Hanna et al. 1993, 1994). Although this line of inquiry is still in its infancy, it is hoped that LeTx will provide a specific pharmacological tool to dissect key regulatory events during macrophage activation. The molecular target for the lethal factor within macrophages remains elusive, but is likely to be the key to understanding the dramatic responses of macrophages to LeTx.

References

Arora, N. and Leppla, S. H. (1994). Fusions of anthrax toxin lethal factor with shiga toxin and diphtheria toxin enzymatic domains are toxic to mammalian cells. *Infect. Immun.*, **62**, 495561.

Bragg, T. S. and Robertson, D. L. (1989). Nucleotide sequence and analysis of the lethal factor gene (lef) from *Bacillus anthracis*. *Gene*, **81**, 45–54.

Cataldi, A., Labruyere, E., and Mock, M. (1990). Construction and characterization of a protective antigen-deficient *Bacillus anthracis* strain. *Mol. Microbiol.*, **4**, 1111–17.

Freidlander, A. M. (1986). Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *J. Biol. Chem.*, **261**, 7123–6.

Freidlander, A. M. (1990). In *Trafficking of bacterial toxins* (ed. C.B. Saelinger), pp. 121–38, CRC Press, Boca Raton.

Hanna, P. C., Acosta, D., and Collier, R. J. (1993). On the role of macropahges in anthrax. *Proc. Natl. Acad. Sci. USA*, **90**, 10198–210.

Hanna, P. C., Kruskal, B. A., Ezekowitz, R. A. B., Bloom, B. R., and Collier, R. J. (1994). Role of macrophage oxidative burst in the action of anthrax lethal toxins. *Mol. Medicine*, **1**, 7–17.

Klimpel, K. R., Molloy, S. S., Thomas, G., and Leppla, S. H. (1992). Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. *Proc. Natl. Acad. Sci. USA*, **89**, 10277–81.

Klimpel, K. R., Arora, N., and Leppla, S. H. (1994). Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. *Mol. Microbiol.*, **13**, 1093–100.

Kochi, S. K., Schiavo, G., Mock, M., and Montecucco, C. (1994). Zinc content of the *Bacillus anthracis* lethal factor. *FEMS Microbiol. Lett.*, **124**, 343–8.

Koehler, T. M. and Collier, R. J. (1991). Anthrax toxin protective antigen: low-pH-induced hydrophobicity and channel formation in liposomes. *Mol. Microbiol.*, **5**, 1501–6.

Kraulis, P. (1991). MOLSCRIPT: a program for viewing ribbon action of anthrax lethal toxins. *J. Appl. Crystallogr.*, **24**, 946–50.

Leppla, S. H. (1988). Production and purification of anthrax toxin. *Meth. Enzymol.* ,**165**, 103–16.

Leppla, S. H. (1991). The antrax toxin complex. In *Sourcebook of bacterial toxins* (ed. J. Alouf and J. H. Freer), pp. 277–302, Academic Press, New York.

Leppla, S. H., Freidlander, A. M., and Cora, E. M. (1988). Proteolytic activation of anthrax toxin bound to cellular receptors. In *Bacterial Protein Toxins* (eds. Fehrenbach, F. Alouf, J., Falmagne, P., Goebel, W., Jeljaszewicz, J., Jurgen, D., and Rappuoli, R. pp. 111–12, Gustav Fischer, Stuttgart.

Lincoln, R. E. and Fish, D. C. (1970). In *Microbial toxins III* (eds. T.C. Montie, Kadis, S., and Ajl, S. J.), pp. 361–414, Academic Press, New York.

Milne, J. C. and Collier, R. J. (1993). pH-dependent permeabilization of the plasma membrane of mammalian cells by anthrax protective antigen. *Mol. Microbiol.*, **10**, 647–53.

Milne, J. C., Furlong, D., Hanna, P. C., Wall, J. S., and Collier, R. J. (1994). Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J Biol. Chem.*, **269**, 20607–12.

Milne, J. C., Blanke, S. R., Hanna, P. C., and Collier, R. J. (1995). Protective antigen-binding domain of anthrax lethal factor mediates translocation of a heterologous protein fused to its amino- or carboxy-terminus. *Mol. Microbiol.*, **15**, 661–6.

Pezard, C., Berche, P., and Mock, M. (1991). Contribution of individual toxin components to virulence of *Bacillus anthracis*. *Infect. Immun.*, **59**, 3472–7.

Turnbull, P. C. (1991). Anthrax vaccines: past, present and future. *Vaccine*, **9**, 533–9.

Welkos, S. L., Keener, T. J., and Gibbs, P. H. (1986). Differences in susceptibility of inbred mice to *Bacillus anthracis*. *Infect. Immun.*, **51**, 795–800.

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Leukocidins and gamma lysins (*Staphylococcus* sp.)

Staphylococcal leukocidins and γ-lysins form a family of bi-component toxins. Two synergic components, each one inactive by itself, bind with high affinity to the membrane of the target cell and assemble a pore, probably oligomeric. Pore formation in leukocytes triggers secondary events like degranulation, secretion, phospholipase activation, DNA degradation, and leukotriene release. In vivo *they induce a strong inflammatory response.*

Staphylococcus aureus strains produce two kinds of bi-component toxins designated as leukocidins and γ-lysins (Freer and Arbuthnott 1983; Noda and Kato 1991).

The Panton–Valentine (PV) leukocidins are two single polypeptide chains of 32 and 34.5 kD, called LukS and LukF respectively (S and F stand for slow and fast eluting during preparation). As single components they are inactive but act synergistically to induce damage of selected white cells: monocytes, macrophages, and polymorphonucleated cells (neutrophils), but not lymphocytes.

Gamma-lysins are also synergic toxins albeit with some differences. The *hlg* locus encodes three proteins: HlgA, HlgB, and HlgC. HlgB can act synergically with either HlgA or HlgC not only to attack leukocytes but also to lyse red blood cells.

As the DNA sequences of these proteins have become available a number of notions became clear:

(1) leukocidins and γ-lysins share strong sequence homology and form a family;

(2) LukS, HlgC, and HlgA form a subfamily (identified as class S components), LukF and HlgB form a second subfamily (identified as class F components);

(3) most toxins previously identified as leukocidins (except the Panton–Valentine) are in fact identical to γ-lysins (LukS to HlgC and LukF to HlgB), see Table 1.

Their relevance as a virulence factor has been firmly established, by epidemiology and in animal models. From 309 clinical isolates of *S. aureus* 99 per cent carried the *hlg* locus and 2 per cent both the *hlg* and the *luk*-PV loci

Table 1 The family of synergic leukotoxins from *Staphylococcus* sp.

Toxin	Acronym[a]	Sp.	Strain	Accession numbers[b]	Chain length (AA)	Reference
S-component						
γ-lysin A	γ1, HlgA	aureus	Smith 5R	L01055 (EMBL)	281	1
γ-lysin A	HlgA	aureus	ATCC 49775	X81586 (EMBL)	280	2
γ-lysin A	HII, HlgA	aureus	RIMD310925	D42143 (EMBL); P31714 (PIR)	280	3
γ-lysin C	γ2, HlgC	aureus	Smith 5R	L01055 (EMBL)	286	1
leukocidin S	LukS, HlgC	aureus	RIMD310925	JN0626 (PIR)	286	3
leukocidin S-R	LukS-R, HlgC	aureus	P83	A49234 (PIR)	286	4
leukocidin S-PV	LukS-PV	aureus	ATCC 49775	X72700 (EMBL)	286	2
leukocidin S	LukS-I	intermedius	ATCC 51874	X79188 (EMBL); S44944 (PIR)	281	5
leukocidin M	LukM	aureus	P83		280	6
F-component						
γ-lysin B	HlgB	aureus	Smith 5R	L01055 (EMBL)	300	1
leukocidin F	LukF, HlgB	aureus	RIMD310925	JN0627 (PIR)	298	3
leukocidin F-R	LukF-R, HlgB	aureus	P83	X64389 (EMBL); B49234 (PIR)	300	4
leukocidin F-PV	LukF-PV	aureus	ATCC 49775	X72700 (EMBL)	301	2
leukocidin F	LukF-I	intermedius	ATCC 51874	X79188 (EMBL); S44945 (PIR)	300	5

[a] The second acronym is the one proposed to become definitive. [b]Accession numbers are referred to the following data bases: EMBL: DNA sequences; PIR: protein identification resource. References: 1, Cooney *et al.* 1993; 2, Prévost *et al.* 1995*c*; 3, Rahman *et al.* 1993; 4, Supersac *et al.* 1993; 5, Prévost *et al.* 1995*a*; 6, Choorit *et al.* 1995.

(Prévost *et al.* 1995*b*). These last strains were the most pathogenic.

The mode of action of PV-leukocidins has now been reasonably established. Since the PV strain produces both leukocidins and γ-lysins all mixed couples were tried and some were found to be active. All toxic couples always contain one class S component and one class F component (see Table 2). The binding of the two components is sequential. The S component binds first and then triggers the binding of the F component. The binding of the S component is tight and occurs with high affinity only to certain cells implying the presence of a protein receptor. The two components finally assemble a pore on the membrane of the cell, which in the presence of Ca^{2+} is selective for divalent cations. Formation of the pore seems to involve toxin oligomerization (Noda and Kato 1991; Finck-Barbançon *et al.* 1993), possibly a trimerization (Ozawa *et al.* 1995). Pore formation triggers a number of secondary events: degranulation, secretion, activation of phospholipase A_2, release of leukotriene B_4 (with chemotactic effects on neutrophils and eosinophils), and DNA fragmentation (Hensler *et al.* 1994). All these effects involve a toxin-induced Ca^{2+} influx. γ-Lysins are also hemolytic towards a number of mammalian species, although human red blood cells are only sensible to the couple HlgA–HlgB. Other activities such as ADP-ribosylation and phospholipase and protease activity have been reported, but their relevance has to be demonstrated. It is not clear at present how they relate to the described pore-forming action.

The permeabilizing activity of γ-lysins extends also to model membranes, such as liposomes and planar bilayers (Ferreras *et al.* 1996). In planar lipid membranes they form a water-filled pore, rather similar to the one of α-toxin. This suggests that γ-lysins can interact with the lipid bilayer without the absolute requirement for a receptor.

The family name of synergohymenotropic toxins (SHT), standing for synergic toxins acting on membranes, has been proposed, but still awaits general acceptance.

S. aureus leukocidins and γ-lysins share sequence homology with α-toxin (see entry in this volume p. 10; Cooney *et al.* 1993), and in part also with *Clostridium perfringens* β-toxin which is found in type B and type C strains (Hunter *et al.* 1993).

Table 2 Leukotoxic and hemolytic activity of the synergic leukotoxins from *Staphylococcus aureus* (ATCC 49775)
(a) Leukotoxic activity (U/mg)

	LukS	HlgC	HlgA
LukF	7×10^7	2×10^7	3×10^7
HlgB	2×10^7	2×10^6	8×10^5

(b) Hemolytic activity (U/mg)

	LukS	HlgC	HlgA
LukF	$<2 \times 10^2$	$<2 \times 10^2$	2.2×10^4
HlgB	$<2 \times 10^2$	1.6×10^6	5.2×10^7

Leukotoxic activity was determined on human PMN adherent to glass and expressed as the amount inducing 50 per cent permeabilization.
Hemolytic activity, was determined on rabbit red blood cells, and expressed as the amount inducing 50 per cent hemolysis.
Erythrocytes, at a concentration of 1 per cent were incubated with variable amount of toxins for 1 hour at 37 °C.
Data were taken from Prévost *et al.* (1995*c*).

Purification and sources

Purification protocols for leukocidins and γ-lysins can be found in Freer and Arbuthnott (1983), however the most recent and effective procedure consists of three steps (Finck-Barbançon *et al.* 1991): ammonium sulfate precipitation from bacterial culture supernatant followed by two passages of cation-exchange chromatography, the first on carboxymethyl-Trysacryl-M and the second on a Mono-S column (Pharmacia). These toxins are not commercially available.

Toxicity

Data on toxicity of PV-leukocidins and γ-lysins throughout the literature are contradictory, mainly due to the different level of purification of the single components. In general it is now accepted that they are not lethal (Freer and Arbuthnott 1983). PV strains produce both leukocidins and γ-lysins and accordingly, taking one class S component and one class F component, six different synergic combinations were tested. By intradermal injection in rabbit it was found that the most active couple was LukS–LukF, inducing acute inflammation and skin necrosis starting from doses of 300 ng; HlgA–LukF had a similar effect, but at doses of 3000 ng; the other four couples, i.e. HlgC–LukF, LukS–HlgB, HlgC–HlgB, and HlgA–HlgB, were only able to induce inflammatory lesions, from doses of 300 ng (Prévost *et al.* 1995*c*). Leukotoxic and hemolytic potency of the six couples are reported in Table 2.

Use in cell biology

Similarly to α-toxin staphylococcal leukocidins could be used to selectively permeabilize cells to small molecules, if applied in the absence of Ca^{2+}, or selectively to Ca^{2+} ions in its presence (Finck-Barbançon *et al.* 1993). Class S components can be used to selectively tag or label certain types of white cells (Meunier *et al.* 1995) without harming them. Upon subsequent addition of the class F component these cell lines can be permeabilized or even destroyed, depending on the dosage used. For other applications of their pore-forming ability (e.g. *perforated-patch, stimulated on–off switching*, etc.) see the entry α-toxin (p. 10).

References

Choorit, W., Kaneko, J., Muramoto, K., and Kamio, Y. (1995). Existence of a new protein component with the same function as the LukF component of leukocidin or gamma-lysin and its gene in *Staphylococcus aureus* P83. *FEBS Lett.*, **357**, 260–4.

Cooney, J., Kienle, Z., Foster, T. J., and O'Toole, P. W. (1993). The gamma-hemolysin locus of *Staphylococcus aureus* comprises three linked genes, two of which are identical to the genes of the F and S component of leukocidin. *Infect. Immun.*, **61**, 768–71.

Ferreras, M., Menestrina, G., Foster, T. J., Colin, D. A., Prévost, G., and Piemont, Y. (1996). Permeabilisation of lipid bilayers by *Staphylococcus aureus* gamma-toxins. *Zentralbl. Bakteriol. Suppl.*, **26**, (in press).

Finck-Barbançon, V., Prévost, G., and Piemont, Y. (1991). Improved purification of leukocidin from *Staphylococcus aureus* and toxin distribution among hospital strains. *Res. Microbiol.*, **142**, 75–85.

Finck-Barbançon, V., Duportail, G., Meunier, O., and Colin, D. A. (1993). Pore formation by a two-component leukocidin from *Staphylococcus aureus* within the membrane of human polymorphonucleated leukocytes. *Biochim. Biophys. Acta*, **1182**, 275–82.

Freer, J. H. and Arbuthnott, J. P. (1983). Toxins of *Staphylococcus aureus*. *Pharmacol. Therapeut.*, **19**, 55–106.

Hensler, T., König, B., Prévost, G., Piemont, Y., Köller, M., and König, W. (1994). Leukotriene B_4 generation and DNA fragmentation induced by leukocidin from *Staphylococcus aureus*: protective role of granulocytes-macrophage colony-stimulating factor (GM-CSF) and G-CSF for human neutrophils. *Infect. Immun.*, **62**, 2529–35.

Hunter, S. E. C., Brown, J. E., Oyston, P. C. F., Sakurai, J., and Titball, R. W. (1993). Molecular genetic analysis of beta-toxin of *Clostridium perfringens* reveals sequence homology with alpha-toxin, gamma toxin, and leukocidin of *Staphylococcus aureus*. *Infect. Immun.*, **61**, 3958–65.

Meunier, O., Falkenrodt, A., Monteil, H., and Colin, D. A. (1995). Application of flow cytometry in toxinology: pathophysiology of human polymorphonuclear leukocytes damaged by a pore-forming toxin from *Staphylococcus aureus*. Cytometry, **21**, 241–7.

Noda, M. and Kato, I. (1991). Leukocidal toxins. In *Sourcebook of bacterial protein toxins* (ed. J. E. Alouf and J. H. Freer), pp. 243–51, Academic Press, London.

Ozawa, T., Kaneko, J., and Kamio, Y. (1995). Essential binding of LukF of staphylococcal gamma-hemolysin followed by the binding of HgII for the hemolysis of human erythrocytes. Biosci. Biotech. Biochem., **59**, 1181–3.

Prévost, G., Bouakkam, T., Piemont, Y., and Monteil, H. (1995*a*). Characterisation of synergohymenotropic toxin produced by *Staphylococcus intermedius*. *FEBS Lett*, **376**, 135–40.

Prévost, G., Couppié, P., Prévost, P., Gayet, S., Petiau, P., Cribier, B., *et al.* (1995*b*). Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. *J. Med. Microbiol.*, **42**, 237–45.

Prévost, G., Cribier, B., Couppié, P., Petiau, P., Supersac, G., Finck-Barbançon, V., *et al.* (1995*c*). Panton-Valentine leucocidin and gamma-hemolysin from *S. aureus* ATCC 49775 are encoded by distinct genetic loci and have different biological activities. *Infect. Immun.*, **63**, 4121–9.

Rahman, A., Izaki, K., and Kamio, Y. (1993). Gamma-hemolysin genes in the same family with lukF and lukS genes in methicillin resistant *Staphylococcus aureus*. Biosci. Biotech. Biochem., **57**, 1234–6.

Supersac, G., Prévost, G., and Piemont, Y. (1993). Sequencing of leucocidin R from *Staphylococcus aureus* P83 suggests that staphylococcal leucocidins and gamma-hemolysin are members of a single two-component family of toxins. *Infect. Immun.*, **61**, 580–7.

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6

Toxins affecting membrane traffic

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Introduction

Life of eukaryotic cells is made possible by an intense vesicular membrane traffic, highly organized in terms of space and kinetics. Ligands, ions, fluids, and portions of the plasma membrane are continuously taken into the cell via endocytosis, and are then addressed to different destinations via sorting compartments. Conversely, proteins, lipids, and glycoconjugates, synthetized in the endoplasmic reticulum and modified in the Golgi apparatus are addressed to their intracellular destination, or are packed inside vesicles for export. Exocytosis can be constitutive for molecules that are to be exported at a basal rate, or regulated for molecules that are to be released in response to appropriate stimuli. The most finely tuned exocytic process is the release of neurotransmitter at the presynaptic membrane. Neurotransmitter is contained within small synaptic vesicles of similar size (about 50 nm in diameter) which are packed at synaptic terminals. The large majority of these vesicles are bound to the cytoskeleton and are not available for immediate release. Some vesicles are bound to the cytosolic face of the presynaptic membrane at active zones which include voltage gated calcium channels. They are ready to release their content in the intersynaptic space. However, at micromolar calcium, only occasionally one of them fuses with the presynaptic membrane, giving rise to a mepp (miniature end plate potential). The membrane depolarization that is at the basis of the transmission of the nerve impulse, opens presynaptic calcium channels. This leads to a local increase of calcium concentration which triggers, within 100–300 μs, the fusion of the synaptic vesicle with the plasma membrane, with the ensuing release of neurotransmitter. The proteins involved in the docking–priming–fusion of small synaptic vesicles with the presynaptic membrane are being characterized. They show a high degree of conservation from yeasts to man and this underlies a similar general organization of the multi-subunit molecular apparatus which mediates the vesicle–membrane fusion process. Three of these proteins: VAMP/synaptobrevin, SNAP-25, and syntaxin are the very specific target of the zinc-dependent proteolytic activity of eight neurotoxins produced by bacteria of the genus Clostridium. The end result of the action of these neurotoxins is always the same: neuroexocytosis, this highly specialized and regulated final step of vesicular traffic, is blocked. These toxins can be used in biochemical knock-out experiments as described in the various entries.

Recently, it has emerged that another bacterial protein toxin interferes with membrane trafficking. The *Helicobacter pylori* cytotoxin causes a massive vacuolar degeneration of cells. Available evidence indicate that the toxin alters, in a rab7 dependent way, the size and dimension of the late endosomal compartment. Late endosomes receive proteins and membranes both from early endosomes and from the trans-Golgi network. Materials leave late endosomes to lysosomes or to the TGN. Though the molecular mechanism of action of the *H. pylori* cytotoxin is still unknown, an entry is included here because of its potential usefulness in studying the endocytic pathway. Given the fundamental importance of a coordinated vesicle movement within cells, it is not unexpected that other toxins will be found to interfere with such cellular events.

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Tetanus neurotoxin (*Clostridium tetani*)

Tetanus neurotoxin is a 150 kDa protein produced by toxigenic strains of Clostridium tetani. *It binds specifically to neuronal cells and enters the cytosol, where it cleaves specifically VAMP/synaptobrevin at a single peptide bond. This selective proteolysis prevents the assembly of the neuroexocytosis apparatus, and release of neurotransmitter evoked by membrane depolarization cannot take place.*

Tetanus neurotoxin (TeNT) is the cause of the spastic paralysis of tetanus (van Heyningen 1968; Montecucco and Schiavo 1995). It is produced as a single polypeptide chain of 150 kDa (1315 amino acid residues, sequence accession number to the Swiss Prot data bank: P04958|TETX_CLOTE). As shown in Fig. 1, it consists of three 50 kDa domains. Selective proteolysis generates within the bacterial culture the active di-chain toxin with a single interchain disulfide bond, whose reduction leads to total loss of neurotoxicity (Schiavo *et al.* 1990). The di-chain toxin is the commercially available form. The heavy chain (H, 100 kda) is composed of two domains. H_C is responsible for the neurospecific binding to a yet unknown presynaptic nerve terminal protein receptor. H_N is involved in the membrane translocation of the L chain in the cytosol (L, 50 kDa). In the course of the pathogenesis of tetanus, TeNT is internalized at the presynaptic terminal of the neuromuscular junction and migrates retroaxonally, inside the motorneuron, to the spinal cord (Schwab *et al.* 1979). It is then released in the intersynaptic space between the motorneuron and the inhibitory interneuron (Renshaw cell), it penetrates this latter cell via intracellular acidic compartments (Williamson and Neale 1994) and blocks their neuroexocytosis. This trafficking of TeNT ends with the specific intoxication of the Renshaw cells of the spinal cord only when TeNT is present at doses of about 1 ng/kg in the mouse. This is the case of clinical tetanus which is characterized by a spastic paralysis. This type of paralysis follows the impairment of the Renshaw cell dependent system that ensures balanced skeletal muscle contraction. In contrast, TeNT at higher doses (> 500 ng/kg in the mouse) causes a flaccid paralysis (Matsuda *et al.* 1982), i.e. it blocks acetylcholine release at the neuromuscular junction, because it becomes capable of entering the moto neuron terminal. TeNT has also been injected into selected brain areas to produce an epilectic-like syndrome (Bagetta *et al.* 1991).

TeNT L chain is composed of 447 amino acid residues (Eisel *et al.* 1986; Fairweather and Lyness 1986), and its sequence shows two regions of strong homology with those of the other clostridial neurotoxins. An amino terminal part of the molecule is likely to be responsible for the specific binding of VAMP (see below). In the middle of the L chain there is a 20 residue long segment, which contains the zinc-binding motif of zinc-endopeptidases. There is a single zinc atom bound to the L chain of TeNT and it is essential for activity (Schiavo *et al.* 1992a).

Purification and sources

TeNT is isolated from culture supernatants of *Clostridium tetani* by ammonium sulfate precipitation followed by

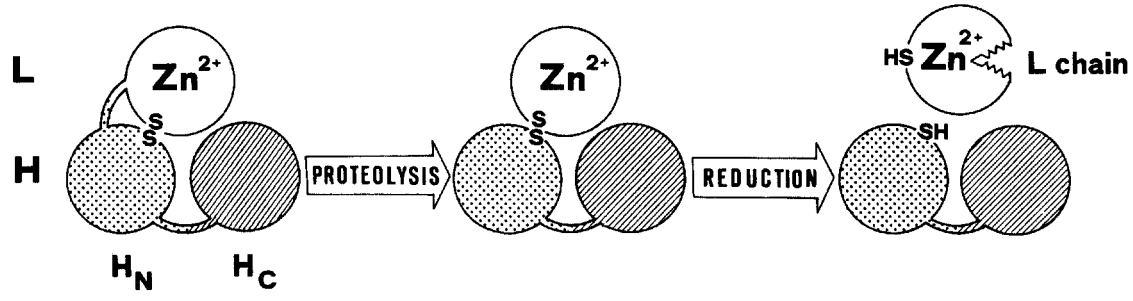

Figure 1. Scheme of the structure and mechanism of activation of tetanus neurotoxin. The toxin is released by *Clostridium tetani* as an inactive single polypeptide chain of 150 kDa, composed of three 50 kDa domains, connected by protease-sensitive loops. Activation follows a selective proteolytic cleavage which generates two disulfide-linked chains: L (50 kDa) and H (100 kDa) and reduction of the single interchain disulfide bond. The three domains play different functional roles in cell penetration: H_C is responsible for cell binding and H_N for cell penetration. Reduction takes place inside the nerve cell and liberates the activity of the L chain in the cytosol. L is a zinc-endopeptidase specific for VAMP/synaptobrevin.

chromatography (Matsuda and Yoneda 1975; Schiavo and Montecucco 1995). During this procedure it is also possible to obtain some free L chain, which can be purified to homogeneity via isoelectrofocusing or HPLC (Weller *et al.* 1989; De Filippis *et al.* 1995). The L chain of TeNT has also been produced by expression in *E. coli* (Yamasaki *et al.* 1994). H_C is obtained by selective proteolysis of TeNT with papain followed by chromatography (Helting and Zwisler 1977; Matsuda *et al.* 1989). TeNT and H_C can be purchased from Alomone Labs, Boehringer, Calbiochem and List. Purity should be controlled by SDS-PAGE.

Toxicity

Toxicity is tested by intraperitoneal injection of different amounts of toxin. Mouse LD_{50} is 0.2–0.5 ng/kg and that of man is estimated to be even lower (Payling-Wright 1955; Gill 1982). TeNT toxicity varies in different animal species and this is due to different binding at the neuromuscular junction and/or to mutations at the site of action of TeNT inside cells. The use of this toxin does not present problems to operators vaccinated with tetanus toxoid vaccine. If more than ten years have elapsed from the last vaccine injection, it is advisable to have a single booster injection. The toxin is very sensitive to oxidants and hence a simple wash with diluted hypochlorite solution is sufficient to eliminate residual TeNT after experiments.

Use in cell biology

The cellular receptor for TeNT is not known, nor its pathway into the cell cytosol. In general terms, neuronal cells in culture, including synaptosomes, internalize TeNT when present in the extracellular at concentrations in the 1–100 nanomolar range. It should be noticed that the TeNT L chain is a stable enzyme and hence can perform its action on many VAMP molecules. It is not unreasonable to assume that one molecule of toxin is able to intoxicate a synapse, as one molecule of diphtheria toxin light chain is able to kill a cell. Non-neuronal cells have to be permeabilized or injected to allow the access of the L chain to the cytosol (Penner *et al.* 1986; Anhert-Hilger *et al.* 1989). An alternative is that of expressing a gene encoding the L chain in a cell or in a transgenic animal (Sweeney *et al.* 1995). TeNT is a zinc-endopeptidase specific for VAMP/synaptobrevin. The basis of this specificity is a double interaction between TeNT and two separate segments of VAMP: a segment present in the middle of the cytosolic portion of VAMP and a segment containing the Q-F peptide bond that will be cleaved (Schiavo *et al.* 1992*b*; Rossetto *et al.* 1994). As shown in Fig. 2, all the three known human isoforms of VAMP are cleaved (Schiavo *et al.* 1992*b*; McMahon *et al.* 1993). VAMPs of other species may not be cleaved by TeNT depending on the conservation of both the toxin binding segment and the toxin cleavage site. VAMP-1 of chicken and rats have a V in place of Q and are not cleaved (Schiavo *et al.* 1992*b*; Patarnello *et al.* 1993). This amino acid change at the site of proteolysis was suggested to be at the basis of the resistance of chicken and rats to tetanus (Patarnello *et al.* 1995). Such a VAMP difference can be exploited to study the role of the different isoforms by comparing results obtained with TeNT in mouse cells, whose VAMP-1 is cleaved, with those obtained with rat cells, whose VAMP-1 is not cleaved. The syb isoform of *Drosophila* VAMP is not cleaved by TeNT, though its cleavage site is the same as that of human VAMP (Sweeney *et al.* 1995). However, syb has a critical G for D substitution in the toxin recognition site.

VAMP proteolysis *in vitro* is assayed by SDS-PAGE and Coomassie blue staining (Schiavo *et al.* 1992*a*,*b*). *In vivo*,

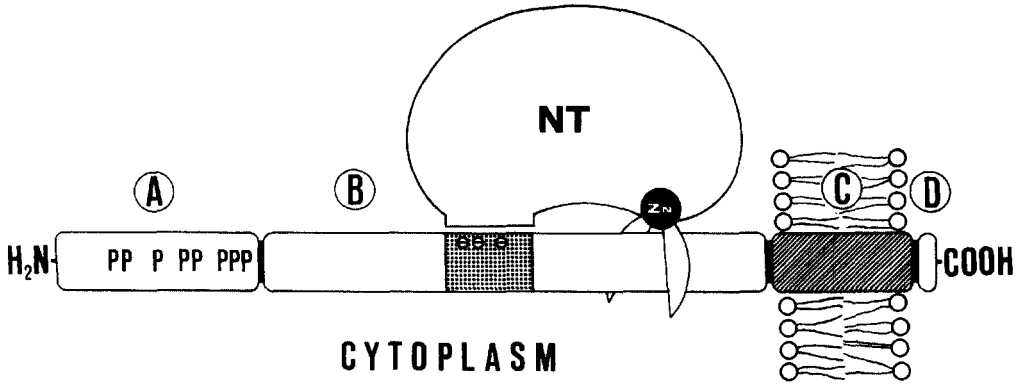

Figure 2. Structure of VAMP and site of proteolysis by tetanus neurotoxin. Three isoforms of VAMP are known: 1 and 2 have a similar size, but differ in the proline-rich amino-terminal domain A, whereas cellubrevin has a shorter A domain. VAMPs are bound to different types of intracellular vesicles via a transmembrane hydrophobic part (C) and have a short intraluminal tail (D). The intermediate portion (B) is highly conserved among species and isoforms and contains the negatively charged site of toxin recognition (dashed) and the site of cleavage by tetanus neurotoxin (NT). The other clostridial neurotoxins recognize their substrate via a similar dual interaction (see following entries).

it is assayed by immunoblotting or immunofluorescence following the disappearance of the VAMP staining (Galli *et al.* 1994).

Because of its ability to perform a trans-synaptic migration, H_C is frequently used as a marker of retro-axonal transport to map neuronal routes from the PNS to the CNS by coupling it to horse radish peroxidase or gold particles (Cabot *et al.* 1991).

References

Anhert-Hilger, G., Weller, U., Dauzenroth, M. E., Habermann, E., and Gratzl, M. (1989). The tetanus toxin light chain inhibits exocytosis. *FEBS Lett.*, **242**, 245–8.

Bagetta, G., Nisticò, G., and Bowery, N. G. (1991). Characteristic of tetanus toxin and its exploitation in neurodegenerative studies. *Trends Pharmacol. Sci.*, **12**, 285–9.

Cabot, J. B., Mennone, A., Bogan, N., Carroll, J., Evinger, C., and Erichsen, J. T. (1991). Retrograde, trans-synaptic and transneuronal transport of fragment C of tetanus toxin by sympathetic preganglionic neurons. *Neuroscience*, **40**, 805–23.

De Filippis, V., Vangelista, L., Schiavo, G., Tonello, F., and Montecucco, C. (1995). Structural studies on the zinc-endopeptidase light chain tetanus neurotoxin. *Eur. J. Biochem.*, **229**, 61–9.

Eisel, U., Jarausch, W., Goretzki, K., Henschen, A., Engels, J., Weller, U., *et al.* (1986). Tetanus toxin: primary structure, expression in *E. coli* and homology with botulinum toxins. *EMBO J.*, **5**, 2495–502.

Fairweather, N. F. and Lyness, V. A. (1986). The complete nucleotide sequence of tetanus toxin. *Nucleic Acids Res.*, **14**, 7809–12.

Galli, T., Chilcote, O., Mundingl, T., Binz, T., Niemann, H. and De Camilli, P. (1994). Tetanus toxin-mediated cleavage of cellubrevinimpairs neuroexocytosis of transferrin receptor-containing vesicles in CHO cells. *J. Cell Biol.*, **125**, 1015–24.

Gill, D. M. (1982). Bacterial toxins: a table of lethal amounts. *Microbiol. Rev.*, **46**, 86–94.

Helting, T. B. and Zwisler, O. (1977). Structure of tetanus toxin. *J. Biol. Chem.*, **252**, 187–93.

McMahon, H. T., Ushkaryov, Y. A., Edelmann, L., Link, E., Binz, T., Niemann, H., *et al.* (1993). Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature*, **364**, 346–9.

Matsuda, M. and Yoneda, M. (1975). Isolation and purification of two antigenically active, complementary polypeptide fragments of tetanus neurotoxin. *Infect. Immun.*, **12**, 1147–53.

Matsuda, M., Sugimoto, N., Ozutsumi, K., and Hirai, T. (1982). Acute botulinum-like intoxication by tetanus neurotoxin in mice. *Biochem. Biophys. Res. Commun.*, **104**, 799–805.

Matsuda, M., Lei, D., Sugimoto, N., Ozutsumi, K., and Okabe, T. (1989). Isolation, purification and characterization of fragment B, the NH_2–terminal half of the heavy chain of tetanus toxin. *Infect. Immun.*, **57**, 3588–93.

Montecucco, C. and Schiavo, G. (1995) Structure and function of tetanus and botulinum neurotoxins. *Quart. Rev. Biophys.*, **28**, 423–72.

Patarnello, T., Bargelloni, L., Rossetto, O., Schiavo, G., and Montecucco, C. (1993). Neurotransmission and secretion. *Nature*, **364**, 581–2.

Payling-Wright, G. (1955). The neurotoxins of *Clostridium botulinum* and *Clostridum tetani*. *Pharmacol. Rev.*, **7**, 413–65.

Penner, R., Neher, E., and Dreyer, F. (1986). Intracellularly injected tetanus toxin inhibits exocytosis in bovine adrenal chromaffin cells. *Nature*, **324**, 76–7.

Rossetto, O., Schiavo, G., Montecucco, C., Poulain, B., Deloy, F., Lozzi, L., and Shone, C. C. (1994). SNARE motifs and neurotoxins. *Nature*, **372**, 415–16.

Schiavo, G. and Montecucco, C. (1995). Tetanus and botulism neurotoxins: isolation and assay. *Methods Enzymol.*, **248**, 643–52.

Schiavo, G., Papini, E. Genna, G. and Montecucco, C. (1990). An intact interchain disulfide bond is required for the neurotoxicity of tetanus toxin. *Infect. Immun.*, **58**, 4136–41.

Schiavo, G., Poulain, B., Rossetto, O., Benfenati, F., Tauc, L., and Montecucco, C. (1992*a*). Tetanus toxin is a zinc protein and its inhibition of neurotrasmitter release and protease activity depend on zinc. *EMBO J.*, **11**, 3577–83.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B. R., *et al.* (1992*b*). Tetanus and botulinum-B neurotoxins block neurotransmitter release by a proteolytic cleavage of synaptobrevin. *Nature*, **359**, 832–5.

Schwab, M. E., Suda, K., and Thoenen, H. (1979). Selective retrograde trans-synaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport. *J. Cell Biol.*, **82**, 798–810.

Sweeney, S. T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C. J. (1995). Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. *Neuron*, **14**, 341–51.

van Heyningen, W. E. (1968). *Tetanus. Sci. Am.*, **218**, 69–77.

Weller, U., Dauzenroth, M.-E., Meyer Heringdorf, D. and Habermann, E. (1989). Chains and fragments of tetanus toxin. *Eur. J. Biochem.*, **182**, 649–56.

Williamson, L. C. and Neale, E. A. (1994). Bafilomycin A1 inhibits the action of tetanus toxin in spinal cord neurons in cell culture. *J. Neurochem.*, **63**, 2342–5.

Yamasaki, S., Baumeister, A., Binz, T., Blasi, J., Link, E., Cornille, F., *et al.* (1994). Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. *J. Biol. Chem.*, **269**, 12764–72.

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VAMP-specific botulinum neurotoxins

Botulinum neurotoxins are a group of closely related protein toxins (seven different serotypes A–G) produced by different bacterial strains of the genus Clostridium. All of them show absolute tropism for the neuromuscular junction, where they bind still unidentified receptors in a strictly serotype specific manner. This binding step is followed by the entry of the toxin into the cytoplasm of the motorneurons and by specific proteolytic cleavage of intracellular targets. Four out of seven serotypes of botulinum neurotoxins cleave VAMP/synaptobrevin, a protein of small synaptic vesicles. This results in a loss of function of the neuroexocytosis machinery and thus a blockade of transmitter release.

Botulinum neurotoxins (BoNT A–G) are the causative agents of the flaccid paralysis of botulism (Hatheway 1995) and are produced by toxigenic strains of *Clostridium botulinum* and less frequently *Cl. barati* and *Cl. butyricum* (Hall *et al.* 1985; Aureli *et al.* 1986). BoNTs are produced as a large complex of 300–900 kDa containing the neurotoxin (150 kDa) and additional components, such as hemagglutinin (300 kDa) and a nontoxic peptide (Sakaguchi 1983). These additional proteins play a role in the stabilization of the neurotoxins to the gut enviroment, their natural port of entry. BoNTs are synthesized as an inactive polypeptide chain (protoxin) that is activated by proteolysis at an exposed loop, generating the active di-chain form of the toxin (DasGupta 1994). This form is composed of the heavy chain (H, 100 kDa) responsible for the interaction of the toxin to specific receptor(s) present at the neuromuscular junction and for the translocation of the light chain (L, 50 kDa) into the cytoplasm. A critical step for the activation of the zinc-endopeptidase activity of the L chain is the reduction of the interchain disulfide bridge (Montecucco and Schiavo 1995).

The L chains of BoNTs are composed of about 450 AA with an overall homology of 32–61 per cent (Campbell *et al.* 1993). The highest homology is concentrated in the amino-terminal region, probably involved in the substrate recognition (Rossetto *et al.* 1994) and in a central region that contains the zinc-binding motif His-Glu-Xxx-Xxx-His of zinc endopeptidases (Vallee and Auld 1990). This region coordinates a zinc atom necessary for the *in vivo* activity of these toxins (Schiavo *et al.* 1992*a*). BoNT/B, D, F, and G (BoNT/B, 1291 AA, GeneBank accession number M81186; BoNT/D, 1276 AA, GeneBank accession number X54254; BoNT/F, 1274 AA, GeneBank accession number M92906; BoNT/G, 1300 AA, GeneBank accession number X74162) cleave VAMP/synaptobrevin at four distinct peptide bonds within the conserved cytoplasmic loop (Fig. 1)(Montecucco and Schiavo 1995).

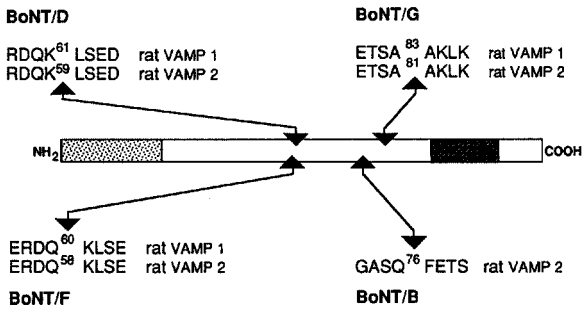

Figure 1. The botulinum neurotoxins B, D, F, and G cleavage sites on VAMP/synaptobrevin. The numbers indicate the new carboxy-terminus of the VAMP fragments generated by the different toxins. The dotted area and the hatched box represent the proline-rich carboxy-terminal domain and the transmembrane portion of VAMP–synaptobrevin, respectively.

Purification and sources

BoNT/B, D, F, and G are isolated from bacterial culture as 300–900 kDa crystalline protein complex by acid precipitation and citrate extraction of the pellet, followed by ammonium sulfate fractionation (Schiavo *et al.* 1992*a*, 1993*a*,*b*, 1994). The crude neurotoxins are purified by chromatography on SP-Sephadex. BoNT/B and F are further purified by immobilized metal ion chromatography (Pharmacia), while BoNT/D are separated from the nontoxic protein component by ion exchange chromatography on MONO Q (Pharmacia)(Schiavo *et al.* 1993*b*). BoNT/G is purified by gel filtration chromatography (Schiavo *et al.* 1994). BoNT/B, D, and F can be purchased from Calbiochem, Sigma, or Wako.

Botulinum neurotoxin (150 kDa) serotypes D and F are usually puried from bacterial cultures as di-chain fully active toxins, while BoNT/B and G are purified in the inactive single chain form. They are activated to the di-chain active form by trypsin cleavage (DasGupta 1994).

Toxicity

Toxicity is tested by intraperitoneal or intravenous injection in mice. Mouse LD_{50}s are in the range 0.1–0.5 ng/kg and are variable in different animal species

(Wright 1955). The variety of serotypes presents a serious problem to the experimentors for the lack of a widespread vaccination. Long term immunization can be obtained by the injection of a pentavalent toxoid vaccine (serotypes A, B, C, D, E; CDC, Atlanta). No adequate immunological protection or serotherapy are available for serotypes F and G.

Use in cell biology

Di-chain BoNTs are internalized by primary neurons in culture and by a variety of neuronal cell lines. Brain cortex synaptosomes also show an efficient uptake mechanism for BoNTs (McMahon *et al.* 1992). In addition clostridial neurotoxins sensitivity can be induced by incubation of noncompetent cells with gangliosides (Bartels and Bigalke 1992). Single chain neurotoxins or purified L chains are completely ineffective if applied extracellularly. Other nonneuronal cells or sub-cellular fractions (i.e. neurohypophysial terminals) that do not present specific toxin receptors, have to be permeabilized to allow the entry of the toxin into the cytoplasm. The permeabilization process can be realized with streptolysin O (Anhert-Hilger *et al.* 1989), with detergents (Lawrence *et al.* 1994), electroporation (Bartels and Bigalke 1992), or micro-injection (Poulain *et al.* 1990). Both the purified L chain or the di-chain toxin can be used in these cases. To test toxin activity on purified membrane preparations (i.e. synaptic vesicles), the di-chain toxins must be reduced just before use with dithiothreitol (Schiavo *et al.* 1992*b*). Intoxication with BoNTs could be performed also by microinjection of BoNT-specific mRNAs (Mochida *et al.* 1990) or by targeted expression of BoNTs light chain in *Drosophila*, similarly to that recently obtained with tetanus toxin (Sweeney *et al.* 1995).

BoNT/B, D, F, and G are zinc-endopeptidases specific for VAMP/synaptobrevin, a small membrane protein present in various tissues in three isoforms (1, 2, and 3 or cellubrevin). The four BoNTs cleave all three VAMP isoforms at four distinct sites (Fig. 1) (Montecucco and Schiavo 1995). In some animal species, polymorphism at the cleavage sites confers resistance to BoNT/B and tetanus toxin as in the case of rat and chicken VAMP 1 (Patarnello *et al.* 1993).

BoNTs can be used in animals to induce a toxic denervation of skeletal muscle and to study the following processes of nerve sprouting and re-innervation (Frangez *et al.* 1994).

References

Anhert-Hilger, G., Bader, M. F., Bhakdi, S., and Gratzl, M. (1989). Introduction of macromolecules into bovine adrenal medullary chromaffin cells and rat pheochromocytoma cells (PC12) by permeabilization with streptolysin O. Inhibitory effect of tetanus toxin on catecholamine secretion. *J. Neurochem.*, **52**, 1751–8.

Aureli, P., Fenicia, L., Pasolini, B., Gianfranceschi, M., McCroskey, L. M., and Hatheway, C. O. (1986). Two cases of type E infant botulism in Italy caused by neurotoxigenic *Clostridium butyricum*. *J. Infect. Dis.*, **154**, 207–11.

Bartels, F. and Bigalke, H. (1992). Restoration of exocytosis occurs after inactivation of intracellular tetanus toxin. *Infect. Immun.*, **60**, 302–7.

Campbell, K., Collins, M. D., and East, A. K. (1993). Nucleotide sequence of the gene coding for *Clostridium botulinum* (*Clostridium argentinense*) type G neurotoxin: genealogical comparison with other clostridial neurotoxins. *Biochim. Biophys. Acta*, **1216**, 487–91.

DasGupta, B. R. (1994). Structures of botulinum neurotoxin, its functional domains, and perspectives on the cristalline type A toxin. In *Therapy with botulinum toxin*, (ed J. Jankovic and M. Hallett), pp. 15–39, Marcel Dekker, New York.

Frangez, R., Dolinsek, J., Demsar, F., and Suput, D. (1994). Chronic denervation caused by botulinum neurotoxin as a model of a neuromuscular disease. *Ann. N.Y. Acad. Sci.*, **710**, 88–93.

Hall, J. D., McCroskey, L. M., Pincomb, B. J., and Hatheway, C. O. (1985). Isolation of an organism resembling *Clostridium barati* which produces type F toxin from an infant with botulism. *J. Clin. Microbiol.*, **21**, 654–5.

Hatheway, C. L. (1995). Botulism: the present status of the disease. *Curr. Top. Microbiol. Immunol.*, **195**, 55–75.

Lawrence, G. W., Weller, U., and Dolly, J. O. (1994). Botulinum A and the light chain of tetanus toxin inhibit distinct stages of Mg.ATP-dependent catecholamine exocytosis from permeabilized chromaffin cells. *Eur. J. Biochem.*, **222**, 325–33.

McMahon, H. T., Foran, P., Dolly, J. O., Verhage, M., Wiegand, V. M., and Nicholls, D. G. (1992). Tetanus toxin and botulinum toxin A and B inhibit glutamate, gamma-aminobutyric acid, aspartate, and met-enkephalin release from synaptosomes. Clues to the locus of action. *J. Biol. Chem.*, **267**, 21338–43.

Mochida, S., Poulain, B., Eisel, U., Binz, T., Kurazono, H., Niemann, H., *et al.* (1990). Molecular biology of clostridial toxin: expression of mRNAs encoding tetanus and botulinum neurotoxins in *Aplysia* neurons. *J. Physiol. (Paris).*, **84**, 278–84.

Montecucco, C. and Schiavo, G. (1995). Structure and function of tetanus and botulinum neurotoxins. *Curr. Top. Microbiol. Immunol.* **195**, 257–74.

Patarnello, T., Bargelloni, L., Rossetto, O., Schiavo, G., and Montecucco, C. (1993). Neurotransmission and secretion. *Nature*, **364**, 581–2.

Poulain, B., Mochida, S., Wadsworth, J. D., Weller, U., Habermann, E., Dolly, J. O., *et al.* (1990). Inhibition of neurotransmitter release by botulinum neurotoxins and tetanus toxin at *Aplysia* synapses: role of constituent chains. *J. Physiol. (Paris)*, **84**, 247–61.

Rossetto, O., Schiavo, G., Montecucco, C., Poulain, B., Deloye, F., Lozzi, L., *et al.* (1994). SNARE motif and neurotoxin recognition. *Nature*, **372**, 415–16.

Sakaguchi, G. (1983). *Clostridium botulinum* toxins. *Pharmacol. Ther.*, **19**, 165–94.

Schiavo, G., Rossetto, O., Santucci, A., DasGupta, B. R., and Montecucco, C. (1992*a*). Botulinum neurotoxins are zinc proteins. *J. Biol. Chem.*, **267**, 23479–83.

Schiavo, G., Benfenati, F., Poulain B., Rossetto O., Polverino de Laureto, P., DasGupta B. R. *et al.* (1992*b*). Tetanus and botulinum B neurotoxins block neurotransmitter release by a proteolytic cleavage of synaptobrevin. *Nature*, **359**, 832–5.

Schiavo, G., Shone, C. C., Rossetto, O., Alexandre, F. C. G., and Montecucco, C. (1993a). Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. *J. Biol. Chem.*, **268**, 11516–19.

Schiavo, G., Rossetto, O., Catsicas, S., Polverino de Laureto, P., DasGupta, B. R., Benfenati, F. *et al.* (1993*b*). Identification of the nerve-terminal targets of botulinum neurotoxins serotypes A, D and E. *J. Biol. Chem.*, **268**, 23784–7.

Schiavo, G., Malizio, C., Trimble, W. S., Polverino de Laureto, P., Milan, G., Sugiyama, H., *et al.* (1994). Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala/Ala peptide bond. *J. Biol. Chem.*, **269**, 20213–16.
Sweeney, S. T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C. J. (1995). Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron*, **14**, 341–51.
Vallee, B. L. and Auld, D. S. (1990). Zinc coordination, function and structure of zinc enzymes and other proteins. *Biochemistry*, **29**, 5647–59.
Wright, G. P. (1955). The neurotoxins of *Clostridium botulinum* and *Clostridium tetani*. *Pharmacol. Rev.*, **7**, 413–65.

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Botulinum neurotoxins type A and E (*Clostridium botulinum*)

Botulinum neurotoxins type A and E are produced by toxigenic strains of Clostridium botulinum. *They bind specifically to the presynaptic membrane of cholinergic neurons and penetrate into the cytosol, where they cleave specifically SNAP-25 at the Q_{197}-R_{198} (type A) and R_{180}-I_{181} (type E) peptide bonds. This selective proteolysis impairs the functional participation of SNAP-25 in the neuroexocytosis machine and the release of acetylcholine evoked by membrane depolarization cannot take place.*

Botulinum neurotoxins are structurally related protein toxins (BoNTs) produced by Clostridia in seven different serotypes: A, B, C, D, E, F, and G. BoNTs penetrate motorneurons at the neuromuscular junction and block acetylcholine release, thus causing the flaccid paralysis of botulism. They impair neurotransmission via the selective cleavage of proteins of the synaptic vesicles docking and fusion complex. BoNT/A and E are involved in human botulism and cleave SNAP-25 (synaptosomal-associated protein of 25 kDa), a protein bound to the cytosolic face of the plasma membrane. Botulinum neurotoxins A and E are produced by bacteria of the genus *Clostridium* and are responsible for the flaccid paralysis of botulism (Hatheway 1995). They are synthesized as a single inactive polypeptide chain of 150 kDa complexed with non-neurotoxic components and released by bacterial lysis. Bacterial or tissue proteases cleave the neurotoxin within an exposed loop and generate the active di-chain form composed of a heavy-chain (H, 100 kDa) and a light chain (L, 50 kDa) bridged by a single interchain disulphide bond. BoNTs bind the presynaptic membrane via the heavy chain and enter the neuron cytosol, where the light chain exerts its proteolytic activity (Montecucco and Schiavo 1995). The gene encoding for BoNT/A is located on a plasmid of variable size (1295 amino acid residues, GeneBank accession number X52066), whereas the gene that codes for BoNT/E is on a bacteriophage (1250 amino acid residues, GeneBank accession number X62089) (Eklund *et al.* 1989; Minton 1995).

The L chains of BoNT/A and BoNT/E, similarly to the L chains of the other clostridial neurotoxins, contain one atom of zinc bound to the zinc binding motif of zinc-endopeptidases HExxH, which is included in a highly conserved region (Schiavo *et al.* 1992). This atom of zinc is essential for the catalytic activity of BoNT/A and /E, which consists of the cleavage of SNAP-25 at Q_{197}-R_{198} and R_{180}-I_{181}, respectively (Blasi *et al.* 1993; Schiavo *et al.* 1993*a*). This leads to the complete inhibition of neurotransmitter release as well as to the inhibition of several other exocytotic events (Sadoul *et al.* 1995). The BoNT/A block of neuroexocytosis can be by-passed by alpha-latrotoxin (Gansel *et al.* 1987).

■ Purification and sources

BoNT/A and E are isolated from bacterial culture by acid precipitation and citrate extraction of the pellet, followed by ammonium sulfate fractionation (Shone and Tranter 1995). The crude toxins are purified by chromatography on SP-Sephadex. They are freed from contaminating clostridial proteinases by immobilized metal ion affinity chromatography (IMAC, Pharmacia) (Rossetto *et al.* 1992). BoNT/A and E can be purchased from Calbiochem, Wako, and Sigma. BoNT/A is usually purified from bacterial culture as di-chain fully active toxin, whereas BoNT/E is purified as the inactive single chain form, which is converted into the active di-chain form by

nicking with trypsin (Sathyamoorthy and DasGupta 1985). BoNT/A is largely used as a therapeutic agent for dystonias, strabismus, and other syndromes, where a depression of neuromuscular junction activity is sought (Jankovic and Hallett 1994). For such a use, it is sold by Allergan (USA) with the product name BOTOX and by Porton (UK) with the trade name of Dysport. Moreover, Associated Synapse (USA) has received the orphan drug status to market BoNT/A.

Toxicity

Toxicity is usually evaluated by intraperitoneal or intravenous injection of serially diluted neurotoxin solutions in mice. Mouse LD_{50} of BoNT/A and E is in the range of 0.1–1 ng/kg and varies greatly in different animal species (Payling-Wright 1955). These neurotoxins are estimated to be even more potent in humans: the human lethal dose may lie in the range 2000–4000 mouse lethal dose. Hence, they have to be manipulated with great care. It is sufficient to use them in a contained place, to protect hands with rubber gloves, and to avoid needles. Wastes should be collected and treated with diluted hypochlorite solutions that quickly inactivate these toxins. Alternatively, operators can be vaccinated with the pentavalent toxoid vaccine prepared with toxin serotypes A, B, C, D, E, which can be obtained through the Botulism Laboratory of the CDC (Atlanta, USA). The California Department of Health Sciences (fax 510-5402646) has recently developed a human-derived botulinum antitoxin.

Use in cell biology

BoNT/A and E as well as the other botulinum neurotoxins bind to specific and distinct receptors located on the motor neuron plasmalemma at the neuromuscular junction (Dolly *et al.* 1984). The cellular receptors for BoNT/A and E are not known, but different receptors must be implicated in the binding of these two BoNTs to account for the lack of competition of these two serotypes and for the different sensitivity of animal species. Primary neurons in culture, neuronal-derived cell lines and brain cortex synaptosomes are able to internalize the di-chain BoNT/A and E (Sanchez-Prieto *et al.* 1987; Blasi *et al.* 1993; Schiavo *et al.* 1993a; Osen-Sand *et al.* 1996). Non-neuronal cells have to be injected or permeabilized to allow the access of the L chain to the cytosol (Penner *et al.* 1986; Boyd *et al.* 1995; Sadoul *et al.* 1995).

BoNT/A and E are zinc-endopeptidases that specifically recognize SNAP-25 and cleave it at single, distinct sites within the carboxyl-terminal region. BoNT/A hydrolyses the Gln197–Arg198 peptide bond, whereas BoNT/E cleaves between Arg180 and Ile181 (Schiavo *et al.*, 1993b) (Fig. 1). Thus, BoNT/A and E cleave within 20 residues of each other at the SNAP-25 COOH-terminus suggesting that this protein region plays a fundamental role in the recognition and/or regulation of the multi-subunit neuroexocytosis machinery. BoNT/A and E are increasingly used to probe the role of SNAP-25 in several cell

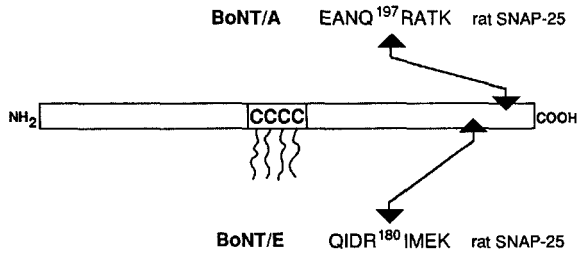

Figure 1. Cleavage of SNAP-25 with botulinum neurotoxins A and E. SNAP-25 is a 206 residue long protein with a characteristic cysteine quartet present in the middle of the chain. These cysteines are palmitoylated. BoNT/A and E cleave SNAP-25 at two different peptide bonds within the carboxyl-terminal. Synapse poisoned with BoNT/A can be induced to release neurotransmitter by alpha-latrotoxin (see p. 233).

processes including exocytosis, axonal growth, synapse stability, and membrane repair (Boyd *et al.* 1995; Sadoul *et al.* 1995; Osen-Sand *et al.* 1996).

After the report appeared in 1980 that BoNT/A injection is very effective in strabismus, the therapeutic use of this neurotoxin has been extended to a variety of diseases, which benefit from a functional paralysis of the neuromuscular junction (Jankovic and Hallett 1994). Currently, the other BoNT types are under clinical test.

References

Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., *et al.* (1993). Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature*, **365**, 160–3.

Boyd, R. S., Duggan, M. J., Shone, C. C., and Foster, K. A. (1995). The effect of botulinum neurotoxins on the release of insulin from insulinoma cell lines HIT-15 and RINm5F. *J. Biol. Chem.*, **270**, 18216–18.

Dolly, J. O, Black, J., Williams, R. S., and Melling, J. (1984). Acceptors for botulinum neurotoxin reside on motor nerve terminals and mediate its internalization. *Nature*, **307**, 457–60.

Eklund, M. W., Poysky, F. T., and Habig, W. H. (1989). Bacteriophages and plasmids in *Clostridium botulinum* and *Clostridium tetani* and their relationship to production of toxins. In: *Botulinum neurotoxins and tetanus toxin* (ed. L. L. Simpson), pp. 25–51, Academic press, New York.

Gansel, M., Penner, R., and Dreyer, F. (1987). Distinct sites of action of clostridial neurotoxins revealed by double poisoning of mouse motor-nerve terminals. *Pflügers Archiv*, **409**, 533–9.

Hatheway, C. L. (1995). Botulism: The present status of the disease. In *Clostridial neurotoxins* (ed. C. Montecucco), *Curr. Top. Microbiol. Immunol.*, **195**, 55–75.

Jankovic, J. and Hallett, M. (ed.) (1994). *Therapy with botulinum toxin*, Marcel Dekker, New York.

Minton, N. P. (1995). Molecular genetics of clostridial neurotoxins. *Curr. Top. Microbiol. Immunol.*, **195**, 161–94.

Montecucco, C. and Schiavo, G. (1995). Structure and function of tetanus and botulinum neurotoxins. *Quart. Rev. Biophys.*, **28**, 423–72.

Osen-Sand, A., Staple, J. K., Naldi, E., Schiavo, G., Rossetto, O., Petitpierre, S., *et al.* (1996). Common and distinct

fusion-proteins in axonal growth and transmitter release. *J. Comp. Neurol.*, **367**, 222–234.
Payling-Wright, G. (1955). The neurotoxins of *Clostridium botulinum* and *Clostridium tetani*. *Pharmacol. Rev.*, **7**, 413–65.
Penner, R., Neher, E., and Dreyer, F. (1986). Intracellularly injected tetanus toxin inhibits exocytosis in bovine adrenal chromatin cells. *Nature*, **324**, 76–7.
Rossetto, O., Schiavo, G., Polverino de Laureto, P., Fabbiani, S., and Montecucco, C. (1992). Surface topography of hystidine residues of tetanus toxin probed by immobilized-metal-ion affinity chromatography. *Biochem. J.*, **285**, 9–12.
Sadoul, K., Lang, J., Montecucco, C., Weller, U., Catsicas, S., Wollheim, C., *et al.* (1995). SNAP-25 is expressed in islets of Lagerhans and is involved in insulin release. *J. Cell. Biol.*, **128**, 1019–28.
Sanchez-Prieto, J., Shira, T. S., Evans, D., Ashton, A., Dolly, J. O., and Nicholls, D. (1987). Botulinum toxin A blocks glutamate exocytosis from guinea-pig cerebral cortical synaptosomes. *Eur. J. Biochem.*, **165**, 675–81.
Sathyamoorthy, W. and DasGupta, B. R. (1985). Separation, purification, partial characterization and some properties of the heavy and light chains of botulinum neurotoxin types A, B and E. *J. Biol. Chem.*, **260**, 10461–6.
Schiavo, G., Rossetto, O., Santucci, A., DasGupta, B. R., and Montecucco, C. (1992). Botulinum neurotoxins are zinc proteins. *J. Biol. Chem.*, **267**, 23479–83.
Schiavo, G., Rossetto, O., Catsicas, S., Polverino de Laureto, P., DasGupta, B. R., Benfenati, F., *et al.* (1993*a*). Identification of the nerve-terminal targets of botulinum neurotoxins serotype A, D and E. *J. Biol. Chem.*, **268**, 23784–7.
Schiavo, G., Santucci, A., DasGupta, B. R., Metha, P. P., Jontes, J., Benfenati, F., *et al.* (1993*b*). Botulinum neurotoxins serotype A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. *FEBS Lett.*, **335**, 99–103.
Shone, C. C. and Tranter, H. S. (1995). Growth of Clostridia and preparation of their neurotoxins. *Curr. Top. Microbiol. Immunol.*, **195**, 143–60.

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Botulinum neurotoxin type C (*Clostridium botulinum*)

Botulinum neurotoxin type C is a protein of 150 kDa produced by toxigenic Clostridium botulinum *type C strains that blocks neurotransmitter release. This neurotoxin binds specifically to nerve terminals and is then translocated to the cytosol where it cleaves syntaxin, and SNAP-25 presynaptic membrane proteins involved in the exocytotic machinery acting as a t-SNARE.*

Botulinum neurotoxin C (BoNT/C) is one of the seven neurotoxins produced by the anaerobic bacteria *Clostridium botulinum*, and are responsible for the flaccid paralysis characteristic of botulism. Its structural gene is located on the genome of bacteriophages (GenBank accession number X53751), which produce a 150 kDa protein of 1290 amino acids (Swiss-Prot accession number P18640) responsible for the neurotoxigenicity of the strain (Eklund *et al.* 1989). This neurotoxin is synthesized by the bacteria as a nonactive single chain protein and is complexed with nontoxigenic components, including hemagglutinin and nonhemagglutinin proteins, L toxin (500 kDa), or nonhemagglutinin component, M toxin (300 kDa) (Tsuzuki *et al.* 1992). Each toxin dissociates in alkaline conditions into the neurotoxin (150 kDa, BoNT/C1, also named S toxin) and nontoxic components. Single chain neurotoxin acquires full toxicity by proteolysis at one third of its length from its amino terminal. That nicking renders a di-chain molecule, formed by a heavy (100 kDa) and a light (50 kDa) chain that are linked together by a disulfide bond (see Fig. 1). BoNT/C1 light

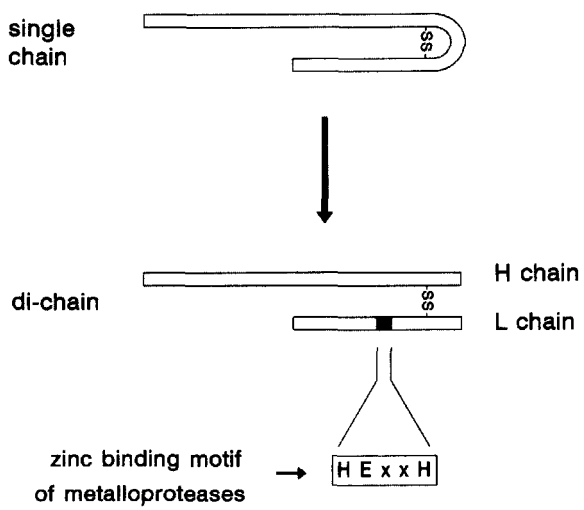

Figure 1. Molecular structure of BoNT/C.

chain exhibits the sequence motif HExxH (Fig. 1) found in a variety of Zn^{2+}-dependent metalloproteases and common to all clostridial neurotoxins, tetanus, and botulinum neurotoxins (Montecucco and Schiavo 1993; Niemann *et al.* 1994), which was shown to account for their final toxic intracellular effect.

In addition to BoNT/C, *Clostridium botulinum* type C strains (and also D strains) produce the *Clostridium botulinum* C2 toxin (binary toxin) and the botulinum C3 ADP-ribosyltransferase (exoenzyme C3), which are not neurotoxigenic. The botulinum C2 toxin gene is located at the bacterial genome, whereas the botulinum C3 ADP-ribosyltransferase gene is located at the bacteriophage DNA (see entry p. 36).

Purification and sources

Botulinum toxin type C is extracted from bacterial cultures (Schiavo *et al.* 1995) or precipitated from culture supernatants by acid (Matsuda *et al.* 1986) or ammonium sulfate (Tsuzuki *et al.* 1992) followed by liquid chromatography. BoNT/C dissociates from nontoxic components under alkaline conditions and further ion exchange chromatography (Matsuda *et al.* 1986; Schiavo *et al.* 1995). Heavy and light chains can be isolated under reducing conditions. Botulinum toxin type C is available from Sigma, Wako, or Calbiochem in its 500 000 kDa (L toxin) form.

Toxicity

Toxicity is evaluated by intraperitoneal injections of a dilution series and expressed as mouse LD_{50}. Lethal toxicity can also be tested by the time-to-death method by intravenous injection of appropriately diluted samples. Mouse LD_{50} is calculated from a calibration curve made with pure BoNT/C (Kondo *et al.* 1984). Toxicity ranges from 0.4×10^7 LD_{50}/mg of toxin (L toxin) to $14–21 \times 10^7$ LD_{50}/mg of toxin (BoNT/C).

Use in cell biology

BoNT/C specifically blocks neurotransmitter release from nerve terminals, as tested at neuromuscular junction and mammal brain synaptosomes. This specificity on neuronal preparations depends on the presence of distinct acceptors on the cell surface (Agui *et al.* 1983; Yokosawa *et al.* 1989).

Briefly, BoNT/C mechanism of action consists of a binding step, reversible and temperature-independent, mediated by the heavy chain, that is followed by a temperature-dependent internalization of both chains into an acidic compartment, and translocation of the light chain to the cytosol (Murayama *et al.* 1987; Niemann 1991). Once in the cytosol, the light chain exerts its toxic effect on neurotransmitter release. This blocking action is associated with its Zn^{2+}-dependent endopeptidase

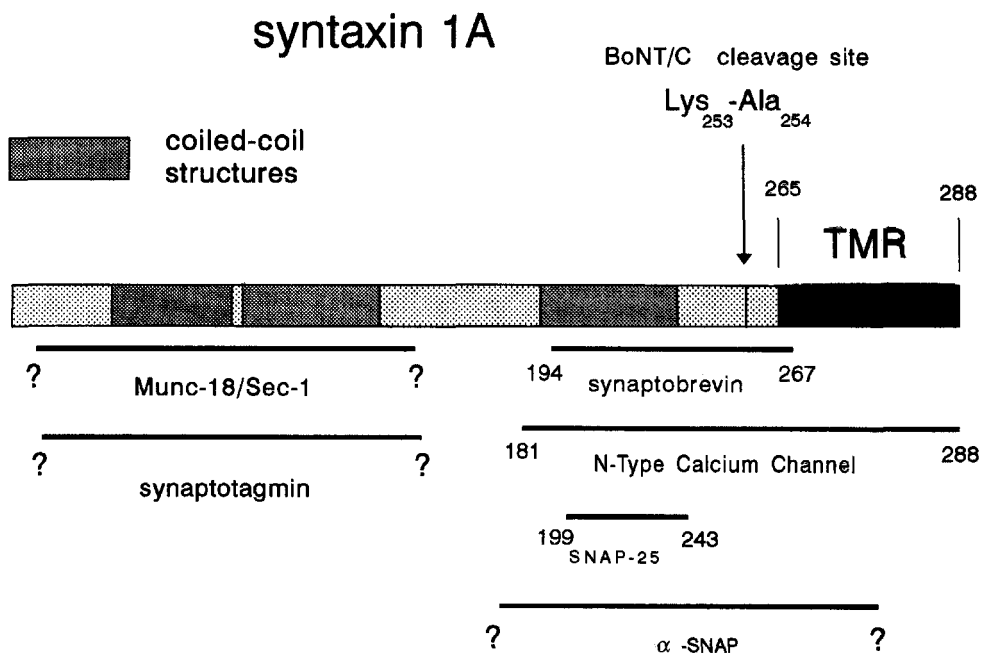

Figure 2. Schematic representation of syntaxin 1A showing: the transmembrane region (TMR); binding domains to other synaptic proteins such as synaptotagmin, Munc 18 (Hata *et al.* 1993), synaptobrevin (Calakos *et al.* 1994), SNAP-25 (Chapman *et al.* 1994), α-SNAP (McMahon and Südhof 1995), and N-type calcium channel (Sheng *et al.* 1994); BoNT/C cleavage site on syntaxin; and coiled-coil structures which are thought to mediate protein–protein interactions (Chapman *et al.* 1994).

activity on syntaxin 1a and 1b (Blasi *et al.* 1993; Schiavo *et al.* 1995), which act as a t-SNARE (Söllner *et al.* 1993). Syntaxins (Fig. 2) are cleaved near the carboxyl-terminal region at the Lys^{253}-Ala^{254} bond in syntaxin 1A and Lys^{252}-Ala^{253} in syntaxin 1B, respectively (Schiavo *et al.* 1995). Interestingly, BoNT/C preferentially cleaves a pool of syntaxin present in synaptic vesicle fraction isolated from poisoned brain synaptosomes (Walch-Solimena *et al.* 1995). Some other non-neuronal members of the syntaxin family, besides syntaxin 1, are also cleaved when incubated *in vitro* with BoNT/C (Schiavo *et al.* 1995), suggesting that BoNT/C light chain would disrupt the action of syntaxin in cells other than neurons. However, non-neuronal cells should be permeabilized or alternatively, BoNT/C light chain injected into the cytosol, to circumvent their absence of specific receptors at the plasma membrane. Recently, BoNT/C has been shown also to cleave SNAP-25 near the site of cleavage of BoNT/A (Williamson *et al.* 1996). BoNT/C is the sole neurotoxin known to cleave two substrates.

BoNT/C is also the only neurotoxin to show cytotoxic effect on primary neuronal cell cultures (Osen-Sand *et al.* 1996).

References

Agui ,T., Syuto, B., Oguma, K, Iida, H., and Kubo, S. (1983). Binding of *Clostridium botulinum* type C neurotoxin to rat brain synaptosomes. *J. Biochem. (Tokyo)*, **94**, 521–7.

Blasi, J., Chapman, E. R., Yamasaki, S., Binz, T., Niemann, H., and Jahn, R. (1993). Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *EMBO J.*, **12**, 4821–8.

Calakos, N., Benett, M. K., Peterson, K. E., and Scheller, R. H. (1994). Protein–protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science*, **263**, 1146–9.

Chapman, E. R., An, S., Barton, N., and Jahn, R. (1994). SNAP-25, a t-SNARE which binds to both syntaxin and synaptobrevin via domains that may form coiled coils. *J. Biol. Chem.*, **269**, 27427–32.

Eklund, M. W., Poysky, F. T., and Habig, W. H. (1989). Bacteriophages and plasmids in *Clostridium botulinum* and *Clostridium tetani* and their relationship to production of toxins. *Botulinum neurotoxin and tetanus toxin* (ed. L. L. Simpson), pp. 26–47, Academic Press, San Diego.

Hata, Y., Slaughter, C. A., and Südhof , T. C. (1993). Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. *Nature*, **366**, 347–51.

Kondo, H., Shimizu, T., Kubonoya, M., Izumi, N., Takahashi, M., and Sakaguchi, G. (1984). Titration of botulinum toxins for lethal toxicity by intravenous injection into mice. *Jpn. J. Med. Sci. Biol.*, **37**, 131–5.

McMahon, H. T. and Südhof, T. C. (1995). Synaptic core complex of synaptobrevin, syntaxin and SNAP-25 forms high affinity alpha-SNAP binding site. *J. Biol. Chem.*, **270**, 2213–17.

Matsuda, M., Ozutsumi, K., Pei-Ying, D., and Sugimoto, N. (1986). Rapid method for purification of *Clostridium botulinum* type C neurotoxin by high performance liquid chromatography (HPLC). *Eur. J. Epidemiol.*, **2**, 265–72.

Montecucco, C. and Schiavo, G. (1993). Tetanus and botulinum neurotoxins. A new group of zinc proteases. *TiBS*, **18**, 324–7.

Murayama, S., Umezawa, J., Terajima, J., Syuto, S., and Kubo, S. (1987). Action of botulinum neurotoxin on acetylcholine release from rat brain synaptosomes: putative internalization of the toxin into synaptosomes. *J. Biochem. (Tokyo)*, **102**, 1355–64.

Niemann, H. (1991) Molecular biology of clostridial neurotoxins. *Sourcebook of bacterial protein toxins* (ed. J. E. Alouf and J. H. Freer), pp. 303–48, Academic Press, London.

Niemann, H., Blasi, J., and Jahn, R. (1994) Clostridial neurotoxins: new tools for dissecting exocytosis. *Trends Cell Biol.*, **4**, 179–85.

Osen-Sand, A., Staple, J. K., Naldi, E., Schiavo, G., Rossetto, O., *et al.* (1996). Common and distinct fusion proteins in axonal growth and transmitter release. *J. Comp. Neurol.*, **367**, 222–34.

Schiavo, G., Shone, C. C., Bennett, M. K., Scheller, R. H., and Montecucco, C. M. (1995). Botulinum neurotoxin type C cleaves a single Lys–Ala bond within the carboxyl-terminal region of syntaxins. *J. Biol. Chem.*, **270**, 10566–70.

Sheng, Z., Rettig, J., Takahashi, M., and Catterall, W. A. (1994). Identification of syntaxin-binding site on N-type calcium channels. *Neuron*, **13**, 1303–13.

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. *et al.* (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature*, **362**, 318–24.

Tsuzuki, K., Kimura, K., Fujii, N., Yokosawa, N., and Oguma, K. (1992). The complete nucleotide sequence of the gene coding for the nontoxic-nonhemagglutinin component of *Clostridium botulinum* type C progenitor toxin. *Biochem. Biophys. Res. Commun.*, **183**, 1273–9.

Walch-Solimena, C., Blasi, J., Edelmann, L., Chapman, E. R., Fischer von Mollard, G., and Jahn, R. (1995). The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *J. Cell Biol.*, **128**, 637–45.

Yokosawa, N., Kurokawa, Y., Tsuzuki, K., Syuto, B., Fujii, N., Kimura, K. *et al.* (1989). Binding of *Clostridium botulimun* type C neurotoxin to different neuroblastoma cell lines. *Infect. Immun.*, **57**, 272–7.

Williamson, L. C., Halpern, J. L., Montecucco, C., Brown, J. E., and, Neale, E. A. (1996). Clostridial neurotoxins and substrate proteolysis in intact neurons. *J. Biol. Chem.*, **271**, 7694–9.

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Vacuolating cytotoxin (*Helicobacter pylori*)

The vacuolating cytotoxin (VacA) is a protein secreted into the culture supernatant by Type I strains of Helicobacter pylori, *a recently discovered bacterium that causes gastric ulcer and that has been associated with gastric tumors. The toxin has been shown to play a major role in disease. When incubated with epithelial cells, VacA causes massive formation of vacuoles that have a membrane marker typical of late endosomes.* In vivo, *purified VacA causes cell vacuolization, tissue damage, and ulcer formation. The identification of the target molecule(s) of this toxin may lead to a further dissection of the rules that govern the traffick of intracellular vesicles in eukaryotic cells.*

VacA is a protein that when purified from the culture supernatant of Type I *Helicobacter pylori* strains (Leunk *et al.* 1988; Cover and Blaser 1992; Crabtree *et al.* 1992; Telford *et al.* 1994a; Marchetti *et al.* 1995) has a molecular weight between 600000–700000 Da. In the Electron microscope, the protein appears as flower-shaped hexamers and heptamers, formed by identical monomers (Fig. 1) (Lupetti *et al.* 1996). The gene codes for a precursor polypeptide of 140000 Da (Telford *et al.* 1994*b*), of which the amino-terminal of 95000 Da forms the VacA monomer, while the carboxy-terminal region is involved

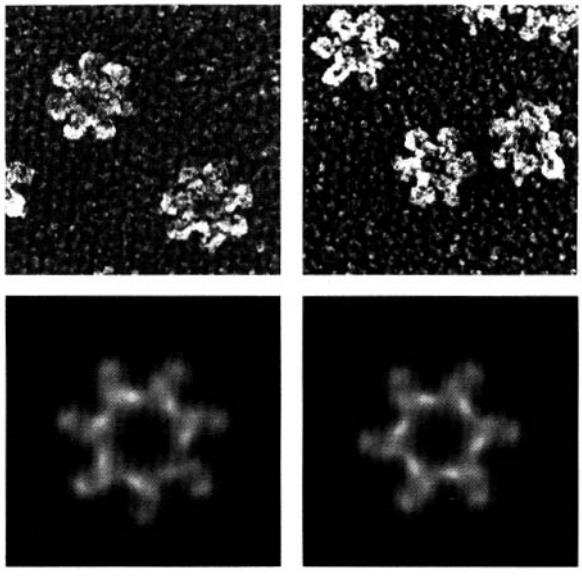

Figure 1. Vacuolating cytotoxin structure. The upper panels show examples of the heptameric and hexameric structures of VacA as observed in electron micrographs of quick-freeze, deep-etched preparations. The oligomers are approximately 30 nm in diameter with a 10–12 nm central cavity. The lower panels show the result of image clustering and averaging of many molecules of VacA. In these images, the subunit structure is easily discerned. (For further details see Lupetti *et al.* 1996.)

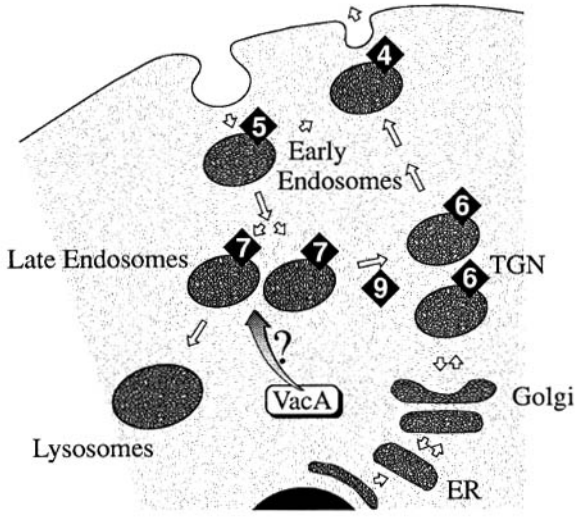

Figure 2. Vacuolating cytotoxin action.

in secretion of the toxin into the culture medium. Once secreted into the extracellular space, the 95 000 Dalton monomer can be proteolytically cleaved at a specific site, forming two subunits of 37 000 and 58 000 Da, that may represent the A and B subunits typical of the AB type of bacterial toxins. One of the subunits is presumed to be involved in binding the eukaryotic cell receptor and facilitating the toxin internalization. The other subunit is presumed to act on and inactivate a molecule of eukaryotic cells that regulates a crucial step of vesicle trafficking. Recent data have shown that vacuoles formed by this toxin contain a membrane marker typical of the late endosomes (Rab7) (Papini *et al.* 1994; Papini *et al.* 1997). The vacuolar proton pump is essential for the formation of the vacuoles, which can be inhibited either by inhibitors of the proton pump such as Bafilomycin A1 (Papini *et al.* 1993), or by a monoclonal antibody against a subunit of the proton pump (Papini *et al.* 1996). Although not yet fully elucidated at the molecular level, the action of the toxin is very likely to be similar to that shown in Fig. 2. Expression of the entire VacA gene, and of each of the subunits has been achieved in *Escherichia*

coli, however, none of the recombinant molecules were found to cause vacuolization of epithelial cells, or to form the heptameric toxin.

Purification and sources

Crude toxin activity capable of inducing vacuolization in eukaryotic cells can be found in concentrated culture supernatants of Type 1 *H. pylori*, or in water extracts of *H. pylori* cells grown in agar plates. The bacteria themselves are able to induce strong vacoulization if incubated with the cells. The purified heptameric molecule can be obtained by ammonium sulfate precipitation of the supernatant followed by Matrex cellufine sulfate chromatography and gel filtration (Manetti *et al.* 1995). The purified protein is not available commercially but can be supplied in small quantities by the authors of this entry.

Toxicity

The toxin causes vacuolization in epithelial cells at a concentration of 5μg/ml. Exposure of the toxin to low pH (from 5.5 to 1.5) for a few minutes is sufficient to activate the toxin (de Bernard *et al.* 1995). The presence of 5 mM ammonia in the medium is essential to obtain a visible vacuolization. Intragastric administration of 5 μg of purified VacA in mice caused loss of gastric gland architecture, cell necrosis, and gastric ulceration (Telford *et al.* 1994*b*).

Use in cell biology

The molecular mechanism of VacA action is still unknown, but interesting potential applications of this cytotoxin in cell biology can already be envisaged. In fact, VacA causes an alteration of the endocytic pathway resulting in the selective swelling of late endosomes or prelysosomal structures characterized by the presence of both V-ATPase and the small GTP-binding protein Rab7 (Papini *et al.* 1994; Papini *et al.* 1997). Hence, the target of VacA action appears to be a factor involved in regulation of membrane flow and/or fusion between endosomes. Similar examples from other systems in which cellular vacuolation can be induced by overexpression of dominant positive mutants of Rab5 (Stenmark *et al.* 1994), reinforce the notion that VacA modifies a fundamental effector in membrane traffick. In particular, because of its apparent effect on prelysosomal compartments (Papini *et al.* 1994), VacA may help to clarify structural and functional aspects of the endocytic pathway.

References

Cover, T. L. and Blaser, M. J. (1992). Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J. Biol. Chem.*, **267**, 10570–5.

Crabtree, J. E., Figura, N., Taylor, J. D., Bugnoli, M., Armellini, D. and Tompkins, D. S. (1992). Expression of 120 kiloDalton protein and cytotoxicity in *Helicobacter pylori*. *J. Clin. Pathol.*, **45**, 733–40.

de Bernard, M., Papini, E., Defilippis, V., Gottardi, E., Telford, J., Manetti, R., *et al.* (1995). Low pH activates the vacuolating toxin of *Helicobacter pylori*, which becomes acid and pepsin resistant. *J. Biol. Chem.*, **270**, 23937–40.

Leunk, R. D., Johnson, P. T., David, B. C., Kraft, W. G., and Morgan, D. R. (1988). Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *J. Med. Microbiol.*, **26**, 93–9.

Lupetti, P., Heuser, J. E., Manetti, R., Massari, P., Lanzavecchia, S., Bellon, P. L., *et al.* (1996). Oligomeric and subunit structure of the *Helicobacter pylori* vacuolating cytotoxin. *J. Cell Biol.*, **133**, 801–7.

Manetti, R., Massari, P., Burroni, D., de Bernard, M., Marchini, A., Olivieri, R., *et al.* (1995) *Helicobacter pylori* cytotoxin: Importance of native conformation for induction of neutralizing antibodies. *Infect. Immun.*, **63**, 4476–80.

Marchetti, M., Aricò, B., Burroni, D., Figura, N., Rappuoli, R., and Ghiara, P. (1995). Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science*, **267**, 1655–8.

Papini, E., Bugnoli, M., de Bernard, M., Figura, N., Rappuoli, R., and Montecucco, C. (1993). Bafilomycin A1 inhibits *Helicobacter pylori*-induced vacuolization of HeLa cells. *Mol. Microbiol.*, **7**, 323–37.

Papini, E., De Bernard, M., Milia, E., Bugnoli, M., Zerial, M., Rappuoli, R., *et al.* (1994). Cellular vacuoles induced by *Helicobacter pylori* originate from late endosomal compartments. *Proc. Natl. Acad. Sci. USA*, **91**, 9720–4.

Papini, E., Satin, B., Bucci, C., de Bernard, M., Telford, J, Rappuoli, R., *et al.* (1997). The small GTP-binding protein Rab7 is essential for cellular vacuolation induced by *Helicobacter pylori*. *EMBO J.* **16**(1):15–24.

Papini, E., Gottardi, E., Satin, B., de Bernard, M., Massari, P., Telford, J., *et al.* (1996). The vacuolar ATPase proton pump is present on intracellular vacuoles induced by *Helicobacter pylori*. *J. Med. Microbiol.*, **45**, 84–9.

Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lutcke, A., Gruenberg, J., and Zerial, M. (1994). Inhibition of Rab5 GTPase activity stimulates membrane fusion in endocytosis. *EMBO J.*, **13**, 1287–96.

Telford, J. L., Covacci, A., Ghiara, P., Montecucco, C., and Rappuoli, R. (1994*a*). Unravelling the pathogenic role of *Helicobacter pylori* in peptic ulcer: potential new therapies and vaccines. *Tibtech*, **12**(10), 420–6.

Telford, J. L., Ghiara, P., Dell'Orco, M., Comanducci, M., Burroni, D., Bugnoli, M., *et al.* (1994*b*). Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J. Exp. Med.*, **179**, 1653–8.

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7

Sodium channel targeted toxins

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Introduction

For defence and offense in the natural world, evolution and human design have placed a high premium on rapid knock-down. Sedentary or slow animals which defend themselves from or prey upon faster animals, plants that produce chemical defences against herbivores, and farmers who wish to reduce to a minimum crop damage due to infestation with pest insects and other species, all utilize toxic substances that act on the nervous system (Benson 1992). The sodium channel is a primary site of action for these compounds because of its central role in action potential conduction and because it is apparently highly conserved in function, although less so in molecular structure, throughout the animal kingdom (Trimmer and Agnew 1989). For example, tetrodotoxin (TTX), a nonpeptidergic component of puffer fish venom, is famous for the potent, rapid, and highly specific action in blocking axonal action potentials that has made it a favourite research tool among electrophysiologists. DDT, a compound of wholly chemical inspiration which was for forty years an inexpensive, safe, broad spectrum insecticide has its insecticidal effect by slowing sodium channel inactivation (Narahashi and Haas 1967). Taken off the market in recent years due to its persistence and consequent deleterious accumulation at the top of the food chain, DDT has been replaced in large part by the pyrethroids, which also have their site of action on the sodium channel.

This chapter provides a description of the polypeptide toxins that act on the sodium channel and which were originally purified from animal venoms, together with a summary of their uses in cell biology. With the recent rapid improvements in polypeptide isolation and purification techniques, and modern methods of synthesis, a rapidly increasing range of peptidergic toxins is being added to the synthetic, heterocyclic, and alkaloid toxins that formerly predominated as research tools. These polypeptides provide important means for identifying, purifying, and characterizing ion channels and receptors, and for probing their quaternary structure and the molecular basis of receptor and channel function. To place the polypeptide toxins in context, a brief summary of the structure of the sodium channel is given, together with an outline of the sites of action of both polypeptide and nonpeptide toxins. For greater detail, readers are referred to the sections dealing with the specific toxin groups and to a number of excellent reviews which are cited in the text.

Structure of the sodium channel

The neuronal sodium channel is composed of three polypeptide subunits designated α (260 kDa), β1 (36 kDa), and β2 (33 kDa) (Catterall 1984). Heterologous expression of the α subunit alone produces functional channels similar but not identical to native channels. All the toxin binding sites identified to date appear to be located on the α subunit which consists of four repeated, structurally homologous domains (I–IV), each composed of 300–400 amino acid residues. Each domain contains six putatively transmembrane segments (S1–S6), and a seventh region (SS1–SS2) which probably lies within the membrane. The latter has been proposed to possess a β-sheet conformation, and biochemical and site directed mutagenesis have suggested that the binding sites of both TTX and the α-scorpion toxins are associated with this β sheet.

Toxin sites of action on the sodium channel

The toxins acting on sodium channels have been classified according to their putative sites of action (Catterall 1977; Blumenthal 1995), the approach that is adhered to in the following outline, but mode of action would serve equally well (e.g. occluders, activators, and stabilizers; Strichartz *et al.* 1987; Strichartz and Castle 1990).

Site 1. Tetrodotoxin, saxitoxin, and μ-conotoxin

The heterocyclic guanidine, TTX, has been known since the turn of the century to block impulse conduction in motor axons (Kao 1966), and its mechanism of action was first demonstrated in voltage clamp studies on squid and lobster giant axons (Narahashi *et al.* 1964; Nakamura *et al.* 1965). TTX and saxitoxin (STX), a structurally related compound produced by a dinoflagellate, bind to the sodium channel with high affinity, a property that is unaltered during detergent solubilization. These compounds have thus provided excellent probes for purification of the channel protein which in turn has led to the molecular cloning of a variety of sodium channels and elucidation of their functional properties at the molecular level (Sigworth 1994).

TTX and STX bind to a site accessible from the external face of the channel. The amino acid residues involved have yet to be defined, but they are thought to be located on the intramembrane loop SS1–SS2, an area shown by site-directed mutagenesis to be important in ion selectivity as well as the binding of channel-specific toxins (Noda *et al.* 1989; Satin *et al.* 1992).

The 22 residue polypeptide toxin, μ-conotoxin (or geographutoxin), is a competitive inhibitor of the binding of STX and its derivatives to the sodium channels of muscle but not nerve. Its binding is also voltage-dependent, in

contrast to that of TTX and STX. This toxin thus distinguishes between nerve and muscle sodium channels which differ very little in their kinetics and their responses to TTX and STX. Nevertheless, cardiac 'TTX-resistant' sodium channels are also unaffected by μ-conotoxin, so that there is little doubt that μ-conotoxin and the structurally unrelated TTX/STX toxins all bind to the same or overlapping sites on the channel (Gray and Olivera 1988). The predatory activity of the venomous *Conus* snails is beautifully illustrated in a review by Olivera *et al.* (1990) and the cell biological applications of the μ-conotoxins are described by Cruz below (see p. 128).

Site 2. Batrachotoxin, grayanotoxin, veratridine, and aconitine

Batrachotoxin (BTX) was originally isolated from the skin secretions of poisonous frog species, while grayanotoxin, veratridine, and aconitine are plant-derived alkaloids. They are lipid-soluble and all act on a binding site that influences the gating mechanism of the sodium channel and which is allosterically coupled to the TTX binding site and also to that of the α-scorpion toxin discussed below (Catterall 1977). These compounds induce persistent activation of the channel and consequent depolarization of the neuronal membrane, and seem to reduce the ion selectivity of the channel. However, the detailed mechanisms underlying these effects may differ. BTX can act from either side of the membrane, suggesting that its binding site might lie within the channel segments traversing the cell membrane, and binds much more rapidly to the open state of the channel. Catterall (1977) proposed that the alkaloids bind preferentially to the activated conformation of the sodium channel, shifting the channel open/closed equilibrium towards the open state, perhaps in the form of a shift in the voltage-dependence of channel activation (Huang *et al.* 1984).

The pyrethroid insecticides are synthetic analogues of the pyrethrins, neurotoxins isolated from chrysanthemum flowers, with sufficient persistence for their agricultural purpose but not so much that they accumulate dangerously either in the soil or the food chain. They also exhibit a species-specific toxicity that leaves certain important beneficials unaffected. A high production cost due to their varied and complex structures is the main drawback of these compounds. The mode of action of the pyrethoids is similar to that of the alkaloids. They bind preferentially to open sodium channels and produce a steady-state sodium current, but may bind to two sites, one or both different from the alkaloid binding site.

Sites 3 and 4. Scorpion and sea anemone toxins

Scorpion and anemone toxins constitute the majority of the polypeptide toxins summarized in this chapter. Sodium channel-specific toxins from the venoms of these species typically but not exclusively consist of only about 60–70 and 46–49 amino acid residues, respectively. No post-translational modifications or unusual amino acids are found in these toxins, in contrast to the μ-conotoxins in which amino acids with *trans*-4-hydroxyproline residues and the amination of the C-terminus have been identified (Strichartz *et al.* 1987). All of these toxins contain disulfide bonds that are essential for their biological activity.

The scorpion toxins fall into two distinct groups distinguished from one another on the basis of binding assays and their electrophysiological actions. The α-scorpion toxins (from *Androctonus* and *Leiurus* venoms) are described by Martin-Eauclaire and Bougis below (see p. 118). These polypeptides delay or abolish channel inactivation by binding to Site 3, and thus prolong the action potential duration in muscle and nerve. The β-scorpion toxins (from *Centruroides* and *Tityrus* venoms) shift the voltage-dependence of activation to more negative potentials as a result of binding to Site 4, inducing spontaneous neuronal activity. Binding of the alpha-scorpion toxins is membrane potential-dependent with K_D values in the nanomolar range at the resting potential. In contrast, β-scorpion toxin binding is membrane potential-independent and exhibits lower affinities. In addition to their modes of action, the scorpion toxins can be classified according to their amino acid composition and sequence homologies (Dufton and Rochat 1984). The two groups of scorpion toxins have in common a dense core containing three strands of antiparallel β-sheet and a short strand of alpha-helix. The details of the non α scorpion toxins are given by Rochat (p. 120) and two groups of insect-selective scorpion toxins, excitatory and depressant, are described by Zlotkin (p. 123 and p. 126, respectively). The concept of expressing the genes coding for insect-selective polypeptide toxins in baculoviruses or organ, specifically in transgenic cultivated plants, enjoys widespread popularity as a crop protection alternative to chemical insecticides (Vaeck *et al.* 1987; Stewart *et al.* 1991; Tomalski and Miller 1991; Hammock *et al.* 1993) and, indeed, genetically improved baculovirus insecticides have reached the stage of field trials (e.g. Cory *et al.* 1994).

Site 3 is also the site of action of polypeptide toxins purified from the venoms of a variety of sea anemone species, detailed below by Shibata and Norton (p. 131 and p. 134, respectively). The anemone toxins are generally smaller than the α-scorpion toxins and have three rather than four disulfide bridges. In contrast to the scorpion toxins, they possess a core of twisted, four-stranded antiparallel β-pleated sheet indicating that they are structurally unrelated to the scorpion toxins with which they also lack sequence homology. Nevertheless, both groups bind to Site 3, exhibit similar pharmacologies, and displace one another from their binding site. The β-scorpion toxins, despite their structural similarities to the α-scorpion toxins, bind to the sodium channel at Site 4 which is distinct from the binding site of the α-scorpion toxins and the anemone toxins.

Sites 3 and 4, the polypeptide toxin binding sites, are structurally distinct from the TTX Site 1 and alkaloid Site 2 locations. Nevertheless, both groups of sites are found

on the α subunit of the sodium channel and a photoactivatable derivative of *Leiurus* toxin labels residues on SS1-SS2 of domains I and IV of the channel. In some cells, the β1 subunit is also photolabelled, suggesting that the polypeptide binding site might be located near the α/β1 interface.

Other sodium channel toxins and binding sites

Spider venoms have yielded a rich assortment of toxins. Nonpeptide examples are the polyamines, a group of acylpolyamine/amino acid hybrids that are similar in structure and mode of action to wasp toxins and act on a variety of ion channels (Scott *et al.* 1993). The peptidergic ω-agatoxins, like the ω-conotoxins, act primarily and specifically on certain subtypes of calcium channel (Olivera *et al.* 1994). Another group of polypeptide toxins from spider venoms are active on the sodium channel and are covered by Adams and Norris (p. 130). The μ-agatoxins from the venom of *Agelenopsis* are cysteine rich polypeptides that cause irreversible paralysis and repetitive action potentials originating in presynaptic axons or nerve terminals. This induced excitation is blocked by TTX, suggesting an effect on the sodium channel (Adams *et al.* 1989).

The list of binding sites summarized above is very likely to be lengthened as new toxins are characterized (Wu and Narahashi 1988). The lipid-soluble dinoflagellate toxins, brevetoxin, ciguatoxin, and maitotoxin probably bind to one or more novel sites since brevetoxin, for example, is not displaced in binding assays by toxins that bind to Sites 1–4. Similarly, a polypeptide δ-conotoxin, selectively active on molluscs and designated TxVIA, appears to have a distinct binding site mediating its mode of action on sodium channel inactivation (Fainzilber *et al.* 1994).

References

Adams, M. E., Herold, E. E., and Venema, V. J. (1989). Two classes of channel-specific toxins from funnel web spider venom. *J. Comp. Physiol.*, A **164**, 333–42.

Benson, J. A. (1992). Natural and synthetic toxins at insect receptors and ion channels: the search for insecticide leads and target sites. In *Neurotox '91. Molecular basis of drug and pesticide action* (ed. I. R. Duce), pp. 57–70, Elsevier Applied Science, London and New York.

Blumenthal, K. (1995). Ion channels as targets for toxins. In *Cell physiology source book* (ed. N. Sperelakis), pp. 389–403, Academic Press, San Diego.

Catterall, W. A. (1977). Activation of the action potential Na^+ ionophore by neurotoxins. *J. Biol. Chem.*, **252**, 8669–76.

Catterall, W. A. (1984). The molecular basis of neuronal excitability. *Science*, **223**, 653–61.

Cory, J. S., Hirst, M. L., Williams, T., Hails, R. S., Goulson, D., Green, B. M., Carty, T. M., Possee, R. D., Cayley, P. J., and Bishop, D. H. L. (1994). Field trial of a genetically improved baculovirus insecticide. *Nature*, **370**, 138–40.

Dufton, M. J. and Rochat, H. (1984). Classification of scorpion toxins according to amino acid composition and sequence. *J. Mol. Evol.*, **20**, 120–7.

Fainzilber, M., Kofman, O., Zlotkin, E., and Gordon, D. (1994). A new neurotoxin receptor site on sodium channels is identified by a contoxin that affects sodium channel inactivation in molluscs and acts as an antagonist in rat brain. *J. Biol. Chem.*, **269**, 2574–80.

Gray, W. R. and Olivera, B. M. (1988). Peptide toxins from venomous *Conta* snails. *Ann. Rev. Biochem.*, **57**, 665–700.

Hammock, B. D., McCutchen, B. F., Beetham, J., Choudary, P. V., Fowler, E., Ichinose, R., *et al.* (1993). Development of recombinant viral insecticides by expression of an insect-specific toxin and insect-specific enzyme in nuclear polyhedrosis viruses. *Arch. Insect Biochem. Physiol.*, **22**, 315–44.

Huang, L. Y. M., Moran, N., and Ehrenstein, G. (1984). Gating kinetics of batrachotoxin-modified sodium channels in neuroblastoma cells determined from single-channel measurements. *Biophys. J.*, **45**, 313–22.

Kao, C. Y. (1966). Tetrodotoxin, saxitoxin, and their significance in the study of excitation phenomena. *Pharmacol. Rev.*, **18**, 997–1049.

Nakamura, Y., Nakajima, S., and Grundfest, H. (1965). The action of tetrodotoxin on electrogenic components of squid giant axons. *J. Gen. Physiol.*, **48**, 985–96.

Narahashi, T. and Haas, H. G. (1967). DDT: interaction with membrane conductance changes. *Science*, **157**, 1438–40.

Narahashi, T., Moore, J. W., and Scott, W. R. (1964). Tetrodotoxin blockage of sodium conductance increase in lobster giant axon. *J. Gen. Physiol.*, **47**, 965–74.

Noda, M., Suzuki, H., Numa, S., and Stuhmer, W. (1989). A single point mutation confers tetrodotoxin and saxitoxin insensitivity on the sodium channel II. *FEBS Lett.*, **259**, 213–16.

Olivera, B. M., Hillyard, D. R., Rivier, J., Woodward, S., Gray, W. R., Corpuz, G., *et al.* (1990). Conotoxins: targeted peptide ligands from snail venoms. In *Marine toxins. Origin, structure, and molecular pharmacology* (ed. S. Hall and G. Strichartz), ACS Symposium Series No. 418, pp. 256–78, American Chemical Society, Washington DC.

Olivera, B. M., Miljanich, G. P., Ramachandran, J., and Adams, M. E. (1994). Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins. *Ann. Rev. Biochem.*, **63**, 823–67.

Satin, J., Kyle, J. W., Chen, M., Bell, P., Cribbs, L. L., Fozzard, H. A., *et al.* (1992). A mutant of TTX-resistant cardiac sodium channels with TTX-sensitive properties. *Science*, **256**, 1202–5.

Scott, R. H., Sutton, K. G., and Dolphin, A. C. (1993). Interactions of polyamines with neuronal ion channels. *Trends Neurosci.*, **16**, 153–60.

Sigworth, F. (1994). Voltage gating ion channels. *Quart. Rev. Biophys.*, **27**, 1–40.

Stewart, L. M. D., Hirst, M., Ferber, M. L., Merryweather, A. T., Cayley, P. J., and Possee, R. D. (1991). Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature*, **352**, 85–8.

Strichartz, G. and Castle, N. (1990). Pharmacology of marine toxins. Effects on membrane channels. In *Marine toxins. Origin, structure, and molecular pharmacology* (ed. S. Hall and G. Strichartz), ACS Symposium Series No. 418, pp. 2–20, American Chemical Society, Washington DC.

Strichartz, G., Rando, T., and Wang, G. K. (1987). An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Ann. Rev. Neurosci.*, **10**, 237–67.

Tomalski, M. D. and Miller, L. K. (1991). Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. *Nature*, **352**, 82–5.

Trimmer, J. S. and Agnew, W. S. (1989). Molecular diversity of voltage-sensitive Na channels. *Ann. Rev. Physiol.*, **51**, 401–18.

Vaeck, M., Reynaerts, A., Höfte, H., Jansens, S., De Beuckeleer, M., Dean, C., *et al.* (1987). Transgenic plants protected from insect attack. *Nature*, **328**, 33–7.
Wu, C. H. and Narahashi, T. (1988). Mechanism of action of novel marine neurotoxins on ion channels. *Ann. Rev. Pharmacol. Toxicol.*, **28**, 141–61.

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α-Scorpion toxins

The α-scorpion toxins were the first scorpion toxins to be characterized. They are 7000 Da polypeptides with four disulfide bridges. They bind with high affinity to the voltage-sensitive sodium channel of excitable cells, thereby impeding the normal channel operation. The toxin binds to site 3 and binding is abolished when the membrane is depolarized.

α-Toxins can be considered as the cause of the scorpion venom toxicity for mammals and particularly human beings, in the 'Old World' (Miranda *et al.* 1970). For example, 90 per cent of the toxicity for the mouse in the venom of *Androctonus australis* from North Africa is due to only 2 per cent of the weight of the crude venom. The toxic fractions are α-toxins (named AaH I, AaH II, AaH III according to their chromatographic behaviour) (Martin and Rochat 1986). These toxins are constituted by a single polypeptide chain of 60 to 70 amino acid residues reticulated by four disulfide bridges (Miranda *et al.* 1970). Their three dimensional structure is well documented: the AaH II structure has been determined at high resolution by X-ray crystallography (Fontecilla-Camps *et al.* 1988) and AaH III by ^{1}H-nuclear magnetic resonance (NMR) (Mikou *et al.* 1992). The toxin molecules have 2 1/2 turns of α-helix and short segments of anti-parallel β-sheets. These elements of secondary structure are joined by two disulfide bridges. The third bridge links loops and the fourth bridge links the NH_2-terminus and COOH-terminus regions. The differences in the activities of α-toxins (compared to that of β-toxins) seems to depend on changes in the length and orientation of loops protuding from the dense core of secondary elements. A surface hydrophobic patch, mostly determined by a cluster of aromatic residues and located on one side of the molecule, may be involved in the interaction of toxins with membrane components. α-Scorpion toxins, like sea anemone toxins, bind to site 3 of the voltage-sensitive sodium channels of excitable cells (i.e., nerve, muscle, and heart cells),

```
                  10        20        30        40        50        60        70        80
                   |         |         |         |         |         |         |         |
AaH I     -KRDGYIVYPN-NCVYHCVPP-----CDGLCKKN-GG-SSGSCSFLVPSGLACWC-KDLP-DNVPIKDT--SRK-CT-
AaH I'    -KRDGYIVYPN-NCVYHCIPP-----CDGLCKKN-GG-SSGSCSFLVPSGLACWC-KDLP-DNVPIKDT--SRK-CT-
AaH I''   -KRDGYIVYPN-NCVYHCVPP-----CDGLCKKN-GG-SSGSCSFLVPSGLACWC-KDLP-DNVPIKDT--SRK-CTR
AaH IV    -GRDGYIVDSK-NCVYHCYPP-----CDGLCKKN-GA-KSGSCGFLVPSGLACWC-NDLP-ENVPIKDP--SDD-CHK
AaH III   -VRDGYIVNSK-NCVYHCVPP-----CDGLCKKN-GA-KSGSCGFLIPSGLACWCVA-LP-DNVPIKDP--SYK-CHS

AaH II    -VKDGYIVDDV-NCTYFCGR---NAYCNEECTKL-KG-ESGYCQWASPYGNACYCYK-LP-DHVRTKGP--GR--CH--*
Bot III   -VKDGYIVDDR-NCTYFCGR---NAYCNEECTKL-KG-ESGYCQWASPYGNACYCYK-VP-DHVRTKGP--GR--CN--*
Bot XI    -LKDGYIVDDR-NCTYFCGT---NAYCNEECVKL-KG-ESGYCQWVGRYGNACWCYK-LP-DHVRTVQA--GR--CR--*
LQQ V     -LKDGYIVDDK-NCTFFCGR---NAYCNDECKKK-GG-ESGYCQWASPYGNACWCYK-LP-DRVSIKEK--GR--CN--*
Amm V     -LKDGYIIDDL-NCTFFCGR---NAYCDDECKKK-GG-ESGYCQWASPYGNACWCYK-LP-DRVSIKEK--GR--CN--*
CsE V     -KKDGYPVDSG-NCKYECLK---DDYCNDLCLER-KA-DKGYCYW-GKV--SCYC--GLP-DNSPTKT--SG-K-CNPA
                   |         |         |         |         |         |         |         |
                  10        20        30        40        50        60        70        80
```

AaH, *Androctonus australis* Hector; Amm, *Androctonus mauretanicus mauretanicus*; Bot, *Buthus occitanus tunetanus*; Lqq, *Leiurus quinquestriatus quinquestriatus*; CsE, *Centruroides sculpturatus* Ewing.* marks α-amidation.

Figure 1. Amino acid sequences of the main α-toxins.

Table 1 Biological characterization of α-toxins

Structural group	Toxin	$K_{0.5}$ (nM)	LD_{50} (i.c.v.) ng/20 g mouse	LD_{50} (s.c.) μg/20 g mouse
1	AaH I	4.5	10.0	0.3
	AaH I′	4.5	10.0	0.3
	AaH I″	4.5	10.0	0.3
	AaH III	3.0	7.0	0.5
	AaH IV	50.0	18.0	2.5
2	AaH II	0.2	0.5	0.2
	Lqq V	1.0	2.5	0.5
	Bot III	0.5	1.1	0.5
	Bot XI	30.0	600.0	54.0
	Amm V	not tested	3.5	0.8
	CsE V	>100.0	not tested	not tested

The LD_{50} are obtained in male C57 Bl/ 6 by subcutaneous (s.c.) or intracerebroventricular (i.c.v.) injection; $K_{0.5}$ is obtained by competition on rat brain synaptosomes with the radioiodinated α-toxin of reference, i.e, ^{125}I-AaH II.

responsible for the rapid depolarization phase of the action potential. Their binding depends on membrane potential (Catterall *et al.* 1976). The site of attachment of α-scorpion toxins on the α-subunit of rat brain Na^+ channel has been determined. It appears to be formed by peptide segments located in extracellular loops of both domains I and IV (Tejedor and Catterall 1988).

Purification and sources

α-Scorpion toxins are routinely purified from *buthidae* venom by conventional chromatography, i.e. a first step of molecular filtration through sephadex G_{50}, followed by one or several steps of ion-exchange chromatography. RP-HPLC (C_8 or C_{18}) can be used for analytical purification. The main source of α-toxins are 'Old World' scorpion venoms, particularly from *Androctonus australis, Androctonus mauretanicus,* and *Leiurus quinquestriatus.* They can be found as traces in some 'New World' scorpion venoms, like those from *Tityus serrulatus* and *Centruroides sculpturatus.* The toxins are not commercially available.

Use in cell biology

α-Toxins induce a prolongation of the action potential and this effect can be abolished by lowering the extracellular Na^+ concentration. Voltage-clamp analyses have been performed on the node of Ranvier from frog myelinated nerve fibres, neuroblastoma cells, rat skeletal muscle, chick *biventer cervicis* muscle, and chick embryonic heart muscle cells in culture. Similar modifications of sodium inactivation are observed in nonvertebral cell membrane, like the giant axons of lobster and squid. α-Scorpion toxin sensitive Na^+ channels can be detected in several types of nonexcitable cells. ^{125}I-labelled α-scorpion toxins can be used as high affinity tools to investigate the voltage-sensitive Na^+ channel of excitable membranes, but their affinity is reduced by membrane depolarization (Catterall *et al.* 1976; Martin-Eauclaire and Couraud 1995). Covalent labelling of site 3 with a photoaffinity derivative of Lqq is possible (Tejedor and Catterall 1988; Martin-Eauclaire and Couraud 1995).

Toxicity

Toxicity is tested by subcutaneous (17–150 μg/kg) or intracerebro-ventricular (25–1500 ng/kg) injection into the mouse. Workers must avoid contact of the toxins with mucous membranes and must not swallow the lyophilysed toxin.

Antibodies

Polyclonal antibodies raised in rabbit against the toxins of *Androctonus australis* and a monoclonal antibody against AaH II have been described (Granier *et al.* 1989) but are not commercially available. Numerous polyclonal antibodies have been prepared against synthetic peptides corresponding to part of the toxin sequences and have provided information on structure–function relationships and allowed epitope mapping (Granier *et al.* 1989).

Genes

Complete complementary DNA sequences are available for AaH I, AaH I′, AaH II, and AaH III (J05102). C-terminal extensions are observed, such as GR for AaH II, which is α-amidated, and R for the other AaH toxins (Bougis *et al.* 1989). The promoter structure and intron–exon organization of AaH I′ gene have been determined (X76135) (Delabre *et al.* 1995).The gene transcriptional unit is 793 bp long and a unique 425 bp intron is located near the end of the signal peptide.

References

Bougis, P. E., Rochat, H., and Smith, L. A. (1989). Precursors of *Androctonus australis* scorpion neurotoxin: structure of

precursors, precessing outcomes and expression of a functional recombinant toxin II. *J. Biol. Chem.*, **264**, 19259–65.
Catterall, W. A., Ray, R., and Morrow, C. (1976). Membrane potential dependent binding of scorpion toxin to the action potential sodium ionophore. *Proc. Natl. Acad. Sci. USA*, **73**, 2682–6.
Delabre, M. L., Pasero, P., Marilley, M. and Bougis, P. E. (1995). Promoter structure and intron–exon organization of a scorpion α-toxin gene. *Biochemistry*, **34**, 6729–36.
Fontecilla-Camps, J., Habersetzer-Rochat, C., and Rochat H. (1988). Toxin II from the scorpion *Androctonus australis* Hector. Orthorhombic crystals, three-dimensional structure and structure-function relationships. *Proc. Natl. Acad. Sci. USA*, **85**, 7443–7.
Granier, C., Novotny, J., Fontecilla-Camps, J. C., Fourquet, P., El Ayeb, M., and Bahraoui, M. (1989). The antigenic structure of a scorpion toxin. *Mol. Immunol.*, **26**, 503–13.
Martin, M. F. and Rochat, H. (1986). Large scale purification of the venom from the scorpion *Androctonus australis* Hector. *Toxicon*, **24**, 1131–9.
Martin-Eauclaire, M. F. and Couraud, F. (1995). Scorpion neurotoxins: effects of mechanisms. *N.Y.*, **22**, 683–716.
Mikou, A., La Plante, S., Guittet, E., Lallemand, J. Y., Martin-Eauclaire, M. F., and Rochat, H. (1992). Toxin III of the scorpion *Androctonus australis* Hector: Proton nuclear magnetic resonance assignments and secondary structure. *J. Biomol. NMR*, **2**, 57–70.
Miranda, F., Kupeyan, C., Rochat, H., Rochat, C., and Lissitsky, S. (1970). Purification of animal neurotoxins: isolation and characterization of eleven neurotoxins from the venoms of the scorpions *Androctonus australis* Hector, *Buthus occitanus tunetanus*, and *Leiurus quinquestriatus quinquestriatus*. *Eur. J. Biochem.*, **16**, 514–23.
Tejedor, F. G. and Catterall, W. A. (1988). Site of covalent attachment of α-scorpion toxin derivatives in domain I of the sodium channel α-subunit. *Proc. Natl. Acad. Sci. USA*, **85**, 8742–6.

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β-Scorpion toxins

The β-toxins are generally present in the venom of scorpions of the New World where they are responsible for scorpionism. Like α-toxins, they are made of 60 to 66 residues with four disulfide bridges. They bind with very high affinity to a specific site, site 4, on the sodium channel, modifying its activation. Its binding on rat brain synaptosomes is independent of membrane potential.

β-Toxins are responsible for the life hazard constituted, in Central and South America, by scorpion stings. They are qualitatively and quantitatively the most important polypeptides of the venom. They are made of one single polypeptide chain of 60 to 66 amino acid residues crosslinked by four disulfide bridges (Possani *et al.* 1981; Bechis *et al.* 1984; Martin-Eauclaire *et al.* 1987; Ceard *et al.* 1992; Zamudio *et al.* 1992). The structure of variant 3 from *Centruroides sculpturatus* Ewing has been determined at high resolution by X-ray crystallography (Fontecilla-Camps *et al.* 1980). This protein although poorly active on mouse has an amino acid sequence very close to that of the most potent β-toxins: it shows a common structure made up of one α-helix and one triple stranded β-sheet maintained in position by two conserved disulfide bridges (Cys-25 and Cys-51, Cys-29 and Cys-53). Two more disulfide bridges link in one hand loops protruding from this region (Cys-16 and Cys-41) and, on the other hand, the C-terminal end of the chain to the N-terminal region (Cys-12 and Cys-72). On one side of the molecule, a hydrophobic surface exists, which has been implicated in the high affinity binding of β-toxins to their pharmacological target (Fontecilla-Camps *et al.* 1980). β-Toxins were defined as those toxins which behave like toxin II of *Centruroides Suffusus suffusus* (Css II): they bind to site 4 of the voltage-dependent sodium channel present in rat brain synaptosomes (Jover *et al.* 1980) modifying the activation process and inducing repetitive firing on frog myelinated nerve on voltage-clamp conditions (Couraud *et al.* 1982; Martin-Eauclaire and Couraud 1995).

It has been recently shown that the high specific binding of toxin IV of *Centruroides Suffusus suffusus*, a β-toxin, at receptor site 4 on purified sodium channels can be restored by reconstitution into phospholipids vesicles (Thomsen *et al.* 1995). This binding was unaffected by membrane potential or by neurotoxins that bind at sites 1–3 or 5, consistent with the binding behaviour of β-toxins to the sodium channel (Jover *et al.* 1980; Martin-Eauclaire and Couraud 1995).

Ts VII, a potent β-toxin purified from *Tityus serrulatus* venom (Bechis *et al.* 1984) is also known as toxin γ

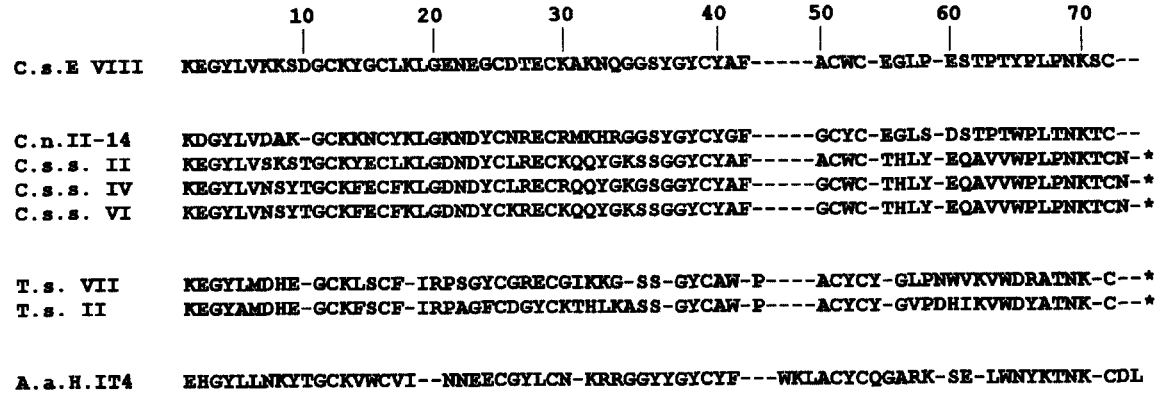

Figure 1. Amino acid sequences of some β-toxins. Data taken from Martin-Eauclaire and Couraud (1995) except for Css IV and VI: Sampieri *et al.* personal communication. C.s.E, *Centruroides sculpturatus* Ewing; C.n., *Centruroides noxius*; C.s.s., *Centruroides suffusus suffusus*; T.s., *Tityus serrulatus;* A.a.H., *Androctonus australis* Hector; * –CO–NH_2 C-terminal end.

(Possani *et al.* 1981). This toxin was shown to be also toxic to insects and to bind selectively, with high affinity, to the insect sodium channel (De Lima *et al.* 1986, 1989). This double specificity has been attributed to a high flexibility of the molecule, leading to better fitting to the different receptor sites (Loret *et al.* 1990). A cDNA encoding Ts VII was cloned: the amino acid sequence of the derived precursor shows that in addition to the removal of a signal peptide a complex maturation is necessary to obtain the C-terminal end of the toxin (Martin-Eauclaire *et al.* 1992).

AaH IT4 is an insect toxin purified from the venom of *Androctonus australis* Hector. This protein was shown to compete with AaH II (an α-toxin), Css II (a β-toxin), and AaH IT (an insect toxin) for binding to their respective sites on the mammal and insect sodium channels. The sequence of this toxin has common features with β-toxins. Moreover, it only recognizes anti-β-toxin antibodies. On these bases, AaH ITIV has been proposed to represent an ancestral scorpion toxin (Loret *et al.* 1991).

Purification

β-Toxins have been purified from the venoms of scorpions belonging to the *Centrurinae* and *Tityinae*. The purification process is most often a succession of gel filtration and ion exchange chromatographies of the water extract of the venom (Possani *et al.* 1981; Bechis *et al.* 1984; Martin-Eauclaire *et al.* 1987; Ceard *et al.* 1992; Zamudio *et al.* 1992). When only small amounts of toxin have to be prepared RP-HPLC might well be used with good results (Céard *et al.* 1992).

Antibodies

Polyclonal antibodies against New World scorpion venoms exist as they are produced for serotherapy which is the only specific treatment for scorpionism. Polyclonal as monoclonal antibodies (Zamudio *et al.* 1992) have been produced against β-toxins or peptides derived from them. The situation appears to be more complex than with α-toxins when studying the crossreactivity between β-toxins. This might be due to greater amino acid sequence variation in potentially antigenic regions (De Lima *et al.* 1993).

Toxicity

Toxicity is measured by determining the LD_{50} on mouse (C57Bl6, male) using two methods of injection: subcutaneous (s.c.) and intracerebroventricular (i.c.v.).

Direct contact of the venom or purified toxin with mucous membranes must be avoided. Handling of dry powders must be done in a fume cupboard.

Use in cell biology

Css II, Css IV, Css VI, and TsVII may be radioiodinated and used in binding experiments as specific markers of receptor site 4 of the sodium channel (Jover *et al.* 1980; Couraud *et al.* 1982; De Lima *et al.* 1986; Martin-Eauclaire

Table 1

Toxin	s.c. mg/20 g	i.c.v. ng/20 g
Css II	0.5	5
Css IV	2.3	2.4
Css VI	1.7	1.2
Ts II	3.7	6.0
Ts VII	4.7	0.6
AaH IT4	–	20.0

et al. 1987; De Lima *et al.* 1989; Thomsen *et al.* 1995). Photoreactive derivatives of ^{125}I-β toxins may be used to define better receptor site 4: both α and β1 subunits of the sodium channel are still good candidates, depending on the β-toxin used (Darbon *et al.* 1983; Jover *et al.* 1988).

References

Bechis, G., Sampieri, F.,Yuan, P. M., Brando, T., Martin-Eauclaire, M. F., Diniz, C. R., *et al.* (1984). Amino acid sequence of toxin VII, a β-toxin from the venom of the scorpion *Tityus serrulatus*. *Biochem. Biophys. Res. Commun.*, **122**, 1146–53.

Ceard B., De Lima, M. E., Bougis, P. E., and Martin-Eauclaire, M. F. (1992). Purification of the main β-toxin from *Tityus serrulatus* scorpion venom using high performance liquid chromatography. *Toxicon*, **30**, 105–10.

Couraud, F., Jover, E., Dubois, J. M., and Rochat, H. (1982). Two types of scorpion toxin receptor sites, one related to the activation, the other to the reactivation of the action potential sodium channel. *Toxicon*, **20**, 9–16.

Darbon, H., Jover, E., Couraud, F., and Rochat, H. (1983). Photoaffinity labeling of α and β scorpion toxin receptors associated with rat brain sodium channel. *Biochem. Biophys. Res. Commun.*, **115**, 415–22.

De Lima, M. E., Martin-Eauclaire, M. F., Diniz, C. R., and Rochat, H. (1986). *Tityus serrulatus* toxin VII bears pharmacological properties of both β toxin and insect toxin from scorpion venoms. *Biochem. Biophys. Res. Commun.*, **139**, 296–302.

De Lima, M. E., Martin-Eauclaire, M. F, Hue, B., Loret, E., Diniz, C. R., and Rochat, H. (1989). On the binding of two scorpion toxins to the central nervous system of the cockroach *Periplaneta americana*. *Insect Biochem.*, **19**, 413–22.

De Lima, M. E., Martin-Eauclaire, M. F., Chavez-Olortegui, C., Diniz, R., and Granier, C. (1993). *Tityus serrulatus* scorpion venom toxins display a complex pattern of antigenic reactivity. *Toxicon*, **31**, 223–7.

Fontecilla-Camps, J. C., Almassy, R. J., Suddath, F. L., Watt, D. D., and Bugg, C. E. (1980). Three dimensional structure of a protein from scorpion venom. A new structural class of neurotoxins. *Proc. Natl. Acad. Sci. USA*, **77**, 6496–500.

Jover, E., Couraud, F., and Rochat, H. (1980). Two types of scorpion neurotoxins characterized by their binding to two separate receptor sites on rat brain synaptosomes. *Biochem. Biophys. Res. Commun.*, **95**, 1607–14.

Jover, E., Massacrier, A., Cau, P., Martin, M. F., and Couraud, F. (1988). The correlation between Na^+ channel submits and scorpion binding sites. *J. Biol. Chem.*, **263**, 1542–8.

Loret, E.P., Sampieri, F., Roussel, A., Granier, C., and Rochat, H. (1990). Conformational flexibility of a scorpion toxin active on mammals and insects: a circular dichroism study. *Proteins, Struc. Funct. Genet.*, **8**, 164–72.

Loret, E. P., Martin-Eauclaire, M. F., Mansuelle, P., Sampieri, F., Granier, C., and Rochat, H. (1991). An anti-insect toxin purified from the scorpion *Androctonus australis* Hector also acts on the α and β sites of the mammalian sodium channel: sequence and circular dichroism study. *Biochemistry*, **30**, 633–40.

Martin-Eauclaire, M. F., Garcia y Perez, L. G., El Ayeb, M., Kopeyan, C., Bechis, G., Jover, E., *et al.* (1987). Purification and chemical and biological characterization of seven toxins from the Mexican scorpion, *Centruroides suffusus suffusus*. *J. Biol. Chem.*, **262**, 4452–9.

Martin-Eauclaire, M. F., Ceard, B., Ribeiro, A. M., Diniz, C. R., Rochat, H., and Bougis, P. E. (1992). Molecular cloning and nucleotide sequence analysis of a cDNA encoding the main β-neurotoxin from the venom of the South American scorpion *Tityus serrulatus*. *FEBS Lett.*, **302**, 220–2.

Martin-Eauclaire, M. F. and Couraud, F. (1995). Scorpion neurotoxins: effects of mechanisms. *N.Y.*, **22**, 683–716.

Possani, L. D., Martin, B. M., Mocha-Morales, J., and Svendsen, I. (1981). Purification and chemical characterization of the major toxins from the venom of the Brazilian scorpion *Tityus serrulatus*. *Lutz and Mello. Carlberg Res. Commun.*, **46**, 195–265.

Thomsen, W., Martin-Eauclaire, M. F., Rochat, H., and Catterall, W. A. (1995). Reconstitution of high-affinity binding of a β-scorpion toxin to neurotoxin receptor site 4 on purified sodium channels. *J. Neurochem.*, **65**, 1358–64.

Zamudio, F., Saavedra, R., Martin, B. M., Gurrola-Brionps, G., Herion, P., and Possani, L. D. (1992). Amino acid sequence and immunological characterization with monoclonal antibodies of two toxins from the venom of the scorpion *Centruroides noxius* Hoffmann. *Eur. J. Biochem.*, **204**, 281–92.

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Excitatory insect selective neurotoxins from scorpion venoms

The excitatory insect toxins are single-chained polypeptides of about 70 amino acids crosslinked by four disulfide bridges, three of which appear to possess homologous positions to those of the α-scorpion toxins which affect mammals. The toxins interact with various intact neuronal preparations through a single class of noninteracting, high affinity (K_D = 1–2 nM), and low capacity (1.2–2.0 pmol/mg membranal protein) binding sites. The rapid excitatory paralysis of insects promoted by these toxins is due to the induction of repetitive electrical activity of motorneurons.

The lethal and paralytic capacity of Buthinae scorpion venoms to insects can be attributed substantially to their inclusion of the excitatory insect selective neurotoxins (Zlotkin 1986).

Four separate excitatory insect toxins have been isolated so far by conventional methods of column chromatography from the venoms of the following Buthinae scorpions: *Androctonus australis* Hector (AaH IT, Zlotkin *et al.* 1971*a* and *b*), *Buhotus judaicus* (BJ IT1, Lester *et al.* 1982), *Androctonus mauretanicus* (Am IT1, Zlotkin *et al.* 1979), and *Leinrus quinguestriatus quinguestriatus* (Lqq IT1, Zlotkin *et al.* 1985). AaH IT was the first insect selective toxin to be identified and studied and its covalent structure is presented in Fig. 1 (Darbon *et al.* 1982).

Purification and chemistry

AaH IT was purified (Zlotkin *et al.* 1971*a*) by a sequence of five steps, including water extract, dialysis, and recycling gel filtration on Sephadex G50 columns followed by a column of an anion exchanger and finally cation exchanger. The latter were eluted in equilibrium conditions by the volatile ammonium acetate buffers (Zlotkin *et al.* 1971*a*). Its amino acid sequence was established by phenylisothiocyanate degradation of several protein derivatives and proteolytic fragments in a liquid protein sequencer using either a 'protein' or a 'peptide' program. The position of the four disulfide bridges were deduced by analysis of proteolytic peptides before and after performic oxidation, and by partial labelling of the half cystine residues with [^{14}C]-iodoacetic acid and determining the specific radioactivities of the *S*-[^{14}C]-carboxymethylated phenylthiohydantoin cysteines. As shown (Fig. 1) AaH IT is a single-chained polypeptide composed of seventy amino acids (M_r = ~8 kDa) cross-linked by four disulfide bridges (Darbon *et al.* 1982).

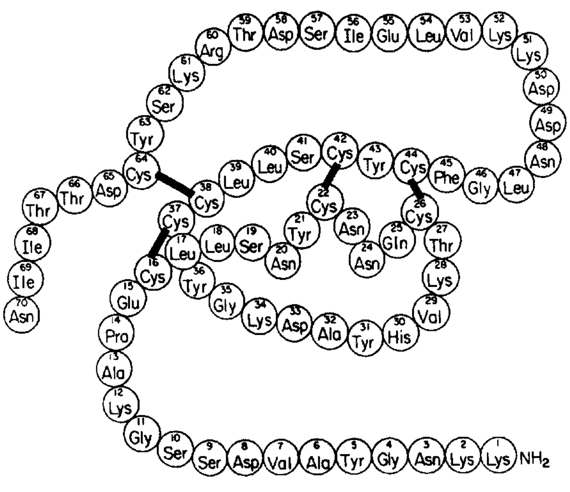

Figure 1. Schematic diagram of the complete covalent structure of the AaH IT (Darbon *et al.* 1982).

Mode of action

AaH IT strict selectivity for insects has been documented by toxicity assays, electrophysiological studies, and binding assays (Zlotkin 1986). The latter have shown that synaptosomal vesicles derived from the CNS of locusts, crickets, flies and fly larvae all possess a single class of noninteracting binding sites of high affinity (K_D = 1–2 nM) and low capacity (0.5–2.0 pmol/mg of membranal protein; Gordon *et al.* 1984, 1985).

The fast excitatory paralysis induced by AaH IT is a result of a presynaptic effect, namely, the induction of repetitive firing of the insect's motor nerves with resulting massive and uncoordinated stimulation of the respective skeletal muscles (Walther *et al.* 1976). The neuronal repetitive activity is attributed to an exclusive and specific modification of sodium conductance by the toxin (Pelhate and Zlotkin 1981, 1982) (Fig. 2(a)), exclusively in insect neuronal membranes. The insect specificity of AaH IT indicates a certain structural and functional uniqueness associated with sodium conductance in insect nervous systems. As such, AaH IT and related toxins may serve as (a) pharmacological tools for the study of excitability in

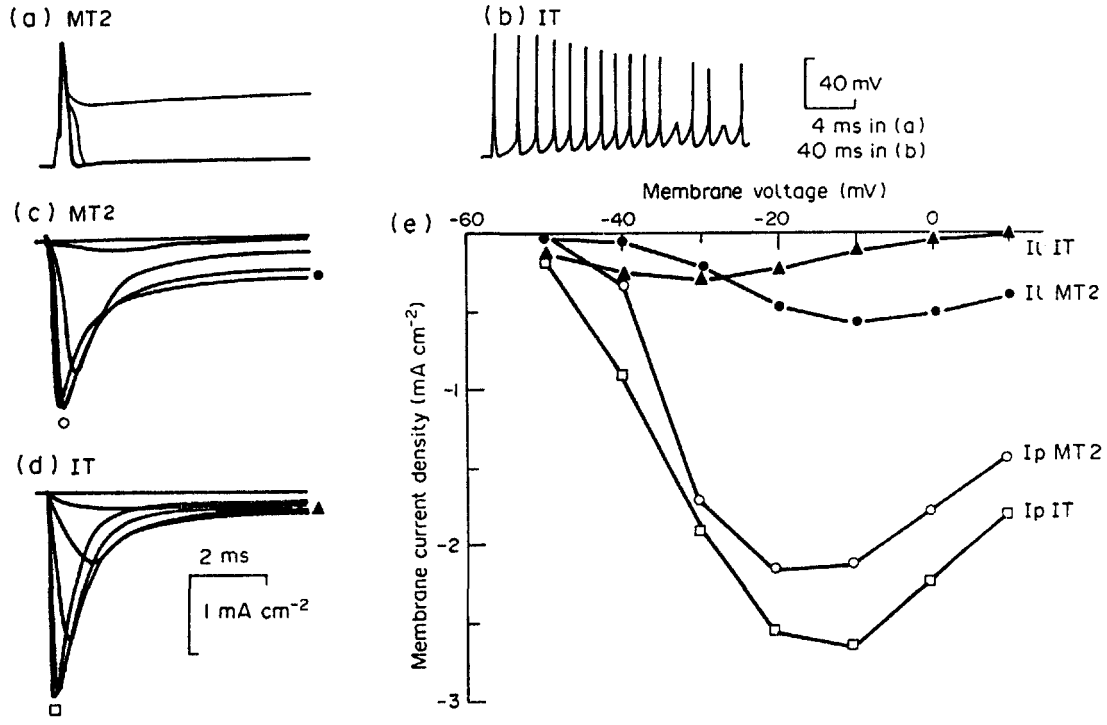

Figure 2. The effects of AaH IT and AaM T2 (AaM II is the α-scorpion toxin strongly affecting mammals, see the entry by Martin-Eauclaire and Bougis, p. 118) on the action potentials and sodium currents recorded from the isolated axon of the cockroach CNS. Current-clamp experiments. (a) 3.5 μM of AaM T2 (b) 1.3 μM of AaH IT. Voltage clamp experiments: The preparation was pretreated by 4-aminopyridine thus suppressing the potassium currents. (c) and (d), sodium currents following the treatment by 3.5 and 1.3 μM of AaM T2 and IT, respectively corresponding to pulses from E_h = 60 mV to E_m = 10 mV in 10 mV steps. (e) Current voltage relations of the data obtained in (c) and (d). Ip: Na^+ peak current, Il: sodium late, steady state current (from Pelhate and Zlotkin 1981).

insects and (b) as models for the design of selective biopesticides in the future.

Structure-function relationships

In a separate study several isotoxins of AaH IT (the AaH IT1 and AaH IT) were isolated and studied (Loret *et al.* 1990). AaH IT2 was sequenced and found to differ in four amino acid positions from AaH IT (Darbon *et al.* 1984) and possessed an equal potential for paralysing fly larvae. The basic amino acid residues of AaH IT1, which differ from those of AaH IT by one amino acid residue, were selectively chemically modified. Six derivatives were characterized. Their toxicity toward fly larvae and cockroaches was determined, and their affinity for the AaH IT1 synaptosomal receptor from cockroach nerve cord was measured. Modification of His-30, Lys-34, and Arg-60 showed no significant effect on biological activity. However, the modification of Lys-28 or Lys-51 demonstrated that these two amino acids are important for toxicity. Furthermore, simultaneous modifications of both Lys-28 and Lys-51 led to a cumulative decrease in biological activity. AaH IT1 and AaH IT2 show similar CD spectra. The secondary structure of AaH IT2 was estimated from circular dichroism data. Results showed that this class of toxin should possess an additional α-helical region and a β-sheet strand, not found in toxins active on mammals. Attempts to localize these secondary structural features in the amino acid sequence of AaH IT2 indicated that these two regions would be located within the last 20 C-terminal amino acid residues. From these studies on secondary structures, it is possible to consider that toxins active on insects are more structurally constrained than those active on mammals; a decreased molecular flexibility may be, at least partially, responsible for the observed specificity of these toxins for the insect sodium channel. Furthermore, the two α-helices found in insect toxins enclosed the two conserved Lys-28 and Lys-51 and might thus be implicated in the toxic site of insect toxins (Loret *et al.* 1990).

Applicability

The applicability of the insect selective neurotoxins has been recently exemplified in the preparation and employment of recombinant baculoviruses expressing the AaH IT toxin. Lepidopterons larvae infected by the recombinant virus reveal an accelerated lethality and a significant reduction of damage to agricultural crops (McCutchen *et al.* 1991; Maeda *et al.* 1991; Stewart *et al.* 1991; Cory *et al.* 1994).

References

Cory, J. S., Hirst, M. L., Williams, T., Halls, R. S., Gouson, D., Green, B. M., *et al.* (1994). Field trial of genetically improved baculovirus insecticide. *Nature (Lond.)*, **123**, 138–40.

Darbon, H., Zlotkin, E., Kopeyan, C., Van Rietschoten, J., and Rochat, H. (1982). Covalent structure of the insect toxin of the North African scorpion. *Androctomus australis* Hector. *Int. J. Pept. Protein Res.*, **23**, 320–30.

Gordon, D., Jover, E., Couraud, F., and Zlotkin, E. (1984). The binding of the insect selective neurotoxin (AaIT) from scorpion venom to the locust synaptosomal membranes. *Biochim. Biophys. Acta*, **178**, 349–58.

Gordon, E., Zlotkin, E., and Catterall, W. A. (1985). The binding of an insect selective neurotoxin and saxitoxin to insect neuronal membranes. *Biochim. Biophys. Acta*, **821**, 130–4.

Lester, D., Lazarorici, P., Pelhate, M. and Zlotkin, E. (1982). Two insect toxins from the venom of the scorpion *Buthotus judaicus*: purification characterization and action. *Biochim. Biophys. Acta*, **701**, 370–81.

Loret, E., Mansuelle, P., Rochat, H., and Granier, C. (1990). Neurotoxins active on insects: amino acid sequences, chemical modifications and secondary structure estimation by circular dichroism of toxins from the scorpion *Androctomus australis* Hector. *Biochemistry*, **29**, 1492–501.

McCutchen, B. F., Chondary, P. V., Crenshaw, R., Maddox, D., Kamita, S. G., Palekar, N., *et al.* (1991). Development of a recombinant baculovirus expressing an insect selective neurotoxin: potential for pest control. *Bio/Technology*, **9**, 848–52.

Maeda, S., Volrath, S. L., Hanzlik, T. N., Harper, S. H., Majima, K., Maddox, D. L., *et al.* (1991). Insecticidal effects of an insect-specific neurotoxin expressed by a recombinant baculovirus. *Virology*, **184**, 777–80.

Pelhate, M. and Zlotkin, E. (1981). Voltage dependent slowing of the turn off of N^+ current in the cockroach giant axon induced by the scorpion venom insect toxin. *J. Physiol. (Lond.)*, **319**, 3–1.

Pelhate, M. and Zlotkin, E. (1982). Actions of insect toxin and other toxins derived from the venom of the scorpion *Androctonus australis* in isolated giant axons of the cockroach (*Periplaneta Americana*). *J. Exp. Biol.*, **97**, 67–77.

Stewart, L. M. D., Hirst, M., Ferber, M. L., Merryweather, A. T., Cayley, P. J., and Possee, R. D. (1991). Construction of an improved baculovirus insecticide containing an insectspecific toxin gene. *Nature (Lond.)*, **352**, 85–8.

Walther, C., Zlotkin, E., and Rathmayer, W. (1976). Actions of different toxins from the scorpion *Androctonus australis* on a locust nerve–muscle preparation. *J. Insect Physiol.*, **22**, 1187–94.

Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M. F., and Rochat, H. (1985). An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site. *Arch. Biochem. Biophys.*, **240**, 877–87.

Zlotkin, E. (1986). The intereaction of insect selective neurotoxins from scorpion venoms with insect neuronal membranes In *Neuropharmacology and pesticide action* (ed. M. G. Ford, G. G. Lunt, R. C. Recy, and P. N. R. Usherwook), pp. 352–83, Ellis Horwood, London.

Zlotkin, E., Miranda, F., Kupeyan, C. and Lissitzky, S. (1971b). A new toxic protein in the venom of the scorpion *Androctonus australis* Hector. *Toxicon*, **9**, 9–13.

Zlotkin, E., Rochat, H., Kupeyan, C., Miranda, F., and Lissitzky, S. (1971*a*). Purification and properties of the insect toxin from the venom of the scorpion *Androctonus australis* Hector. *Biochimie (Paris)*, **53**, 1073–8.

Zlotkin, E., Teitelbaum, Z., Rochat, H., and Miranda, F. (1979). The insect toxin from the venom of the scorpion *Androctonus mauretanicas*. Purification characterization and specificity. *Insect Biochem.*, **9**, 347–54.

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Depressant insect selective neurotoxins from scorpion venoms

The depressant insect selective neurotoxins are single-chained polypeptides of 61–64 amino acids which reveal a dual mode of action. Their induced progressive flaccid paralysis of insects is preceded by a short transient period of excitability. The flaccid paralysis of insects, induced by the depresssant toxins is a consequence of the blockage of evoked action potentials and neuromuscular junctions.

The depressant insect-toxins, upon injection, induce a slow, progressively developing flaccid paralysis of blowfly larvae, which reveal a prolonged–extended shape of their body in contrast to the shortened–contracted body shape induced by the excitatory toxins (see above entry; Fig. 1; Zlotkin 1983).

Depressant toxins were isolated from the venom of the following Buthinae scorpions: *Buthotus judaicus* (Bj IT2; Lester *et al.* 1982), *Leiurus quinqauestriatus quinquestriatus*, (Lqq 2; Zlotkin *et al.* 1985), *Leiurus quinquestriatus hebraeus* (Lqh IT2; Zlotkin *et al.* 1991).

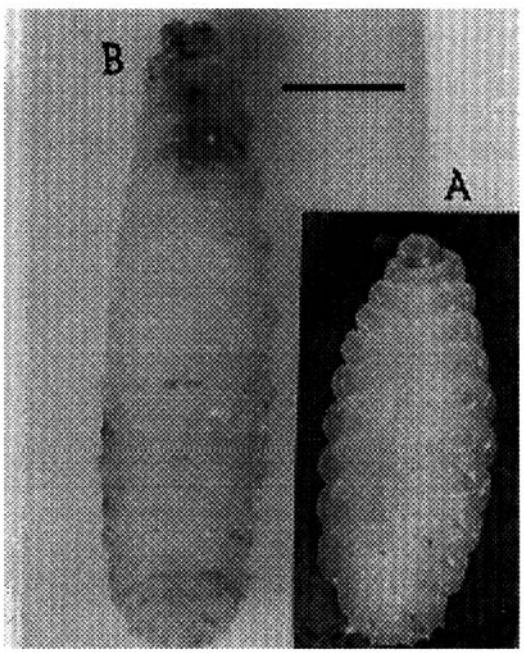

Figure 1. Responses of *Sarcophaga falculata* blowfly larvae to the injection of various scorpion venoms and their derived insect toxins. A. Typical contraction paralysis as induced by various buthid scorpion venoms as well as the excitatory insect selective toxins. B. Typical flaccid–extended paralysis as induced by the depressant insect selective toxins. Bar corresponds to 3.8 mm (from Zlotkin 1983).

Chemistry

The depressant toxins were isolated from electrically milked and lyophilized crude scorpion venoms by the aid of conventional methods of column abromatyraphy, as exemplified by the purification of Lqh IT2 toxin. The latter included Sephadex G-50 recycling gel filtration, anion exchanger (DEAE-Seph. 'Pharmacia', Sweden), cation exchanger (CM-cellulose, C52-Whatman, England), followed by a reversed phase (C18) column in an HPLC system as a final step (Zlotkin *et al.* 1991). The primary structures were determined by automated Edman degradations of reduced vinyl pyridilated, desalted, and proteolytically digested peptides (Zlotkin *et al.* 1991). Fig. 2 presents the primary structures of the three depressant toxins as compared to two excitatory insect toxins (AaH IT, Lqq IT1) and two alpha toxins derived from the same scorpion venom (Lqq 4, Lqq 5).

Mode of action

The effects of the depressant toxins on neuronal excitability were studied on the giant axon of the American cockroach (Lester *et al.* 1982; Zlotkin *et al.* 1985) and the neuromuscular preparation of the prepupal *Musca domestica* (Zlotkin *et al.* 1991).

It has been shown that the flaccid paralysis of insects induced by the depressant toxins is preceded by a short transient burst of repetitive activity from a presynaptic origin (motor nerves) similar to the excitatory toxins (Zlotkin *et al.* 1991).

The flaccid paralysis of insects is a consequence of:

1. A blockage of the evoked action potentials attributed to two separate mechanisms: firstly to the suppression of the activatable sodium conductance as revealed by voltage clamp data and secondly by depolarization of the axonal membrane attributed to the increase of the resting sodium permeability (Lester *et al.* 1982; Zlotkin *et al.* 1985).
2. Blockage of the neuromuscular junction due to a strong reduction in neurotransmitter release due to the deplorazatory effect of the depressant toxins at the terminal branches of the motor nerves (Zlotkin *et al.* 1991, 1993).

AMINO ACID SEQUENCE

	1	2	3	4	5	6	7
	1 0	0	0	0	0	0	0
LqhIT2	...DGYIKRR	DGCKVACLIG	NEG.CDKECK	AYGG.SYGYC	...WTWGLAC	WCEGLPDDKT	WKS.ETNTCG .....
LqqIT2	...DGYIRKR	DGCKLSCLFG	NEG.CNKECK	SYGG.SYGYC	...WTWGLAC	WCEGLPDEKT	WKS.ETNTCG .....
BjIT2	...DGYIRKK	DGCKVSCIIG	NEG.CRKECV	AHGG.SFGYC	...WTWGLAC	WCENLPDAVT	WKS.STNTCG .....
AaIT	.KKNGYAVDS	SGKAPECLLS	N..YCNNQCT	KVHYADKGYC	CLL.....SC	YCFGLNDDKK	VLEISDTRKS YCDTTIIN
LqqIT1	.KKNGYAVDS	SGKAPECLLS	N..YCYNECT	KVHYADKGYC	CLL.....SC	YCVGLSDDKK	VLEISDARKK YCDFVTIN
Lqq4	GVRDAYIADD	KNCVYTC.GS	NS.YCNTECT	KNGAE.SGYC	QWLGKYGNAC	WCIKLPDKVP	IRI..PGKCR .....
Lqq5	.LKDGYIVDD	KNCTFFC.GR	NA.YCNDECK	KKGGE.SGYC	QWASPYGNAC	WCYKLPDRVS	IKE..KGRCN .....

Figure 2. Comparison of scorpion toxin amino acid sequences. The depressant insect toxin sequences are compared with those of the excitatory toxins, AaIT(AaH IT) and Lqq IT1 and the alpha toxins Lqq 4 and Lqq 5 (see entry by Martin-Eauclaire and Bougis). The sequences were aligned for maximum similarity with the aid of the University of Wisconsin Genetics Computing Group Profile Analysis (from Zlotkin *et al.* 1991).

cDNA clones

A partial cDNA clone, pBjTb61, encoding the Bj IT2 was isolated by using degenerate oligonucleotide probes and PCR. The isolated fragment of 125 bp long was cloned and its determined sequence was found to correspond to a segment of the Bj IT2 polypeptide (Gurevitz *et al.* 1990). This partial cDNA clone was used to demonstrate the venom gland specific transcription of the gene encoding Bj IT2 and for analysis of the transcriptional product of the gene encoding Lqh IT2 (Zilberberg *et al.* 1992).

The insert in plasmid pBj1Tb61 (Gurevitz *et al.* 1990) was used as a probe to screen cDNA libraries constructed from the poly (A)$^+$RNA derived from the telsons of *B. iudaicus* and *L.q. hebraeus* scorpions. Several clones encoding the respective depressant toxins were isolated from each library and the insects sequenced (Zilberberg *et al.* 1991).

The existence of the depressant toxin cDNA clones may enable their employment in the reinforcement of recombinant baculoviruses expressing the excitatory insect selective neurotoxins (Stewart *et al.* 1991; Cory *et al.* 1994). Such a possibility may be useful due to the synergic interaction recently shown to occur between depressant and excitatory insect toxins in paralysis induction of the economically important lepidopterous larvae (Herrmann *et al.* 1995).

References

Cory, J. S., Hirst, M. L., Williams, T., Halls, R. S., Gouson, D., Green, B. M., *et al.* (1994). Field trial of genetically improved baculovirus insecticide. *Nature (Lond.)*, **370**, 138–40.

Gurevitz, M., Zlotkin, E., and Zilberberg, N. (1990). Characterization of the transcript for a depressant insect selective neurotoxin gene with an isolated cDNA clone from the scorpion *Buthotus iudaicus*. *Febs. Lett.*, **219**, 229–32.

Herrmann, R., Moskowitz, H., Zlotkin, E., and Hammock, B. D. (1995). Positive cooperativity among insecticidal scorpion neurotoxins. *Toxicon*, **33**, 1099–102.

Lester, D., Lazarovici, P., Pelhate, M., and Zlotkin, E. (1982). Two insect toxins from the venom of the scorpion *Buthotus judaicus*: purification, characterization and action. *Biochim. Biophys. Acta.*, **701**, 370–81.

Stewart, L. M. D., Hirst, M., Ferber, M. L., Merryweather, A. T., Cayley, P. J., and Possee, R. D. (1991). Construction of an improved baculovirus insecticide containing an insect specific toxin gene. *Nature (Lond.)*, **352**, 85–8.

Zilberberg, N., Zlotkin, E., and Gurevitz, M. (1991). The cDNA sequence of a depressant insect selective neurotoxin from the scorpion *Buthotus iudaicus*. *Toxicon*, **29**, 1155–8.

Zilberberg, N., Zlotkin, E., and Gurevitz, M. (1992). Molecular analysis of cDNA and the transcript encoding the depressant insect selective neurotoxin of the scorpion *Leiurus quinquestriatus hebraeus*. *Insect Biochem.*, **22**, 199–203.

Zlotkin, E. (1983). Insect selective toxins derived from scorpion venoms: an approach to insect neuropharmacology. *Insect Biochem.*, **13**, 219–36.

Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M. F., and Rochat, H. (1985). An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site. *Arch. Biochem. Biophys.*, **240**, 877–87.

Zlotkin, E., Eitan, M., Bindokas, V. P., Adams, M. E., Moyer, M., Burkhart, W., *et al.* (1991). Functional duality and structural uniqueness of depressant insect-selective neurotoxins. *Biochemistry*, **30**, 4814–21.

Zlotkin, E., Gurevitz, M., Fowler, E., and Adams, M. E. (1993). Depressant insect selective neurotoxins from scorpion venom: chemistry, action and gene cloning. *Arch. Ins. Biochem. Physiol.*, **22**, 55–73.

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μ-Conotoxins (*Conus geographus*)

μ-Conotoxins GIIIA, GIIIB, and GIIIC are basic neuropeptides containing 22 amino acid residues. They are found in the venom of Conus geographus, *the species of marine snails responsible for several human fatalities. The μ-conotoxins cause paralysis of vertebrates by binding to neurotoxin Site I and blocking the pore of the voltage-sensitive Na channels in skeletal muscle.*

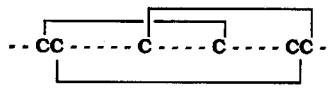
μ-Conotoxins are small basic peptides that inhibit the action potential of skeletal muscle by blocking voltage-gated sodium channels (Cruz *et al.* 1985; Gray *et al.* 1988). Three major homologues and four minor under hydroxylated forms (see Table 1) have been found in the venom of the most dangerous *Conus* species, *C. geographus* (Sato *et al.* 1983; Cruz *et al.* 1985). The peptides contain 22 amino acid residues including three hydroxyprolines and six cysteines. The presence of several Lys and Arg residues renders μ-conotoxins highly positive: a charge of +6 in GIIIA and +7 in GIIIB and GIIIC. The major peptide is μ-conotoxin GIIIA or geographutoxin I; μ-conotoxin GIIIB (geographutoxin II) and μ-conotoxin GIIIC occur in smaller amounts. The minor forms appear to be the post-translational processing intermediates of GIIIA and GIIIB, which contain proline instead of hydroxyproline at either position 6 or 7.

μ-Conotoxins have been shown to compete with saxitoxin and tetrodotoxin for neurotoxin Site I of the channel (Moczydlowski *et al.* 1986; Ohizumi *et al.* 1986; Yanagawa *et al.* 1986). Comparison of the activities of various synthetic analogues of GIIIA have consistently indicated the importance of Arg^{13} for the blocking of the Na channel pore (Sato *et al.* 1991; Becker *et al.* 1992; Wakamatsu *et al.* 1992). The reported three-dimensional structure has the three pairs of disulfide bonds at the center of a 20-Å ellipsoid (Lancelin *et al.* 1991). Seven cationic side chains of lysine and arginine residues and three hydroxyprolines protrude radially from the peptide backbone. The residues most important for activity (Arg^{13}, Lys^{16}, Hyp^{17}, and Arg^{19}) are all on one side of μ-conotoxin GIIIA, the surface that presumably binds with the active site of the channel .

Table 1 Structure of μ-conotoxins

μ-Conotoxin	Amino acid sequence
GIIIA	RD**CC**TOOKK**C**KDRQ**C**KOQR**CC**A*
[Pro^6]GIIIA	RD**CC**TPOKK**C**KDRQ**C**KOQR**CC**A*
[Pro^7]GIIIA	RD**CC**TOPKK**C**KDRQ**C**KOQR**CC**A*
GIIIB	RD**CC**TOORK**C**KDRR**C**KOMK**CC**A*
[Pro^6]GIIIB	RD**CC**TPORK**C**KDRR**C**KOMK**CC**A*
[Pro^7]GIIIB	RD**CC**TOPRK**C**KDRR**C**KOMK**CC**A*
GIIIC	RD**CC**TOOKK**C**KDRR**C**KOL K**CC**A*

Disulfide linkages



* The asterisks indicate that the α-carboxyl groups are amidated. Except for O (*trans*-4-hydroxyproline), the standard one-letter amino acid code is used. The amino acid sequences are from Cruz *et al.* (1985) and Sato *et al.* (1983).

Purification and sources

μ-Conotoxins GIIIA, GIIIB, and GIIIC can be isolated from *C. geographus* by size fractionation on Sephadex G-25 followed by a series of HPLC runs using reverse phase C18 column, eluted with a gradient of acetonitrile in 0. 1 per cent trifluoroacetic acid (Cruz *et al.* 1985). μ-Conotoxins GIIIA and GIIIB are available from Sigma Chemical Company.

Activity

The K_D of GIIIA obtained using batrachotoxin-activated sodium channels in lipid bilayers is around 100 nM (Cruz *et al.* 1985). The μ-conotoxins of *C. geographus* have a high specificity for the muscle subtype of Na channels; they are believed to be the most selective ligands for the neurotoxin site I of skeletal muscle sodium channels (Gonoi *et al.* 1987). GIIIA and GIIIB have been shown to discriminate by ~1000-fold between skeletal muscle and neuronal subtypes of voltage sensitive sodium channels inserted in lipid bilayers. GIIIB (Gonoi *et al.* 1987), is more selective for skeletal muscle TTX-sensitive vs. insensitive Na channel than TTX itself (a discrimination factor of ~10 000-fold compared to ~200-fold for TTX).

Very recently, a μ-conotoxin was found by Olivera and co-workers (Shon *et al.* 1996) in *Conus purpurascens* by sequencing the toxin gene. The chemically synthesized new conotoxin, μ-PIIIA has been shown to reversibly block the type II Na channel of rat brain, which is TTX sensitive but μ-GIIIA resistant.

Use in cell biology

μ-Conotoxin GIIIA has been used by a number of investigators for blocking action potentials when studying

inherent end plate potentials (EPPs) and miniature end plate potentials (MEPPs) in neuromuscular preparations of vertebrates (Hong and Chang 1989; Prior *et al.* 1993). Its high selectivity for muscle sodium channels has made it the choice reagent for this purpose.

Together with saxitoxin and tetrodotoxin, μ-conotoxin GIIIA has also been used as a pharmacological tool for characterizing clones of voltage-sensitive sodium channels (Chen *et al.* 1992). Of the two isoforms of VSSCs cloned from rat skeletal muscle, rSKM1 is sensitive to both tetrodotoxin and GIIIA whereas rSKM2 is not. Denervated and developing skeletal muscle express rSKM2, which is believed to be identical to the TTX-insensitive Na channels from heart. The VSCC clone from human heart, hH1 is resistant to both TTX and GIIIA (Gellens *et al.* 1992). The binding of the μ-conotoxins to mutants of sodium channels has been used to map out amino acid residues important for their interaction with neurotoxin site I of VSSCs (Stephan *et al.* 1994; Dudley *et al.* 1995).

References

Becker S., Prusak-Sochaczewski, E., Zamponi, G., Beck-Sickinger, A. G., Gordon, R. D., and French, R. J. (1992) Action of derivatives of μ-conotoxin GIIIA on Na channels. Single amino acid substitutions in the toxin separately affect association and dissociation rates. *Biochemistry*, **31**, 8229–38.

Chen, L. Q., Chahine, M., Kallen, R. G., Barchi, R. L., and Horn, R. (1992). Chimeric study of sodium channels from rat skeletal and cardiac muscle. *FEBS Lett.*, **309**, 253–7.

Cruz, L. J., Gray, W. R, Olivera, B. M., Zeikus, R. D., Kerr, L., Yoshikami, D., *et al.* (1985). *Conus geographus* toxins that discriminate between neuronal and muscle Na channels. *J. Biol. Chem.*, **260**, 9280–8.

Dudley, S. C. Jr., Todt, H., Lipkind, G., and Fozzard, H. A. (1995). A muconotoxin-insensitive Na^+ channel mutant: possible localization of a binding site at the outer vestibule. *Biophys. J.*, **69**, 1657–65.

Gellens, M. E., George, A. L., Chen, L. Q., Chahine, M., Horn, R., Barchi, R. L., *et al.* (1992). Primary structure and functional expression of the human cardiac tetrodotoxin-insensitive voltage-dependent sodium channel. *Proc. Natl. Acad. Sci. USA*, **89**, 554–8.

Gonoi, T., Ohizumi, Y., Nakamura, H., Kobayashi, J., and Catterall, W. A. (1987). The *Conus* geographutoxin II distinguishes between two functional sodium channel subtypes in rat muscle cells developing *in vitro*. *J. Neurosci.*, **7**, 1728–31.

Gray, W. R., Olivera, B. M., and Cruz, L. J. (1988). Peptide toxins from venomous *Conus* snails. *Annu. Rev. Biochem.*, **57**, 665–700.

Hong, S. J. and Chang, C. C. (1989). Use of geographutoxin II μ-conotoxin for the study of neuromuscular transmition in mouse. *Brit. J. Pharmacol.*, **97**, 934–40.

Lancelin, J. -M., Kohda, D., Tate, S., Yanagawa, Y., Abe, T., Satake, M., *et al.* (1991) Tertiary structure of conotoxin GIIIA in aqueous solution. *Biochemistry*, **30**, 6908–16.

Moczydlowski, E., Olivera, B. M., Gray, W. R., and Strichartz, G. R. (1986). Discrimation of muscle and neuronal Na-channel subtypes by binding competition between ^{3}H-saxitoxin and μ-conotoxins. *Proc. Natl. Acad. Sci. USA*, **83**, 5321–5.

Ohizumi, Y., Nakamura, H., Kobayashi, J., and Catterall, W. A. (1986). Specific inhibition of [^{3}H]saxitoxin binding to skeletal muscle sodium channels by geographutoxin II, a polypeptide channel blocker. *J. Biol. Chem.*, **261**, 6149–52.

Prior, C., Dempster, J., and Marshall, I. G. (1993). Electrophysiological analysis of transmission at the skeletal neuromuscular junction. *J. Pharmacol. Toxicol. Methods*, **30**, 1–17.

Sato, S., Nakamura, H., Ohizumi, Y., Kobayashi, J., and Yoshimasa, H. (1983). The amino acid sequences of homologous hydroxyproline-containing myotoxins from the marine snail *Conus geographus* venom. *FEBS Lett.*, **155**, 277–80.

Sato, K., Ishida, Y., Wakamatsu, K., Kato, R., Honda, H., Ohizumi, Y., *et al.* (1991) Active site μ-conotoxin GIIIA, a peptide blocker of muscle Na channels. *J. Biol. Chem.*, **266**, 16989–91.

Shon, K. -J., Yoshikami, D., Marsh, W., Jacobsen, R. B., Gray, W. R., Floresca, C. C. Z., *et al.* (1996). μ-Conotoxin PIIIA, a new peptide for discriminating among tetrodotoxin-sensitive Na channel subtypes. Submitted for publication.

Stephan, M. M., Potts, J. F., and Agnew, W. S. (1994). The mul skeletal muscle sodium channel mutation E403Q eliminates sensitivity to tetrodotoxin but not to mu-conotoxins GIIIA and GIIIB. *J. Membr. Biol.*, **137**, 1–8.

Wakamatsu, K., Kohda, D., Hatanaka, H., Lancelin, J. M., Ishida, Y., Oya, M., *et al.* (1992) Structure–activity relationships of μ-conotoxin GIIIA: structure determination of active and inactive Na channel blocker peptides by NMR and simulated annealing calculations. *Biochemistry*, **31**,12577–84.

Yanagawa, Y., Abe, T., and Satake, M. (1986). Blockade of [^{3}H]lysine-tetrodotoxin binding to sodium channel proteins by conotoxin GIII. *Neurosci. Lett.*, **64**, 7–12.

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μ-Agatoxins (*Agelenopsis aperta*)

μ-Agatoxins are insecticidal peptide toxins from venom of the American funnel web spider, Agelenopsis aperta. *All μ-agatoxins are toxic to insects, but lack biological activity against vertebrates* in vitro *or* in vivo. *Evidence to date suggests that the μ-agatoxins modify the kinetic behavior of insect voltage-gated sodium channels.*

μ-Agatoxins are a group of homologous polypeptide toxins from venom of the American funnel web spider, *Agelenopsis aperta* (Adams *et al.* 1989*b*; Skinner *et al.* 1989). Six toxins, μ-Aga-I to VI (Fig. 1), which range in size from 36–38 amino acids, have been isolated and sequenced. The amino acid sequences of μ-agatoxins are highly conserved and the spacing of cysteine residues, which form four internal disulfide bonds, is identical among all of the toxins. The μ-agatoxins cause convulsive paralysis when injected into insects (*Musca domestica*, *Manduca sexta*, *Heliothis virescens*). This behaviour is correlated with the appearance of repetitive action potentials in motorneurons. (Adams *et al.* 1989*b*) and increased spontaneous neurotransmitter release from motor nerve terminals (Adams *et al.* 1989*a*). Elevation of spontaneous transmitter release is reversed by treatment with tetrodotoxin, suggesting that the μ-agatoxins target sodium channels. Several curtatoxins isolated from the related spider *Hololena curta* are identical or similar to the μ-agatoxins (Stapleton *et al.* 1990).

Whole cell patch clamp experiments (Norris *et al.* 1995) show that μ-agatoxins modify the voltage sensitivity and kinetics of sodium channel inactivation, but have no effect on mammalian sodium channels at micromolar concentrations.

Purification and sources

μ-Agatoxins are purified from the milked venom of *A. aperta* using reversed-phase liquid chromatography techniques (RPLC) (Adams *et al.* 1989*b*; Skinner *et al.* 1989). Although μ-agatoxins are not particularly susceptible to oxidation, they should be stored between –20 and –80 °C to prevent significant degradation.

Although none of the μ-agatoxins are commercially available at this time in purified form, the *Agelenopsis aperta* venom containing the toxins can be purchased from Spider Pharm, 407 E. Bristol Rd, Feasterville, PA 19053 ; Tel: 215-355-8295; Fax: 215-355-8447.

Toxicity

Injection of μ-agatoxins into insects leads to excitability and convulsive paralysis. Toxicity data (LD_{50} values) are as follows (Adams *et al.* 1989*b*; Skinner *et al.* 1989):

LD_{50} (*Musca domestica*)	
μ-Aga-I,III,IV,V, and VI	0.1–0.6 μg/g
μ-Aga-II	5.8 μg/g
LD_{50} (*Manduca sexta*)	
μ-Aga-I-VI	28–78 μg/g

Intracranial injections of μ-agatoxins into mice result in no detectable symptoms or toxicity (Adams, unpublished data). There are no reports to date of significant mammalian toxicity on oral or i/v administration. However, very little toxicity data has been generated regarding this group of toxins.

Uses

The high selectivity of these toxins for insect sodium channels makes them potentially useful for elucidation of differences between insect and mammalian sodium channels. Their high insect selective toxicity makes them

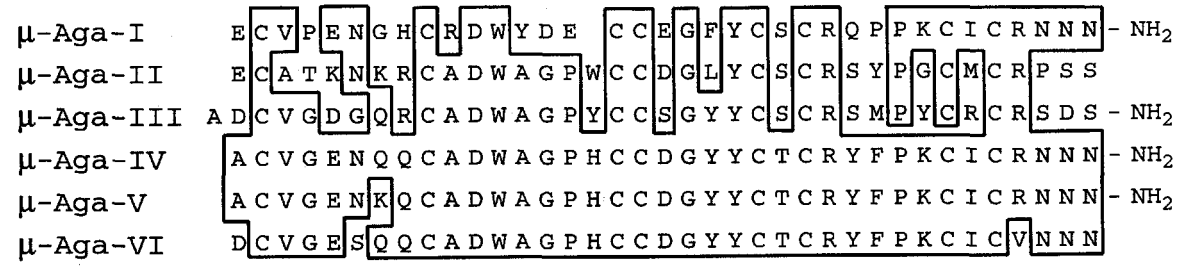

Figure 1. Amino acid sequences of μ-agatoxins. Amino acids within the box are identical between peptides (adapted from Skinner *et al.* 1989).

candidates for development as active components of engineered biological pesticides.

References

Adams, M. E., Bindokas, V. P., and Zlotkin, E. (1989a). Synaptic toxins from arachnid venoms: probes for new insecticide targets. In *Insecticide action. From molecule to organism* (ed. T. Narahashi and J. E. Chambers), pp. 189–203, Plenum, New York.

Adams, M. E., Herold, E. E., and Venema, V. J. (1989*b*). Two classes of channel-specific toxins from funnel web spider venom. *J. Comp. Physiol. [a]*, **164**(3), 333–42.

Norris, T. M., Lee, D., and Adams, M. E. (1995). Unpublished data.

Skinner, W. S., Adams, M. E., Quistad, G. B., Kataoka, H., Cesarin, B. J., Enderlin, F. E., *et al.* (1989). Purification and characterization of two classes of neurotoxins from the funnel web spider, *Agelenopsis aperta*. *J. Biol. Chem.*, **264**(4), 2150–5.

Stapleton, A., Blankenship, D. T., Ackermann, B. L., Chen, T. M., Gorder, G. W., Manley, G. D., *et al.* (1990). Curtatoxins. Neurotoxic insecticidal polypeptides isolated from the funnel-web spider *Hololena curta*. *J. Biol. Chem.*, **265**(4), 2054–9.

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Anthopleurin-A, -B, and -C (anemone toxin)

Anthopleurin-A (AP-A), -B (AP-B), and -C (AP-C) are polypeptides isolated from different sea anemone. AP-A, -B, and -C contain 49, 50, and 47 amino acids, respectively. These polypeptides have a potent cardiostimulant action without affecting heart rate and arterial blood pressure. In addition, the polypeptides have excitatory and inhibitory effects on the smooth muscles and nerves. Electrophysiological studies indicate that the polypeptides inhibit the sodium channel inactivation through the binding to the receptor site 3 of muscle and nerve sodium channels increasing the Na^+ influx across the cell membrane.

Table 1 summarizes the properties of the three anthopleurins. All three substances are very soluble in water and quite stable at pH 1.0–8.0, but lose positive inotropic activity increasingly fast as the pH rises above 8.0. Figure 1 indicates the amino acid sequences of AP-A (Tanaka *et al.* 1977) and AP-C, and the partial sequence of the major constituent of AP-B.

The cysteine crosslinks in AP-A have been determined to be 4–46, 6–36, and 29–47 as shown in Fig. 2. Conformational studies have been carried out using laser Raman, circular dichroism, fluorescence spectral studies, and Chou-Fasman calculations (Ishizaki *et al.* 1979) as well as carbon-13 nuclear magnetic resonance spectrometry (Norton and Norton 1979).

Table 1 Properties of three anthopleurins

	AP-A	AP-B	AP-C
Molecular weight	5183	~5000	4875
Number of amino acids	49	~50	47
Isoelectric point (pI)	8.2	9.05	8.03
R_f (pH 3.7 gel)	0.46	0.64	0.49
Concentration in anemone	10–40 ppm	3 ppm	25–30 ppm

```
      1                   5                  10                  15
AP-A Gly-Val-Ser-Cys-Leu-Cys-Asp-Ser-Asp-Gly-Pro-Ser-Val-Arg-Gly-

AP-C  .   .  Pro  .   .   .   .   .   .   .   .   .   .   .   .

AP-B  .   .  Pro  .   .   .   .   .   .   .   .  Pro-Asn  .   .

      16                  20                  25                  30
Ap-A Asn-Thr-Leu-Ser-Gly-Thr-Leu-Trp-Leu-Tyr-Pro-Ser-Gly-Cys-Pro-

AP-C  .   .   .   .   .  Ile  .   .   .   .   .  Ala  .   .   .

AP-B  .   .   .   .   .  Ile  .   .  Phe-Ala-Pro-Ser  .  (X)  .

      31                  35                  40                  45
AP-A Ser-Gly-Trp-His-Asn-Cys-Lys-Ala-His-Gly-Pro-Thr-Ile-Gly-Trp-

AP-C  .   .   .   .   .   .   .   .   .   .   .   .   .   .   .

AP-B (X)  .   .  (X) not finished

      46          49
AP-A Cys-Cys-Lys-Gln

AP-C  .   .   .   .
```

Figure 1. Amino acid sequences of AP-A, AP-B, and AP-C.

Chemical blocking studies have shown that the histidines, arginine, tyrosine, and tryptophans are probably not involved in the active site; one or more of the carboxyls are involved; one or both lysines may be involved since isopropylation of the NH_2 group does not affect positive inotropic activity, but the bulky and base-neutralizing trinitrophenyl group abolishes activity (Newcomb *et al.* 1980). It could well be that the function of the 'active site', the region at 7-ASP, 9-ASP, and 37-LYS, is not active because it loosely chelates and transfers Ca^{2+}, but rather because the site is important in inducing a secondary effect of transferring Ca^{2+} by a presently unknown mechanism (Fig. 2).

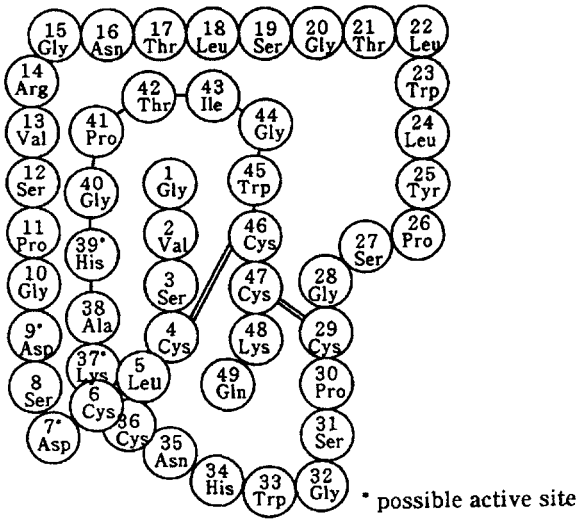

Figure 2. Structure of AP-A.

Purification and sources

AP-A and AP-B were both isolated from *Anthopleura xanthogrammica* (Brandt) and AP-C from *A. elegantissima* (Brandt). After standing for about a month the ethanol preservative is decanted from the animals and the drugs isolated from the preservative. The crude extract is subjected at first to gel permeation chromatography on Sephadex G-50, ion-exchange chromatography on CM-Sephadex C-25, and desalting with G-50 and G-10 to give about 65 per cent pure AP-A and less pure AP-B (Norton *et al.* 1976). To obtain analytically pure AP-A the additional steps of gradient elution with pyridine and acetic acid on sulfoethyl cellulose and the use of Sephadex G-25 as an adsorption chromatographic column are employed (Norton *et al.* 1978). Using essentially these same procedures, AP-B could not be obtained in analytically pure form (Norton *et al.* 1978), in fact recent high pressure liquid chromatography experiments on a C-18 reverse phase column indicate two major and two relatively minor components based on 254 and 280 nm UV absorbance. AP-C was isolated in an analytically pure form by a procedure similar to that for AP-A (Norton *et al.* 1978).

Toxicity

The toxicities of AP-A, AP-B, and AP-C are determined in albino mice. The lethal doses in 50 per cent of the animals (LD_{50}) are taken at 24 hour periods and calculated by the Litchfield and Wilcoxon method. The evaluation is made using intraperitoneal injection of five to seven doses administered to seven groups of mice (seven mice in each group). The LD_{50}s of each of these three peptides are found to be in the range of 350–550 μg/kg (Shibata *et al.* 1976). In addition, the lethal doses of AP-A and ouabain in a dog open chest experiment are 19.3 ±

1.6 μg/kg and 236.2 ± 21.9 μg/kg respectively (Scriabine *et al.* 1979).

Biological activity

Polypeptides isolated from sea anemone have been shown to produce a positive inotropic effect in isolated cardiac tissues of rabbit (Shibata *et al.* 1976, 1978; Kodama *et al.* 1981), anesthetized cats and dogs (Shibata *et al.* 1976), and conscious dogs without other major hemodynamic effect (Blair *et al.* 1978; Scriabine *et al.* 1979). The positive inotropic effect of AP-A occurred independent of any changes in heart rate or mean arterial blood pressure and is not mediated by the release of catecholamines from sympathetic nerves (Shibata *et al.* 1976). The cardiotonic effect of AP-A is maintained even during hypoxia or in the presence of a glucose-free medium in isolated rabbit ventricular muscle (Kodama *et al.* 1981). Further, AP-A produces a positive inotropic response in both normal and ischemic myocardium without changing heart rate, left ventricular systolic or aortic blood pressure (Gross *et al.* 1985). In addition, polypeptides cause powerful excitatory and inhibitory actions in the ileum, taenia caeci, and vas deferens of guinea-pig (Norton *et al.* 1981; Ohizumi and Shibata 1981, 1982). The polypeptides-induced contractions of intestinal smooth muscles are due to the excitation of cholinergic nerves, while that of the vas deferens is caused by NE release from adrenergic nerve endings. The polypeptides-induced relaxation of the taenia caeci is due to the excitation of adrenergic nerves, while the relaxation of the ileum is mediated through nonadrenergic inhibitory mechanisms. In synaptosomes from rat brain, AP-A releases ATP and Na^+, presumably resulting from the opening of Na^+ channels.

The electrophysiological studies in cardiac and smooth muscles and nerves indicate that the polypeptides have a major effect on prolonging the action potential duration of the Na^+ channel of cell membranes. This is explained by the inhibition of Na channels inactivation. This would cause an increased Na^+ entry and indirectly, by activating the Na^+ and Ca^{2+} exchange mechanisms, result in increased Ca^{2+} concentration and excitatory effect. Also, AP-A may also act by increased translocation of intracellular Ca (Low *et al.* 1979; Hashimoto *et al.* 1980; Kudo and Shibata 1980; Kodama *et al.* 1981; Muramatsu *et al.* 1990).

Conclusion

Most of the known sea anemone toxins are specific for the fast Na^+ channel of the excitable membrane of nerve, smooth muscle, and cardiac muscle cells tested. The Na^+ channels appear to be a privileged structure for the action of neurotoxic molecules of natural origin. Sea anemone toxins are useful in the investigation and interpretation of the structure, function, and differentiation of the Na channel at the molecular level.

Limited amounts of AP-A and AP-B can be obtained from Dr Shoji Shibata, Department of Pharmacology, School of Medicine, University of Hawaii.

References

Blair, R. W., Peterson, D. R., and Bishop, V. S. (1978). The effects of anthopleurin-A on cardiac dynamics in conscious dogs. *J. Pharmacol. Exp. Ther.*, **207**, 271–6.

Gross, G. J., Walters, D. C., Hardman, H. F., and Shibata, S. (1985). Cardiotonic effects of anthopleurin-A (AP-A), a polypeptide from a sea anemone, in dogs with a coronary artery stenosis. *Eur. J. Pharmacol.*, **110**, 271–6.

Hashimoto, K., Ochi, R., Hashimoto, K., Inui, J., and Miura, Y. (1980). The ionic mechanism of prolongation of action potential duration of cardiac ventricular muscle by anthopleurin-A and its relationship to the inotropic effect. *J. Pharmacol. Exp. Ther.*, **215**, 479–85.

Ishizaki, H., Mckay, R. H., Norton, T. R., Yasunobu, K. T., Lee, J., and Tu, A. T. (1979). Conformational studies of peptide heart stimulant anthopleurin-A: laser Raman circular dichroism fluorescence spectral studies and Chou-Fasman calculations. *J. Biol. Chem.*, **254**, 9651–6.

Kodama, I., Toyama, T., Shibata, S., and Norton, T. R. (1981). Electrical and mechanical effects of anthopleurin-A a polypeptide from a sea anemone on isolated rabbit ventricular muscle under conditions of hypoxia and glucose-free medium. *J. Cardiovasc. Pharmacol.*, **3**, 75–86.

Kudo, Y. and Shibata, S. (1980). The potent excitatory effect of a novel polypeptide, anthopleurin-B, isolated from sea anemone (*Anthopleura xanthogrammica*) on the frog spinal cord. *J. Pharmacol. Exp. Ther.*, **214**, 443–8.

Low, P. A., Wu, C. H., and Narahashi, T. (1979). The effect of anthopleurin-A on crayfish giant axon. *J. Pharmacol. Exp. Ther.*, **210**, 417–21.

Muramatsu, I., Saito, K., Ohmura, T., Kigoshi, S., and Shibata, S. (1990). Supersensitivity to tetrodotoxin and lidocaine of anthopleurin-A-treated Na^+ channels in crayfish giant axon. *Eur. J. Pharmacol.*, **186**, 41–7.

Newcomb, R., Seriguchi, D. G., Norton, T. R., and Yasunobu, K. T. (1980). Effects of chemical modification of anthopleurin-A, a peptide heart stimulant. In *Frontiers in protein chemistry* (ed. D. T. Liu, L. Mamiya, and K. T. Yasunobu), pp. 539–50, Elsevier, Amsterdam.

Norton, R. S. and Norton, T. R. (1979). Natural abundance carbon-13 nuclear magnetic resonance study of anthopleurin-A, a cardiac stimulant from the sea anemone, *Anthopleura xanthogrammica*. *J. Biol. Chem.*, **254**, 10220–6.

Norton, T. R., Shibata, S., Kashiwagi, M., and Bentley, J. (1976). The isolation and characterization of cardiotonic polypeptide anthopleurin-A from the sea anemone *Anthopleura xanthogrammica*. *J. Pharm. Sci.*, **65**, 1368–74.

Norton, T. R., Kashiwagi, M., and Shibata, S. (1978). Anthopleurin-A, B and C, cardiotonic polypeptides from the sea anemones. In *Drugs and food from sea* (ed. P. N. Kaul and C. J. Sindermann), pp. 37–50, The University of Oklahoma Press, Norman, Oklahoma.

Norton, T. R., Ohizumi, Y., and Shibata, S. (1981). Excitatory effect of a new polypeptide (anthopleurin-B) from sea anemone on the guinea-pig vas deferens. *Br. J. Pharmacol.*, **74**, 23–8.

Ohizumi, H. and Shibata, S. (1981). Possible mechanism of the dual action of new polypeptide (anthopleurin-B) from sea anemone in the isolated guinea-pig ileum and taenia caeci. *Br. J. Pharmacol.*, **72**, 239–45.

Ohizumi, Y. and Shibata, S. (1982). Nature of anthopleura-B-induced release of norepinephrine from adrenergic nerves. *Am. J. Physiol.*, **363**, 273–81.
Scriabine, A., Van Arman, C. G., Morgan, G., Morris, A. A., Bennett, C. D., and Bobidar, N. R. (1979). Cardiotonic effects of anthopleurin-A, a polypeptide from a sea anemone. *J. Cardiovasc. Pharm.*, **1**, 571–83.
Shibata, S., Norton, T. R., Izumi, T., Matsuo, T., and Katsuki, S. (1976). A polypeptide (AP-A) from sea anemone (*Anthopleura xanthogrammica*) with potent positive inotropic action. *J. Pharmacol. Exp. Ther.*, **199**, 298–309.
Shibata, S., Izumi, T., Seriguchi, D. G., and Norton, T. R. (1978). Further studies on the positive inotropic effect of the polypeptide anthopleurin-A from sea anemone. *J. Pharmacol. Exp. Ther.*, **205**, 683–92.
Tanaka, M., Haniu, M., Yasunobu, K. T., and Norton, T. R. (1977). Amino acid sequence of the *Anthopleura xanthogrammica* heart stimulant, anthopleurin-A. *Biochemistry*, **16**, 204–8.

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Anemone toxins (type II)

Type II toxins from sea anemones are polypeptides of molecular mass about 5 kDa which interact with the voltage-gated sodium channel of excitable tissue, delaying its inactivation and prolonging the action potential. All of the known toxins are active in crustacea but several are only weakly active in mammals.

Sea anemones produce a family of closely related cardioactive and neurotoxic polypeptides having molecular masses of approximately 5 kDa. These toxins have been classified into two groups, type I toxins from the family Actiniidae (*Actinia* sp. and *Anthopleura* sp.) and type II toxins from the family Stichodactylidae (*Heteractis* sp. and *Stichodactyla* sp.) (Kem 1988). The amino acid sequences of several type II toxins are shown in Fig. 1.

These two classes of toxin are similar with respect to the locations of their disulfide bridges and a number of residues thought to play a role in biological activity or maintenance of the tertiary structure, but they are distinguishable on the basis of sequence similarity (> 60 per cent within each type, but < 30 per cent between the two types) and immunological crossreactivity. Some of the distinguishing features of the type II toxins are a triplet of acidic residues at positions 6–8, an extension of basic residues at the C-terminus, and the substitution of acidic or polar residues for the aromatic residues at positions 22 and 42 in the type I toxins. Other toxins have been isolated which do not fall neatly into either class. Examples are calitoxin, which has His at position 13 instead of Arg and has His–Glu–Ala as its C-terminus (Cariello *et al.* 1989) and *Actinia equina* toxin I (Lin *et al.* 1996), which resembles the type I toxins but has a seven-residue insert following residue 24 (according to the type I numbering scheme) and shows differences in some of the residues conserved throughout the type I toxins (Norton 1991).

Toxins of both types share a common mode of action in binding to the voltage-gated sodium channel of excitable tissue (Catterall 1988; Wann 1993) and delaying channel inactivation. This interaction results in a prolongation of the action potential.

The three-dimensional structures in solution of three type I toxins, AP-A (Pallaghy *et al.* 1995), ATX Ia (Widmer *et al.* 1989), and AP-B (Monks *et al.* 1995), and one type II toxin, Sh I (Fogh *et al.* 1990; Wilcox *et al.* 1993), have been determined using ^{1}H NMR data. In each case the structure consists of a four-stranded, anti-parallel β-sheet, connected by three loops (as illustrated for Sh I in Figs 2

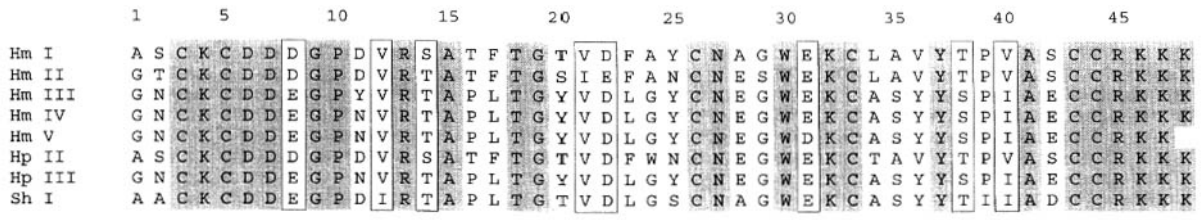

Figure 1. Amino acid sequences of type II sea anemone toxins (Kem 1988; Norton 1991). Residues that are identical are shaded grey and those that are conservatively substituted are boxed.

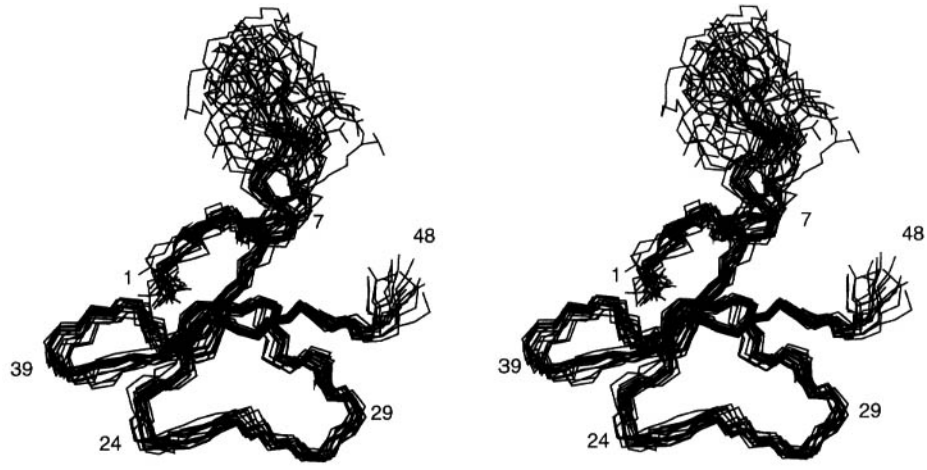

Figure 2. Stereo view of the backbones of the 20 best NMR-derived structures of Sh I (Wilcox *et al.* 1993; Brookhaven Protein Data Bank entry no. 1SHI). The structures are superimposed over the backbone heavy atoms (N, C^{α}, C) of residues 1–7 and 16–47.

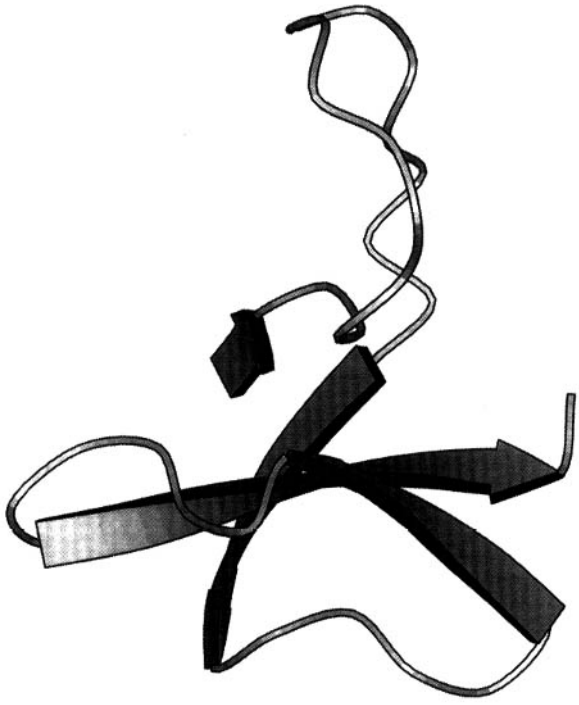

Figure 3. Richardson-style diagram of the structure of Sh I. The structure closest to the average over the 20 structures in Fig. 2 is shown; the view is related to that in Fig. 2 by a slight rotation about the vertical axis. The diagram was generated using the program MOLSCRIPT (Kraulis 1991).

and 3). The largest loop, encompassing residues 7–16 in Sh I, is less well defined by the NMR data than the rest of the structure (Fig. 2) because of a lack of long-range NMR restraints between residues in the loop and those in the well-defined bulk of the molecule. Although Sh I is currently the only type II toxin for which a high-resolution structure is available, NMR data on three other type II toxins from the genus *Heteractis* (Wemmer *et al.* 1986; Pease *et al.* 1989; Hinds and Norton 1993) imply that their structures in solution are similar to those of Sh I and the type I toxins AP-A and ATX Ia. This suggests that the wide range of potency and species specificity among these toxins reflects the presence or absence of key residues rather than gross structural differences.

Numerous studies have been undertaken of structure–function relationships within this family of toxins (Kem 1988; Norton 1991) but interpretation of the data, the majority of which comes from chemical modification studies, is complicated by the use of different toxins and different bioassays. Nevertheless, it is thought that the positive charges of one or more of the amino groups, the carboxylates of one or more Asp or Glu residues near the start of the first loop, and probably the guanidinium group of Arg13 in the first loop are required for full activity. In the case of Sh I, Pennington *et al.* (1990*a*) showed, using a series of synthetic peptide analogues with single amino acid substitutions, that Asp6, Asp7, and Glu8 were each essential for activity, with > 10^4-fold losses in toxicity resulting from their replacement by Asn or Gln, respectively. Substitution of Asp11 by Asn or of Lys4 by *N*-acetyl Lys reduced the toxicity by 120-fold and 80-fold, respectively.

Selective proteolysis of Sh I adjacent to either Arg13 or Lys32 abolished neurotoxicity in crabs (Monks *et al.* 1994). Cleavage adjacent to Arg13 in the type I toxin anthopleurin-A also eliminated activity (Norton 1991).

Alternative names

Unlike the type I toxins, many of which have been assigned descriptive names, the type II toxins are generally referred to by their systematic names (e.g. Hm I refers to toxin I from *Heteractis macrodactylus*). The Hm toxins are sometimes referred to as RTX toxins and the Hp toxins as Rp (reflecting the older genus name of *Radianthus*).

Purification and sources

All of the toxins in Fig. 1 were isolated originally as polypeptides from the relevant species of anemone. Literature references for these toxins are given in Norton (1991). Sh I has been synthesized (Pennington *et al.* 1990b) and is available from Bachem Bioscience Inc (King of Prussia, PA 19406, USA). At least some samples of naturally occurring Sh I are amidated at the C-terminus (Wilcox *et al.* 1993), although this appears to be unimportant for activity as the natural and synthetic toxins are equipotent in crabs.

Toxicity

These toxins display a wide range of tissue and species specificity, as illustrated in Table 1. There appears to be a crude inverse correlation between activity in crustacea and mice.

Uses in cell biology

Sea anemone toxins bind to site 3 on the voltage-gated sodium channel (Catterall 1988; Wann 1993), delaying channel inactivation and prolonging the action potential. The only other toxins currently know to bind to this site are the scorpion α-toxins (Kem 1988; Norton 1991). The binding sites for the type I and type II toxins are similar (if not identical), but the type I toxins have been available for longer and have been investigated more extensively; as a result the type I toxins tend to be used more often in electrophysiological and pharmacological experiments.

The type II anemone toxins (Schweitz *et al.* 1985) and scorpion α-toxins (Frelin *et al.* 1984) have similar affinities for TTX-sensitive and TTX-insensitive sodium channels in rat neuroblastoma cells and skeletal myotubes, respectively, whereas the sodium channels of rat cardiac cells in culture, which have a low affinity for tetrodotoxin (TTX), have a particularly high affinity for type I toxins (Renaud *et al.* 1986).

The activity of some of the anemone toxins on non-mammalian (primarily crustacean) sodium channels has stimulated their evaluation as potential biopesticides. ATX II and Sh I, for example, are both potent toxins on locust nerves (Ertel *et al.* 1995). In this type of application the lack of activity of type II toxins such as Sh I on mammalian nerve preparations becomes an advantage.

References

Cariello, L., De Santis, A., Fiore, F., Piccoli, R., Spagnuolo, A., Zanetti, L., *et al.* (1989). Calitoxin, a neurotoxic peptide from the sea anemone *Calliactis parasitica*: amino acid sequence and electrophysiological properties. *Biochemistry*, **28**, 2484–9.

Catterall, W. A. (1988). Structure and function of voltage-sensitive ion channels. *Science*, **242**, 50–61.

Ertel, E. A., Wunderler, D. L., and Cohen, C. J. (1995). Locust neuronal Na channels are highly sensitive to natural toxins. *Biophys. J.*, **68**, A153.

Fogh, R. H., Kem, W. R., and Norton, R. S. (1990). Solution structure of neurotoxin I from the sea anemone *Stichodactyla helianthus*. A nuclear magnetic resonance, distance geometry and restrained molecular dynamics study. *J. Biol. Chem.*, **265**, 13016–28.

Frelin, C., Vigne, P., Schweitz, H., and Lazdunski, M. (1984). The interaction of sea anemone and scorpion neurotoxins with tetrodotoxin-resistant Na^+ channels in rat myoblasts. A comparison with Na^+ channels in other excitable and non-excitable cells. *Mol. Pharmacol.*, **26**, 70–4.

Table 1 Pharmacological properties of type II sea anemone toxins

Toxin	LD_{50} (μg/kg)			K_D on rat brain synaptosomes (nM)	EC_{50} on rat heart muscle (nM)
	Crab	Mouse i.p.	Mouse i.c.		
Hm I		3000			
Hm II		1650			
Hm III	85	25			
Hm IV		40			
Hm V		350			
Hp I	36	145	1.5	900	3000
Hp II	15	4200	12	$>10^5$	5000
Hp III	10	53	2.4	300	4000
Hp IV	90	40	2.0	10 000	1300
Sg I	7	>2000	>3600	>10 000	
Sh I	0.5–3	>15 000	116	40 000	>8000

Modified from Norton (1991), where original literature references are cited.

Hinds, M. G. and Norton, R. S. (1993). Sequential ^{1}H-NMR assignments of neurotoxin III from the sea anemone *Heteractis macrodactylus* and structural comparison with related toxins. *J. Protein Chem.*, **12**, 371–8.
Kem, W. R. (1988). Sea anemone toxins: structure and action. *In The biology of Nematocysts* (ed. D. Hessinger and H. Lenhoff), pp. 375–405, Academic Press, New York.
Kraulis, P. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.*, **24**, 946–50.
Lin, X., Ishida, M., Nagashuma, Y., and Shiomi, K. (1996). A polypeptide toxin in the sea anemone *Actinia equina* homologous with other sea anemone sodium channel toxins: isolation and amino acid sequence. *Toxicon*, **34**, 57–65.
Monks, S. A., Gould, A. R., Lumley, P. E., Alewood, P. F., Kem, W. R., Goss, N. H. *et al.* (1994). Limited proteolysis study of structure–function relationships in Sh I, a polypeptide neurotoxin from a sea anemone. *Biochim. Biophys. Acta*, **1207**, 93–101.
Monks, S. A., Pallaghy, P. K., Scanlon, M. J., and Norton, R. S. (1995). Solution structure of the cardiostimulant polypeptide anthopleurin-B and comparison with anthopleurin-A. *Structure*, **3**, 791–803.
Norton, R. S. (1991). Structure and structure–function relationships of sea anemone proteins that interact with the sodium channel. *Toxicon*, **29**, 1051–84.
Pallaghy, P. K., Scanlon, M. J., Monks, S. A., and Norton, R. S. (1995). Three dimensional structure in solution of the polypeptide cardiac stimulant anthopleurin-A. *Biochemistry*, **34**, 3782–94.
Pease, J. H. B., Kumar, N. V., Schweitz, H., Kallenbach, N. R., and Wemmer, D. E. (1989). NMR studies of toxin III from the sea anemone *Radianthus paumotensis* and comparison of its secondary structure with related toxins. *Biochemistry*, **28**, 2199–204.
Pennington, M. W., Kem, W. R., and Dunn, B. M. (1990a). Synthesis and biological activity of six monosubstituted analogs of a sea anemone polypeptide neurotoxin. *Peptide Res.*, **3**, 228–32.
Pennington, M. W., Kem, W. R., Norton, R. S., and Dunn, B. M. (1990b). Chemical synthesis of a neurotoxic polypeptide from the sea anemone *Stichodactyla helianthus*. *Int. J. Peptide Prot. Res.*, **36**, 335–43.
Renaud, J.-F., Fosset, M., Schweitz, H., and Lazdunski, M. (1986). The interaction of polypeptide neurotoxins with tetrodotoxin-resistant Na^+ channels in mammalian cardiac cells. Correlation with inotropic and arrhythmic effects. *Eur. J. Pharmacol.*, **120**, 161–70.
Schweitz, H., Bidard, J.-N., Frelin, C., Pauron, D., Vijverberg, H. P. M., Mahasneh, D. M., *et al.* (1985). Purification, sequence and pharmacological properties of sea anemone toxins from *Radianthus paumotensis*. A new class of sea anemone toxins acting on the sodium channel. *Biochemistry*, **24**, 3554–61.
Wann, K. T. (1993). Neuronal sodium and potassium channels: structure and function. *Brit. J. Anaesth.*, **71**, 2–14.
Wemmer, D. E., Kumar, N. V., Metrione, R. M., Lazdunski, M., Drobny, G., and Kallenbach, N. R. (1986). NMR analysis and sequence of toxin II from the sea anemone *Radianthus paumotensis*. *Biochemistry*, **25**, 6842–9.
Widmer, H., Billeter, M., and Wüthrich, K. (1989). The three-dimensional structure of the neurotoxin ATX Ia from *Anemonia sulcata* in aqueous solution by nuclear magnetic resonance spectroscopy. *Proteins*, **6**, 357–71.
Wilcox, G. R., Fogh, R. H., and Norton, R. S. (1993). Refined structure in solution of the sea anemone neurotoxin Sh I. *J. Biol. Chem.*, **268**, 24707–19.

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Calitoxins

Calitoxin (CLX), isolated from Calliactis parasitica, *is a highly toxic peptide, whose amino acid (AA) sequence (IECKCEGDAPDLSHMTGTVYFSCKGGDGSWSKCLTYTA VADCCHEA) differs greatly from that of all sea anemone toxins isolated so far. The polypeptide chain contains 46 AA residues, with a molecular mass of 4886 Da and a pI of 5.4. Despite the differences in primary structure, the electrophysiological effects of CLX on crustacean neuromuscular transmission are very similar to those of the other anemone toxins. Indeed, CLX slows down sodium current inactivation, which in turn prolongs the axonal action potentials leading to a prolonged depolarization of the nerve terminals, thereby causing massive transmitter release. Two genes (*clx-1 *and* clx-2*) coding for two highly homologous calitoxins were isolated and characterized from a* C. parasitica *genomic library. The* clx-1 *gene encodes the already known calitoxin sequence, designated CLXI, whereas a single base-pair substitution in the coding region of* clx-2 *is responsible for a single AA replacement in position 6 (Glu → Lys) of a new peptide named CLXII. The structural organization of the two genes is very similar. The open reading frame (ORF) of both* clx-1 *and* clx-2 *codes for a precursor peptide of 79 AA, whose N-terminus has the feature of a signal peptide, while the C-terminus corresponds to the sequences of mature CLXI and CLXII.*

Sea anemones, in common with other members of the phylum Cnidaria (Coelenterata), possess numerous tentacles containing specialized stinging cells or cnidocytes. These stinging cells are equipped with organelles known as 'nematocysts' that contain small threads that are forcefully everted when stimulated mechanically or chemically. Anemones use this venom apparatus in the capture of prey, as well as for defence against predators.

Peptides and proteins figure prominently amongst the various classes of sea anemone toxins isolated and characterized to date. It is now recognized that this group of toxins consists of three classes of polypeptides, two made up of molecules containing 46–49 AA residues (type 1), and one of shorter polypeptides containing 27–31 residues (type 2). All these polypeptides have a paralysing effect on the sodium channels exerted through a prolonged depolarization of the nerve terminals, thereby causing massive transmitter release.

We isolated and characterized a new long polypeptide, calitoxin (CLX) from the sea anemone *Calliactis parasitica* (Cariello *et al.* 1989). The toxin contains 46 amino acid residues and exerts similar presynaptic effects on crustacean nerve muscle preparation as the other anemone toxins. However, its AA sequence is significantly different from those of both type I and type 2 toxins. For example, it lacks the functionally important Arg-14 residue (having instead an His residue at position 15), the halfcystine residue normally at position 29 is at position 26 and the C-terminal sequence is His–Glu–Ala in contrast to the basic C-terminal sequence found in type 1 and, even more markedly, type 2 toxins was confirmed by two-dimensional nuclear magnetic resonance studies (Norton and Cariello, unpublished data).

By analysing a genomic library prepared from the sea anemone *C. parasitica* we isolated and characterized (Spagnulo *et al.* 1994) two genes (*clx-1* and *clx-2*) coding for two highly homologous calitoxins. The *clx-1* gene sequence, positioned between nucleotides 1021–1158 encodes calitoxin. The comparison of the calitoxin AA sequence with that deduced from the nucleotide sequence of *clx-1* showed a single discrepancy: the Glu residue in position 45, as determined by the Edman procedure, is in fact a Gln, as assessed by DNA sequencing. This discrepancy may be due to a deamidation event occurring during the Edman reaction and/or the purification procedure. The *clx-2* gene sequence, positioned between nucleotides 1313–1450 codes for a peptide almost identical to that encoded by the *clx-1* gene: a single base substitution in position 1328 of *clx-2* is responsible for the replacement of the Glu residue with a Lys residue in position 6 of the mature peptide. This calitoxin-like peptide will be henceforth referred to as calitoxin II (CLXII), whereas the already known toxin is designated calitoxin I (CLXI).

The structural organization of the two genes is very similar. Two introns interpose between three exonic regions, whose sequences show a high degree of identity (95 per cent). The precise location of intervening sequences was obtained by RNAase protection assay, RT-PCR, and RACE analyses. The sequences located at the exon–intron boundaries correspond to the classical splice sequences (Breathnach and Chambon 1981). The translation initiation site at Met –33 is located in the second exon, while the entire translated sequence spans over the second and the third exon. In both genes, the ORF is interrupted by the nonsense TAA triplet situated mmediately after the calitoxin coding sequence.

The homology between the two genes is not restricted to the ORF, it extends to the upstream and downstream flanking regions. A putative CAAT element and a TATA box motif are located 65 bp and 30 bp, respectively, upstream from the transcription initiation site in both genes. The polyadenylation signal is located at corresponding sites in the transcription unit of the *clx-1* and *clx-2* genes. Next to the translation initiation site is a hydrophobic peptide (52 per cent of nonpolar AA

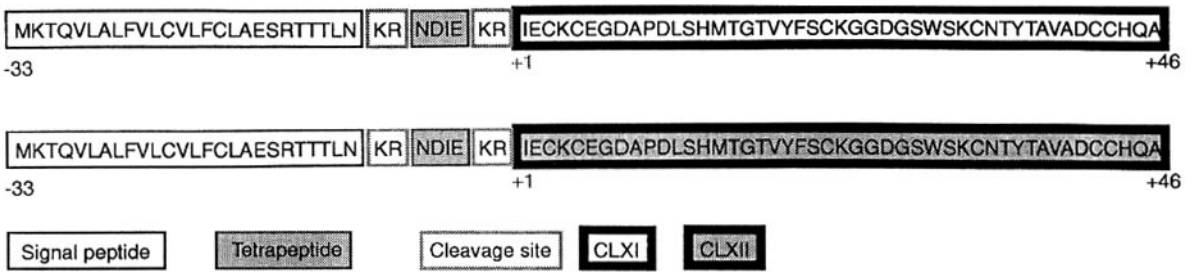

Figure 1. The CLX precursor sequences deduced from the gene sequences, based on the assumption that translation begins at the first in-frame methionine of the long ORF. +1 indicates the start CLX; −33 indicates the signal peptide's start.

residues), situated from position −32 to −9, whose sequence is almost identical in the two translated products. The presence of a positive charge to the NH_2-terminus, Lys at position −32, followed by a core of 13 hydrophobic residues are features distinctive of signal peptides of several secreted proteins (Watson 1984). The presence of two dibasic AA sequences situated at positions −7, −6 and −2, −1 strongly suggests that these sequences represent specific cleavage sites for trypsin-like enzymes, whose action would trigger the release of mature calitoxins I and II from their respective precursors. Although such cleavage sites have been found in several precursors of bioactive peptides (Turner 1986), the results reported here provide the first evidence of the existence of a precursor molecule in the toxin biosynthetic pathway.

The finding that *C. parasitica* toxins are processed from precursors suggests that inactive precursors are stored in the cnidocytes (the cells specialized for toxin production) until a specific signal induces maturation events that lead to the release of the biologically active peptides. Moreover, complete processing of both calitoxin precursors at the two putative cleavage sites would cause the release of a tetrapeptide possibly endowed with some biological meaning.

The presence of non-homologous sequences at the 5′- and 3′-ends of sequenced DNA fragments suggests a non-allelic origin of the two genes. Therefore, the finding in *C. parasitica* of two different genes coding for two homologous toxins might imply different biological functions of the two neurotoxic peptides. Indeed, three toxins (ATXI, ATXH, and ATXV) have been isolated from *Anemonia sulcata* extracts; these share a 60 per cent to 90 per cent sequence identity but exhibit differences in toxicity and in animal species selectivity (Norton 1991). In this context, we isolated only CLXI from the *C. parasitica* extracts, when we monitored the paralysing activity (Cariello *et al.* 1989). Various hypotheses can be invoked to explain these findings: CLXII is much less active than CLM or it produces different effects when injected in crabs; CLXII is expressed at a very low degree; or CLXII precursor processing occurs only under special circumstances.

Purification and toxicity assay

The sea anemones, *C. parasitica*, collected in the Bay of Naples, were stirred for 1 h at room temperature in distilled water (1:1 w/v). The resulting suspension was centrifuged at 18 000 rpm for 1 h with a SS 34 Sorvall rotor. The pellet was treated as before until no activity was present in the supernatant. The combined supernatants, diluted with distilled water to 4 mΩ-1 and adjusted to pH 8.8, were chromatographed on a column of QAE-Sephadex A-25 equilibrated with 0.01 M Tris–HCl, pH 7.8, containing 0.06 M NaCl. The toxic activity was eluted with 0.2 M ammonium acetate, pH 5.0. After lyophilization, the toxic material was further purified by gel filtration on a column of Sephadex G-50 superfine, equilibrated with 0.1 M ammonium acetate, pH 5.0, and eluted with the same buffer. The active fractions were combined and fractionated further by RP-HPLC on a Beckman instrument using a semipreparative column (l × 25 cm, 5Wn particle size, ODS). The elution was performed with a 20-min linear gradient (from 5 to 60 per cent of solvent B) consisting of solvent A (0.1 per cent TFA) and solvent B (acetonitrile with 0. 1 per cent TFA) at a flow rate of 3 mlmin. The homogeneity of CLX was ascertained by way of analytical RP-HPLC using the same elution system, and by chromatofocusing.

References

Breathnach, R. and Chambon, P. (1981). Organization and expression of eucaryotic split genes coding for proteins. *Ann. Rev. Biochem.*, **50**, 349–83.

Cariello, L., de Santis, A., Fiore, F., Piccoli, R., Spagnuolo, A., Zanetti, L., *et al.* (1989). Calitoxin, a neurotoxic peptide from the sea anemone *Calliactis parasitica*: amino acid sequence and electrophysiological properties. *Biochemistry*, **28**, 2484–9.

Norton, R. S. (1991). Structure and structure–function relationships of sea anemone proteins that interact with the sodium channel. *Toxicon*, **29**, 1051–84.

Spagnuolo, A., Zanetti, L., Cariello, L., and Piccoli, R. (1994). Isolation and characterization of two genes encoding

calitoxins, neurotoxic peptides from *Calliactis parasitica* (Cnidaria). *Gene*, **138**, 187–91.
Turner, A. J. (1986). Processing and metabolism of nanopeptides. *Assays Biochem.*, **22**, 69–119.
Watson, M. E. E. (1984). Compilation of published signal sequences. *Nucleic Acid Res.*, **12**, 5145–64.

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8

Potassium channel-blocking toxins

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Introduction

Toxins which bind with high specificity to potassium (K) channels and thereby alter their activity, are important tools in studies on the structure and function of K channels. Over the recent years, we have witnessed a rapid progress in the isolation and characterization of K channel-blocking peptide toxins. For many years, researchers working in the K channel field were envious of the plethora of toxins that were available to specifically block various calcium, sodium, and ligand-gated ion channels. As outlined in the following section of this book, this situation has been changed by work in many laboratories which have been able to discover and to isolate various specific K channel-blocking toxins from venoms extracted from bees, scorpions, sea anemones, spiders, and snakes.

The purpose of this introduction is to outline briefly what some of the peptide toxins may have in common in terms of their structure and function, and to discuss their usefulness as tools in studying K channel function. According to their relatedness in primary sequence, five different peptide-toxin families may be recognized: the apamin family containing members isolated from bee venoms, the charybdotoxin family isolated from scorpion venoms, the dendrotoxin family isolated from snake venoms, the hanatoxins isolated from spider venom, and the sea anemone toxins. It is obvious that the families have been given nonsystematic and sometimes whimsical names which say more about the original discoverers of the peptide toxins than about the molecules themselves. Because of this unsatisfactory situation, C. Miller recently suggested in his excellent review on the structure and function of the charybdotoxin family of K channel-blocking peptides a formalized nomenclature (Miller 1995). In analogy to the now widely accepted K channel nomenclature, he proposed to abbreviate K channel-blocking peptide toxins as K-toxins (KTx) and in particular the charybdotoxin family members as α-KTx*m. n*, where *m* refers to the subfamily and *n* to the member within the given subfamily. For example, α-KTx1.1 would be charybdotoxin, α-KTx2.1 noxiustoxin etc. as outlined in Table 2 below.

Apamin and related peptides

The apamin family consists of three relatively small peptides (Table I): apamin, mast cell degranulating peptide (MCDP), and tertiapin (Habermann 1984). The structural hallmarks of these peptide toxins are two intramolecular disulfide bridges which affix an amino-terminal β-turn structure of four amino acids to a ~10 amino acid long carboxy terminal α helix (Fig. 1) (Pease and Wemmer 1988). Two arginine residues (R13, R14) and glutamine 17, which protrude from the a helical surface are apparently essential for the toxicity of apamin (Vincent *et al.* 1975; Labbe-Jullie *et al.* 1991). Similar residues may also underlie the toxicity of MCDP. This observation may imply that the interaction side of toxins of the apamin family is formed by residues protuding from the α helical backbone. Thus, the structures of the interaction surface of apamin-like toxins most likely differ from those of members of the charybdotoxin and dendrotoxin families as outlined below. Accordingly, K channels may have mutual interacting, yet discrete binding sites for charybdotoxins, dendrotoxins, and apamin-like toxins. This suggestion is supported by reports that MCDP inhibits dendrotoxin (Toxin 1) and β-bungarotoxin binding to chick and rat brain membranes in a noncompetitive manner (Rehm *et al.* 1988; Schmidt *et al.* 1988), and that the receptor binding sites for dendrotoxin and MDCP on voltage-activated K_v1. 1 channels may be overlapping, but are not identical (Stocker *et al.* 1991). In contrast to MCDP, which blocks some members of the K_v1 family of voltage-activated K channels (Pongs 1992*a*), apamin blocks the small conductance (SK) Ca-activated K channels, also known as apamin-sensitive K channels (Dreyer 1990; Strong 1990). As discussed in the entry on apamin, apamin is unique among the K channel-blocking toxins, since it is the only peptide toxin which blocks SK channels.

Table 1 Comparison of amino acid sequences of apamin, mast cell degranulating peptide (MCDP), and tertiapin (Habermann 1984)

Toxin	Amino acid sequence
	* * *
Apamin	**C N C K** – – A P E T A L **C** A R R **C** Q Q H – NH_2
MCDP	I K **C N C K R** H V **I** K **P H** I **C** R **K** I **C G K** N – NH_2
Tertiapin	A L **C N C** N **R** – I **I** I **P H** M **C** W **K** K **C G K** K

Residues essential for apamin toxicity (Vincent *et al.* 1975; Labbe-Julie *et al.* 1991) have been marked with asterisks. Identical amino acids are bold. – Gaps were introduced for optimal sequence alignment.

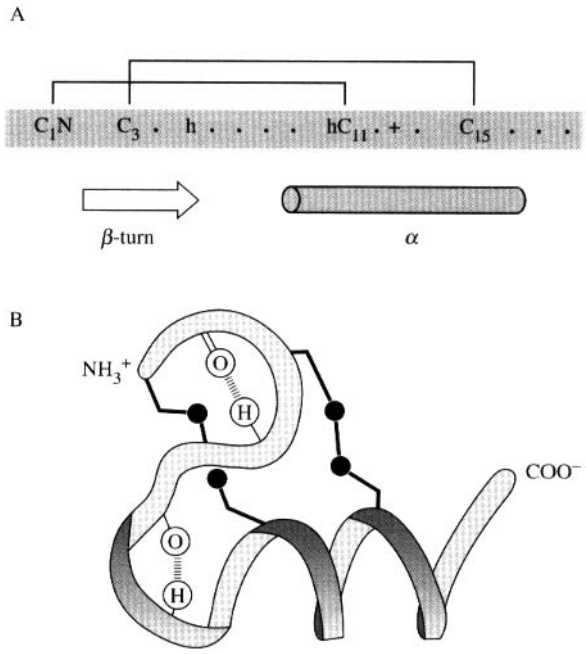

Figure 1. A: Schematic consensus sequence of apamin-like potassium-channel blocking peptides (Habermann 1984) (C, cysteine; N, asparagine; h, hydrophobic amino acid). Disulfide bridges are indicated by lines. Arrow under the sequence indicates β-turn and grey cylinder α-helix (α) according to the backbone structure depicted in B. B: NH_3^+ and COO indicate amino- and carboxy-terminal end, respectively. The structure has been adopted from Pease and Wemmer (1988).

The scorpion toxins

Most of the presently known peptide toxins are members of the charybdotoxin (α-KTx) family (for details see the entries on charybdotoxin, kaliotoxin, and margatoxin, respectively). Thus, it is not surprising that most of our knowledge stems from studies on α-KTx peptides, which are shown in Table 2. The three-dimensional structures of several α-KTx peptides have been elucidated by multidimensional nuclear magnetic resonance methods (Bontems *et al.* 1991; Johnson and Sugg 1992; Fernandez *et al.* 1994; Krezel *et al.* 1995). From these studies it emerges that (α-KTx peptides have a characteristic fold of their polypeptide backbone. One hallmark is again the occurrence of intramolecular disulfide bonds; this time three (Fig. 2) in comparison to the two in the apamin toxin family. The disulfide bonds make up the internal volume of the α-KTx peptide. The other hallmark is a three stranded β-sheet juxtaposed to a short two- to three-turn α-helix held into place by the disulfide bonds (Fig. 2). Possibly, leiurotoxin (Chicchi *et al.* 1988) which has been placed into the α-KTx family (see Table 2) has a similar fold, except that the β-sheet may only be two stranded lacking the aminoterminal nonconserved β-strand (β1 in Fig. 2). But note that leiurutoxin competes with apamin binding sites (Chicchi *et al.* 1988), whereas the other α-KTx peptides may not do so.

The most important residues of α-KTx peptides, that constitute the interaction side, reside in the most conserved carboxy terminal α-KTx sequence (for review, see Miller 1995). It forms the loop connecting the second and third β-sheet as well as part of the β-sheet (see Fig. 2). Using the charybdotoxin numbering convention, much of the interaction side is made up by the sequence G_{26}–K_{27}–C_{28}–M(I)$_{29}$–N(G)$_{30}$–X_{31} –K_{32}–C_{33}±$_{34}$–C_{35}, (X, any amino acid residue; ±, charged amino acid residue) (Miller 1995). The toxicity of α-KTx peptides crucially depends on the presence of lysine 27. The side chain of this amino acid residue protrudes from the flat peptide toxin surface into solution and appears to be a sort of molecular mimic. The positively charged ε-amino group resembles a tethered potassium ion looking for its K channel bait. Somewhat like a sticky finger the lysine is able to enter the outer mouth of a K channel pore and thereby to plug the channel. Binding apparently takes place to the open

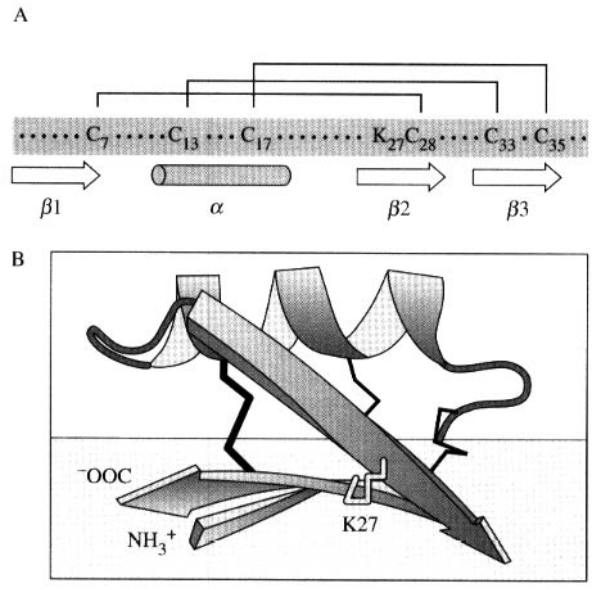

Figure 2. A: Schematic consensus sequence of charybdotoxin-like potassium-channel blocking peptides summarized in Table 2 (C, cysteine; K, lysine). Disulfide bridges are indicated by lines. Conserved areas of secondary structure are indicated under the sequence by arrows for β-sheet and a grey cylinder for α-helix (α). B: Schematic representation of α-KTx1.1 backbone fold. Disulfide bridges are sandwiched between an α-helix on top and a β-sheet at the bottom. NH_3^+ and COO indicate amino- and carboxy-terminal end, respectively. Critical K27, which most likely projects into the potassium channel pore, is explicitely indicated. The structure has been adopted from Miller (1995) and Bontems *et al.* (1991).

Table 2 Alignment of K channel-blocking scorpion toxins

Common name	Amino acid sequence	α-KTx name
Charybdotoxin-type: subfamily 1		
CTx	ZFTNVSCTTSKE-CWSVCQRLHNTSRG-KCMNKKCRCYS	1.1
Lg2	ZFTQESCTASNQ-CWSICKRLHNTNRG-KCMNKKCRCYS	1.2
IbTx	ZFTDVDCSVSKE-CWSVCKDLFGVDRG-KCMGKKCRCYQ	1.3
LbTx	VFIDVSCSVSKE-CWAPCKAAVGTDRG-KCMGKKCKCY?	1.4
Noxiustoxin-type: subfamily 2		
NTx	TIINVKCT-SPKQCSKPCKELYGSS AGAKCMNGKCKCYNN	2.1
MgTx	TIINVKCT-SPKQCLPPCKAQFGQS AGAKCMNGKCKCYP	2.2
CITx1	ITINVKCT-SPQQCLRPCKDRFGQHAGGKCI NGKCKCYP	2.3
Kaliotoxin-type: subfamily 3		
KITx	GVEINVKCSGSP-QCLKPCKDA-GMRFG-KCMNRKCHCTP?	3.1
AgTx2	GVPINVSCTGSP-QCIKPCKDA-GMRFG-KCMNRKCHCTPK	3.2
AgTx3	GVPINVPCTGSP-QCIKPCKDA-GMRFG-KCMNRKCHCTPK	3.3
AgTx1	GVPINVKCTGSP-QCLKPCKDA-GMRFG-KCI NGKCHCTPK	3.4
KITx2	VRIPVSCKHSG-QCLKPCKDA-GMRFG-KCMNGKCDCTPK	3.5
Tytiustoxin-type: subfamily 4?		
TyKα	VFINAKCRGSPE-CLPKCKEAIGKAAG-KCMNGKCKCYP	4.1
Leiurotoxin-type: subfamily 5?		
LeTx	AFCNL-RM-CQLSCRSL-GL-LG-KCIGDKCECVKH	5.1

Alignment of published scorpion toxin sequences, extended by leiurotoxin. Abbreviations for common names are shown at left and proposed formal α-KTx nomenclature (Miller 1995) at right. – Gaps were introduced for optimal sequence alignment.

as well as closed channel. Extensive structure/function analyses combining reciprocal *in vitro* mutagenesis of several α-KTx peptides and K channels combined with electrophysiological studies have come up with this conclusion. Furthermore, the results of these studies were used to derive a footprint of α-KTx peptide residues that are in close contact with matching residues in the K channel vestibule. With the known three-dimensional structure of α-KTx peptides in hand, these studies also provided important topological information on the possible three-dimensional structure of K channel vestibules within a resolution of 5 Å (Fig. 3) (Goldstein *et al.* 1994; Stampe *et al.* 1994; Aiyar *et al.* 1995; Hidalgo and MacKinnon 1995). There is fairly good agreement among the various studies, which have been carried out with α-KTx1.1, α-KTx2.2, α-KTx3.1, and α-KTx3.2 and *Shaker* or K_v1.3 channels, respectively. However, it is important to recognize that more or less subtle differences may exist between the close-contact topologies which have been derived for the different α-KTx – K channel pairs. For example, the most important residues at the α-KTx1.1 interaction surface are K27, M29, and N30 (Goldstein *et al.* 1994). These three residues together with R34 and Y36 were localized as close-contact residues at the level of the receptor floor of the *Shaker* channel vestibule. In the case of α-KTx3.1, toxin residues G10, R24, F25, M29, N30, R31, and T36 were localized as close-contact residues at the level of the receptor floor of K_v1.3 channels (Aiyar *et al.* 1995). Some extra (weak-contact) residues have been mapped in the larger interaction surface, i. e. T8/9 in the case of *Shaker* channels (Goldstein *et al.* 1994), T8, W14, and K31 in the case of K_v1.3 channels (Aiyar *et al.* 1995), and F2 and W14 in the case of BK channels (Stampe *et al.* 1994). The latter are only blocked by α-KTx peptides which contain F2 and W14 residues, that is by members of the α-KTx1.1 subfamily. This may suggest that these two residues are important determinants for the toxicity of BK channel blocking peptides.

Although the topologies of the interaction side between *Shaker* related K channels and α-KTx peptides have been mapped in such great detail, it is not possible (yet) to predict from the primary sequence of the K channel H5 sequence, which probably wholly makes up the outer K channel vestibule, whether the particular K channel is sensitive to α-KTx block or not. This unsettling point may be illustrated by three examples. Neutralization of K427 in *Shaker* by asparigine greatly increases the channel's sensitivity to toxin block (Stocker and Miller 1994). In contrast, placement of a positive charge at the homologous position in K_v1.3 (N382K), produces no appreciable change in toxin block (Aiyar *et al.* 1995). In a second example, the replacement of G380 with histidine renders K_v1.3 resistant to α-KTx3.1 (Aiyar *et al.* 1995), whereas K_v1.1, which has a histidine at the equivalent position (Stuhmer *et al.* 1989), is highly sensitive to α-KTx3.1 block (Grissmer *et al.* 1994). A third example represents the replacement of G380 of K_v1.3 by glutamine. This mutation makes K_v1.3 resistant to α-KTx1.1 and α-KTx3.1 (Aiyar *et al.* 1995), but not to α-KTx 2.1 and α-KTx2.2, while K_v1.2, which has a glutamine at the equivalent position (Stuhmer *et al.* 1989), is highly sensitive to α-KTx1.1 and α-KTx2.1, but resistant to -KTx3.1

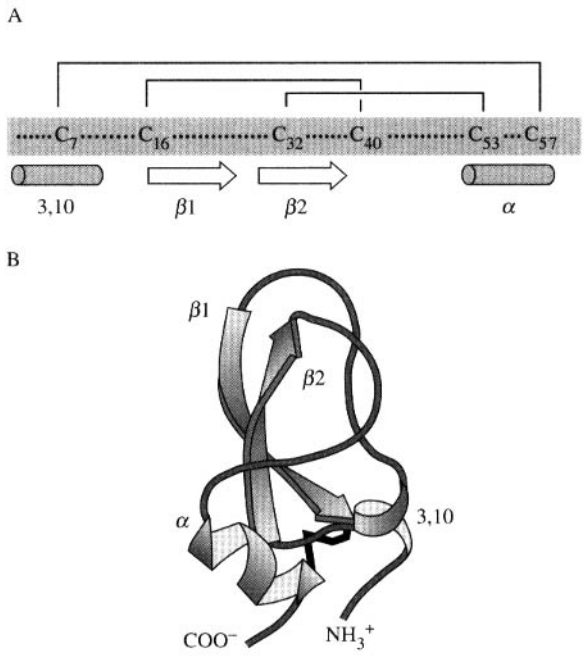

Figure 3. A: Schematic consensus sequence of dendrotoxin like potassium-channel blocking peptides. (C, cysteine). Disulfide bridges are indicated by lines. Conserved areas of secondary structure are indicated under the sequence by arrows for β-sheet and by grey cylinders for 3, 10- and α-helix, respectively.
B: Schematic representation of dendrotoxin I backbone fold. Secondary structures are marked: $\beta 1$, $\beta 2$, β-sheet; α, α-helix, 3, 10, 3, 10-helix. NH_3^+ and COO^- indicate amino and carboxy-terminal ends, respectively. The structure has been adopted from Lancelin *et al.* (1994).

(Grissmer et *al.* 1994). These examples indicate that slight sequence variations, which may not influence otherwise the gating and conductance properties of the K channel, may have important consequences for the channel's toxin susceptibility. The molecular basis underlying the varied toxin susceptibilities may be in several cases purely sterical, i. e. a residue in the channel's vestibule is 'in the way'. In other cases, the varied toxin susceptibilities may also reflect subtle structural differences both of the toxins and the channel vestibules, which may add up to nonmatching topologies at the interaction surface in one case, but not in another. Clearly, one would like to have the same detailed structural information on K channel vestibules as is available for α-KTx peptides for an even better understanding of toxin – K channel specificity.

Dendrotoxins

The dendrotoxin family represents a group of 57 to 60 amino acid long snake toxins which are related in sequence and structure to β-bungarotoxin and pancreatic trypsin inhibitor. Eight dendrotoxin isoforms have been isolated from the venom of Dendroaspis snakes (for a sequence alignment of the isoforms see the entry on dendrotoxins). This family has been recently enlarged by 'kalicludin', a homologous peptide isolated from the sea anemone *Anemonia sulcata* (see the entry on sea anemone potassium channel toxins). The structure of dendrotoxins has been investigated by crystallographic and multidimensional nuclear magnetic resonance methods (Skarzynski 1992; Foray et *al.* 1993; Lancelin et *al.* 1994). The dendrotoxin peptide backbone fold, which is very similar to that of pancreatic trypsin inhibitor, is based on three regular secondarystructure motifs (Fig. 3): a small aminoterminal 3,10-helix, a hairpin two-stranded β-sheet and a carboxyterminal three-turn α-helix. The three motifs are joined by three disulfide bridges that connect the 3,10-helix and the β-sheet with the α-helix and, respectively, the two loops in front and behind the β-sheet. The hairpin between the two β-strands contains a suspicious lysine (K28 in toxin K), which is conserved among the dendrotoxins, but absent in the sequence related trypsin inhibitor peptides. As the lysine protrudes from the toxin surface into solution, it might also function as a tethered K channel bait similarly to the conserved α-KTx lysine. However, recent site-directed mutagenesis of α-dendrotoxin has revealed that the equivalent lysine residue 30 in α-dendrotoxin is not essential for dendrotoxin binding (Danse et *al.* 1994). Another lysine candidate residue may be K19, which is also solvent exposed and conserved among the dendrotoxins. The variant dendrotoxins appear to bind to *Shaker* related K channels with differing affinities (Pongs 1992a). Yet detailed studies are not available. From the limited information that is available it seems that the interaction surface between dendrotoxin and the K channel vestibule is very similar to those between α-KTx peptides and K channels. Towards the rim of the vestibule the interaction surface for dendrotoxins may be extended further, since amino acid residues, two to three places upstream of G380 in $K_v1.3$, have been found to influence the toxin susceptibility of *Shaker*-related channels (Hurst et *al.* 1991; Stocker et *al.* 1991; Gross et *al.* 1994).

Hanatoxins

All of the K channel-blocking toxins, that have been discussed so far, target either the *Shaker*-related K_v1 subfamily of voltage-activated K channels and/or Ca-activated K channels. Thus, it would be highly desirable to search for other toxins which target the large body of remaining K channels, e. g. the K_v2–K_v6 subfamilies, the *eag* voltage-activated K channels or the inwardly rectifying channels. Recently, one such search has resulted in the isolation of two peptides, hanatoxin (HaTx) 1 and 2, from the venom of a Chilean tarantula (Swartz and MacKinnon 1995).

The two peptides inhibit the *Shab*-related $K_v2.1$ channel (Frech et *al.* 1989). Also, the *Shal*-related $K_v4.2$ channel (Baldwin et *al.* 1991) was sensitive to HaTx. The

sequences of the two 35 amino acid long HaTxs and the spacing of the six cysteine residues distinguishes them from the above described toxin families. The HaTx sequences are distantly related to grammotoxin, a member of the conotoxins, which inhibits voltage-activated Ca channels (Lampe *et al.* 1993). Clearly, they represent a new category of K channel-blocking peptides and thus may be the founding members of a newly emerging important family of K channel inhibitors. The binding site for HaTx on $K_v2.1$ channels is apparently different from those for the α-KTx and dendrotoxin peptides. Presumably, HaTx interacts with other regions of the K channels, which are important for their activity.

Sea anemone toxins

As described in one of the subsequent entries, three 35 to 37 residue long peptide toxins have been isolated from sea anemones (*Sh*K, AsK, BgK) (Karlsson *et al.* 1992; Aneiros *et al.* 1993; Castaneda *et al.* 1995). The toxins inhibit *Shaker*-related voltage-activated K channels, in particular $K_v1.3$ channels with high affinity (Schweitz *et al.* 1995). In a way, it is peculiar that $K_v1.3$ channels stand out, most of the time, as the K channels with the highest toxin susceptibility. The primary sequences and their cysteine spacing make the sea anemone toxins clearly distinguishable from all the other toxins. Also, the formation of a disulfide bridge between the first and last cysteine in the sequence is not found in the other peptide toxins. Thus, these toxins may represent yet another distinct family of K channel-blocking toxins. The interaction of sea anemone toxins with K channels has not yet been studied in great detail. From the present information that is available (see the entry on sea anemone potassium channel toxins) it emerges, that sea anemone toxins may have K channel binding sites overlapping with those for charybdotoxin and dendrotoxin. The sea anemone toxins apparently also have a critical lysine residue (K22 in *Sh*K). One may speculate that this lysine functions similarly to the one in α-KTx peptides representing the central pore-plugging residue.

Conclusions

We have witnessed a steady and remarkable increase in the number of K channel-blocking peptides, which make it possible to specifically inhibit an increasing number of cloned K channels. The information, that has been gained over the recent years, may be an important guide for the design and the development of therapeutically useful drugs like immunosuppressants in transplantation medicine, anti-arrhythmica in cardiovascular treatments, or insulin-secretion regulators in diabetes. However, it should always be kept in mind that the pharmacological phenotype of a homomultimeric cloned K channel is a laboratory artefact. Whether, and even if at all, this *in vitro* pharmacology is relevant for native K channels found in excitable cells has to be carefully examined in each case.

This final paragraph, will briefly discuss some of the problems which one may encounter in the application of toxins as pharmacological tools to study native K channels. Voltage-activated as well as Ca-activated K channels are heteroligomeric complexes assembled from four membrane-inserted pore-forming α-subunits and auxiliary β-subunits (Knaus *et al.* 1994; Pongs 1995). The stoichiometry seems to be $\alpha_4\beta_4$. Furthermore, the different members of one α-subunit (and β-subunit) subfamily may coassemble with each other to form variant K channels having toxin susceptibilities that are distinct from those of the corresponding homomultimeric K channels (MacKinnon 1991; Pongs 1992*b*). Although subunit assemblies seem to be restricted to within subunit subfamilies and may not occur among members of different subunit subfamilies (Shen and Pfaffinger 1995), there are still a staggering number of possible native K channels with similar electrophysiological properties but different subunit compositions. Whether all of the possible combinations are realized in the brain is a matter of conjecture. Immunocytochemical in conjunction with biochemical data suggest that differing heteromultimeric assemblies are found in the different areas and neurons of the mammalian brain (Sheng *et al.* 1993; Wang *et al.* 1993; Veh *et al.* 1995). Also, unrelated subunit compositions may yield K channels mediating K currents with very similar or even undistinguishable properties. This situation makes it very difficult to use the various toxins for dissecting out distinct K currents in neurons. Such studies may only be fruitful if the number of different K channel subunits and their combinations are known for the neuron under study. Though there is still a long way to go towards this goal, the progress in the pharmacological and structural analysis of toxin–K channel interactions looks encouraging and promises a bright future.

References

Aiyar, J., Withka, J. M., Rizzi, J. P., Singleton, D. H., Andrews, G. C., Lin, W., *et al.* (1995) Topology of the pore-region of a K^+ channel revealed by the NMR-derived structures of scorpion toxins. *Neuron*, **15**, 1169–81.

Aneiros, A., Garcia, I., Martinez, J. R., Harvey, A. L., Anderson, A,J, Marshall, D. L., *et al.* (1993). A potassium channel toxin from the secretion of the sea anemone *Bunodosoma granulifera*. Isolation, amino acid sequence and biological activity. *Biochem. Biophys. Acta*, **1157**, 86–92.

Baldwin, T. J., Tsaur, M. L., Lopez, G. A., Jan, Y. N., and Jan, L. Y. (1991). Characterization of a mammalian cDNA for an inactivating voltage-sensitive K^+ channel. *Neuron*, **7**, 471–83.

Bontems, F., Roumestand, C., Gilquin, B., Menez, A., and Toma, F. (1991). Refined structure of charybdotoxin: common motifs in scorpion toxins and insect defensins. *Science*, **254**, 1521–3.

Castaneda, O., Sotolongo, V., Amor, A. M., Stocklin, R., Anderson, A. J., Harvey, A. L., *et al.* (1995). Characterizaiton of a potassium channel toxin from the Carribean sea anemone *Stichodactyla helianthus*. *Toxicon*, **33**, 606–13.

Chicchi, G., Gimenez-Gallego, G., Ber, E., Garcia, M. L., Winquist, R., and Cascieri, M. A. (1988). Purification and characterization of a unique, potent inhibitor of apamin binding from *Leiurus quinquestriatus hebreus* venom. *J. Biol. Chem.*, **263**, 10192–7.

Danse, J. M., Rowan, E. G., Gasparini, S., Ducancel, F., Vatanpour, H., Young, L. C., *et al.* (1994). On the site by which α-dendrotoxin binds to voltage-dependent potassium channels: site-directed mutagenesis reveals that the lysine triplet 28–10 is not essential for binding. *FEBS Lett.*, **356**, 153–8.

Dreyer, F. (1990). Peptide toxins and potassium channels. *Rev. Physiol. Biochem. Pharmacol.*, **115**, 93–136.

Femandez, I., Romi, R., Szendeffy, S., Martin-Eauclaire, M. F., Rochat, H., Van Rietschoten, J., *et al.* (1994). Kaliotoxin (1–37) shows structural differences with related potassium channel blockers. *Biochemistry*, **33**, 14256–63.

Foray, M. F., Lancelin, J. M., Hollecker, M., and Marion, D. (1993). Sequence-specific ^{1}H-NMR assignment and secondary structure of black mamba dendrotoxin-I, a highly selective blocker of voltage-gated potassium channels. *Eur. J. Biochem.*, **211**, 813–20.

Frech, G. C., VanDongen, A. M., Schuster, G., Brown, A. M., and Joho, R. H. (1989). A novel potassium channel with delayed rectifier properties isolated from rat brain by expression cloning. *Nature*, **340**, 642–5.

Goldstein, S. A. N., Pheasant, D. J., and Miller, C. (1994). The charybdotoxin receptor of a *Shaker* K^+ channel: peptide and channel residues mediating molecular recognition. *Neuron*, **12**, 1377–88.

Grissmer, S., Nguyen, A. N., Aiyar, J., Hanson, D. C., Mather, R. J., Gutman, G. A., *et al.* (1994). Pharmacological characterization of five cloned voltage-gated PC channels, types K_v1.1, 1.2, 1.3, 1.5 and 3.1, stably expressed in mammalian cell lines. *Molec. Pharmacol.*, **45**, 1227–34.

Gross, A., Abramson, T., and MacKinnon, R. (1994). Transfer of the scorpion toxin receptor to an insensitive potassium channel. *Neuron*, **13**, 961–6.

Habermann, E. (1984). Apamin. *Pharmac. Ther.*, **25**, 255–70.

Hidalgo, P. and MacKinnon, R. (1995). Revealing the architecture of a K^+ channel pore through mutant cycles with a peptide inhibitor. *Science*, **251**, 942–4.

Hurst, R. S., Busch, A. E., Kavanaugh, M. P., Osborne, P. B., North, R. A., and Adelman, J. P. (1991). Identification of amino acid residues involved in dendrotoxin block of rat voltage-dependent potassium channels. *Mol. Pharmacol.*, **40**, 572–6.

Johnson, B. A. and Sugg, E. E. (1992). Determination of the three-dimensional structure of iberiatoxin in solution by ^{1}H nuclear magnetic resonance spectroscopy. *Biochemistry*, **31**, 8151–9.

Karlsson, E., Aneiros, A., Castaneda, O., and Harvey, A. L. (1992). Potassium channel toxins from sea anemones. In *Recent advances in toxicology research* (ed. P. Gopalakrishankone and C. K. Tan), Vol. 2, pp 378–91, Nat. Univ., Singapore.

Knaus, H. G., Garcia-Calvo, M., Kaczorowski, G. J., and Garcia, M. L. (1994) Subunit composition of the high-conductance calcium-activated potassium channel from smooth muscle, a representative of the *m*Slo and *slowpoke* family of potassium channels. *J. Biol. Chem.*, **269**, 3921–4.

Krezel, A., Kasibahatla, C., Hidalgo, P., MacKinnon, R., and Wagner, G. (1995). Solution structure of the potassium channel inhibitor agitoxin 2: caliper for probind channel geometry. *Protein Sci.*, **4**, 1478–89.

Labbe-Jullie, C., Granier, C., Albericio, F., Defendi, M. L.,Ceard, B., Rochat, H., *et al.* (1991). Binding and toxicity of apamin. Characterization of the active site. *Eur. J. Biochem.*, **196**, 639–45.

Lampe, R. A., Defeo, P. A., Davison, M. D., Young, J., Herman, J. L., Spreen, R. C., *et al.* (1993). Isolation and pharmacological characterization of ω-conotoxin SIA, a novel peptide inhibitor of neuronal voltage sensitive calcium channel responses. *Mol. Pharmacol.*, **44**, 451–60.

Lancelin, J.-M., Foray, M.-F., Hollecker, M., and Marion, D. (1994) Proteinase inhibitor homologues as potassium channel blockers. *Struct. Biol.*, **1**(4), 246–50.

MacKinnon, R. (1991). Determination of the subunit stoichiometry of a voltage-activated potassium channel. *Nature*, **350**, 232–5.

Miller, C. (1995). The charybdotoxin family of PC channel-blocking peptides. *Neuron*, **15**, 5–10.

Pease, J. H. and Wemmer, D. E. (1988). Solution structure of apamin determined by nuclear magnetic resonance and distance geometry. *Biochemistry*, **27**, 8491–8.

Pongs, O. (1992*a*). Structural basis of voltage-gated K^+ channel pharmacology. *Trends Pharmac. Sci.*, **13**, 359–65.

Pongs, O. (1992*b*) Molecular biology of voltage-dependent potassium channels. *Phys. Rev.*, **72**, S69–88.

Pongs, O. (1995). Regulation of the activity of voltage-gated potassium channels by β subunits. *Neurosci.*, **7**, 137–46.

Rehm, H., Bidard, J. N., Hugues, S., and Lazdunski, M. (1988). The receptor site for bee venom mast cell degranulating peptide. Affinity labelling and evidence for a common molecular target for mast cell degranulating peptide and dendrotoxin I, a snake toxin active on K^+ channels. *Biochemistry*, **27**, 1827–32.

Schmidt, R. R., Betz, H., and Rehm, H. (1988). Inhibition of α-bungaratoxin binding to brain membranes by mast cell degranulating eptide, toxin I and ethylene glycol bis(b-aminoethylether)-*N,N,N,N*-tetracetic acid. *Biochemistry*, **27**, 963–7.

Schweitz, H., Bruhn, T., Guillemare, E., Moinier, D., Lancelin, J. M., Beress, L., *et al.* (1995). Kalicludines and kaliseptine: two different classes of sea anemone toxins for voltage-sensitive K^+ channels. *J. Biol. Chem.*, **270**, 25121–26.

Shen, V. and Pfaffinger, P. (1995). Molecular recognition and assembly sequences involved in the subfamily-specific assembly of voltage-gated K^+ channel subunit proteins. *Neuron*, **14**, 625–33.

Sheng, M., Liao, Y. J., Jan, Y. N., and Jan, L. Y. (1993). Presynaptic A-currents based on hetefomultimeric K^+ channels detected *in vivo*. *Nature*, **365**, 72–4.

Skarzynski, T. (1992). Crystal structure of α-dendrotoxin from the green mamba venom and its comparison with the structure of bovine pancreatic trypsin inhibitor. *J. Mol. Biol.*, **224**, 671–80.

Stampe, P., Kolmakova-Partensky, L., and Miller, C. (1994). Intimations of K^+ channel structure from a complete functional map of the molecular surface of charybdotoxin. *Biochemistry*, **33**, 443–50.

Stocker, M. and Miller, C. (1994). Electrostatic distance geometry in a PC channels vestibule. *Proc. Natl. Acad. Sci. USA*, **91**, 9509–13.

Stocker, M., Pongs, O., Hoth, M., Heinemann, S. H., Stiihmer, W., Schreter, K. H. *et al.* (1991). Swapping of functional domains in voltage-gated K^+ channels. *Proc. R. Soc. London Ser. B*, **245**, 101–7.

Strong, P. N. (1990). Potassium channel toxins. *Pharmacol. Ther.*, **46**, 137–62.

Stuhmer, W, Ruppersberg, J. P., Schröter, K. H., Sakmann, B., Stocker, M., Giese, P., *et al.* (1989). Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain. *EMBO J.*, **8**, 3235–44.

Swartz, K. J. and MacKinnon, R. (1995). An inhibitor of the K_v2.1 potassium channel isolated from the venom of a Chilean tarantula. *Neuron*, **15**, 941–9.

Veh, R. W., Lichtinghagen, R., Sewing, S., Wunder, F., Grumbach, I., and Pongs, O. (1995). Immunohistochemical localization of five members of the K_vI channel subunits: Contrasting

subcellular locations and neuron-specific co-localizations in rat brain. *Eur. J. Neurosci.*, **7**, 2189–205.
Vincent, J. P., Schweitz, H., and Lazdunski, M. (1975). Structure–function relationships and site of action of apamin, a neurotoxic polypeptide of bee venom with an action on the central nervous system. *Biochemistry*, **14**, 2521–5.
Wang, H., Kunkel, D., Martin, T., Schwartzkroin, P., and Tempel, B. (1993). Heteromultimeric K^+ channels in terminal juxtaparanodal regions of neurones. *Nature*, **365**, 75–9.

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Kaliotoxin

Kaliotoxin (KTX) is a single 4-kDa polypeptide chain, reticulated by three disulfide bridges. KTX displays some sequence similarity with other scorpion-derived inhibitors of various K^+ channels. The toxin is able to block the intermediate conductance Ca^{2+}-activated K^+ channel of nerve cells from the mollusc Helix pomatia, *and various voltage-dependent K^+ channels from rat brain.*

KTX was originally described as an inhibitor of the intermediate conductance Ca^{2+}-activated K^+ channel (IKCa) of nerve cells from the mollusc *Helix pomatia*. Single channel experiments performed on IKCa excised from their Helix neurons showed that KTX acted exclusively at the outer face of the channel. KTX was first purified from the venom of the Moroccan scorpion *Androctonus mauretanicus mauretanicus* (Crest *et al.* 1992). It is a single 4-kDa polypeptide chain showing amino acid sequence homology with other scorpion-derived inhibitors of Ca^{2+}-activated or voltage-gated K^+ channels. Comparison of these sequences led to the identification of a short amino acid sequence (26–33) which may be implicated in the toxin channel interaction.

Later, KTX and shorter peptides, (KTX (25–35) amide and KTX (26–32) amide), were successfully synthesized (Romi *et al.* 1993). These peptides expressed no KTX activity but were able to compete in binding experiments with the ^{125}I-KTX bound to its receptor on rat brain synaptosomes. They were further shown to antagonize both the toxicity and blocking activity of the KTX. It was concluded that the highly conserved region may contain a low affinity binding site essential for potassium channel recognition.

Further competition assays between ^{125}I-KTX and different toxins (dendrotoxin, charybdotoxin, MCD peptide, iberiotoxin, apamin) for binding to rat brain synaptosomes suggested that KTX also interacts with voltage-gated K^+ channels (Romi *et al.* 1993). Electrophysiological experiments performed using five cloned voltage-gated K^+ channels (K_v) stably expressed in mammalian cell lines showed that KTX exhibits a high specificity for the $K_v1.3$ (Grissmer *et al.* 1994). Further investigations of the KTX

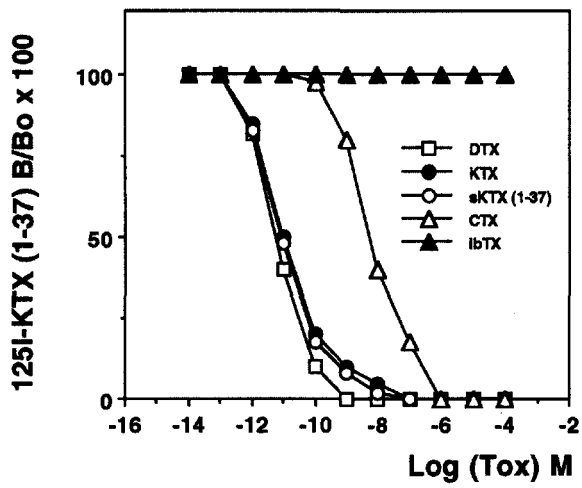

Figure 2. Effects of various toxins on the ^{125}I-KTX binding to rat brain synaptosomal membrane. KTX (black dots); synthetic $KTX_{(1-37)}$ (white dots); DTX, α-dendrotoxin from mamba snake (open squares); CTX, charybdotoxin from the scorpion *Leiurus quinquestriatus* (open triangles); IbTX, iberiotoxin from the scorpion *Buthus tamulus* (black triangles).

KTX	**GVEINVKCSGSPQCLKPCKDAGMRFGKCMNRKCHCTPK**
KTX2	**-VRIPVSCKHSGQCLKPCKDAGMRFGKCMNGKCDCTPK**
KTX3	**-VGIPVSCKHSGQCIKPCKDAGMRFGKCMNRKCDCTPK**

Figure 1. Amino acid sequences of KTX, KTX_2 and KTX_3.

activities showed that the toxin was also able to block the Gardos channel of erythrocytes, an intermediate conductance Ca^{2+}-activated K^+ channel (Brugnara 1995). In contrast, KTX is unable to block the big conductance Ca^{2+}-activated K^+ channel (BKCa) of motor nerve terminals in mouse *triangularis sterni* nerve–muscle preparations (Harvey *et al.* 1995).

The kaliotoxin family has recently been enlarged by the purification of two new toxins from the venom of two other *Buthidae*: KTX_2 and KTX_3. KTX_2 was isolated from *Androctonus australis* scorpion venom and shares 76 per cent identity with KTX (Laraba-Djebari *et al.* 1994).

The differences between the two peptides are in the NH_2-terminal region and the residues 31 and 34 located in the region involved in the channel recognition. KTX_3 has been purified from the venom of *Buthus tunetanus* and differs from KTX_2 at only two positions.

Using degenerate primers, a 370-base pair cDNA encoding the KTX_2 precursor was amplified by polymerase chain reaction from a cDNA library of *Androctonus australis* venom glands. It encodes a presumed signal peptide of 22 residues followed by the sequence of the mature peptide.

Purification

KTX has been purified to homogeneity from the venom of the scorpion *Androctonus mauretanicus mauretanicus* from Morocco, by one step of RP18-HPLC (Crest *et al.* 1992).

KTX_2 has been obtained from the venom of *Androctonus australis* Hector and Garzonii from Algeria and Tunisia (Laraba-Djebari *et al.* 1994). KTX_3 has been recently purified from the venom of *Buthus tunetanus* (to be published). Synthetic KTX and truncated analogues are commercially available.

Toxicity

KTX is not toxic for mice when injected subcutaneously (up to 200 μg per mouse, i.e. 10 mg/kg). The LD_{50} of the peptide after intraventricular (i.c.v.) injection is 24 ng for a mouse (i.e. 1.2 mg/kg). LD_{50s} of KTX_2 and KTX_3 by i.c.v. injection are 5 times higher.

Activities

In electrophysiological experiments, KTX suppressed the IKCa current of nerve cells from the mollusc *Helix pomatia* with a K_d of 20 nM (Crest *et al.* 1992). It did not affect the BKCa of the motor nerve terminals in experiments performed on the left *triangularis sterni* nerve–muscle preparations. In a mammalian cell line which stably expresses five cloned K^+ channels ($mK_v1.1$, $rK_v1.2$, $mK_v1.3$, $hK_v1.5$, $mK_v3.1$) KTX showed a high affinity (0.6 nM) for $K_{v1.3}$, a low affinity for $K_v1.1$ (41 nM) and no affinity (> 1 mM) for the other K_v (Grissmer *et al.* 1994). ^{125}I-KTX can be used for binding experiments.

Antibodies

Polyclonal antibodies have been raised in rabbit against KTX (Laraba-Djebari *et al.* 1994) but are not commercially available.

Gene

The complete cDNA sequence of the KTX_2 precursor is published (Laraba-Djebari *et al.* 1994).

References

Brugnara, C., Armsby, C. C., De Franceschi, L., Crest, M., Martin-Eauclaire, M. F., and Alper, S. L. (1995). Ca^{2+}-activated channels of human and rabbit erythrocytes display distinctive patterns of inhibition by venom peptide toxins. *J. Biol. Memb.*, **147**, 71–82.

Crest, M., Jaquet, G., Gola, M., Zerrouk, H., Benslimane, A., Rochat, H., *et al.* (1992). Kaliotoxin, a novel peptidyl inhibitor of neuronal BK-type Ca^{2+}-activated K^+ channels characterized from *Androctonus mauretanicus mauretanicus* venom. *J. Biol. Chem.*, **267**, 1640–7.

Grissmer, S., Nguyen, A., Aiyar, J., Hanoon, D. C., Mather, R. J., Gutman, G. A., *et al.* (1994). Pharmacological characterization of five cloned voltage-gated K^+channels, type $K_V1.1$, 1.2, 1.3, 1.5 and 3.1 stably expressed in mammalian cell lines. *Mol. Pharmacol.*, **45**, 1227–34.

Harvey, A. L., Vantapour, H., Rowan, E. G., Vita, C., Menez, A., and Martin-Eauclaire, M. F. (1995). Structure–activity studies on scorpion toxins that block potassium channel. *Toxicon*, **33**, 425–36.

Laraba-Djebari, F., Legros, C., Ceard, B., Romi, R., Mansuelle, P., Jacquet, G., *et al.* (1994). The kaliotoxin family enlarged – Purification, characterization and precursor nucleotide sequence of KTX_2 from *Australis australis* venom. *J. Biol. Chem.*, **269**, 32835–43.

Romi, R., Crest, M., Gola, M., Sampieri, F., Jacquet, G., Zerrouk, H., *et al.* (1993). Synthesis and characterization of kaliotoxin. Is the 26–32 sequence essential for potassium channel recognition ? *J. Biol. Chem.*, **268**, 26302–9.

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Scyllatoxin (*Leiurus quinquestriatus hebraeus*)

Scyllatoxin is a peptidic toxin obtained from the scorpion Leiurus quinquestriatus hebraeus. *Its biological target is the apamin-sensitive Ca^{2+}-dependent K^+ channels. Chemical synthesis of scyllatoxin leads to a iodinable (TYR2) derivative of the toxin which can be used for biochemical approaches. Spatial structure has been investigated in 2D NMR techniques.*

Scyllatoxin (sequence accession number to the Swiss Prot databank: P16341 SCKL LEIQH) is a peptidic neurotoxin which specifically blocks low conductance Ca^{2+}-dependent K^+ channels (sK) (Chicchi *et al.* 1988; Auguste *et al.* 1990). These channels have previously been demonstrated to be associated with apamin receptors, another peptidic toxin with 18 amino acids purified from bee venom (Hugues *et al.* 1982*a*,*b*). Scyllatoxin is composed of 31 amino acids and is reticulated by three disulphide bridges. Structure–function studies have shown that Arg6, Arg13, and His31 are crucial for toxin/receptor interaction and for biological activity of the toxin. Chemical modification of Lys20, 25, 30 residues or Glu27 does not alter binding capacity but seriously alters biological activity (Auguste *et al.* 1992*a*). The spatial structure of scyllatoxin has been determined using 2D NMR and data obtained have shown that Arg6 and Arg13 were positioned in space in a way which mimics the active centre of apamin which is constituted of Arg13 and Arg14, two contiguous arginine residues (Martins *et al.* 1990). The brain distribution of scyllatoxin receptors is similar to that of apamin receptors, which is not surprising since both toxins have the same biological target. However, scyllatoxin is able to discriminate between three classes of receptors which it recognizes with different affinities: less than 70 pM for high affinity receptors; between 100 and 500 pM for medium affinity receptors; and more than 800 pM for low affinity receptors (Auguste *et al.* 1992*a*). An endogeneous equivalent of scyllatoxin exists in mammalian tissues (Auguste *et al.* 1992*b*).

Purification and sources

Scyllatoxin is obtained from the venom of the scorpion *Leiurus quinquestriatus hebraeus*. Two purification procedures have been reported using several HPLC purification steps to prepare homogeneous scyllatoxin (Chicchi *et al.* 1988; Auguste *et al.* 1990). The purification procedure described by Auguste *et al.* (1990) leads to two isoforms of scyllatoxin (1) a C-terminal amidated form which binds with a best affinity to its receptors and, (2) a C-terminal carboxylate form with lower affinity. Chemical synthesis of scyllatoxin has been described (Auguste *et al.* 1990) and leads to reasonable amounts of native toxin with yields around 10 per cent.

Iodination

Since native scyllatoxin does not contain Tyr residue, iodination of the natural toxin bears on His31, which is important for scyllatoxin activity and leads to an inactive labelled derivative. To obtain biologically active labelled scyllatoxin it is then necessary to iodinate a synthetic scyllatoxin in which Phe2 has been replaced by a Tyr. This analogue keeps all biological properties of native scyllatoxin and can be used for biochemical investigations (Auguste *et al.* 1990).

References

Auguste, P., Hugues, M., Gravé, B., Gesquières, J. C., Maes, P., Tartar, A., *et al.* (1990). Leiurotoxin I (scyllatoxin), a peptide ligand for Ca^{2+}-activated K^+ channels: chemical synthesis, radiolabelling and receptor characterization. *J. Biol. Chem.*, **265**, 4753–9.

Auguste, P., Hugues, M., Mourre, C., Moinier, D., Tartar, A., and Lazdunski, M. (1992*a*). Scyllatoxin, a blocker of Ca^{2+}-activated K^+ channels: structure–function relationships and brain localization of the binding sites. *Biochemistry*, **38**, 648–54

Auguste, P., Hugues, M., Borsotto, M., Thibault, J., Romey, G., Coppola, T., *et al.* (1992*b*). Characterization and partial purification from pheochromocytoma cells of an endogenous equivalent of scyllatoxin, a scorpion toxin which blocks small conductance Ca^{2+}-activated K^+ channels. *Brain Res.*, **599**, 230–6.

Chicchi, G. G., Gimenez-Gallego, G., Ber, E., Garcia, M. L., Winquist, R., and Cascieri, M. A. (1988). Purification and characterization of a unique potent inhibitor of apamin binding from *Leiurus quinquestriatus hebraeus* venom. *J. Biol. Chem.*, **263**, 10192–7.

Hugues, M., Duval, D., Kitabgi, P., Lazdunski, M., and Vincent, J.-P. (1982*a*). Preparation of a pure monoiodo-derivative of the bee venom neurotoxin apamin and its binding properties to rat brain synaptosomes. *J. Biol. Chem.*, **257**, 2762–9.

Hugues, M., Romey, G., Duval, D., Vincent, J.-P., and Lazdunski, M. (1982*b*). Apamin as selective blocker of the calcium-dependent potassium channel in neuroblastoma cells. Voltage-clamp and biochemical characterization of the toxin receptor. *Proc. Natl. Acad. Sci. USA*, **79**, 1308–12.

Martins, J.-C., Zhang, W., Tartar, A., Lazdunski, M., and Borremans, F. A. M. (1990). Solution conformation of leiurotoxin I (scyllatoxin) by ^{1}H nuclear magnetic resonance. Resonance assignment and secondary structure. *FEBS Lett.*, **260**, 249–53.

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Apamin (honey bee *Apis mellifera*)

Apamin is a highly basic, 18-residue peptide (MW 2027) that blocks small-conductance, Ca^{2+}-activated K^+ channels at nanomolar concentrations and induces generalized convulsions. It is the smallest polypeptide toxin known.

Apamin represents not more than 2 per cent of dry weight of honey bee crude venom (Habermann 1972). The apamin molecule shares considerable sequence and structural characteristics with MCD peptide. It is crosslinked by two disulfide bridges between Cys1/Cys11 and Cys3/Cys15. NMR studies showed that the N-terminal residues Asn2–Ala5 form a β-turn and the residues Ala9–Gln17 on the C-terminus are in an α-helical structure (Pease and Wemmer 1988). Residues that are essential for biological activity are Arg13, Arg14 (Vincent *et al.* 1975) and Gln17 (Labbe-Jullie *et al.* 1991). Peripheral application of apamin is sufficient to induce the characteristic symptoms of central apamin poisoning: extreme hypermotility and hyperexcitability culminating in generalized convulsions (Habermann 1972, 1977, 1984). The molecular mechanism of these actions is the blockade of small-conductance, Ca^{2+}-activated ('apamin-sensitve') K^+ channels (Dreyer 1990; Strong 1990; Garcia *et al.* 1991).

■ Purification and sources

Apamin was first purified by Habermann and Reiz (Habermann and Reiz 1965) using gel filtration followed by acidic ion exchange chromatography. Apamin can be purchased from Alomone Labs, Bachem, Calbiochem, Latoxan, and RBI. It is offered either as native or synthetic product. The central effects of apamin can be used as a test for its biological activity (Habermann 1972).

■ Toxicity

Toxicity of apamin is tested by intravenous or intraventricular injection. After peripheral application of at least 0. 5 mg/kg, mice display the typical neurotoxic symptoms. Mouse LD_{50} is 4 mg/kg when administered intravenously but about 3000-fold lower for intraventricular application (Habermann 1972, 1977).

■ Use in cell biology

First evidence that apamin blocks Ca^{2+}-activated K^+ channels came from experiments measuring the K^+ loss from hepatocytes and red blood cells (Banks *et al.* 1979; Burgess *et al.* 1981). Numerous electrophysiological studies then established apamin as a highly selective and potent blocker of the Ca^{2+}-activated, TEA-resistant, d-tubocurarine-sensitive, nearly voltage-independent K^+ channel of small conductance (SK channel) in a variety of cell types, both excitable and nonexcitable (Dreyer 1990; Strong 1990; Garcia *et al.* 1991). Thus, apamin can be regarded as an outstanding tool to identify this ion channel type. Very high affinity binding sites for apamin with dissociation constants (K_D) in the range of 20–50 pM exist in brain, in agreement with its neurotoxicity. The binding to peripheral organs is much less, except that high affinity binding sites were further detected in liver, colon, adrenal cortex, smooth muscles, and neuroblastoma cell preparations. Binding of apamin to its acceptor sites is inhibited by the scorpion toxin leiurotoxin I (= scyllatoxin) which also blocks SK channels in various preparations (Dreyer 1990; Strong 1990; Garcia *et al.* 1991).

The cDNA of an apamin binding protein of porcine vascular smooth muscle was cloned (Sokol *et al.* 1994). The gene (denoted Kcal 1. 8) codes for a 438-amino-acid protein with four potential transmembrane domains and no significant sequence homology to any known ion channels or receptors. Very recently, SK channels were cloned from rat and human brain (Köhler *et al.* 1996). Cloned rat SK channels exhibit high sensitivity to apamin with an inhibition constant (K_i) of about 60 pM.

Apamin incorporated into a lipid bilayer forms voltage-dependent, cation-selective ion channels with properties indistinguishable from those of the ion channels formed by MCD peptide in lipid bilayers (Kondo *et al.* 1992).

References

Banks, B. E., Brown, C., Burgess, G. M., Burnstock, G., Claret, M., Cocks, T. M., *et al.* (1979). Apamin blocks certain neurotransmitter-induced increases in potassium permeability. *Nature*, **282**, 415–17.

Burgess, G. M., Claret, M., and Jenkinson, D. H. (1981). Effects of quinine and apamin on the calcium-dependent potassium permeability of mammalian hepatocytes and red cells. *J. Physiol (Lond.)*, **317**, 67–90.

Dreyer, F. (1990). Peptide toxins and potassium channels. *Rev. Physiol. Biochem. Pharmacol.*, **115**, 93–136.

Garcia, M. L., Galvez, A., Garcia-Calvo, M., King, V. F., Vazquez, J., and Kaczorowski, G. J. (1991). Use of toxins to study potassium channels. *J. Bioenerg. Biomembr.*, **23**, 615–46.

Habermann, E. (1972). Bee and wasp venoms. *Science*, **177**, 314–22.

Habermann, E. (1977). Neurotoxicity of apamin and MCD peptide upon central application. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **300**, 189–91.

Habermann, E. (1984). Apamin. *Pharmacol. Therap.*, **25**, 255–70.

Habermann, E. and Reiz, K. G. (1965). Ein neues Verfahren zur Gewinnung der Komponenten von Bienengift, insbesondere des zentral wirksamen Peptids Apamin. *Biochem. Z.*, **341**, 451–66.

Köhler, M., Hirschberg, B., Bond, C. T., Kinzie, J. M., Marrion, N. V., Maylie, J., *et al.* (1996). Small-conductance, calcium-activated potassium channels from mammalian brain. *Science*, **273**, 1709–14.

Kondo, T., Ikenaka, K., Fujimoto, I., Aimoto, S., Kato, H., Ito, K., *et al.* (1992). K^+ channel involvement in induction of synaptic enhancement by mast cell degranulating (MCD) peptide. *Neurosci. Res.*, **13**, 207–16.

Labbe-Jullie, C., Granier, C., Albericio, F., Defendini, M. L., Ceard, B., Rochat, H., *et al.* (1991). Binding and toxicity of apamin. Characterization of the active site. *Eur. J. Biochem.*, **196**, 639–45.

Pease, J. H. and Wemmer, D. E. (1988). Solution structure of apamin determined by nuclear magnetic resonance and distance geometry. *Biochemistry*, **27**, 8491–8.

Sokol, P. T., Hu, W., Yi, L., Toral, J., Chandra, M., and Ziai, M. R. (1994). Cloning of an apamin binding protein of vascular smooth muscle. *J. Protein Chem.*, **13**, 117–28.

Strong, P. N. (1990). Potassium channel toxins. *Pharmacol. Ther.*, **46**, 137–62.

Vincent, J. P., Schweitz, H., and Lazdunski. M. (1975). Structure–function relationships and site of action of apamin, a neurotoxic polypeptide of bee venom with an action on the central nervous system. *Biochemistry*, **14**, 2521–5.

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MCD peptide (honey bee *Apis mellifera*)

Mast cell degranulating peptide (MCD peptide) is a highly basic, 22-residue peptide (MW 2588) that blocks voltage-gated K^+ channels at nanomolar concentrations and has epileptogenic potency. Quite distinct from this, it causes mast cell degranulation (hence its name) and histamine release.

Like apamin, MCD peptide constitutes not more than 2 per cent of dry weight of honey bee crude venom (Habermann 1972). The MCD peptide molecule possesses two disulfide bridges between Cys3/Cys15 and Cys5/Cys19. NMR spectroscopy studies demonstrated that the N-terminal residues Ile1–Cys5 form a β-turn and the residues His13–Asn22 on the C-terminus are in an α-helical structure (Kumar *et al.* 1988). This conformation is very similar to that of apamin. In contrast to apamin, MCD peptide is barely neurotoxic when applied intravenously, but intraventricular injection results in arousal and, at higher concentrations, epileptiform seizures leading ultimately to death (Habermann 1977). These actions are brought about by the blockade of a subtype of the delayed outward rectifier K^+ channel (Dreyer 1990; Strong 1990; Garcia *et al.* 1991). Distinct from its central action, MCD peptide causes degranulation of mast cells and subsequent histamine release. Furthermore, MCD peptide has been reported to possess anti-inflammatory activity in the carrageenin-induced oedema model of the rat hind paw (Ziai *et al.* 1990) but this effect arises from mast cell degranulation *in vivo* (Banks *et al.* 1990).

Purification and sources

MCD peptide was originally purified by gel filtration and two consecutive steps of cation exchange chromatography (Breithaupt and Habermann 1968). It can be purchased from Alomone Labs, Bachem, Calbiochem, Latoxan, and RBI. It is offered either as native or synthetic product. Degranulation of mast cells and subsequent histamine release can be used as a test for its biological activity (Breithaupt and Habermann 1968).

Toxicity

Toxicity of MCD peptide is tested by intraventricular injection. Mouse LD_{50} for central application is 10 μg/kg,

which is a 10-times higher dose compared to apamin (Habermann 1977).

Use in cell biology

Electrophysiological studies with MCD peptide conducted on rat nodose ganglion cells showed that it acts, like dendrotoxin, on a voltage-dependent, almost non-inactivating outward rectifier K^+ current but not on the transient, rapidly inactivating A-type K^+ current (Stansfeld *et al.* 1987). Dendrotoxin-sensitive K^+ channels with delayed rectifier properties cloned from rat brain cDNA and expressed in *Xenopus* oocytes are also sensitive to MCD peptide (Stühmer *et al.* 1988). Together with other reports, this confirmed the idea that MCD peptide and some members of the dendrotoxin family act on the same K^+ channel type (Dreyer 1990; Strong 1990; Garcia *et al.* 1991). Various binding studies revealed common acceptor sites for MCD peptide, dendrotoxin I, and dendrotoxin α (Dreyer 1990; Strong 1990; Garcia *et al.* 1991). In addition, the solubilized acceptors for MCD peptide and dendrotoxin I from rat brain were found to have almost identical molecular weights (about 77 kDa) and properties (Rehm *et al.* 1988).

Treatment of mast cells with MCD peptide leads to an increase in the intracellular Ca^{2+} concentration followed by degranulation. This effect can be blocked by pertussis toxin pretreatment, suggesting an activation of perussis toxin-sensitive G-proteins as the mechanism of action of MCD peptide in mast cells (Fujimoto *et al.* 1991).

MCD peptide incorporated into a lipid bilayer forms voltage-dependent, cation-selective ion channels with the same properties as the ion channels formed by apamin in lipid bilayers (Kondo *et al.* 1992).

References

Banks, B. E., Dempsey, C. E., Vernon, C. A., Warner, J. A., and Yamey, J. (1990). Anti-inflammatory activity of bee venom peptide 401 (mast cell degranulating peptide) and compound 48/80 results from mast cell degranulation *in vivo*. *Br. J. Pharmacol.*, **99**, 350–4.

Breithaupt, H. and Habermann, E. (1968). Mastzelldegranulierendes Peptid (MCD-Peptid) aus Bienengift: Isolierung, biochemische und pharmakologische Eigenschaften. *Naunyn-Schmiedebergs Arch. Pharmak. u. Exp. Pathol.*, **261**, 252–70.

Dreyer, F. (1990). Peptide toxins and potassium channels. *Rev. Physiol. Biochem. Pharmacol.*, **115**, 93–136.

Fujimoto, I, Ikenaka, K., Kondo, T., Aimoto, S., Kuno, M., and Mikoshiba, K. (1991). Mast cell degranulating (MCD) peptide and its optical isomer activate GTP binding protein in rat mast cells. *FEBS Lett.*, **287**, 15–18.

Garcia, M. L., Galvez, A., Garcia-Calvo, M., King, V. F., Vazquez, J., and Kaczorowski, G. J. (1991). Use of toxins to study potassium channels. *J. Bioenerg. Biomembr.*, **23**, 615–46.

Habermann, E. (1972). Bee and wasp venoms. *Science*, **177**, 314–22.

Habermann, E. (1977). Neurotoxicity of apamin and MCD peptide upon central application. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **300**, 189–91.

Kondo, T., Ikenaka, K., Fujimoto, I., Aimoto, S., Kato, H., Ito, K., *et al.* (1992). K^+ channel involvement in induction of synaptic enhancement by mast cell degranulating (MCD) peptide. *Neurosci. Res.*, **13**, 207–16.

Kumar, N. V., Wemmer, D. E., and Kallenbach, N. R. (1988). Structure of P401 (mast cell degranulating peptide) in solution. *Biophys. Chem.*, **31**, 113–19.

Rehm, H., Bidard, J.-N., Schweitz, H., and Lazdunski, M. (1988). The receptor site for the bee venom mast cell degranulating peptide. Affinity labeling and evidence for a common molecular target for mast cell degranulating peptide and dendrotoxin I, a snake toxin active on K^+ channels. *Biochemistry*, **27**, 1827–32.

Stansfeld, C. E., Marsh, S. J., Parcej, D. N., Dolly, J. O., and Brown, D. A. (1987). Mast cell degranulating peptide and dendrotoxin selectively inhibit a fast-activating potassium current and bind to common neuronal proteins. *Neuroscience*, **23**, 893–902.

Strong, P. N. (1990). Potassium channel toxins. *Pharmacol. Ther.*, **46**, 137–62.

Stühmer, W., Stocker, M., Sakmann, B., Seeburg, P., Baumann, A., Grupe, A., *et al.* (1988). Potassium channels expressed from rat brain cDNA have delayed rectifier properties. *FEBS Lett.*, **242**, 199–206.

Ziai, M. R., Russek, S., Wang, H. C., Beer, B., and Blume, A. J. (1990). Mast cell degranulating peptide: a multi-functional neurotoxin. *J. Pharm. Pharmacol.*, **42**, 457–61.

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Charybdotoxin and iberiotoxin (*Leiurus quinquestriatus* var. *hebraeus* and *Buthus tamulus*)

Charybdotoxin and iberiotoxin are 37 amino acid peptides isolated from scorpion venoms. Both peptides have a rigid structure as a result of the presence of three disulfide bridges. Charybdotoxin inhibits a variety of potassium channels (i.e. K 1.3 voltage-dependent channel of the Shaker family, and a variety of Ca^{2+}-activated K^+ channels), whereas iberiotoxin is highly selective for the high-conductance Ca^{2+}-activated K^+ channel.

Charybdotoxin (ChTX) and iberiotoxin (IbTX) are 37 amino acid peptides containing six Cys residues that form three disulfide bridges (Gimenez-Gallego *et al.* 1988; Galvez *et al.* 1990). Both peptides have their N-terminal residue blocked in the form of pyroglutamic acid and share 68 per cent homology in their amino acid sequences (Fig. 1). The three-dimensional structure of these peptides has been determined in solution by NMR techniques (Bontems *et al.* 1991; Johnson and Sugg 1992). The backbone of both peptides is identical. The surfaces of ChTX and IbTX are formed by three small antiparallel strands that are linked to a helix region by the three disulfide bonds. Most of the residues are exposed to the solvent, except for the Cys residues. It is interesting to note that all critical residues of ChTX necessary for its interaction with K^+ channels are located on one face of the molecule, well separated from the unimportant amino acids (Stampe *et al.* 1994). The critical residues of ChTX are all conserved in IbTX and, therefore, it is expected that the corresponding face of IbTX is the one involved in close interaction with the high-conductance Ca^{2+}-activated K^+ (maxi-K) channel.

Both ChTX and IbTX bind in the external vestibule of K^+ channels and block ion conduction by physical occlusion of the pore (MacKinnon and Miller 1988; Giangiacomo *et al.* 1992). Binding of both toxins is driven by electrostatic mechanisms, especially in the case of ChTX which has a net charge of +5. In single channel recordings of maxi-K channels reconstituted in planar lipid bilayers, addition of either toxin to the extracellular face of the channel leads to the appearance of silent periods interdispersed between bursts of normal channel activity. These silent periods have been interpreted as times in which a single toxin molecule is bound to the channel to block ion conduction. Since toxin binding is a freely reversible process, toxin dissociation would lead to normal channel activity since the kinetics of channel gating are faster than those of toxin binding. Analysis of single channel data in the presence of increasing concentrations of toxin confirms that toxin binding occurs through a simple bimolecular reaction. The silent periods observed in the presence of IbTX are of longer duration than those produced by ChTX, suggesting that IbTX must overcome a higher energy barrier in order to dissociate from the channel. Under normal physiological salt concentrations, blockade of maxi-K channels occurs at toxin concentrations of 1–5 nM.

A particularly interesting toxin residue involved in channel inhibition is Lys27. Neutralization of this residue eliminates the observed voltage-dependence of toxin binding (Park and Miller 1992). It has been suggested that Lys27 lies physically close to the channel pore entrance; K^+ ions moving from inside can approach a site along the conduction pathway whose occupancy destabilizes bound toxin via electrostatic repulsion with the Σ-amino group of Lys27.

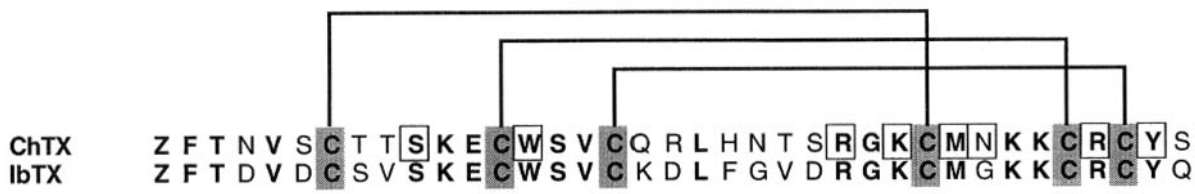

Figure 1. Comparison of amino acid sequences of charybdotoxin (ChTX) and iberiotoxin (IbTX). Identical residues are bold, and the disulfide bonds are indicated. The residues in ChTX that are critical for interaction with the maxi-K channel are boxed. (Stampe *et al.* 1994).

Purification and sources

ChTX and IbTX were originally purified from crude venom of the scorpions *Leiurus quinquestriatus* var. *hebraeus* and *Buthus tamulus*, respectively, by a combination of ion-exchange and reversed-phase chromatography (Gimenez-Gallego *et al.* 1988; Galvez *et al.* 1990). Alternatively, it is possible to produce both toxins by either solid-phase synthesis or by expression in *E. coli* (Sugg *et al.* 1990; Park *et al.* 1991). This latter approach allows not only production of large toxin quantities, but also generation of point mutants for structure–activity relationship studies. ChTX and IbTX can also be purchased from Peptides International, Peninsula Laboratories, Calbiochem, Bachem Bioscience, Sigma, Latoxan, and Alomone Labs. The purity of these preparations should be checked by reversed-phase HPLC. In addition, ^{125}I-ChTX can be purchased from DuPont NEN.

Toxicity

Intravenous injection of either ChTX or IbTX in rats up to 150 μg/kg are without significant effects. A transient and reversible elevation in blood pressure has been observed with either toxin, presumably the result of increased peripheral neurotransmitter release. It is expected that icv injection of the toxins would cause more pronounced effects, especially with ChTX, because of the predicted enhancement in neurotransmitter release in the CNS.

Use in cell biology

Since ChTX is not absolutely selective for only a single K^+ channel (i.e. it inhibits K 1.3, maxi-K channels and small conductance Ca^{2+}-activated K^+ channels with high potency), its use in cell biology to determine the physiologic role that a particular K^+ channel plays in cell function should be viewed with caution. IbTX, on the other hand, appears to be a selective inhibitor of maxi-K channels, so it has been useful in *in vitro* studies to determine the role that this channel plays in different tissues. The myogenic activity of guinea pig bladder and taenia coli smooth muscles is markedly enhanced by IbTX, suggesting, that in these tissues, maxi-K channels are a major repolarization pathway following Ca^{2+} entry during action potential induced cell depolarization (Suarez-Kurtz *et al.* 1991). However, in other guinea pig smooth muscle tissues, such as uterus and portal vein, IbTX has no effect, despite the fact that the presence of maxi-K channels is well documented in these tissues. In guinea pig airway smooth muscle, relaxation of a carbachol-contracted preparation by a number of different agents (β agonists, phosphodiesterase inhibitors, nitroprusside) can be antagonized and even abolished in the presence of IbTX (Jones *et al.* 1993). One possible explanation for these findings is that these relaxation mechanisms in airway involves activation of maxi-K channels. In addition, in airway sensory neurons, inhibition of the e-NANC contractions by μ-opioid agonists, α-adrenergic agonists, or neuropeptide Y can be eliminated by IbTX (Miura *et al.* 1993). These data suggest a role for maxi-K channel agonists as potential therapeutic agents for the treatment of asthma. Finally, it is worth mentioning that IbTX has been used to support the idea that at some synapses, voltage-gated Ca^{2+} channels and maxi-K channels are clustered together at the presynaptic surface (Robitalle *et al.* 1993). Modulation of maxi-K channels in these systems should control the amount of neurotransmitter released.

References

Bontems, F., Roumestand, C., Boyot, P., Gilquin, B., Doljansky, Y., Menez, A., *et al.* (1991). Three-dimensional structure of natural charybdotoxin in aquous solution by ^{1}H-NMR. Charybdotoxin possesses a structural motif found in other scorpion toxins. *Eur. J. Biochem.*, **196**, 19–28.

Galvez, A., Gimenez-Gallego, G., Reuben, J. P., Roy-Contancin, L., Feigenbaum, P., Kaczorowski, G. J., *et al.* (1990). Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion *Buthus tamulus*. *J. Biol. Chem.*, **265**, 11083–90.

Giangiacomo, K. M., Garcia, M. L., and McManus, O. B. (1992). Mechanism of iberiotoxin block of the large-conductance calcium-activated potassium channel from bovine aortic smooth muscle. *Biochemistry*, **31**, 6719–27.

Gimenez-Gallego, G., Navia, M. A., Reuben, J. P., Katz, G. M., Kaczorowski, G. J., and Garcia, M. L. (1988). Purification, sequence, and model structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. *Proc. Natl. Acad. Sci. USA*, **85**, 3329–33.

Johnson, B. A. and Sugg, E. E. (1992). Determination of the three-dimensional structure of iberiotoxin in solution by ^{1}H nuclear magnetic resonance spectroscopy. *Biochemistry*, **31**, 8151–9.

Jones, T. R., Charette, M. L., Garcia, M. L., and Kaczorowski, G. J. (1993). Interaction of iberiotoxin with beta adrenoceptor agonists and sodium nitroprusside on guinea pig trachea. *J. Applied Physiol.*, **74**, 1879–84.

MacKinnon, R. and Miller, C. (1988). Mechanism of charybdotoxin block of the high-conductance, Ca^{2+}-activated K^+ channel. *J. Gen. Physiol.*, **91**, 335–49.

Miura, M., Belvisi, M. G., Ward, J. K., and Barnes, P. J. (1993). Role of Ca^{2+}-activated K^+ channels in opioid-induced prejunctional modulation of airway sensory nerves. *American Review of Respiratory Disease*, **147**, A815.

Park, C.-S. and Miller, C. (1992). Interaction of charybdotoxin with permeant ions inside the pore of a K^+ channel. *Neuron*, **9**, 307–13.

Park, C. S., Hausdorff, S. F., and Miller, C. (1991). Design, synthesis, and functional expression of a gene for charybdotoxin, a peptide blocker of K^+ channels. *Proc. Natl. Acad. Sci. USA*, **88**, 2046–50.

Robitalle, R., Garcia, M. L., Kaczorowski, G. J., and Charlton, M. P. (1993). Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron*, **11**, 645–55.

Stampe, P., Kolmakova-Partensky, L., and Miller, C. (1994). Intimations of K^+ channel structure from a complete functional map of the molecular surface of charybdotoxin. *Biochemistry*, **33**, 443–50.

Suarez-Kurtz, G., Garcia, M. L., and Kaczorowski, G. J. (1991). Effects of charybdotoxin and iberiotoxin on the spontaneous

motility and tonus of different guinea pig smooth muscle tissues. *J. Pharmacol. Exp. Ther.*, **259**, 439–43.
Sugg, E. E., Garcia, M. L., Reuben, J. P., Patchett, A. A., and Kaczorowski, G. J. (1990). Synthesis and structural characterization of charybdotoxin, a potent peptidyl inhibitor of the high conductance Ca^{2+}-activated K^+ channel. *J. Biol. Chem.*, **265**, 18745–8.

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Margatoxin, noxiustoxin and kaliotoxin (*Centruroides margaritatus, Centruroides noxius, Androctonus mauretanicus*)

Margatoxin and noxiustoxin, 39 amino acid members of a second subset of charybdotoxin-related peptides, and kaliotoxin, a 38 amino acid member of a third subset, are blockers of mammalian voltage-gated potassium channels (K_v) especially $K_v1.3$ in lymphocytes and neural tissue. Noxiustoxin also blocks the mammalian high conductance calcium-activated potassium channel (maxi-K) at high concentrations and kaliotoxin is an inhibitor of a molluscan calcium-activated potassium channel (K_{Ca}).

Margatoxin (MgTX) and noxiustoxin (NxTX) are 39 amino acid peptides which share with charybdotoxin (ChTX) significant sequence homology and such structural motifs as three disulfide bridges, a mid-chain (α-helix), and a C terminal antiparallel β-sheet. MgTX and NxTX comprise the best-studied members of a second class of scorpion venom derived potassium channel blockers based on structure and channel selectivity. One other scorpion toxin that has been included in this class because of sequence homology with MgTX and NxTX is *Centruroides limpidus limpidus* toxin I (Martin *et al.* 1994). This peptide also blocks voltage-gated potassium channels but has not been extensively studied.

NxTX was originally reported to block a squid axon delayed rectifier potassium channel at high concentrations (Carbone *et al.* 1982) and later was reported to inhibit the skeletal muscle maxi-K channel with a K_d of 450 nM (Valdivia *et al.* 1988). However, NxTX is most potent, K_d = 0. 2 nM, in blocking K_vI. 3 voltage-gated K^+ channels in Jurkat cells and K_vI. 3 channels expressed in *Xenopus* oocytes (Sands *et al.* 1989; Swanson *et al.* 1990). NxTX is also quite potent in blocking [^{125}I]ChTX and [^{125}I]$_2$MgTX binding to human T lymphocytes, Jurkat cells and plasma membranes prepared from those cells (Deutsch *et al.* 1991; Slaughter *et al.* 1991; Felix *et al.* 1995). NxTX has been found to inhibit K_vI. 2 with similar potency to its block of K_vI. 3 (Grissmer *et al.* 1994). NxTX has no effect on the low conductance K_{Ca} channels from lymphocytes (Leonard *et al.* 1992).

The very similar MgTX, with 79 per cent sequence identity with NxTX, is more potent in blocking K_vI. 3, K_d of 50 pM in electrophysiological experiments, and has no effect on maxi-K channels nor on the low conductance K_{Ca} of lymphocytes at 1 μM (Leonard *et al.* 1992). MgTX is 100-fold less sensitive on K_vI. 6, and does not affect K_v1. 5 or K_v3. 1 (Garcia-Calvo *et al.* 1993). High resolution 3D structure has been obtained by NMR ^{1}H, ^{13}C, ^{15}N triple-resonance spectroscopy (Johnson *et al.* 1994), and shows a structure similar to ChTX and iberiotoxin (IbTX), but with a slightly longer β-sheet. MgTX has been radiolabelled as either the mono- or di-iodotyrosine derivative (Felix *et al.* 1995; Koch *et al.* 1995). [^{125}I]MgTX binds to a single class of receptor sites under low salt conditions in rat brain (K_d = 0. 1 pM; Koch *et al.* 1995). Binding is optimized in the presence of 5 mM K^+, but is reduced at high K^+ and by Na^+. Under physiological concentrations of NaCl, the di-iodinated form, [^{125}I]$_2$MgTX, is more potent than the mono-iodinated form in binding to Jurkat plasma membranes or human T lymphocytes (K_d = 8 pM vs 30 pM; Felix *et al.* 1995).

Kaliotoxin (KTX; Crest *et al.* 1992), kaliotoxin 2 (Laraba-Djebari *et al.* 1994), and agitoxins 1, 2, and 3 (Garcia *et al.* 1994) are structurally closely related to each other, but differ more significantly from ChTX than MgTX and NxTX, and therefore define a third class of potassium channel inhibiting scorpion peptides. However, among this class there are significant differences in selectivity for channels. While KTX inhibits a *Helix pomatia* K_{Ca} without affecting voltage-gated channels (Crest *et al.* 1992), it has been found to exhibit a 40-fold selectivity for K_vI. 3 (K_d 0. 65 nM) over K_vI. I with little effect on K_vI. 2 and K_vI. 5 in stably transfected cell lines (Grissmer *et al.* 1994). In ad-

dition, KTX is reported to have modest effects on the maxi-K channel in mammalian cells (IC_{50}s of 300, 480, and 4000 nM reported for different tissues; Laraba-Djebari *et al.* 1994). The 3D structure for KTX1-37 was reported to have a shorter, distorted helical region that interacts with opposite sides of the β-sheet. This interaction contrasts with that determined for ChTX, IbTX, and MgTX in which the helix interacts with only one side of the β-sheet.

Purification and sources

NxTX, MgTX, and KTX were originally prepared from *Centruroides noxius* (Possani *et al.* 1982), *C. margaritatus* (Garcia-Calvo *et al.* 1993) and *Androctonus mauretanicus* (Crest *et al.* 1992), respectively, through conventional peptide purification techniques. These peptides have also been prepared by using other methods: MgTX was prepared biosynthetically (Garcia-Calvo *et al.* 1993); MgTX and KTX were prepared by solid phase synthesis (Romi *et al.* 1993; Bednarek *et al.* 1994, respectively).

Both MgTX and KTX are sold by Peptides International. Bachem Bioscience, Inc., sells MgTX and Latoxan and Research Biochemicals International sell KTX.

Toxicity

No known toxicity studies with MgTX or NxTX have been reported to date, although it may be inferred by their effects on neural potassium channels that some toxicity might be expected, especially in the case of direct application. An i. c. v. LD_{50} in mice of 6–9 pmol/mouse has been reported for KTX (Romi *et al.* 1993).

Use in cell biology

Because of their selectivity for K_vI. 3, NxTX and MGTX have been used to explore the role of this channel in human T lymphocyte activation. Since ChTX inhibits the low conductance K_{Ca} channels found in T lymphocytes in addition to inhibiting K_vI. 3, the action of ChTX in blocking T cell activation parameters has always been equivocal. K_vI. 3 is thought to set the resting potential of human T lymphocytes because MgTX, NxTX, and ChTX cause depolarization from −50 to −30 mV as determined by [^{3}H]ltetraphenylphosphonium uptake (Leonard *et al.* 1992). MgTX, NxTX, and ChTX as well as high external K^+ block the mitogen induced increase in Ca^{2+} required for IL-2 production and T cell proliferation (Lin *et al.* 1993). The depolarization is thought to affect the Ca^{2+} influx either by reducing the electrochemical driving force, or possibly by some effect on the Ca^{2+} influx pathway itself. By whichever mechanism, after block of the rise in Ca^{2+}, the final result of the addition of the peptides is the block of IL-2 production and T cell proliferation.

References

Bednarek, M. A., Bugianesi, R. M., Leonard, R. J., and Felix, J. P. (1994). Chemical synthesis and structure function studies of margatoxin, a potent inhibitor of voltage-dependent potassium channels in human T lymphocytes. *Biochem. Biophys. Res. Commun.*, **198**, 619–25.

Carbone, E., Wanke, E., Prestipino, G., Possani, L. D., and Maelicke, A. (1982). Selective blockage of voltage-dependent K^+ channels by a novel scorpion toxin. *Nature (London)*, **296**, 90–1.

Crest, M., Jacquet, G., Gola, M., Zerrouk, H., Benslimane, A., Rochat, H., *et al.* (1992). Kaliotoxin, a novel peptidyl inhibitor of neuronal BK-type Ca^{2+}-activated K^+ channels characterized from *Androctonus mauretanicus mauretanicus* venom. *J. Biol. Chem.*, **267**, 1640–7.

Deutsch, C., Price, M., Lee, S., King, V. F., and Garcia, M. L. (1991). Characterization of high affinity binding sites for charybdotoxin in human T lymphocytes: evidence for association with the voltage-gated K^+ channel. *J. Biol. Chem.*, **266**, 3668–74.

Felix, J. P., Bugianesi, R. M., Abramsom, A. A., and Slaughter, R. S. (1995). Binding of mono- and di-iodinated margatoxin to human peripheral T lymphocytes and to Jurkat plasma membranes. *Biophys. J.*, **68**, 267a.

Garcia, M. L., Garcia-Calvo, M., Hidalgo, P., and MacKinnon, R. (1994). Purification and characterization of three inhibitors of voltage-dependent K^+ channels from *Leiurus quinquestriatus* var. *hebraeus* venom. *Biochemistry*, **33**, 6834–9.

Garcia-Calvo, M., Leonard, R. J., Novick, J., Stevens, S. P., Schmalhofer, W., Kaczorowski, G. J. *et al.* (1993). Purification, characterization, and biosynthesis of margatoxin, a component of *Centruroides margaritatus* venom that selectively inhibits voltage-dependent potassium channels. *J. Biol. Chem.*, **268**, 18866–74.

Grissmer, S., Nguyen, A. N., Aiyar, J., Hanson, D. C., Mather, R. J., Gutman, G. A., *et al.* (1994). Pharmacological characterization of five cloned voltage-gated K^+ channels, types K_vI.l, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. *Mol. Pharmacol.*, **45**, 1227–34.

Johnson, B. A., Stevens, S. P., and Williamson, J. M. (1994). Determinants of the three-dimensional structure of margatoxin by ^{1}H, ^{13}C, ^{15}N triplo-resonance nuclear magnetic resonance spectroscopy. *Biochemistry*, **3**, 15061–70.

Koch, R. O. A., Eberhart, A., Seidl, C. V., Saria, A., Kaczorowski, G. J., Slaughter, R. S., *et al.* (1995). [^{125}I]Margatoxin, an extraordinary high-affinity ligand for voltage-gated K^+ channels in mammalian brain. *Biophys. J.*, **68**, 266a.

Laraba-Djebari, F., Legros, C., Crest, M., Ceard, B., Romi, R., Mansuelle, P., *et al.* (1994). The kaliotoxin family enlarged. *J. Biol. Chem.*, **269**, 32835–43.

Leonard, R. J., Garcia, M. L., Slaughter, R. S., and Reuben, J. P. (1992). Selective blockers of voltage-gated K^+ channels depolarize human T lymphocytes: mechanism of the antiproliferative effect of charybdotoxin. *Proc. Natl. Acad. Sci. USA*, **89**, 10094–8.

Lin, C. S., Boltz, R. C., Blake, J. T., Nguyen, M., Talento, A., Fischer, P. A., *et al.* (1993). Voltage-gated potassium channels regulate calcium-dependent pathways involved in human T lymphocyte activation. *J. Exp. Med.*, **177**, 637–45.

Martin, B. M., Ramirez, A. N., Gurrola, G. B., Nobile, M., Prestipino, G., and Possani, L. D. (1994). Novel K^+-channel-blocking toxins from the venom of the scorpion *Centruroides limpidus limpidus Karsch. Biochem. J.*, **304**, 51–6.

Possani, L. D., Martin, B. M., and Svendsen, I. B. (1982). The primary structure of Noxiustoxin: a K^+ channel blocking peptide, purified from the venom of the scorpion

Centruroides noxius Hoffmann. Carlsberg Res. Commun., **47**, 285–9.
Romi, R., Crest, M., Gola, M., Sampieri, F., Jacquet, G., Zerrouk, H., *et al.* (1993). Synthesis and characterization of kaliotoxin: Is the 26–32 sequence essential for potassium channel recognition? *J. Biol. Chem.*, **268**, 26302–9.
Sands, S. B., Lewis, R. S., and Cahalan, M. D. (1989). Charybdotoxin blocks voltage-gated K^+ channels in human and murine T lymphocytes. *J. Gen. Physiol.*, **93**, 1061–74.
Slaughter, R. S., Shevell, J. L., Felix, J. P., Lin, C. S., Sigal, N. H., and Kaczorowski, G. J. (1991). Inhibition by toxins of charybdotoxin binding to the voltage-gated potassium channel of lymphocytes: correlation with block of activation of human peripheral T-lymphocytes. *Biophys. J.*, **59**, 213a.
Swanson, R., Marshall, J., Smith, J. S., Williams, J. B., Boyle, M. B., Folander, K., *et al.* (1990). Cloning and expression of cDNA and genomic clones encoding three delayed rectifier potassium channels in rat brain. *Neuron*, **4**, 929–39.
Valdivia, H. H., Smith, J. S., Martin, B. M., Coronado, R., and Possani, L. D. (1988). Charybdotoxin and noxiustoxin, two homologous peptide inhibitors of the $K^+(Ca^{2+})$ channel. *FEBS Lett.*, **2**, 280–4.

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Dendrotoxins *(Dendroaspis* species*)*

Dendrotoxins are 57–60 residue proteins isolated from venoms of mamba snakes, Dendroaspis angusticeps, D. polylepis, *and* D. viridis. *They are related to Kunitz-type inhibitors of serine proteases. The dendrotoxins are potent and selective blockers of some sub-types of voltage-dependent K^+ ion channels. They can be used to isolate K^+ channels and to show their tissue distribution.*

Dendrotoxins block certain voltage-dependent K^+ channels in neurones (Harvey and Anderson 1991). They are isolated from venom of three species of African mamba snakes: the Eastern green mamba (*Dendroaspis angusticeps*), the Western green mamba (*D. viridis*), and the black mamba (*D. polylepis*) (Strydom 1973*a*; Harvey and Karlsson 1980). The dendrotoxins are small basic proteins of 57–60 residues in a single polypeptide chain crosslinked by three disulfide bonds (Fig. 1). Six natural variants have been sequenced: two isoforms of α-dendrotoxin and δ-dendrotoxin from *D. angusticeps*, toxin I and toxin K from *D. polylepis*, and Dv-14 from *D. viridis* (Strydom 1973*b*; Joubert and Taljaard 1980; Mehraban *et al.* 1986). Two related toxins, β- and γ-dendrotoxin, from *D. angusticeps* have been partially sequenced (Benishin *et al.* 1988).

The dendrotoxins were discovered because of their ability to facilitate the release of acetylcholine at the neuromuscular junction (Harvey and Karlsson 1980, 1982; Anderson and Harvey 1988), which is a consequence of their ability to block some voltage-dependent K^+ channels in nerve endings (Dreyer and Penner 1987; Anderson and Harvey 1988; Benishin *et al.* 1988). Dendrotoxins block some neuronal K^+ channels but not others, and often induce repetitive action potentials. In studies on cloned K^+ channels, dendrotoxins preferentially block

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        1                 10                  20                  30                  40                  50                  60
1 α-DTx1  Z P R R K L C I L H R N P G R C Y D K I P A F Y Y N Q K K K Q C E R F D W S G C G G N S N R F K T I E E C R R T C I G
2 α-DTx2  Z P R R K L C I L H R D P G R C Y D K I P A F Y Y N Q K K K Q C E R F D W S G C G G N S N R F K T I E E C R R T C I G
3 DpI     Z P L R K L C I L H R N P G R C Y Q K I P A F Y Y N Q K K K Q C E G F T W S G C G G N S N R F K T I E E C R R T C I R K
4 DpK       A A K Y C K L P L R I G P C K R K I P S F Y Y K W K A K Q C L P F D Y S G C G G N A N R F K T I E E C R R T C V G
5 δ-DTx     A A K Y C K L P V R Y G P C K K K I P S F Y Y K W K A K Q C L P F D Y S G C G G N A N R F K T I E E C R R T C V G
6 Dv-14     A A K Y C K L P V R Y G P C K K K I P S F Y Y K W K A K Q C L Y F D Y S G C G G N A N R F K T I E E C R R T C V G
7 β-DTx   - - - G x G c P L T L P F G r - - T x E E N S x Y K - - - - - c L P F L F S G C G G N A N r F Q T I G E c r - - - - -
8 γ-DTx   - - - - - - - - L P A E x G r - - - - Q F x S F Y - - - - - - c L P F L F S G C G G - A - - F Q T I G E c r - - - - -
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Figure 1. Amino acid sequence of the dendrotoxins. Disulphide bonds are Cys7–Cys57, Cys16–Cys40, and Cys32–Cys 53. α-DTx1 is $C_{13}S_2C_3$ of Joubert and Taljaard (1980); α-DTx2 is the variant sequenced recently (Danse *et al.* 1994); δ-DTx is $C_{13}S_1C_3$ of Joubert and Taljaard. The partial sequences of β- and γ-DTx show unidentified residues by dashes or x; lower case letters indicate some uncertainty (Benishin *et al.* 1988).

$K_v1.2$ (IC_{50} 0. 1–17 nM), $K_v1.1$ (IC_{50} 1–20 nM), and $K_v1.6$ (IC_{50} 52 nM), with little effect on $K_v1.3$, $K_v1.4$, $K_v1.5$, $K_v3.1$, or $K_v4.2$ (Pongs 1992; Grissmer *et al.* 1994). As α- and δ-dendrotoxins appear to block different K^+ currents in synaptosomes from those blocked by β- and γ-dendrotoxins (Benishin *et al.* 1988), it is likely that different subtypes of voltage-dependent K^+ channels will have different sensitivities to individual dendrotoxins.

Purification and sources

Dendrotoxins are isolated from the venom of *Dendroaspis angusticeps*, *D. polylepis*, or *D. viridis* by gel filtration followed by cationic ion exchange chromatography (Harvey and Karlsson 1980) or HPLC. With toxins from *D. polylepis* and *D. viridis*, care must be taken to exclude contamination with α-neurotoxins that block nicotinic acetylcholine receptors. α-Dendrotoxin and toxin K have also been produced in *E. coli* and expressed in functional forms (Smith *et al.* 1993; Danse *et al.* 1994). Some dendrotoxins are available from Alomone Labs, Bachem, Calbiochem, ICN, Latoxan, RBI, and Sigma. ^{125}I-α-Dendrotoxin is available from Amersham International.

Toxicity

Dendrotoxins are not very toxic when injected peripherally. The i. v. LD_{50} in mice is about 20–25 μg/g (Joubert and Taljaard 1980). Lethal activity is much higher on direct injection into the CNS: MLD on intracerebroventricular injection in rats is 2. 5 ng/g with α-dendrotoxin and 0. 5 ng/g with toxin I (Mehraban *et al.* 1985). An LD_{50} value of 5 ng/g was estimated with α-dendrotoxin in mice (Danse *et al.* 1994). Dendrotoxins are not likely to be absorbed orally, and they do not present particular problems to laboratory workers.

Use in cell biology

Dendrotoxins can be radiolabelled and used as markers for voltage-dependent K^+ channels. They have been used to isolate K^+ channel proteins from brain membranes (Black *et al.* 1988; Rehm and Lazdunski 1988), and they have been used in autoradiographic studies to show the distribution of K^+ channels in different brain regions (Bidard *et al.* 1989; Pelchen-Matthews and Dolly 1989). Individual dendrotoxins have regional differences in their binding (Awan and Dolly 1991).

References

Anderson, A. J. and Harvey, A. L. (1988). Effects of the potassium channel blocking dendrotoxins on acetylcholine release and motor nerve terminal activity. *Br. J. Pharmacol.*, **93**, 215–21.

Awan, K. A. and Dolly, J. O. (1991). K^+ channel sub-types in rat brain: characteristic locations revealed using β-bungarotoxin, α- and δ-dendrotoxins. *Neuroscience*, **40**, 29–39.

Benishin, C. G., Sorensen, R. G., Brown, W. E., Krueger, B. K., and Blaustein, M. P. (1988). Four polypeptide components of green mamba venom selectively block certain potassium channels in rat brain synaptosomes. *Mol. Pharmacol.*, **34**, 152–9.

Bidard, J.-N., Mourre, C., Gandolfo, G., Schweitz, H., Widmann, C., Gottesmann, C., *et al.* (1989). Analogies and differences in the mode of action and properties of binding sites (localization and mutual interactions) of two K^+ channel toxins, MCD peptide and dendrotoxin I. *Brain Res.*, **495**, 45–57.

Black, A. R., Donegan, C. M., Denny, B. J., and Dolly, J. O. (1988). Solubilization and physical characterisation of acceptors for dendrotoxin and β-bungarotoxin from synaptic membranes of rat brain. *Biochemistry*, **27**, 6814–20.

Danse, J. M., Rowan, E. G., Gasparini, S., Ducancel, F., Vatanpour, H., Young, L. C., *et al.* (1994). On the site by which α-dendrotoxin binds to voltage-dependent potassium channels: site-directed mutagenesis reveals that the lysine triplet 28–30 is not essential for binding. *FEBS Lett.*, **356**, 153–8.

Dreyer, F. and Penner, R. (1987). The actions of presynaptic snake toxins on membrane currents of mouse motor nerve terminals. *J. Physiol.*, **386**, 455–63.

Grissmer, S., Nguyen, A. N., Aiyar, J., Hanson, D. C., Mather, R. J., Gutman, G. A., *et al.* (1994). Pharmacological characterization of five cloned voltage-gated K^+ channels, types $K_v1.1$, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. *Mol. Pharmacol.*, **45**, 1227–34.

Harvey, A. L. and Anderson, A. J. (1991). Dendrotoxins: Snake toxins that block potassium channels and facilitate neurotransmitter release. In *Snake toxins* (ed. A. L. Harvey), pp. 131–64, Pergamon Press, New York.

Harvey, A. L. and Karlsson, E. (1980). Dendrotoxins from the venom of the green mamba, *Dendroaspis angusticeps*. A neurotoxin that enhances acetylcholine release at neuromuscular junctions. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **312**, 1–6.

Harvey, A. L. and Karlsson, E. (1982). Protease inhibitor homologues from mamba venoms: Facilitation of acetylcholine release and interactions with prejunctional blocking toxins. *Br. J. Pharmacol.*, **77**, 153–61.

Joubert, F. J. and Taljaard, N. (1980). The amino acid sequence of two proteinase inhibitor homologues from *Dendroaspis angusticeps* venom. *Hoppe Seylers Z. Physiol. Chem.*, **361**, 661–74.

Mehraban, F., Black, A. R., Breeze, A. L., Green, D. G., and Dolly, J. O. (1985). Identification by cross-linking of a neuronal acceptor protein for dendrotoxin, a convulsant neurotoxin. *FEBS Lett.*, **174**, 116–21.

Mehraban, F., Haines, A., and Dolly, J. O. (1986). Monoclonal and polyclonal antibodies against dendrotoxin: Their effects on its convulsive activity and interactions with neuronal acceptors. *Neurochem. Int.*, **9**, 11–22.

Pelchen-Matthews, A. and Dolly, J. O. (1989). Distribution in the rat central nervous system of acceptor sub-types for dendrotoxin, a K^+ channel probe. *Neuroscience*, **29**, 347–61.

Pongs, O. (1992). Molecular biology of voltage-dependent potassium channels. *Physiol. Rev.*, **72**, S69–88.

Rehm, H. and Lazdunski, M. (1988). Purification and subunit structure of a putative K^+-channel protein identified by its binding properties for dendrotoxins I. *Proc. Natl. Acad. Sci. USA*, **85**, 4919–23.

Smith, L. A., Lafaye, P. J., LaPenotiere, H. F., Spain, T., and Dolly, J. O. (1993). Cloning and functional expression of dendrotoxin K from black mamba, a K^+ channel blocker. *Biochemistry*, **32**, 5692–7.

Strydom, D. J. (1973a). Snake venom toxins. The amino acid sequences of two toxins from *Dendroaspis polylepis polylepis* (black mamba) venom. *J. Biol. Chem.*, **247**, 4029–42.
Strydom, D. J. (1973b). Protease inhibitors as snake venom toxins. *Nature, New Biol.*, **243**, 88–9.

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Sea anemone potassium channel toxins

Three short (35 to 37 residues) sea anemone toxins have been isolated which block voltage dependent potassium channels. ShK has a particularly high affinity for the lymphocyte $K_v1.3$ channel. Analysis of several synthetic ShK analogues reveals different structural requirements for toxin interaction with brain $K_v1.2$ channels versus lymphocyte $K_v1.3$ channels. These toxins can serve as molecular models for designing selective potassium channel blockers.

During screening for dendrotoxin-like compounds in marine organisms, extracts of several sea anemones were found to inhibit the binding of ^{125}I-dendrotoxin I, a probe for voltage dependent potassium channels, to rat brain synaptosomal membranes (Harvey *et al.* 1991; Karlsson *et al.* 1991). Two toxins were later isolated from Caribbean sea anemones, ShK toxin from *Stichodactyla helianthus* (Karlsson *et al.* 1992; Aneiros *et al.* 1993; Castañeda *et al.* 1995) and BgK toxin from *Bunodosoma granulifera* (Karlsson *et al.* 1992). More recently, another toxin has been isolated from *Anemonia sulcata*, which we refer to here as AsK toxin (Schweitz *et al.* 1995). These toxins are known to block voltage-dependent potassium channels.

The K_i-values determined by inhibition of ^{125}I-dendrotoxin I binding to rat brain synaptosomal membranes were 0.3 nM for ShK toxin (0.6 nM for inhibition of ^{125}I-α-dendrotoxin binding), 0.7 nM for BgK toxin, and 10 nM for AsK toxin. From patchclamp experiments an IC_{50} (50 per cent block of potassium channel currents) of 133 pM was obtained for ShK toxin with Jurkat T-lymphocyte (a human lymphoma peripheral T-lymphocyte cell line) $K_v1.3$ channels (Pennington *et al.* 1995). Furthermore, ShK toxin with an IC_{50} of 32 pM displaced ^{125}I-charybdotoxin binding to the lymphocyte $K_v1.3$ channel, indicating an affinity nearly 20 times higher than charybdotoxin (Pennington *et al.* 1995).

All three known short sea anemone potassium channel toxins are similar sized (35 to 37 residues) basic peptides with obvious sequence homology. Each contains six cysteine residues forming three intramolecular disulfide bonds. The toxins represent a new family of potassium channel toxin, since their sequences do not show any homology with any other potassium channel toxin. The disulfide pairings of ShK toxin have been determined (Pohl *et al.* 1995) and are assumed to be the same for the homologues BgK toxin and AsK toxin (Fig. 1). The arrangement of disulfide bonds creates an N- to C-terminal cyclic structure through the disulfide bond pairing Cys3–Cys35. The secondary structure of ShK toxin has approximately 30 per cent α-helix determined by several spectroscopic methods (Kem *et al.* 1996). NMR analysis locates the α-helical regions at residues 14–18 and 21–24 (Tudor *et al.* 1996).

Sea anemones also have other types of potassium channel toxins. Extracts from the sea anemone *Bunodosoma cangium* blocked Ca^{2+}-dependent potassium currents in crayfish muscle and chromaffin cells (Buño and Červeňanský 1995). Two types of potassium channel toxins were recently isolated from the anemone *Anemonia sulcata*. The first type, which we designate as AsK toxin, is homologous with ShK and BgK toxins (Fig. 1). Toxins of the second type, 'Kalicludins' are larger molecules (58–59 residues) homologous with dendrotoxins and also a class of protease inhibitors; these toxins also possess the two biological activities (Schweitz *et al.* 1995).

Two of the short toxins have been recently synthesized, ShK toxin by Pennington *et al.* (1995) at Bachem Bioscience, Inc. (King of Prussia, PA, USA) and also at The Peptide Institute, Inc. (Osaka, Japan) and BgK toxin by Ménez and co-workers (Personal communication) at the CEA, Département d' Ingéniere et d' Etudes des Protéins. The synthetic ShK toxin was actually more potent than the natural toxin. This may be due to more extensive oxidation of the methionine residue during purification of the natural toxin (Pennington *et al.* 1995). Additionally, several monosubstituted synthetic analogues of ShK toxin have been prepared and tested on two different types of potassium channels (Table 1). Analogues with significantly less affinity than the native toxin in displacing

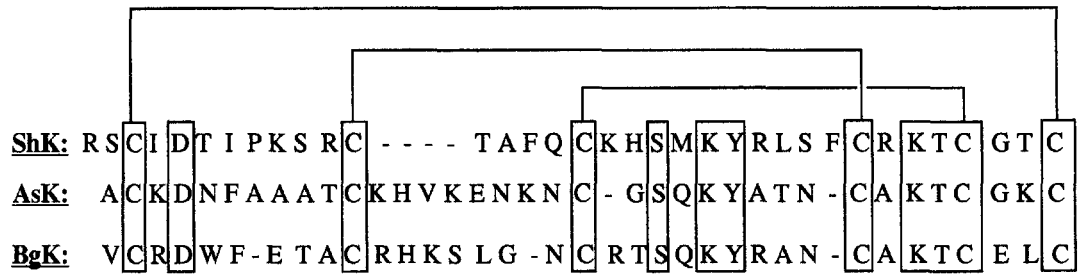

Figure 1. Amino acid sequences of ShK, BgK, and AsK aligned in homology. Common amino acids are boxed. The C-terminal tetrapeptide of BgK was first reported to have the sequence LQCC (Aneiros *et al.* 1993), but later analysis showed the sequence to be CELC. BgK synthesized with this sequence is identical with the native toxin (A. Ménez, personal communication). (A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, Y = Tyr, V = Val, W = Trp.)

Table 1 Ability of ShK toxin analogues to displace binding of charybdotoxin[a] to Jurkat T-lymphocytes and dendrotoxin to rat brain membranes (Pennington *et al.* 1996)

Toxin analogue	Secondary structure[b]	IC_{50} (nM)	
		Lymphocyte ^{125}I-ChTX	Rat Brain ^{125}I-DTX
ShK	Normal	0.04	8
R1S	Normal	0.05	16
D5N	Disordered	N.D.	N.D.
K9Q	Normal	0.31	11
R11Q	Normal	0.64	18
F15A	Normal	0.054	6
F15W	Normal	0.065	6
K22A	Normal	0.30[c]	>1000
Y23F	Normal	0.18[c]	9
Y23S	Disordered	>50[c]	>5000
R24A	Normal	0.04	10

[a] Charybdotoxin, a scorpion toxin that inhibits several types of potassium channels, including Ca^{2+}-activated and voltage-dependent channels (Pongs 1992).
[b] Normal: circular dichroism spectrum identical to that of native ShK. Disordered: greatly different spectrum lacking minima characteristic of α-helix.
[c] Data derived from patchclamp of Jurkat T lymphocyte $K_v1.3$ channels. Using this system, native ShK toxin has an IC_{50} of 133 pM (Pennington *et al.* 1995).

^{125}I-charybdotoxin from $K_v1.3$ channels in Jurkat T-lymphocytes were K9Q (8 × reduction), R11Q (16 × reduction), and K22A (3 × reduction). Thus K9, R11, and K22 probably participate in the binding of ShK toxin to $K_v1.3$ channels. Only the substitution of Ala for Lys at position 22 caused much lower (250 times) affinity for the rat brain potassium channels. The low activities of the D5N and Y23S ShK analogues were probably due to structural perturbations, as their circular dichroism spectra and HPLC behaviour indicated that these peptides had not folded properly.

Thus, ShK toxin utilizes at least some different amino acid residues for binding to these two potassium channels. Alteration of amino acid side chains that interact with only one of the ion channels might increase toxin specificity for one of the ion channels. ShK toxin analogues K9Q and R11Q are more selective than the native toxin for $K_v1.2$ channels. Similarly, the ShK toxin analogue K22A was more selective for $K_v1.3$ channels.

Purification and sources

BgK toxin and ShK toxin were isolated by gel filtration of *mucus (BgK toxin) or whole body extract (ShK toxin) on* Sephadex G-50, HPLC ion-exchange on BioGel TSK SP-5-PW (cation-exchanger with sulfopropyl as ion exchange group), and reverse phase HPLC on a C4 column. Volatile buffers (ammonium acetate and acetonitrile in trifluoroacetic acid for reverse phase HPLC) were used to facilitate recovery by freeze-drying without prior desalting. The toxin content was low, less than 0.5 mg per gram starting material. Synthetic ShK toxin may be purchased from Bachem Bioscience Inc. (King of Prussia, PA) or The Peptide Institute (Osaka, Japan).

Toxicity

The lethal doses for ShK toxin, BgK toxin, and AsK toxin are not known, but like dendrotoxins, they probably have a low lethality by intravenous injection, but are extremely potent when injected within the brain. For *instance, dendrotoxin has an* LD_{50} *i.v. of 38 μg/g mouse* (Strydom 1973) and a minimum lethal dose of only 2.5ng/g body weight when injected into rat brain (Mehraban *et al.* 1985).

Use in cell biology

BgK toxin and ShK toxin, especially analogues with higher specificity than native toxins, should be useful probes for studying voltage-dependent potassium chan-

nels. ShK toxin is readily radiolabelled with ^{125}I, using the chloramine-T method. The nonspecific binding of radioiodinated ShK toxin to rat brain membranes is lower than for ^{125}I-charybdotoxin. Also, specific binding of ^{125}I-ShK toxin displays much less dependence upon pH and ionic strength. These properties make it a superior radioligand for investigations of voltage-gated potassium channels (Mahnir *et al.* submitted). ShK toxin appears to be a competitive inhibitor of charybdotoxin and a noncompetitive inhibitor of dendrotoxin binding to rat brain membranes (Mahnir *et al.* submitted).

Investigations are currently in progress to determine whether the sea anemone short potassium channel toxins affect other types potassium channel besides the delayed rectifier type.

References

Aneiros, A., García, I., Martínez, J. R., Harvey, A. L., Anderson, A. J., Marshall, D. L., *et al.* (1993). A potassium channel toxin from the secretion of the sea anemone *Bunodosoma granulifera*. Isolation, amino acid sequence and biological activity. *Biochim. Biophys. Acta*, **1157**, 86–92.

Buño, W. and Červeñańsky, C. (1995). Selective block of Ca^{2+}-dependent K^+ current in crayfish muscle and chromaffin cells by sea anemone *Bunodosoma cangium* venom. *J. Neurosci.*, in press.

Castañeda, O., Sotolongo, V., Amor, A. M., Stocklin, R., Anderson, A. J., Harvey, A. L., *et al.* (1995). Characterization of a potassium channel toxin from the Caribbean sea anemone *Stichodactyla helianthus*. *Toxicon*, **33**, 606–13.

Harvey, A. L., Anderson, M. J., Rowan, E. J., Marshall, D. L., Castañeda, O., and Karlsson, E. (1991). Dendrotoxin-like activity isolated from sea anemones. *Br. J. Pharmacol.*, **104**, 34P.

Karlsson, E., Adam, A., Aneiros, A., Castañeda, O., Harvey, A. L., Jolkkonen, M., *et al.* (1991). New toxins from marine organisms. *Toxicon*, **29**, 1168.

Karlsson, E., Aneiros, A., Casteneda, O., and Harvey, A. L. (1992). Potassium channel toxins from sea anemones. In *Recent advances in toxinology research* (ed. P. Gopalakrishankone and C. K. Tan), Vol. 2, pp.378–91, Nat. Univ., Singapore.

Kem, W. R., Williams, R. W., Sanyal, G., and Pennington, M. W. Secondary structure of ShK toxin, a potassium channel blocking peptide. *Lett. in Peptide Sci.*, **3**, 69–72.

Mahnir, V. M., Pennington, M. W., and Kem, W. R., Binding of radiolabelled ShK to potassium channels in rat brain membranes. *J. Biol. Chem.*, submitted.

Mehraban, F., Black, A. R., Breeze, A. L., Green, D. G., and Dolly, J. O. (1985). A functional membranous acceptor for dendrotoxin in rat brain: solubilization of the binding component. *Biochem. Soc. Trans.*, **13**, 507–8.

Pennington, M. W., Byrnes, M. E., Zaydenberg, I., Khaytin, I., de Chastonay, J., Krafte, D., *et al.* (1995). Chemical synthesis and characterization of ShK toxin: a potent potassium channel inhibitor from a sea anemone. *Int. J. Peptide Protein Res.*, **46**, 354–8.

Pennington, M. W., Kem, W. R., Mahnir, V. M., Byrnes, M. E., Zaydenberg, I., Khaytin, I., *et al.* (1996). Identification of essential residues in the potassium channel inhibitor ShK toxin. Analysis of monosubstituted analogs. In *Peptides: Chemistry, structure and biology* (ed. P. T. P. Kaumaya and R. S. Hodges), Escom, Leiden, The Netherlands, 192–4.

Pohl, J., Hubalek, F., Byrnes, M. E., Nielsen, K. R., Woods, A., and Pennington, M. W. (1995). Assignment of the three disulfide bonds in ShK toxin: A potent potassium channel inhibitor from the sea anemone *Stichodactyla helianthus*. *Lett. in Peptide Sci.*, **1**, 291–7.

Pongs, O. (1992). Structural basis of voltage-gated K^+ channel pharmacology. *Trends Pharmacol. Sci.*, **13**, 359–65.

Schweitz, H., Bruhn, T., Guillemare, E., Moinier, D., Lancelin, J. M., Béress, L., *et al.* (1995). Kalicludines and kaliseptine: two different classes of sea anemone toxins for voltage sensitive K^+ channels. *J. Biol. Chem.*, **270**, 25121–6.

Strydom, D. J. (1973). Protease inhibitors as snake venom toxins. *Nature New Biol,*. **243**, 88–9.

Tudor, J. E., Pallaghy, P. K., Pennington, M. W., and Norton, R. S. (1996). Solution structure of ShK toxin, a novel potassium channel inhibitor from a sea anemone. *Nat. Struct. Biol.*, **3**, 317–20.

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9

Calcium channel targeted toxins

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Introduction

Voltage-gated Ca^{2+} channels are multisubunit complexes composed of a channel-forming and voltage-sensing $\alpha 1$ subunit and several regulatory and/or auxiliary subunits. They constitute a complex family of channels comprising a large number of different subtypes, which have in common a steep voltage dependence of open probability and a very high selectivity for Ca^{2+} over Na^+ and K^+ ions in physiological solutions.

The structure of the $\alpha 1$ subunit shares a basic design with other voltage-gated ion channels, consisting of six membrane spanning segments (S1 to S6) flanked by cytoplasmic and extracellular loops, with the loop between S5 and S6 (termed H5 sequence, SS1–SS2, or the P region) folding into the membrane to form part of the pore (Fig. 1). As in the case of Na^+ channels this basic design is repeated in four homologous domains. The S4 segment, which contains positively charged residues at every third or fourth position, has been shown to play a major role in voltage sensing in Na^+ and K^+ channels, and is likely to play a similar role in Ca^{2+} channels. Biophysical studies have shown that high selectivity for Ca^{2+} ions is achieved through a high affinity binding site (K_d around 1 μM) located within the pore, close to the external mouth, and recent mutagenesis studies suggest that such a site is formed by four conserved glutamate residues, one glutamate donated by each of the four H5 sequences of 1α (McCleskey 1994; Sather *et al.* 1994).

Since Ca^{2+} ions are intracellular chemical messengers, capable of activating and regulating a wide spectrum of important cellular functions, voltage-gated Ca^{2+} channels are special with respect to other ion channels in that they act as transducers of electrical signals into chemical messages, and as such have crucial roles in the control of important Ca^{2+}-dependent processes (Hille 1992). Thus, the evolutionary proliferation of natural toxins designed to inhibit them selectively does not seem surprising. ω-toxins (in particular the more selective ω-conotoxin-GVIA isolated from *Conus geographus* snail venom and ω-agatoxinIVA isolated from the funnel-web spider *Agelenopsis aperta* venom) have been instrumental in defining different classes of Ca^{2+} channels and have proven to be powerful pharmacological tools for exploring their functional role (Olivera *et al.* 1994).

On the basis of the threshold voltage for activation, voltage-gated Ca^{2+} channels have been divided in low-voltage-activated (LVA or T-type) channels (threshold: –70 to –50 mV) and high-voltage-activated (HVA) Ca^{2+} channels (threshold > –40 mV) (Bean 1989; Bertolino and LLinas 1992). According to pharmacological criteria, HVA Ca^{2+} channels have been classified as dihydropyridine (DHP)-sensitive channels (L-type), ω-CgTx-GVIA-sensitive channels (N-type), and ω-AgaIVA-sensitive channels (P- and Q-type, distinguished on the basis of different sensitivities to ω-AgaIVA: $K_d < 10$ nM for P- and $K_d < 10$ nM for Q-type). An additional component of HVA Ca^{2+} current (R-type) has been identified as the current resistant to DHPs and ω-CTx-MVIIC, a toxin that inhibits slowly and irreversibly P- and Q-type channels and

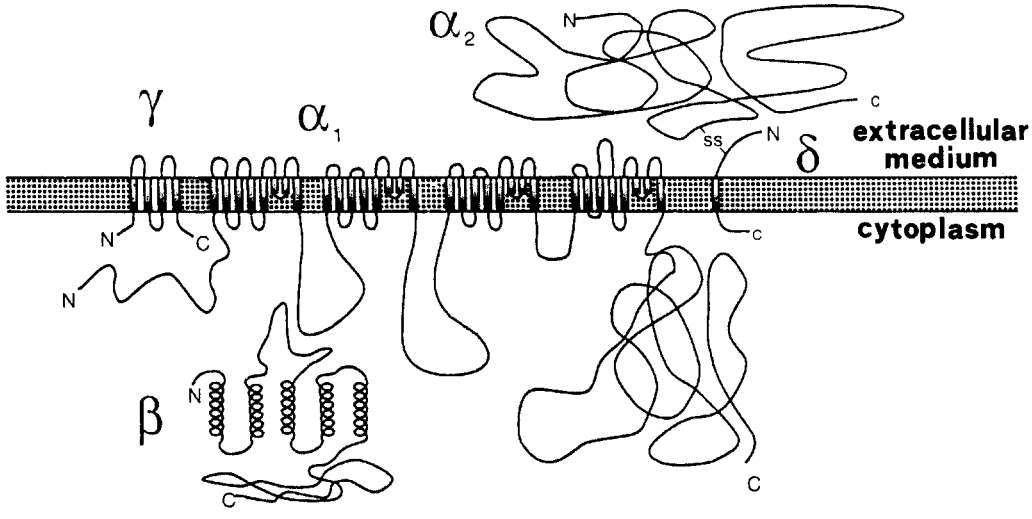

Figure 1. Oligomeric structure of voltage-gated Ca^{2+} channels (modified from Perez-Reyes and Schneider 1994).

inhibits rapidly and reversibly N-type channels (Birnbaumer *et al.* 1994; Olivera *et al.* 1994; Dunlap *et al.* 1995). DHPs, ω-CgTx-GVIA, and ω-AgaIVA have been particularly useful for discriminating among Ca^{2+} channel types because they are quite selective in blocking L-, N-, and P/Q-type channels, respectively, while most other blockers of Ca^{2+} channels (including ω-grammotoxins and other ω-conotoxins and ω-agatoxins, and also FTX a polyamine from Agelenopsis venom) appear to be poorly selective (Olivera *et al.* 1994).

The basic insight into the subunit composition of voltage-gated Ca^{2+} channels has been derived mainly from work carried out with the channel purified from skeletal muscle. The DHP-sensitive Ca^{2+} channel of skeletal muscle is composed of four subunits: α1 (175 kDa), 2α (160 kDa), β (55 kDa), and γ (33 kDa). Of non-L type Ca^{2+} channels detailed subunit structure is known only for N-type channels, which consists of an α1 subunit (230 kDa), a β subunit (57 kDa), an α2δ subunit, and an additional subunit (95 kDa) not present in skeletal muscle (Hofmann *et al.* 1994; Isom *et al.* 1994; Perez-Reyes and Schneider 1994). α2δ subunits are disulfide-linked dimers of α2 and δ subunits: δ contain a transmembrane segment that anchors the dimer to the membrane, while α2 are large glycoproteins probably located extracellularly. γ subunits are believed to be cytoplasmic. The subunit, an integral membrane protein containing four putative transmembrane domains, seems to be unique to skeletal muscle (Fig. 1).

Cloning studies have shown that Ca^{2+} channels α_1 and subunits are encoded by at least six (α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, and the skeletal α_{1S}) and four (β_1, β_2, β_3, β_4) different genes, respectively (Snutch and Reiner 1992; Birnbaumer *et al.* 1994; Perez-Reyes and Schneider 1994; Dunlap *et al.* 1995). Further molecular diversity is created by the existence of multiple isoforms for each gene, generated in many cases by alternative splicing of the genes. Thus at present a minimum of 19 structurally distinct α1 subunits and 9 distinct β are known, most of which are expressed in the brain (Fig. 2). α2δ subunits identified to date are encoded by only one gene, which give rise to multiple splice variants.

Heterologous expression studies have shown that α_{1C} and α_{1D}, like α_{1S}, give rise to DHP-sensitive Ca^{2+} channels, α_{1B} to ω-CgTx-GVIA-sensitive Ca^{2+} channels, α_{1A} to ω-AgaIVA-sensitive Ca^{2+} channels, and α_{1E} to Ca^{2+} channels resistant to all specific inhibitors. The three L-type α1 subunits have 60–70 per cent amino acid identity among each other and only 30–40 per cent identity with the non-L α_1 subunits, which are 50–60 per cent identical with each other: Within the L family, α_{1C} (which is widely expressed in cardiac and smooth muscle and in neuronal and endocrine cells and also nonexcitable cells such as fibroblasts and kidney epithelial cells) and α_{1D} (whose expression is restricted to neuronal and endocrine cells) are more similar to each other than to the skeletal muscle α_{1S}. Within the non-L family, $\alpha_{1A}$and α_{1B} are more similar to each other than α_{1E}. The three non-L α1 subunits are almost exclusively expressed in neuronal and/or neuroendocrine tissues.

Although in many cases α1 subunits alone are sufficient for expression of functional Ca^{2+} channels in heterologous systems, the auxiliary subunits and in particular the β subunits have major effects on Ca^{2+} channel func-

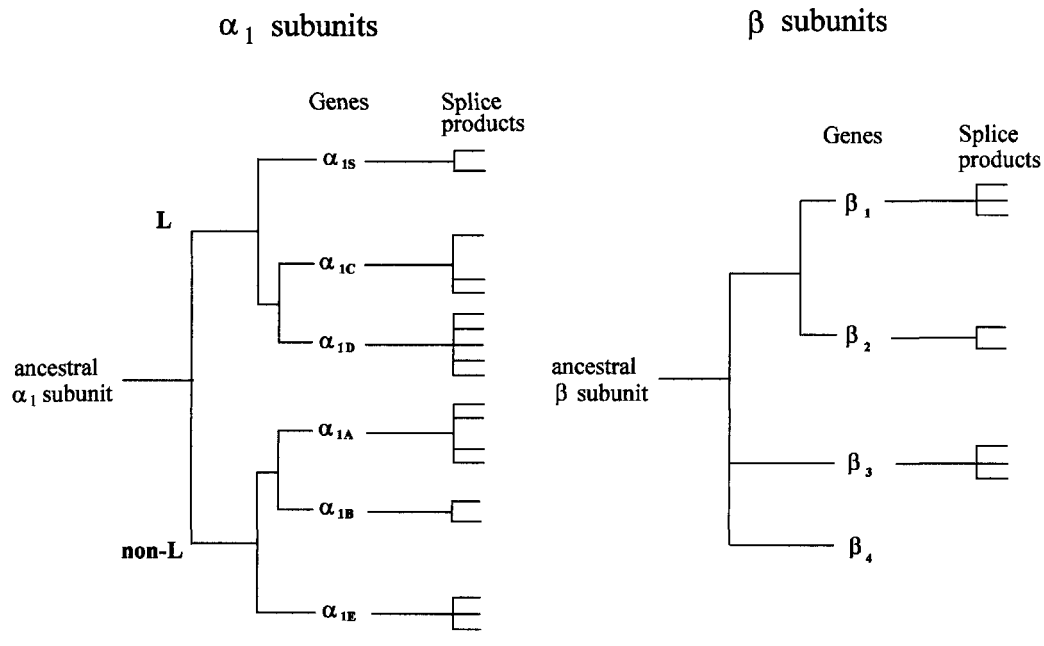

Figure 2. Gene family for α1 and β subunits of voltage-gated Ca^{2+} channels (modified from Dunlap *et al.* 1995).

tion (Hofmann *et al.* 1994; Isom *et al.* 1994). Different subunits in combination with a given $\alpha 1$ subunit give rise to Ca^{2+} channels with very different biophysical properties. Particularly striking are the widely different effects of different β subunits on inactivation kinetics and voltage dependence of steady state inactivation of α_{1A} and α_{1E} (e.g. Stea *et al.* 1994).

Considering the different genes and isoforms for $\alpha 1$ and β subunits and the number of possible subunit combinations, a functional diversity of Ca^{2+} channels much wider than the established pharmacological diversity can be expected in native membranes, especially in the brain which expresses most of the known isoforms. Since there is accumulating evidence from electrophysiological studies in neurons that each of the pharmacological classes of native neuronal Ca^{2+} channels comprises various members with distinct biophysical properties, sometimes coexpressed in the same type of neuron (Forti and Pietrobon 1993), it appears likely that the large potential for combinatorial structural heterogeneity of brain Ca^{2+} channels may actually be fully exploited.

L-type Ca^{2+} channels are the major pathway for voltage-gated Ca^{2+} entry in cardiac and smooth muscle and, as such, play a crucial role in excitation–contraction coupling. Acting as voltage sensors for intracellular Ca^{2+} release from the sarcoplasmic reticulum, they are essential components of the excitation–contraction coupling machinery in skeletal muscle. In endocrine and neuroendocrine cells L-type channels are involved in the control of hormone release. The precise role of L-type channels in neurons is not yet well understood. However, their wide distribution in CNS and their preferential localization in cell bodies and at the base of major dendrites (Westenbroek *et al.* 1990) suggest that they may be critically involved in initiating Ca^{2+}-dependent intracellular regulatory events (e.g. regulation of cellular signalling pathways and gene expression) in response to synaptic excitation (Ghosh and Greenberg 1995).

Despite the extremely large heterogeneity at the molecular and probably also functional level, most native L-type channels described so far have some common biophysical properties, namely a relatively high threshold voltage for activation (–40/–30 mV), a slow voltage-dependent inactivation and a steady state inactivation at relatively positive voltages (> –60 mV), and a single channel conductance of 22–27 pS (Bean 1989; Forti and Pietrobon 1993). Besides the sensitivity to dihydropyridine drugs that define them, L-type channels have in common also the resistance to all ω-toxins except ω-AgaIIIA and the sensitivity to calciseptine (Olivera *et al.* 1994).

N-type and P/Q-type Ca^{2+} channels play a major role in controlling neurotransmitter release at synaptic terminals in both peripheral and central nervous systems. In many synapses N- and P/Q-type channels coexist in individual nerve terminals and control exocytosis jointly (Olivera *et al.* 1994; Dunlap *et al.* 1995).

Even though both ω-CgTx-GVIA-sensitive and ω-AgaIVA-sensitive Ca^{2+} channels with quite different kinetics and voltage-dependent inactivation properties have been reported in different cells, N-type and P/Q-type Ca^{2+} channels have in common a high threshold voltage for activation at –30/–10 mV, which distinguishes them from R-type channels, which are characterized by a lower threshold for activation at –50/–30 mV (Mintz *et al.* 1992; Elmslie *et al.* 1994; Tottene *et al.* 1996; Randall and Tsien 1995, and compare, e.g., Stea *et al.* 1994 and Soong *et al.* 1993 for recombinant channels).

The function of R-type channels in neuronal physiology remains largely unknown, although both their functional properties and their prevailing localization in cell bodies and a subset of dendrites suggest a possible role in the generation of calcium-dependent action potentials and calcium transients in response to excitatory postsynaptic potentials at relatively negative voltages (Yokoyama *et al.* 1995).

LVA or T-type Ca^{2+} channels have a pharmacological profile similar to that of R-type channels with respect to resistance to the known selective antagonists and sensitivity to Ni^{2+} block, but they can be distinguished from R-type channels on the basis of their lower sensitivity to Cd^{2+} block, lower threshold of activation, higher rate of inactivation, and lower single channel conductance and current. T-type channels are present in most excitable cells. They are involved in generation of rhythmic pacemaker activity in cardiac muscle and neurons, and are thought to be responsible for neuronal oscillatory activity that is likely implicated in various brain functions such as wakefulness regulation and motor coordination (Bean 1989; Bertolino and Llinas 1992). The interesting recent demonstration of activation of T-type channels in dendrites of hippocampal neurons by subthreshold EPSPs, suggest a possible important role in synaptic integration and in some forms of plasticity (Magee and Johnston 1995).

References

Bean, B. P. (1989). Classes of Ca^{2+} channels in vertebrate cells. *Annu. Rev. Physiol.*, **51**, 367–84.

Bertolino, M. and LLinas, R. R. (1992). The central role of voltage-activated and receptor operated calcium channels in neuronal cells. *Annu. Rev. Pharmacol. Toxicol.*, **32**, 399–421.

Birnbaumer, L., Campbell, K. P., Catterall, W. A., Harpold, M. M., Hofmann, F., Horne, W. A., *et al.* (1994). The naming of voltage-gated calcium channels. *Neuron*, **13**, 505–6.

Dunlap, K., Luebke, J. I., and Turner, T. J. (1995). Exocytotic Ca^{2+} channels in mammalian central neurons. *Trends Neurosci.*, **18**, 89–98.

Elmslie, K. S., Kammermeier, P. J., and Jones, S. W. (1994). Reevalutation of Ca^{2+} channel types and their modulation in bullfrog sympathetic neurons. *Neuron*, **13**, 217–28.

Forti, L. and Pietrobon, D. (1993). Functional diversity of L-type calcium channels in rat cerebellar neurons. *Neuron*, **10**, 437–50.

Ghosh, A. and Greenberg, M. E. (1995). Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science*, **268**, 239–47.

Hille, B. (1992). *Ionic channels of excitable membranes*, Sinauer, Sutherland, MA.

Hofmann, F., Biel, M., and Flockerzi, V. (1994). Molecular basis for Ca^{2+} channel diversity. *Annu. Rev. Neurosci.*, **17**, 399–418.
Isom, L. L., De Jongh, K. S., and Catterall, W. A. (1994). Auxiliary subunits of voltage-gated ion channels. *Neuron*, **12**, 1183–94.
McCleskey, E. W. (1994). Calcium channels: cellular roles and molecular mechanisms. *Curr. Opin. Neurobiol.*, **4**, 304–12.
Magee, J. C. and Johnston, D. (1995). Synaptic activation of voltage-gated channels in the dendrites of hippocampal pyramidal neurons. *Science*, **268**, 301–7.
Mintz, I. M., Adams, M. E., and Bean, B. P. (1992) P-type calcium channels in rat central and peripheral neurons. *Neuron*, **9**, 85–95.
Olivera, B. M., Miljanich, G. P., Ramachandran, J., and Adams, M. E. (1994). Calcium channel diversity and neurotransmitter release: The ω-conotoxins and ω-agatoxins. *Annu. Rev. Biochem.*, **63**, 823–67.
Perez-Reyes, E. and Schneider, T. (1994). Calcium channels: structure, function, and classification. *Drug Develop. Res.*, **33**, 295–318.
Randall, A. and Tsien, R. W. (1995). Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons. *J Neurosci.*, **15**, 2995–3012.
Sather, W. A., Yang, J., and Tsien, R. W. (1994). Structural basis of ion channel permeation and selectivity. *Curr. Opin. Neurobiol.*, **4**, 313–23.
Snutch, T. P. and Reiner, P. B. (1992). Ca^{2+} channels: diversity of form and function. *Curr. Opin. Neurobiol.*, **2**, 247–53.
Soong, T. W., Stea, A., Hodson, C. D., Dubel, S. F., Vincent, S. R., and Snutch, T. P. (1993). Structure and functional expression of a member of the low voltage-activated calcium channel family. *Science*, **260**, 1133–6.
Stea, A., Tomlinson, W. J., Wah Soong, T., Bourinet, E., Dubel, S. J., Vincent, S. R. *et al.* (1994). Localization and functional properties of a rat brain a_{1A} calcium channel reflect similarities to neuronal Q- and P-type channels. *Proc. Natl. Acad. Sci. USA*, **91**, 10576–80.
Tottene, A., Moretti, A., and Pietrobon, D. (1996). Functional diversity of P-type and R-type calcium channels in rat cerebellar neurons. *J. Neurosci.*, **16**, 6353–63.
Westenbroek, R. E., Ahlijanian, M. K., and Catterall, W. A. (1990). Clustering of L-type Ca^{2+} channels at the base of major dendrites in hippocampal pyramidal neurons. *Nature*, **347**, 281–4.
Yokoyama, C. T., Westenbroek, R. E., Hell, J. W., Soong, T. W., Snutch, T. P., and Catterall, W. A. (1995). Biochemical properties and subcellular distribution of the neuronal class E calcium channel α_1 subunit. *J. Neurosci.*, **15**, 6419–32.

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ω-Conotoxins (*Conus* spp.)

The ω-conotoxins are a family of peptide neurotoxins present in the venoms of many species of predatory cone snails (Conus) *that inhibit voltage-gated Ca channels; the well-characterized ω-conotoxins come from three fish-hunting* Conus *species. All ω-conotoxins are 24–30 amino acids in length, with three disulfide bonds; the best defined are ω-conotoxin* GVIA *(ω*GVIA*) from* Conus geographus *and ω-conotoxins* MVIIA, MVIIC, *and* MVIID *from* Conus magus *venom (ω*MVIA, ω*MVIIC, and ω*MVIID*). In mammalian systems, ω*GVIA *and ω*MVIIA *are remarkably specific for voltage-gated calcium channels that contain the* α_{1B} *subunit.; a second group of ω-conotoxins, including ω*MVIIC *and ω*MVIID *have broader specificity in mammalian systems, and preferentially target calcium channel complexes containing* α_{1A} *subunits. Since calcium channels present at presynaptic termini generally belong to the classes above, ω-conotoxins have become widely used tools for blocking synaptic transmission and studying synapses.*

The ω-conotoxins are a major family of paralytic neurotoxins produced in the venoms of various species of marine gastropods belonging to the genus *Conus*. At this time, the most widely known of these peptides is ω-conotoxin GVIA (ωGVIA), which has 27 amino acids and three disulfide linkages; in many scientific publications, the term ω-conotoxin (and sometimes the even more general 'conotoxin') is used to refer to ω-conotoxin GVIA. (For example, the widely used 'conotoxin-sensitive Ca channel' usually means the channel is sensitive to ωGVIA.) Several other ω-conotoxins have the same pharmacological specificity as ω-conotoxin GVIA in mammalian systems, notably ω-conotoxins MVIIA and SVIA. ωMVIIA is generally more reversible than ωGVIA, while ωSVIA has much lower affinity for mammalian Ca channels and is not widely used. ω-conotoxin SVIA is the smallest member of the family described so far (24 amino acids). The biochemistry, electrophysiology, and pharmacology of these peptides have been comprehensively reviewed (Olivera *et al.* 1994).

Purification and chemical synthesis

The ω-conotoxins were originally identified and purified from the venom of *Conus* (ω-conotoxin GVIA from *Conus geographus* venom, ω-conotoxin MVIIA from *Conus magus* venom). Venoms were collected by dissecting fresh *Conus* specimens, and the dissected venom ducts used as the source of venom. Venom can also be obtained from some *Conus* species by milking (Hopkins *et al.* 1995). The purification of ω-conotoxins from venom has been described (Olivera *et al.* 1984, 1990). More recently, the sequences of ω-conotoxins have been inferred by analysing cDNA clones derived from venom ducts (Hillyard *et al.* 1992; Monje *et al.* 1993).

Although purification from natural sources is always an option, almost all ω-conotoxins used for research, therapeutic, and diagnostic purposes have been chemically synthesized since the first synthesis of these peptides in 1987 (Rivier *et al.* 1987). Several different strategies have been employed to optimize synthesis and folding into the correct disulfide-bonded configuration (Olivera *et al.* 1994).

Toxicity; clinical potential

The α_{1B}-targeted ω-conotoxins (ωGVIA and ωMVIIA, shown in Fig. 1) cause shaking upon intracranial injection into mice, but not death even at concentrations several orders of magnitude above those necessary to give the shaking symptomatology. Indeed, one of these peptides, ω-conotoxin MVIIA is being developed as a potential therapeutic agent for intractable pain. This analgesic application is probably based on the interaction with α_{1B}-containing calcium channels, the channel subtype likely to be the target of opiate receptors. Another potential therapeutic application for this compound for which clinical trials are ongoing is as a neuroprotective agent during hypoxic/ischemic episodes. Patents for such clinical applications have been issued to Neurex Corporation, Menlo Park, California.

However, it should be noted that while the peptides are not toxic to mice, these peptides cause death in lower vertebrates (fish, amphibian, and avian systems). Some mammals may be susceptible following direct injection into the central nervous system (at high doses, lethality is observed in rats).

In contrast, the ω-conotoxins which also inhibit α_{1A}-containing voltage-gated calcium channels (i.e., ωMVIIC and ωMVIID) are lethal to mammals as well as to lower vertebrates. In mammals, lethality occurs only upon intracranial injection; a progressive respiratory distress is observed in intoxicated animals.

Genes

Complete coding sequences are available for ω-conotoxin GVIA (Genbank M84612) (Colledge *et al.* 1992). The precursor sequences of the ω-conotoxins are highly homologous to two other families of *Conus* peptides, the μO- and δ-conotoxins which, respectively, block and increase voltage-gated conductances of certain sodium channel subtypes (McIntosh *et al.* 1995; Shon *et al.* 1995).

Use in cell biology and neuroscience

ω-Conotoxins have become standard tools for inhibiting synaptic transmission; over 1000 publications in the neuroscience literature employed these agents, especially ω-conotoxin GVIA. The peptides have also been used in anatomical studies to localize calcium channels: radiolabelled derivatives for macroscopic mapping and fluores-

I. (Specific for Ca^{2+} channels containing α_{1B} subunits)

ω-conotoxin GVIA	CKSOGSSCSOTSYNCC-RSCNOYTKRCY*
ω-conotoxin MVIIA	CKGKGAKCSRLMYDCCTGSC--RSGKC*

II. (Prefer Ca^{2+} channels containing α_{1A} subunits)

ω-conotoxin MVIIC	CKGKGAPCRKTMYDCCSGSCGRRGK-C*
ω-conotoxin MVIID	CQGRGASCRKTMYNCCSGSC--NRGRC*

Disulfide Bonding of ω-Conotoxins

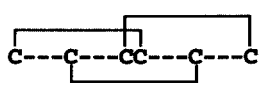

Figure 1. ω-Conotoxins.

cent ω-conotoxin derivatives for presynaptic termini. Antibodies to ω-conotoxins have also been made.

References

Colledge, C. J., Hunsperger, J. P., Imperial, J. S., and Hillyard, D. R. (1992). Precursor structure of omega-conotoxin GVIA determined from a cDNA clone. *Toxicon*, **30**, 1111–16.

Hillyard, D. R., Monje, V. D., Mintz, I. M., Bean, B. P., Nadasdi, L., Ramachandran, J., *et al.* (1992). A new *Conus* peptide ligand for mammalian presynaptic Ca^{2+} channels. *Neuron*, **9**, 69–77.

Hopkins, C., Grilley, M., Miller, C., Shon, K. -J., Cruz, L. J., Gray, W. R., *et al.* (1995). A new family of *Conus* peptides targeted to the nicotonic acetylcholine receptor. *J. Biol. Chem.*, **270**, 22361–7.

McIntosh, J. M., Hasson, A., Spira, M. E., Li, W., Marsh, M., Hillyard, D. R. *et al.* (1995). A new family of conotoxins that blocks voltage-gated sodium channels. *J. Biol. Chem.*, **270**, 16796–802.

Monje, V. D., Haack, J., Naisbitt, S., Miljanich, G., Ramachandran, J., Nasdasdi, L., *et al.* (1993). A new *Conus* peptide ligand for Ca channel subtypes. *Neuropharmacology*, **32**, 1141–9.

Olivera, B. M., McIntosh, J. M., Cruz, L. J., Luque, F. A., and Gray, W. R. (1984). Purification and a sequence of a presynaptic peptide toxin from *Conus geographus* venom. *Biochemistry*, **23**, 5087–90.

Olivera, B. M., Rivier, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., *et al.* (1990). Diversity of *Conus* neuropeptides. *Science*, **249**, 257–63.

Olivera, B. M., Miljanich, G., Ramachandran, J., and Adams, M. E. (1994). Calcium channel diversity and neurotransmitter release: the omega-conotxins and omega-agatoxins. *Ann. Rev. Biochem.*, **63**, 823–67.

Rivier, J., Galyean, R., Gray, W. R., Azimi-Zoonooz, A., McIntosh, J. M., Cruz, L. J., *et al.* (1987). *Neuron*,al calcium channel inhibitors. Synthesis of omega-conotoxin GVIA and effects on 45Ca uptake by synaptosomes. *J. Biol. Chem.*, **262**, 1194–8.

Shon, K., Grilley, M. M., Marsh, M., Yoshikami, D., Hall, A. R., Kurz, B., *et al.* (1995). Purification, characterization, synthesis, and cloning of the lockjaw peptide from *Conus purpurascens* venom. *Biochemistry*, **34**, 4913–18.

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ω-Agatoxins (*Agelenopsis aperta*)

ω-Agatoxins are a diverse group of polypeptide calcium channel antagonists from venom of the American funnel web spider, Agelenopsis aperta. *Four types of ω-agatoxins (Types I, II, III, and IV) ranging in molecular mass from 5–9 kDa are distinguished by amino acid sequence variation and differences in pharmacological specificity for calcium channel subtypes. Block of presynaptic calcium channels by the ω-agatoxins interferes with neurotransmitter release and synaptic transmission in a variety of animal groups.*

Type I

ω-Aga-IA (7791 Da) is a 69 amino acid (Table 1) heterodimeric peptide composed of a 66 amino acid major chain and a three amino acid minor chain connected by a disulfide bond (Adams *et al.* 1990; Santos *et al.* 1992). The major chain contains four internal disulfide bonds. N-terminal amino acid sequences of ω-Aga-IB and IC indicate that they have related structures (Adams *et al.* 1990). ω-Aga-IA blocks presynaptic calcium channels at the insect neuromuscular junction and calcium action potentials in insect neurosecretory neurons (Bindokas and Adams 1989; Bindokas *et al.* 1991). Calcium channel currents in rat dorsal root ganglion neurons are also blocked by ω-Aga-IA (Scott *et al.* 1990).

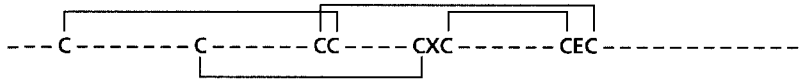
Type II

ω-Aga-IIA (1) is a ~9 kDa polypeptide (Table 1) which blocks insect (Bindokas and Adams 1991), avian (Venema *et al.* 1992), and mammalian (Adams *et al.* 1992) presynaptic calcium channels. A partial amino acid sequence of ω-Aga-IIA has been published (Adams *et al.* 1990).

Type III

ω-Aga-IIIA (8505 Da) is a monomeric peptide composed of 76 amino acids (Table 1) and six internal disulfide bonds (Venema *et al.* 1992). Several toxins closely related to ω-Aga-IIIA also are identified as ω-Aga-IIIB, IIIC, and

Table 1 ω-Agatoxin sequences

	(Major chain)	(Minor chain)
ω-Aga-IA	AKALPPGSV**C**DGNESD**C**K**C**YGKWHK**C**R**C**PWKWHFTGEGP**C**T**C**EKGMKHT**C**ITKLH**C**PNKAEWGLDW	SP**C**
ω-Aga-IIA	G**C**IEIGGD**C**DGYQEKSY**C**Q**CC**RNNGF**C**S... (partial sequence)	
ω-Aga-IIIA	S**C**IDIGGD**C**DGEKDD**C**Q**CC**RRNGY**C**S**C**YSLFGYLKSG**C**K**C**VVGTSAEFQGI**C**RRKARQ**C**YNSDPDK**C**E SHNKPKRR*	
ω-Aga-IVA	KKK**C**IAKDYGR**C**KWGGTP**CC**RGRG**C**I**C**SIMGTN**CEC**KPRLIMEGLGLA	
ω-Aga-IVB	EDN**C**IAEDYGK**C**TWGGTK**CC**RGRP**C**R**C**SMIGTN**CEC**TPRLIMEGLSFA	
Disulfide connectivity (IVA/IVB)		

*Indicates C-terminal amidation

IIID (Ertel *et al.* 1994*a*). ω-Aga-IIIA blocks avian preynaptic calcium channels (Venema *et al.* 1992) and mammalian N-type, L-type, and P-type channels with equal potency (Mintz *et al.* 1991; Mintz 1994) and thus may identify a conserved binding site on a broad spectrum of high threshold calcium channels. This toxin also blocks calcium channel currents in insect central neurons (Ertel *et al.* 1994*a*), but has no effect on presynaptic calcium channels at the insect neuromuscular junction (Bindokas *et al.* 1991). ω-Aga-IIIA blocks mammalian cardiac L-type calcium channel currents without affecting gating current (Ertel *et al.* 1994*b*), suggesting that the toxin probably physically occludes the channel pore. Although ω-Aga-IIIA completely inhibits L-type calcium channels (Mintz *et al.* 1991; Cohen *et al.* 1992), it only partially blocks neuronal N-type and P-type channels in neurons (Mintz *et al.* 1991; Mintz 1994). The toxin also blocks calcium channel α_{1A} (Sather *et al.* 1993) and α_{1E} (Kondo *et al.* 1994) subunits expressed in *Xenopus* oocytes.

ω-Aga-IIIA and the ω-conotoxins appear to have overlapping binding sites on multiple calcium channel subtypes. Evidence for this hypothesis comes from both radioligand binding and electrophysiological experiments. ω-Aga-IIIA prevents the binding of both ω-conotoxin GVIA (N-type-specific ligand) and ω-conotoxin MVIIC (N, P, and O-type ligand) to rat brain membranes (Adams *et al.* 1993a). This data is complemented by electrophysiological experiments showing that ω-Aga-IIIA prevents ω-conotoxin GVIA from blocking N-type channels (Mintz 1994) and MVIIC from blocking P-type channels (McDonough *et al.* 1995).

Type IV

ω-Aga-IVA (5203 Da) and ω-Aga-IVB (5274 Da) are 48 amino acid monomeric peptides (Table 1) with four internal disulfide bonds (Adams *et al.* 1993*b*; Mintz *et al.* 1992*b*). ω-Aga-IVB also has been referred to as ω-Aga-TK (Teramoto *et al.* 1993). Both toxins have been chemically synthesized (Nishio *et al.* 1993; Heck *et al.* 1994; Kuwada *et al.* 1994). A unique aspect of ω-Aga-IVB is its postranslational modification of serine 46 from the *L*- to the *D*-configuration (Heck *et al.* 1994; Kuwada *et al.* 1994). Data on the disulfide bonding pattern, three-dimensional structures, and structure–activity relationships for these peptide toxins are now available (Adams *et al.* 1993b; Nishio *et al.* 1993; Yu *et al.* 1993; Reily *et al.* 1994, 1995; Kim et al. 1995).

The type IV ω-agatoxins are specific, high affinity $K_d \approx 1$nM antagonists of P-type calcium channels in the mammalian brain. The binding site for Type IV ω-agatoxins on P-type calcium channels appears to be separate from that defined by the Type III ω-agatoxins (Adams *et al.* 1993a; Mintz *et al.* 1994). At higher concentrations (>100 nM), ω-Aga-IVA also blocks Q-type calcium currents in rat cerebellar granule neurons (Randall and Tsien 1995). ω-Aga-IVA blocks presynaptic calcium channels in the rat brain (Mintz *et al.* 1992*a, b*; Turner *et al.* 1992, 1993), resulting in suppression of synaptic transmission (Leubke *et al.* 1993; Takahashi and Momiyama 1993; Olivera *et al.* 1994; Wheeler *et al.* 1994), indicating that P- and/or Q-type channels are involved in the regulation of transmitter release at synaptic junctions.

ω-Aga-IVA also has been shown to block calcium channels in insect neurosecretory cells (Bickmeyer 1994) and at neuromuscular junctions of crayfish skeletal muscle (Araque *et al.* 1994). It therefore appears that P-type calcium channels or closely related forms are quite conserved evolutionarily.

Purification and sources

The ω-agatoxins are purified by reversed-phase high-performance liquid chromatography. Protocols for purification of each type have been published as follows: ω-Aga-IA and IIA, Adams *et al.* (1990); ω-Aga-IIIA, Venema *et al.* (1992*b*); ω-Aga-IIIB-D, Ertel *et al.* (1994*a*); ω-Aga-IVA, Mintz *et al.* (1992*b*); ω-Aga-IVB, Adams *et al.* (1993*b*).

ω-Aga-IVA is available from Latoxan or its US affiliate, Accurate Chemical & Scientific Corporation.

Toxicity

None of the ω-agatoxins have been reported to have significant oral or i.v. toxicity. Intracerebrocranial (ICV)

injection of ω-Aga-IIIA into young mice causes convulsions and death (M. E. Adams, unpublished data). ICV injections of ω-Aga-IVA into young mice leads to respiratory failure and death (M. E. Adams, unpublished data).

Uses in cell biology and pharmacology

ω-Aga-IIIA binds to all known high-threshold calcium channels (L, N, O, P, Q, R) (Kondo *et al.* 1994; Mintz 1994). It has been used as a probe for mapping the distribution of these channels in the rat brain (McIntosh *et al.* 1992) and as a tool for revealing calcium channel gating currents (Ertel *et al.* 1994*b*).

ω-Aga-IVA and ω-Aga-IVB are potent and selective antagonists of P-type calcium channels in cerebellar Purkinje neurons, visual cortical neurons, spinal interneurons, and dorsal root ganglion neurons (Mintz *et al.* 1992*a*, *b*; Adams *et al.* 1993*b*). This toxin is useful in defining P-type currents in various types of mammalian neurons, and in determining the contribution of P-type calcium channels to neurotransmitter release in various parts of the nervous system (Turner *et al.* 1992, 1993; Leubke *et al.* 1993; Takahashi and Momiyama 1993; Regehr and Mintz 1994).

References

Adams, M. E., Bindokas, V. P., Hasegawa, L., and Venema, V. J. (1990). Omega-agatoxins: novel calcium channel antagonists of two subtypes from funnel web spider (*Agelenopsis aperta*) venom. *J. Biol. Chem.*, **265**(2), 861–7.

Adams, M. E., Bindokas, V. P., and Venema, V. J. (1992). Probing calcium channels with venom toxins. In *Neurotox '91 the molecular basis of drug and pesticide action* (ed. I. R. Duce), pp. 33–44, Elsevier, Amsterdam.

Adams, M. E., Myers, R. A., Imperial, J. S., and Olivera, B. M. (1993*a*). Toxityping rat brain calcium channels with omega-toxins from spider and cone snail venoms. *Biochemistry*, **32**(47), 12566–70.

Adams, M. E., Mintz, I. M., Reily, M. D., Thanabal, V., and Bean, B. P. (1993*b*). Structure and properties of ω-Aga-IVB, a new antagonist of P-type calcium channels. *Mol. Pharm.*, **44**(4), 681–8.

Araque, A., Clarac, F., and Buno, W. (1994). P-type Ca^{2+} channels mediate excitatory and inhibitory synaptic transmitter release in crayfish muscle. *Proc. Natl. Acad. Sci. USA*, **91**(10), 4224–8.

Bickmeyer, U., Rossler, W., and Wiegand, H. (1994). Omega AGA toxin IVA blocks high-voltage-activated calcium channel currents in cultured pars intercerebralis neurosecretory cells of adult locusta migratoria. *Neurosci. Lett.*, **181**(1–2), 113–16.

Bindokas, V. P. and Adams, M. E. (1989). omega-Aga-I: a presynaptic calcium channel antagonist from venom of the funnel web spider, *Agelenopsis aperta*. *J. Neurobiol.*, **20**(4), 171–88.

Bindokas, V. P., Venema, V. J., and Adams, M. E. (1991). Differential antagonism of transmitter release by subtypes of omega-agatoxins. *J. Neurophysiol.*, **66**(2), 590–601.

Cohen, C. J., Ertel, E. A., Smith, M. M., Venema, V. J., Adams, M. E., and Leibowitz, M. D. (1992). High affinity block of myocardial L-type calcium channels by the spider toxin omega-Aga-toxin IIIA: advantages over 1,4-dihydropyridines. *Mol. Pharmacol.*, **42**(6), 947–51.

Ertel, E. A., Warren, V. A., Adams, M. E., Griffin, P. R., Cohen, C. J., and Smith, M. M. (1994*a*). Type III omega-agatoxins: a family of probes for similar binding sites on L- and N-type calcium channels. *Biochemistry*, **33**(17), 5098–108.

Ertel, E. A., Smith, M. M., Leibowitz, M. D., and Cohen, C. J. (1994*b*). Isolation of myocardial L-type calcium channel gating currents with the spider toxin omega-Aga-IIIA. *J. Gen. Physiol.*, **103**(5), 731–53.

Heck, S. D., Kelbaugh, P. R., Kelly, M. E., and Thadeio, P. F. (1994). Disulfide bond assignment of omega-agatoxins IVB and IVC – Discovery of a d-serine residue in omega-agatoxin IVB. *J. Am. Chem. Soc.*, **116**, 10426–36.

Kim, J. I., Konishi, S., Iwai, H., Kohno, T., Gouda, H., Shimada, I., *et al.* (1995). Three-dimensional solution structure of the calcium channel antagonist omega-agatoxin IVA: consensus molecular folding of calcium channel blockers. *J. Mol. Biol.*, **250**(5), 659–71.

Kondo, A., Soong, T. W., Mbungu, D. N., Olivera, B. M., Snutch, T. P. and Adams, M. E. (1994). Pharmacological profile of the rat α_{1E} calcium channel subunit expressed in *Xenopus* oocytes. *Soc. Neurosci. Abstr.*, **20**, 72.

Kuwada, M., Teramoto, T., Kumagaye, K. Y., Nakajima, K., Watanabe, T., Kawai, T., *et al.* (1994). Omega-agatoxin-TK containing D-serine at position 46, but not synthetic omega-[I-Ser46]agatoxin-TK, exerts blockade of P-type calcium channels in cerebellar Purkinje neurons. *Mol. Pharmacol.*, **46**(4), 587–93.

Leubke, J. L., Dunlap, K., and Turner, T. J. (1993). Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. *Neuron*, **11**, 1–20.

McDonough, S. I., Mintz, I. M., and Bean, B. P. (1995). ω-conotoxin MVIIC block of N-type and P-type calcium channels: interactions with other toxins. *Soc. Neurosci. Abstr.*, **21**, 339.

McIntosh, J. M., Adams, M. E., Olivera, B. M., and Filloux, F. (1992). Autoradiographic localization of the binding of calcium channel antagonist, [^{125}I]omega-agatoxin IIIA, in rat brain. *Brain Res.*, **594**(1), 109–14.

Mintz, I. M. (1994). Block of Ca channels in rat central neurons by the spider toxin omega-Aga-IIIA. *J. Neurosci.*, **14**(5), 2844–53.

Mintz, I. M., Venema, V. J., Adams, M. E., and Bean, B. P. (1991). Inhibition of N- and L-type Ca^{2+} channels by the spider venom toxin omega-Aga-IIIA. *Proc. Natl. Acad. Sci. USA*, **88**(15), 6628–31.

Mintz, I. M., Adams, M. E., and Bean, B. P. (1992*a*). P-type calcium channels in rat central and peripheral neurons. *Neuron*, **9**(1), 85–95.

Mintz, I. M., Venema, V. J., Swiderek, K. M., Lee, T. D., Bean, B. P., and Adams, M. E. (1992*b*). P-type calcium channels blocked by the spider toxin, ω-Aga-IVA. *Nature*, **355**, 827–9.

Nishio, H., Kumagaye, K. Y., Kubo, S., Chen, Y. N., Momiyama, A., Takahashi, T., *et al.* (1993). Synthesis of omega-agatoxin IVA and its related peptides. *Biochem. Biophys. Res. Commun.*, **196**(3), 1447–53.

Olivera, B. M., Miljanich, G., Ramachandran, J., and Adams, M. E. (1994). Calcium channel diversity and neurotransmitter release: The ω-conotoxins and ω-agatoxins. *Ann. Rev. Biochem.*, **63**, 823–67.

Randall, A. and Tsien, R. W. (1995). Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons. *J. Neurosci.*, **15**(4), 2995–3012.

Regehr, W. G. and Mintz, I. M. (1994). Participation of multiple calcium channel types in transmission at single climbing fiber to Purkinje cell synapses. *Neuron*, **12**(3), 605–13.

Reily, M. D., Holub, K. E., Gray, W. R., Norris, T. M., and Adams, M. E. (1994). Structure–activity relationships for P-type calcium channel selective ω-agatoxins. *Nature Structural Biology*, **1**(12), 853–6.

Reily, M. D., Thanabal, V., and Adams, M. E. (1995). The solution structure of ω-Aga-IVB, a P-type calcium channel antagonist from venom of the funnel web spider, *Agelenopsis aperta*. *J. Biomol. NMR*, **5**, 122–32.
Santos, A. D., Imperial, J. S., Chaudhary, T., Beavis, R. C., Chait, B. T., Hunsperger, J. P., *et al.* (1992). Heterodimeric structure of the spider toxin omega-agatoxin IA revealed by precursor analysis and mass spectrometry. *J. Biol. Chem.*, **267**(29), 20701–5.
Sather, W., Tanabe, T., Zhang, J.-F., Mori, Y., Adams, M., and Tsien, R. (1993). Distinctive biophysical and pharmacological properties of class A (BI) calcium channel α_1 subunits. *Neuron*, **11**, 291–303.
Scott, R. H., Dolphin, A. C., Bindokas, V. P., and Adams, M. E. (1990). Inhibition of neuronal Ca^{2+} channel currents by the funnel web spider toxin omega-Aga-IA. *Mol. Pharmacol.*, **38**(5), 711–18.
Takahashi, T. and Momiyama, A. (1993). Different types of calcium channels mediate central synaptic transmission. *Nature*, **366**(6451), 156–8.
Teramoto, T., Kuwada, M., Niidome, T., Sawada, K., Nishizawa, Y., and Katayama, K. (1993). A novel peptide from funnel web spider venom, omega-Aga-TK, selectively blocks, P-type calcium channels. *Biochem. Biophys. Res. Commun.*, **196**(1), 134–40.
Turner, T. J., Adams, M. E., and Dunlap, K. (1992). Calcium channels coupled to glutamate release identified by ω-Aga-IVA. *Science*, **258**, 310–13.
Turner, T. J., Adams, M. E., and Dunlap, K. (1993). Multiple calcium channel types coexist to regulate synaptosomal neurotransmitter release. *Proc. Natl. Acad. Sci. USA*, **90**, 9518–22.
Venema, V. J., Swiderek, K. M., Lee, T. D., Hathaway, G. M., and Adams, M. E. (1992). Antagonism of synaptosomal calcium channels by subtypes of omega-agatoxins. *J. Biol. Chem.*, **267**(4), 2610–15.
Wheeler, D. B., Randall, A., and Tsien, R. W. (1994). Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. *Science*, **264**(5155), 107–11.
Yu, H., Rosen, M. K., Saccomano, N. A., Phillips, D., Volkmann, R. A., and Schreiber, S. L. (1993). Sequential assignment and structure determination of spider toxin omega-Aga-IVB. *Biochemistry*, **32**(48), 13123–9.

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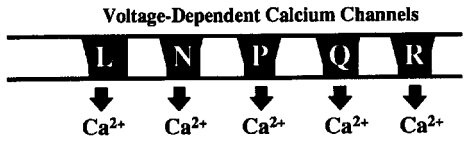
ω-Grammotoxin SIA (*Grammostola spatulata*/Chilean pink tarantula)

ω-Grammotoxin SIA, a structurally constrained peptide isolated from the venom of the tarantula species Grammostola spatulata, *inhibits several high threshold non-L-type voltage-dependent calcium channels. The toxin can reversibly inhibit multiple calcium-mediated processes, including neurotransmitter release and synaptic transmission, with high efficacy. The toxin's biological profile, confirmed with synthetic material, is similar to that of the* Conus magus *snail toxin MVIIC but its kinetic features and molecular site of action are distinct.*

ω-Grammotoxin SIA (ω-GsTx SIA) is a 36 amino acid, single chain peptide containing three disulfide bonds and an amidated carboxy terminus (Lampe *et al.* 1993). The average molecular weight of ω-GsTx SIA, determined by electrospray mass spectrometry (ES-MS), is 4109.2 Da in good agreement with the average theoretical molecular mass of 4109.7 Da. (Lampe *et al.* 1993). Reduction of the peptide results in the anticipated 6 amu shift with a corresponding ES-MS value of 4115.2 Da. Direct confirmation of the cysteine oxidation pattern has not been determined but is presumed to be the alternating Cys 1–4, Cys 2–5, and Cys 3–6 motif characteristic of the *Conus* snail toxins. The toxin is aqueous soluble, relatively temperature insensitive (extended storage as aqueous solution at 4 °C is routine), not readily susceptible to protease digestion and doesn't demonstrate a high propensity to stick to either glass or plastic. The peptide is enriched in basic residues (theoretical isoelectric point, pI, = 8.1) presumed to be necessary for its biological activity. Using F-moc chemistry and random oxidation schemes, synthetic peptide has been prepared, however yields have been low.

ω-GsTx SIA has been documented to inhibit calcium mediated biological responses that are sensitive to ω-conotoxin GVIA, ω-agatoxin IVA (ω-Aga-IVA), and ω-conotoxin MVIIC, but not those sensitive to dihydropyridines (Keith *et al.* 1992, 1995; Lampe *et al.* 1993; Piser *et al.* 1994, 1995, 1996; Turner *et al.* 1995). On the basis of the current classification scheme for high threshold, voltage-dependent calcium channels (VDCC), ω-GsTx SIA inhibits N-, P-, and Q-type but not L-type channels (Fig. 1). Evidence for ω-GsTx SIA inhibition of responses mediated



Nitrendipine	++				
ω-CgTx GVIA		++			
ω-Aga-IVA			++	+	
ω-CmTx MVIIC		+	++	++	
ω-GsTx SIA		++	++	++	?

Figure 1. Pharmacological profile of selected inhibitors of high threshold voltage-dependent calcium channels. ++ Indicates effective and potent inhibition of indicated calcium channel subtype; + indicates effective, but somewhat less potent inhibition; ? indicates that pharmacologically unclassified calcium channel subtype(s) other than L-, N-, P-, or Q-type may be inhibited (see Turner *et al.* 1995; Smith and Cunnane 1995). ω-CgTx GVIA, ω-conotoxin GVIA; ω-Aga-IVA, ω-agatoxin IVA; ω-CmTx MVIIC, ω-conotoxin MVIIC; ω-GsTx SIA, ω-grammotoxin SIA.

by a resistant type (pharmacologically defined) of VDCC has also been reported (Smith and Cunnane 1995; Turner *et al.* 1995). Analysis against cloned VDCC indicates that ω-GsTx SIA inhibits 90 per cent of the peak inward current through α_{1A}-β_{2A} channels with minimal (10 per cent) or no effect on α_{1E}-β_{1B} or α_{1C} channels, respectively (McDonough *et al.* 1996). The block of VDCC by ω-GsTx SIA is reversible and this property is voltage-sensitive (Keith *et al.* 1992, 1995; Piser *et al.* 1994, 1995; McDonough *et al.* 1996). Inhibition of other voltage-dependent ion channels by ω-GsTx SIA has not been documented.

Purification and sources

ω-GsTx SIA is purified from the venom, supplied by Spider Pharm, Inc. (Feasterville, PA, USA), of the Chilean Pink/Rose tarantula species, *Grammostola spatulata,* using a series of reverse phaseHPLC binary gradient elutions composed of acetonitrile as the organic phase.(Keith *et al.* 1992; Lampe *et al.* 1993). Peptide purity, as defined by RP-HPLC, ES-MS, and capillary electrophoresis analyses, is greater than 98 per cent using this protocol. Venom concentration, reproducible across multiple milkings, is approx. 600–800 μM. Synthetic peptide has been prepared by a commercial vendor on a custom basis. Aliquots of either native or synthetic ω-GsTx SIA peptide are obtainable on a limited basis from the authors.

Toxicity

No formal toxicological evaluation of ω-GsTx SIA has been conducted. Preliminary observations within mice, at intrathecal doses of 0.1–1 μg, indicate that cumulative motor disturbances (including loss of righting reflex, hindlimb splaying, tremor/seizure) occur and at the highest dose are lethal. Additionally, similar seizure-prone phenomenon and lethal toxic effects have been observed within mice following i.c.v. dosing at levels exceeding 5–10 μg.

Uses in cell biology

Several features of ω-GsTx SIA inhibition of high threshold VDCC render it useful as a tool to study calcium mediated cell biological responses. These include its kinetic characteristics, its nonselectivity and its mode of action on channel gating. With respect to nonselectivity, ω-GsTx SIA is distinct from the *Agelenopsis aperta* toxin ω-Aga-IIIA in its lack of activity against L-type channels. The VDCC inhibitory profile of ω-GsTx SIA is similar to that of the *Conus magus* snail toxin ω-CmTx MVIIC, however ω-GsTx SIA has a considerably faster association rate (Piser *et al.* 1995; Turner *et al.* 1995), is reversible (Keith *et al.* 1992; Piser *et al.* 1994, 1995, 1996), and potentially inhibits responses in certain systems with greater efficacy than MVIIC (Smith and Cunnane 1996; Turner *et al.* 1995). Preliminary binding observations indicate that ω-GsTxm SIA is not capable of displacing any of the other toxin ligands (i.e. *Conus geographus* snail toxin ω-CgTx GVIA, *Conus magus* snail toxins ω-CmTx MVIIC or ω-CmTx MVIIA, *Agelenopsis aperta* spider toxin -Aga-IVA) used to define the VDCC types at which the peptide produces block. Binding of labelled ω-GsTx SIA has not been performed to determine if any of the functionally overlapping toxins would occlude this interaction. It is known that the Phe-substituted Tyr analogue of ω-GsTx SIA retains activity rendering it a more useful ligand for iodination studies.

Presumably due to its failure to inhibit L-type VDCC, ω-GsTx SIA has shown greater efficacy against calcium mediated responses involving pre-synaptic components/processes (i.e. neurotransmitter release/synaptic transmission) versus isolated whole cell measurements. ω-GsTx SIA has been reported to completely inhibit calcium influx in rat and chick synaptosomes (Lampe *et al.* 1993; Keith *et al.* 1995). It will inhibit > 90 per cent of K^+-stimulated release of aspartate/glutamate from rat hippocampal/cortical tissue (Lampe *et al.* 1993; Keith *et al.* 1995; Turner *et al.* 1995) or norepinephrine from either rat or chick tissue (Lampe *et al.* 1993). Potassium-evoked release of glutamate, as well as basal release of serotonin, was also completely abolished by ω-GsTx SIA when measured *in vivo* in rats (Keith *et al.* 1995). Conversely, whole cell measurements of calcium current or flux in cells possessing a significant L-type component have resulted in less than complete inhibition as expected (Keith *et al.* 1992, 1995; Piser *et al.* 1995).

References

Keith, R. A., DeFeo, P. A., Lampe, R. A., Spence, K. T., Lo, M. M. S., and ffrench-Mullen, J. M. H. (1992). Inhibition of neuronal

calcium channels by a fraction from *Grammostola spatulata* venom. *Pharmacol. Commun.*, **1**, 19–25.
Keith, R. A., Mangano, T. J., Lampe, R. A., DeFeo, P. A., Hyde, M. J., and Donzanti, B. A. (1995). Comparative actions of synthetic ω-grammotoxin SIA and synthetic ω-Aga-IVA on neuronal calcium entry and evoked release of neurotransmitters *in vitro* and *in vivo*. *Neuropharmacol.*, **34**, 1515–28.
Lampe, R. A., DeFeo, P. A., Davison, M. D., Young, J., Herman, J. L., Spreen, R. C., *et al.* (1993). Isolation and pharmacological characterization of ω-grammotoxin SIA, a novel peptide inhibitor of neuronal voltage-sensitive calcium channel responses. *Mol. Pharmacol.*, **44**, 451–60.
McDonough, S. J., Noceti, F., Stefani, E., Lampe, R. A., and Bean, B. P. (1996). Alteration of voltage-dependent gating of P-type and α_{1A} calcium channels by ω-grammotoxin SIA. *Biophys. Soc. Abst.*, in press.
Piser, T. M., Lampe, R. A., Keith, R. A., and Thayer, S. A. (1994). ω-Grammotoxin SIA blocks action-potential-induced Ca^{2+} influx and whole-cell Ca^{2+} current in rat dorsal root ganglion neurons. *Pflügers Archiv*, **426**, 214–20.
Piser, T. M., Lampe, R. A., Keith, R. A., and Thayer, S. A. (1995). ω-Grammotoxin SIA blocks multiple, voltage-gated, Ca^{2+} channel subtypes in cultured rat hippocampal neurons. *Mol. Pharmacol.*, **48**, 131–9.
Piser, T. M., Lampe, R. A., Keith, R. A., and Thayer, S. A. (1996). Complete and reversible block of glutamatergic synaptic transmission by ω-grammotoxin SIA. *Neurosci. Lett.*, in press.
Smith, A. B. and Cunnane, T. C. (1996). ω-Conotoxin GVIA-resistant neurotransmitter release in postganglionic sympathetic nerve terminals. *Neuroscience*, **70**(3), 817.
Turner, T. J., Lampe, R. A., and Dunlap, K. (1995). Characterization of presynaptic calcium channels with ω-conotoxin MVIIC and ω-grammotoxin SIA: Role for a resistant calcium channel type in neurosecretion. *Mol. Pharmacol.*, **47**, 348–53.

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Hololena toxin (*Hololena curta*)

Hololena toxin is an irreversible calcium channel antagonist isolated from the venom of the funnel web spider, Hololena curta. *It is specific for a subpopulation of neuronal calcium channels in* Drosophila.

Hololena toxin was isolated because of its ability to produce a presynaptic block of neuromuscular transmission in *Drosophila* larvae (Bowers *et al.* 1987). The nature of the presynaptic block indicated that the toxin was blocking neuronal calcium channels; this hypothesis has been verified directly using intracellular electrophysiology in *Drosophila* neurons (Leung *et al.* 1989; Leung and Byerly 1991). Hololena toxin is not active on any of the major voltage-dependent currents in *Drosophila* muscle, nor is it active at frog neuromuscular junction (Bowers *et al.* 1987). The isolated activity is only one of several that have now been described from the venom of this funnel web spider (Stapleton *et al.* 1990; Quistad *et al.* 1991; Lundy and Frew 1993; Meinwald and Eisner 1995), making the simple designation, hololena toxin, unavoidably anachronistic.

■ Purification and sources

Venom of *Hololena curta* was obtained by electrophoretic milking with particular care in avoiding contamination from digestive fluids. Purification of the hololena toxin was achieved using routine size exclusion chromatography and C_{18} reverse phase HPLC (Bowers *et al.* 1987). On SDS–polyacrylamide gel electrophoresis (SDS/PAGE), hololena toxin behaves as a disulfide-bonded heterodimer with an estimated molecular weight of 16 000 Daltons (subunits of 7000 and 9000 Daltons). These molecular weight estimates are based on the simplest interpretation of the reduced/non-reduced SDS/PAGE analysis and should be considered tentative until more definitive structural analysis is conducted (Bowers *et al.* 1987). Venom from *Hololena curta* can be purchased from Spider Pharm, Feasterville, PA 19053 USA.

■ Use in cell biology

Analysis of the activity of hololena toxin on cultured *Drosophila* neurons has verified the specificity of this toxin for a subset of neuronal calcium channels (Leung *et al.* 1989; Leung and Byerly 1991). In fact, because hololena toxin blocks only half of the calcium current and a subset of calcium channels in this system, the toxin provided the first compelling evidence for the presence of distinct subpopulations of calcium channels in *Drosophila* neurons (Leung *et al.* 1989; Leung and Byerly 1991). Whether the calcium channels that are blocked in cultured neurons are responsible for neuromuscular transmission has not been determined.

References

Bowers, C. W., Phillips, H. S., Lee, P., Jan, L. Y., and Jan, Y. N. (1987). The identification and purification of an irreversible presynaptic neurotoxin from the venom of the spider, *Hololena curta*. *Proc. Natl. Acad. Sci. USA*, **84**, 3506–10.

Leung, H.-T. and Byerly, L. (1991). Characterization of single calcium channels in *Drosophila* embryonic nerve and muscle cells. *J. Neurosci.*, **11**, 3047–59.

Leung, H.-T., Branton, W. D., Phillips, H. S., Jan, L., and Byerly, L. (1989). Spider toxins selectively block calcium currents in *Drosophila*. *Neuron*, **3**, 767–72.

Lin, J.-W., Rudy, B., and, Llinás, R. (1990). Funnel-web spider venom and a toxin fraction block calcium current expressed from rat brain mRNA in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA*, **87**, 4538–42.

Lundy, P. M. and Frew, R. (1993). Pharmacological characterization of voltage-sensitive Ca^{2+} channels in autonomic nerves. *Eur. J. Pharmacol.*, **231**, 197–202.

Meinwald, J. and Eisner, T. (1995). The chemistry of phyletic dominance. *Proc. Natl. Acad. Sci. USA*, **92**, 14–18.

Quistad, G. B., Reuter, C. C., Skinner, W. S., Dennis, P. A., Suwanrumpha, S., and Fu, E. W. (1991). Paralytic and insecticidal toxins from the funnel web spider, *Hololena curta*. *Toxicon*, **29**, 329–36.

Stapleton, A., Blankenship, D. T., Ackermann, B. L., Chen, T. -M., Gorder, G. W., Manley, G. D., *et al.* (1990). Curtatoxins. *J. Biol. Chem.*, **265**, 2054–9.

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PLTXII *(Plectreurys tristes)*

PLTXII is a 44 amino acid proteolipid found in the venom of the spider Plectreurys tristes. *It specifically and irreversibly blocks voltage dependent Ca^{2+} channels in insect* (Drosophila) *neurons and results in inhibition of neurotransmitter release from presynaptic nerve terminals.*

PLTXII is one member of a family of *Plectreurys* toxins which are potent neurotoxins found in *Plectreurys* spider venom (Branton *et al.* 1987). The peptide chain of the toxin contains 44 amino acids including 10 cysteine residues that probably form five disulfide bonds (Branton *et al.* 1993a) (Swiss-Prot databank:P34079; NCBI Seq ID: 633835), as shown in Fig. 1. The peptide chain is quite polar in character, but the carboxy terminal threonine residue is carboxyamidated and O-palmitoylated, resulting in an amphipathic structure that is soluble in both aqueous and organic media.

All similar toxins in *Plectreurys* venom appear to be similarly acylated at C-terminal threonine or serine residues. The novel, O-palmitoyl moiety is absolutely required for biological activity (Branton *et al.* 1993*a*; Bodi *et al.* 1995). PLTXII is a potent and specific inhibitor of insect *(Drosophila)* neuronal voltage dependent Ca^{2+} channels. At nanomolar concentration, it rapidly and irreversibly blocks synaptic transmission in *Drosophila* by blocking presynaptic Ca^{2+} channels (Branton *et al.* 1987; Leung *et al.* 1989).

Ala Asp Cys Ser Ala Thr Gly Asp Thr Cys Asp His
Thr Lys Lys Cys Cys Asp Asp Cys Tyr Thr Cys Arg
Cys Gly Thr Pro Trp Gly Ala Asn Cys Arg Cys Asp
Tyr Tyr Lys Ala Arg Cys Asp Thr(Pal)amide

Figure 1. The structure of PLTXII.

Purification and sources

PLTXII is purified from electrically milked *Plectreurys tristes* venom (Spider Pharm, Feasterville, PA) by size exclusion chromatography followed by two steps of C18 reversed phase HPLC (Branton *et al.* 1987, 1993*a*). The proteolipid has a high affinity for C18 columns and elutes relatively late in a gradient of increasing organic solvent. The toxin is relatively stable when pure, but care must be taken to avoid loss by adsorption from dilute solution to both hydrophilic and hydrophobic surfaces. Exposure to base results in hydrolysis of the O-palmitoyl ester, loss of biological activity, and much earlier elution from C18 columns. Synthesis is challenging but has been achieved (Branton *et al.* 1993*b*; Bodi *et al.* 1995), and PLTXII is produced commercially by the Peptide Institute (Osaka).

Toxicity

At this time, pure PLTXII has no known toxicity to vertebrates and human envenomaton by *Plectreurys tristes* itself produces only very minor symptoms (Carpenter

et al. 1991). No LD_{50} has been established. The use of PLTXII itself probably does not present a hazard to personnel. However, reasonable care is advised in handling large amounts of toxin and due caution should be exercised with modified analogues of PLTXII or significant amounts of vertebrate toxins which are present in the venom (Feigenbaum *et al.* 1988; Newman *et al.* 1992; Lundy and Frew 1993).

Use in cell biology

PLTXII is a potent blocker of calcium currents and neurotransmitter release in insects and is significant as a generally available calcium channel blocker that is specific for insect calcium channels verses vertebrate channels. It blocks almost all of the current in cultured embryonic *Drosophila* neurons which probably includes contributions from more than one class of voltage dependent calcium channel (Leung *et al.* 1989). The mechanism of action and specific site of action of PLTXII are not known. The novel proteolipid structure is unique among calcium channel toxins and has broad similarities to a variety of vertebrate intracellular regulatory proteins which are also fatty acylated (Branton *et al.* 1993*a*). It is possible that the mechanism of action of PLTXII and other toxins in the same family is unique relative to other calcium channel blockers. The possibility that PLTXII could act at an intracellular site has been suggested. Recent synthesis of PLTXII should allow exploration of these issues.

References

Bodi, J., Nishio, H., Zhou, Y., Branton, W. D., Kimura, T., and Sakakibara, S. (1995). Synthesis of an O-palmitoylated 44-residue peptide amide (PLTXII) blocking presynaptic calcium channels in *Drosophila*. *Peptide Res.*, **8**, 228–35.

Branton, W. D., Kolton, L., Jan, Y. N., and Jan, L. Y. (1987). Neurotoxins from *Plectreurys* spider venoms are potent presynaptic blockers in *Drosophila*. *J. Neurosci.*, **7**, 4195–200.

Branton, W. D., Rudnick, M. S., Zhou, Y., Eccleston, E. D., Fields, G. B., and Bowers, L. D. (1993*a*). Fatty acylated toxin structure. *Nature*, **365**, 496–7.

Branton, W. D., Fields, C. G., VanDrisse, V. L., and Fields, G. B. (1993*b*). Solid phase synthesis of O-palmitoylated peptides. *Tetrahedron Lett.*, **34**, 4885–8.

Carpenter, T. L., Bernacky, B. J., and Stabell, E. E. (1991). Human envenimization by *Plectreurys tristis Simon (Araneae: Plectreuridae)*: a case report. *J. Med. Entomol.*, **28**, 477–8.

Feigenbaum, P., Garcia, M. L., and Kaczorowski, G. J. (1988). Evidence for distinct sites coupled to high affinity omega-cronotoxin receptors in rat brain synaptic plasma membrane vesicles. *Biochem. Biophy. Res. Commun.*, **154**, 298–305.

Leung, H. T., Branton, W. D., Phillips, H. S., Jan, L., and Byerly, L. (1989). Spider toxins selectively block calcium currents in *Drosophila*. *Neuron*, **3**, 767–72.

Lundy, P. M. and Frew, R. (1993). Evidence of mammalian Ca^{2+} channel inhibitors in venom of the spider. *Toxicon*, **31**, 1249–56.

Newman, E. A., Zhou, Y., Rudnick, M. S., and Branton, W. D. (1992). Toxins in *Plectreurys* spider venom are potent blockers of vertebrate calcium channels. *Soc. Neurosci.*, **409** (abstract).

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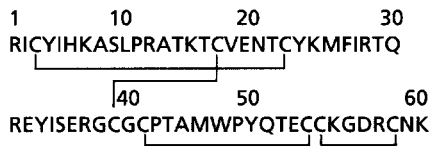
Calciseptine *(Dendroaspis polylepis)*

Calciseptine is a 60-amino acid polypeptide with four disulfide bridges, purified from Dendroaspis polylepis *snake (black mamba) venom. It is a smooth muscle relaxant and an inhibitor of cardiac contractions. It is a specific blocker of L-type calcium channel with a clearly tissue-dependent sensitivity.*

Calciseptine has been purified from the venom of the black mamba *Dendroaspis polylepis polylepis*. It is a single polypeptide chain of 7 kDa (60 amino acid residues with eight cysteines forming four disulfide bridges, sequence accession number to the Swiss Prot databank: P22947 TXCA-DENPO). Its primary structure is indicated below.



Its sequence and its structural organization have similarities with neurotoxins that block the nicotinic receptors and with snake cyto-cardiotoxins (Rees and Bilwes 1993). Calciseptine has been shown to selectively block L-type calcium channels in a variety of cells but with a clearly tissue-dependent sensitivity. Calciseptine blocks L-type calcium current in aortic smooth muscle cells, ventricular cardiac cells, dorsal root ganglion nervous cells, and insulinoma cells. In addition, calciseptine relaxes spontaneous or K^+-evoked smooth muscle contraction and has an acute hypotensive effect in rats (De Weille *et al.* 1991; Watanabe *et al.* 1995) and blocks Bay K 8644-induced cardiac contractions. In contrast to classical L-type calcium blockers, calciseptine neither affects L-type calcium channels nor contraction of skeletal muscle cells. This peptide is totally inactive on other voltage-dependent calcium channels such as N-type and T-type channels (De Weille *et al.* 1991). It has also been reported that calciseptine competitively inhibited the specific binding of [^{3}H]nitrendipine to synaptosomal membranes whereas it enhanced the specific binding of [^{3}H]diltiazem and had no effect on [^{3}H]verapamil binding (Yasuda *et al.* 1993).

Purification and sources

Calciseptine was purified from the crude venom of the black mamba in three steps:

(1) gel filtration;

(2) ion exchange on TSK SP 5PW; and

(3) reverse-phase chromatography on RP18. Details can be found in Schweitz *et al.* (1990), which describes the isolation of all peptides from *Dendroaspis polylepis* venom. In this work, calciseptine is referred to as peptide E3. Purified calciseptine can be purchased from Latoxan, 05150 Rosans, France and Alomone Labs. Ltd. Headquarters, Shatner Center 3, P.O. Box 4287 (91042 Jerusalem, Israel). Synthetic calciseptine is available from Peptide Institute Inc., Terutushi Kimura, Protein Research Foundation 4-1-2 Ina, Minoh-shi (Osaka 562, Japan) (Kuroda *et al.* 1992).

Toxicity

Toxicity was tested on mice by intraperitoneal (ip), intracisternal (ic), or intravenous (iv) injections of different amounts of calciseptine. Toxicity was very weak since LD_{50} (ip) is > 18 mg/kg, LD_{50} (ic) is > 3 mg/kg, and LD_{50} (iv) is 5 mg/kg.

Use in cell biology

Voltage-dependent calcium channels play a central role in many processes of excitable cells such as excitation–contraction coupling, excitation–secretion coupling, activation of second messenger systems, gene expression, or cell death. Voltage-dependent calcium channels have been classified into high-threshold (L-, N-, P-, Q-, R-types) and low-threshold (T-type) voltage-gated channels and distinguished by their biophysical and pharmacological properties.

High-affinity polypeptide toxins blocking these channels are important tools for defining calcium channel subtypes, for visualizing their cellular distribution and understanding their functions.

References

De Weille, J. R., Schweitz, H., Maes, P., Tartar, A., and Lazdunski, M. (1991). Calciseptine, a peptide isolated from black mamba venom, is a specific blocker of the L-type calcium channel. *Proc. Natl. Acad. Sci. USA*, **88**, 2437–40.

Kuroda, H., Chen, Y.-N., Watanabe, T. X., Kimura, T., and Sakakibara, S. (1992). Solution synthesis of calciseptine, an L-type specific calcium channel blocker. *Peptide Res.*, **5**, 265–8.

Rees, B. and Bilwes, A. (1993). Three-dimensional structures of neurotoxins and cardiotoxins. *Chem. Res. Toxicol.*, **6**, 385–406.

Schweitz, H., Bidard, J. N., and Lazdunski, M. (1990). Purification and pharmacological characterization of peptide toxins from the black mamba (*Dendroaspis polylepis*) venom. *Toxicon*, **28**, 847–56.
Watanabe, T. X., Itahara, Y., Kuroda, H., Chen, Y. N., Kimura, T., and Sakakibara, S. (1995). Smooth muscle relaxing and hypotensive activities of synthetic calciseptine and the homologous snake venom peptide FS2. *Japan J. Pharmacol.*, **68**, 305–13.
Yasuda, O., Morimoto, S., Chen, Y., Jiang, B., Kimura, T., Sakakibara, T., *et al.* (1993). Calciseptine binding to 1,4-dihydropyridine recognition site of the L-type calcium channel of rat synaptosomal membranes. *Biochem. Biophys. Res. Commun.*, **194**, 587–94.

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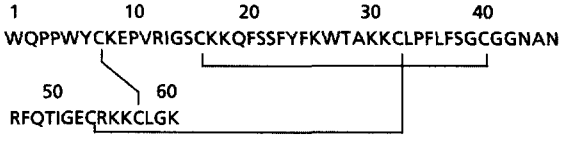
Calcicludine (*Dendroaspis angusticeps*)

Calcicludine is a 60-amino acid polypeptide with three disulfide bridges purified from Dendroaspis angusticeps *snake (eastern green mamba) venom. It is structurally homologous to the Kunitz-type protease inhibitor family. It is a potent blocker of high-threshold calcium channels with the higher affinity for the L-type in cerebellar granule neurons.*

Calcicludine has been purified from the venom of the green mamba *Dendroaspis angusticeps*. It is a polypeptide of 7 kDa (60 amino acid residues with six cysteines forming three disulfide bridges, sequence accession number to PIR databank: A36989). Calcicludine is structurally homologous to Kunitz-type protease inhibitors. Homologies are 40 per cent with dendrotoxins, 29 per cent with the basic pancreatic trypsin inhibitor, and 39 per cent with the protease inhibitor domain of the amyloid b protein (APPI). Its primary structure is shown below.



Despite its structural homology with dendrotoxins, which selectively block a class of voltage-dependent potassium channels, calcicludine specifically blocks most of the high-threshold calcium channels (L-, N-, or P-type) in the 10–100 nM range of concentration (Schweitz *et al.* 1994). The sensitivity of L-, N-, and P-type channels to this toxin is clearly tissue- and species-dependent. For instance, a particular type of L-type Ca^{2+} channel in cerebellar granule cells is much more sensitive to calcicludine (EC_{50} = 0.2 nM) than cardiac L-type channels (EC_{50} = 5 nM) or DRG neuronal L-type channels (EC_{50} = 60–80 nM). A total insensitivity was found for rat skeletal muscle L-type channels. Chicken DRG N-type channels are more sensitive to calcicludine (EC_{50} = 25 nM) than rat DRG N-type channels (EC_{50} = 60–80 nM). Cerebellar granule cell N-type channels are almost insensitive to calcicludine (EC_{50} > 100 nM). P-type calcium current in cerebellar Purkinje neurons have a good sensitivity to calcicludine (EC_{50} = 1–5 nM). Calcicludine is the only toxin capable of blocking L-, N-, and P-type calcium channels. It appears also to be a very selective blocker of a sub-type of L-type calcium channel that is predominantly expressed in cerebellar granule cells.

Binding experiments on rat olfactory bulb membranes using [^{125}I]calcicludine have identified a single class of high affinity sites with a K_d of 15–36 pM without interactions with those of the classical classes of L- and N-type calcium blockers. In the rat brain, high densities of specific [^{125}I]calcicludine binding sites were found in the olfactory bulb, in the molecular layer of the dendate gyrus and the stratum oriens of the CA3 field in the hippocampal formation, and in the granular layer of the cerebellum.

■ Purification and sources

The crude venom of *Dendroaspis angusticeps* (Latoxan, Rosans, France) was dissolved in 1 per cent acetic acid and chromatographed onto a Sephadex G50 column. The peptidic fraction was loaded on a TSK SP5PW column. Peptidic fractions were then eluted with a linear gradient from 1 per cent acetic acid to 1 M ammonium acetate (fractions A to R). Fraction Q was loaded on a Lichrosorb RP18 column and eluted with a linear gradient from 10 per cent to 40 per cent of 0.5 per cent trifluoroacetic acid plus 0.9 per cent triethylamine in acetonitrile. Fraction Q1 is calcicludine. This toxin has been syn-

thetized (Terutoshi Kimura) Peptide Institute Inc. Protein Research Foundation 4-1-2 Ina, Minoh-Shi, Osaka 562, Japan and is commercially available (Alomone Labs Ltd, Headquarters, Shatner Center 3, P.O. Box 4287, Jerusalem 91042, Israel).

Toxicity

Toxicity was tested on mice by intracisternal (ic) and intravenous (iv) injections of different amount of calcicludine. Toxicity was very weak since LD_{50} (ic) is > 1.8 mg/kg and LD_{50} (iv) is 2.5 mg/kg.

Use in cell biology

Voltage-dependent calcium channels play a central role in many processes of excitable cells such as excitation–contraction coupling, excitation–secretion coupling, activation of second messenger systems, gene expression, or cell death. Voltage-dependent calcium channels have been classified into high-threshold (L-, N-, P-, Q-, R-types) and low-threshold (T-type) voltage-gated channels and distinguished by their biophysical and pharmacological properties.

High-affinity polypeptide toxins blocking these channels are important tools for defining calcium channel subtypes, for visualizing their cellular distribution and understanding their functions.

References

Schweitz, H., Heurteaux, C., Bois, P., Moinier, D., Romey, G. and Lazdunski, M. (1994). Calcicludine, a venom peptide of the Kunitz-type protease inhibitor family, is a potent blocker of high-threshold Ca^{2+} channels with a high affinity for L-type channels in cerebellar granule neurons. *Proc. Natl. Acad. Sci. USA*, **91**, 878–82.

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β-Leptinotarsin-h

Leptinotarsin, also known as leptinotoxin, is a protein secreted into the hemolymph of potato beetles of the genus Leptinotarsa. *Both β-leptinotarsin-h from* L. haldemani *and β-leptinotarsin-d from* L. decemlineata *have been shown to be lethal to insects and many different vertebrates on injection. The leptinotoxins cause the release of neurotransmitters from synapses in diverse neuronal preparations. The increase in frequency of miniature end-plate potentials (mepps) caused by leptinotarsin-h has been shown to be of a biphasic nature, the first phase of which is abolished by low Ca^{2+} concentration in the medium. The action of the toxin is saturable and closely resembles transmitter release due to K^+ depolarization. It is thus thought that leptinotarsin-h opens presynaptic voltage-gated Ca^{2+}-channels.*

β-Leptinotarsin-h is a toxic protein present in the hemolymph of the potato beetle, *Leptinotarsa haldemani*. The analogous protein, β-leptinotarsin-d, from *L. decemlineata*, the Colorado potato beetle, is also toxic, but to a lesser extent and so most studies have used the h-type. Purification and SDS–PAGE shows that the proteins have molecular weights of around 57 and 45 kiloDaltons, respectively. Bidimensional PAGE with isoelectric focusing indicate that the protein is slighty acidic (Crosland *et al.* 1984). Neither the protein nor the gene has been sequenced.

Intoxication can only occur when injected; oral ingestion causes no effect, thus it is probable that the protein does not play a defensive role for the potato beetle. Injection of the protein into the thorax of insects causes them to become sluggish, their limbs to become rigid and spread and finally causes death. In vertebrates, symptoms include partial paralysis, decrease in breathing rate, convulsions, and lethargy leading to death (Hsiao and Fraenkel 1969).

Release of neurotransmitter by β-leptinotarsin-h requires the presence of Ca^{2+}. The biphasic increase in miniature end plate potentials (mepps) caused by the toxin is tetrodotoxin insensitive, but the first phase can be abolished by low surrounding Ca^{2+} concentration (McClure *et al.* 1980). Later it was shown that leptinotarsin increases the permeability to Ca^{2+}. Thus it was hypothesized that the toxin acts by propping open voltage-gated Ca^{2+} channels (Crosland *et al.* 1984). Further studies suggested that these actions could be attributed to ionophore activity of leptinotarsin (Madeddu *et al.* 1985*a*,*b*). Finally, the leptinotarsin

evoked-release of transmitter was shown to be saturable and very similar to that seen on depolarization with K^+. In addition, it was shown that Ca^{2+} is required for binding of the neurotoxin to its putative receptor and that its effects can not be blocked by the Ca^{2+} channel antagonist, omega-conotoxin. This is consistent with the initial idea of leptinotarsin acting as a presynaptic Ca^{2+} channel agonist, whilst not excluding the possibility that it may be a receptor mediated Ca^{2+} ionophore (Miljanich *et al.* 1988).

Purification and sources

β-Leptinotarsin-h is purified from the hemolymph of the larva of *L. haldemani* by precipitating with ammonium sulfate and then dialysing. The nondialysable protein is lyophilized, dissolved in a weak phosphate buffer, and centrifuged at 5000 *g* for 10 min at 4 °C. The supernatant is then submitted to a series of chromatography steps. Using a sequence of Sephadex G-150, DEAE-Sephacel, phosphocellulose, and Reactive Blue 2-agarose chromatography the toxin can be extracted to 1050 times purity (Crosland *et al.* 1984).

Toxicity

Leptinotarsin-h has been shown to be toxic to a wide variety of insects, except the potato beetle itself, when injected into the thorax. Also, injection of the hemolymph or the purified toxin is lethal to mice. Intoxication cannot occur orally suggesting that the protein does not have a defensive function and thus potato beetles present no public health risk. Toxicity is tested by injecting intraperitoneally into mice or into the thorax of house flies. The dose of pure toxin required to kill an adult Wilson strain house fly within 4 hours after injection was determined to be 0.6 μg (Hsiao and Fraenkel 1969).

Use in cell biology

The toxin has been shown to cause release of neurotransmitters from a variety of vertebrate nerve terminal preparations including rat (Yoshino *et al.* 1980; Crosland *et al.* 1984), guinea pig (Madeddu *et al.* 1985a), and the marine electric ray (Miljanich *et al.* 1988). Also, leptinotarsin has been successfully used with pheochromocytoma cells (Madeddu *et al.* 1985*b*) and the rat neuromuscular junction (McClure *et al.* 1980), but does not seem to be active in the frog (Hsiao and Fraenkel 1969) or in squid optic lobe synaptosomes. Application of the toxin to immobilized, ^{3}H-choline loaded synaptosomes causes release of radiolabelled acetylcholine (Crosland *et al.* 1984). Unfortunately, the exact location of action of β-leptinotarsin remains elusive and thus it has not successfully been used in dissecting the steps in neurotransmitter release following depolarization.

References

Crosland, R. D., Hsiao, T. H., and McClure, W. O. (1984). Purification and characterization of β-leptinotarsin-h, an activator of presynaptic calcium channels. *Biochemistry*, **23**, 734–41.

Hsiao, T. H. and Fraenkel, G. (1969). Properties of leptinotarsin, a toxic hemolymph protein from the Colorado potato beetle. *Toxicon*, **7**, 119–30.

McClure, W. O., Abbot, B. C., Baxter, D. E., Hsiao, T. H., Satin, L. S., Siger, A., *et al.* (1980). Leptinotarsin: A presynaptic neurotoxin that stimulates release of acetylcholine. *Proc. Natl. Acad. Sci. USA*, **77**(2), 1219–23.

Madeddu, L., Pozzan, T., Robello, M., Rolandi, R., Hsiao, T. H., and Meldolesi J. (1985a). Leptinotoxin-h action in synaptosomes, neurosecretory cells, and artificial membranes: Stimulation of ion fluxes. *J. Neurochem.*, **45**(6), 1708–18.

Madeddu, L., Saito, I., Hsiao, T. H., and Meldolesi, J. (1985*b*). Leptinotoxin-h action in synaptosomes and neurosecretory cells: Stimulation of neurotransmitter release. *J. Neurochem.*, **45**(6), 1719–30.

Miljanich, G. P., Yeager, R. E., and Hsiao, T. H. (1988). Leptinotarsin-d, a neurotoxic protein, evokes neurotransmitter release from, and calcium flux into, isolated electric organ nerve terminals. *J. Neurobiol.*, **19**(4), 373–86.

Yoshino, J. E., Baxter, D. E., Hsiao, T. H., and McClure, W. O. (1980). Release of acetylcholine from rat brain synaptosomes stimulated with leptinotarsin, a new neurotoxin. *J. Neurochem.*, **34**(3), 635–42.

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Taicatoxin (*Oxyuranus scutelatus scutelatus*)

Taicatoxin a complex oligomeric toxin isolated from the venom of the Australian snake Oxyuranus scutelatus scutelatus, *is constituted by three different subunits: an α-neurotoxin-like polypeptide, a neurotoxic phospholipase, and a serine protease inhibitor. It blocks the high threshold Ca^{2+}-channel current of excitable cells in heart and does not affect the low threshold Ca^{2+}-channel current.*

Taicatoxin (TCX) is a complex oligomeric toxin isolated from the venom of the Australian taipan snake *Oxyuranus scutelatus scutelatus*. It contains three different active components: an α-neurotoxin-like peptide with a mol. wt of 8 kDa, a phospholipase of mol. wt 16 kDa, and a serine protease inhibitor of mol. wt 7 kDa, joined by noncovalent bonds, at an approximate stoichiometry of 1:1:4, respectively. The most active form is composed of these three peptides, although the phospholipase and the neurotoxin-like peptides are toxic by themselves.

Purification and sources

Taicatoxin (TCX) can easily be purified from the venom of the Australian taipan snake *Oxyuranus scutelatus scutelatus* by ion exchange chromatography through DEAE-cellulose followed by two steps of CM-cellulose chromatography at pH 4.7 and 6.0, respectively. The phospholipase moiety can be separated by affinity column on a PC-Sepharose column or by Sephadex G-50 (superfine) gel filtration chromatography in the presence of a high salt concentration (1 M NaCl) and alkaline pH (8.2). Specific questions on TCX can be addressed to Dr Lourival D. Possani (see address at the end of this entry).

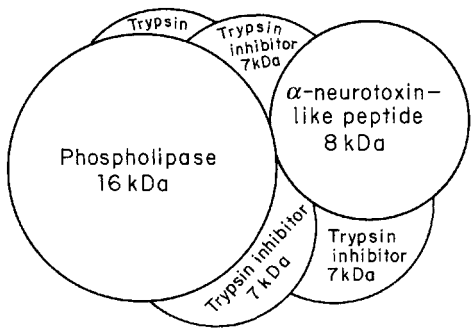

Figure 1. Schematic representation of the minimum subunit composition of TXC. It is composed of one molecule of a phospholipase (16 kDa), a neurotoxic peptide similar to alpha-toxins (8 kDa), and four identical peptides of a trypsin inhibitor (7 kDa).

Structure of taicatoxin

The N-terminal amino acid sequence of the three main components of TCX were determined. The full primary structure of the protease inhibitor moiety was obtained.

Toxicity

Intraperitoneal injection of TCX into mice is lethal within two hours in the range 50–100 ng of TCX/g of mouse (Possani *et al.* 1989, 1992).

Use in cell biology

TCX blocks the high threshold Ca^{2+}-channel current of excitable cells in heart and does not affect the low threshold Ca^{2+}-channel current. The toxin acts at a site that is only accessible extracellularly. The selective Ca^{2+}-channel blockade is reversible and does not modify single channel conductance but only changes the gating mechanism. The binding of the toxin is voltage dependent with higher affinity for inactivated channels (Possani *et al.* 1992). Channel recognition and blockade seems to require at least two of the subunits (α-neurotoxin and protease inhibitor). TCX is not tissue restricted. It affects high threshold Ca^{2+} channels of a variety of muscle cells as well as neurosecretory cells (unpublished observations). TCX can be considered as a valuable probe for the study of the structure–function relationships of Ca^{2+} channels (Brown *et al.* 1987).

References

Brown, A. M., Yatani, A., Lacerda, A. E., Gurrola, G., and Possani, L. D. (1987). Neurotoxins that act selectively on voltage-dependent cardiac calcium channels. *Circulation Research*, **61**, 6–9.

Possani, L. D., Mochca-Morales, J., Martin, B., Yatani, A., and Brown, A. (1989). Taicatoxin, a complex oligomeric protein

from Taipan snake venom, blocks specifically the Ca^{2+} channels of cardiac muscle (abstract). *Toxicon*, **27**, 71–2.
Possani, L. D., Martin, B. M., Yatani, A., Mochca-Morales, J., Zamudio, F. Z., Gurrola, G. B., *et al.* (1992). Isolation and physiological characterization of taicatoxin, a complex toxin with specific effects on calcium channels. *Toxicon*, **30**, 1343–64.

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10

Acetylcholine receptor targeted toxins

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Introduction

The nicotinic acetylcholine receptors (AChRs) are the target of the toxins described in this chapter, which bind to them with high affinity. These toxins were instrumental in AChRs purification experiments in the early 1970s and allowed a comprehensive understanding of their biophysical and functional properties.

AChRs are cationic ion channels whose probability of aperture is increased by acetylcholine (ACh). They belong to a super family of ligand-gated ion channels, which includes $GABA_A$, glycine, and serotonin $5HT_3$ receptors (Stroud *et al.* 1990; Changeux 1993; Devillers-Thiéry *et al.* 1993; Ortells and Lunt 1995).

AChRs are key molecules in cholinergic transmission at the neuromuscular junction, the sympathetic ganglia, the retina, and various brain areas; they can be postsynaptic (muscle and ganglia) or presynaptic, as in a number of CNS nuclei (i.e. striatum).

On the basis of their molecular structure and functional properties, AChRs can be divided into three families: muscular, neuronal, and homomeric (Ortells and Lunt 1995).

Subunits

AChRs are composed of five subunits that delimit a cationic channel. The biophysical and pharmacological properties of AChRs depend on their subunit composition and, so far, 16 subunits have been cloned in different animal species: nine α subunits ($\alpha1$–$\alpha9$), four β subunits ($\beta1$–$\beta4$) and one δ, ε, and γ subunit Fig. 1). Muscle AChRs are formed of $\alpha1$ (Changeux 1993), $\beta1$, δ and γ (embryonal), or ε (adult) subunits; neuronal AChRs are formed of two α ($\alpha2$, $\alpha3$ or $\alpha4$) and three β ($\beta2$ or $\beta4$) subunits (Luetje and Patrick 1991); and homomeric receptors of five α ($\alpha7$, $\alpha8$ or $\alpha9$) subunits (Gotti *et al.* 1994; Elgoyen *et al.* 1994). The agonist and toxin binding site is mainly located in the α subunits, but the contribution of adjacent subunits also affects its fine pharmacology. The different combination of subunits confers to each subtype a peculiar biophysical and pharmacological property (see Fig. 1). The function of $\alpha5$, $\alpha6$, and $\beta3$ subunits still remains elusive. However, $\alpha5$ expressed in heterologous systems with another α and β subunit modifies the pharmacological and biophysical properties of the ion channels (Ramirez-Latorre *et al.* 1996).

The different subunits have a putative similar molecular topology, with four transmembrane domains, a large extracellular hydrophilic N-terminus, and an intracellular C-terminus. Between transmembrane regions 3 and 4 there is a large intracellular loop. The amino acid sequence homology among the subunits is high in the transmembrane domains and very low in the large cytoplasmatic loop, which can be considered the molecular fingerprint of every subunit. This loop contains a number of phosphorylation sites that are important for receptor function and regulation.

Binding sites

The ligand binding site is located at the N-terminus of α subunit in a region created by three loops: the AA sequences 188–201, 135–151, and 85–96 (Devillers-Thiéry *et al.* 1993). This site is characterized by a cysteine doublet (cys 192–193 in Torpedo AChR) that is essential for the binding of nicotinic agents, as their mutation or deletion drastically reduces ligand binding.

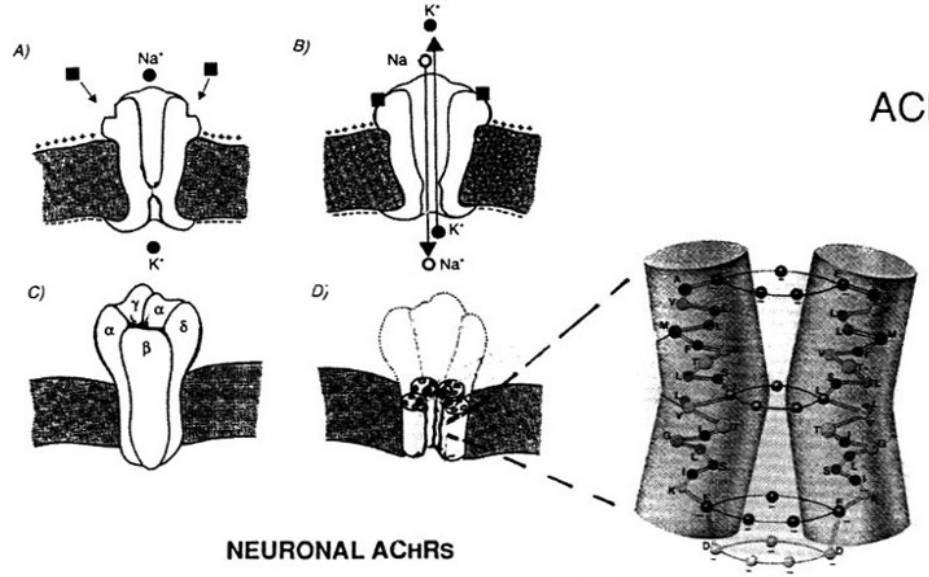
Toxins bind predominantly to the 189–200 site, but also bind to other sites that are less related to the specificity of the binding but important for its stabilization. Synthetic peptides with the same AA sequence as the Torpedo 188–201 toxin binding site, bind α-bungarotoxin, albeit with a lower affinity than the wild receptor (Gotti *et al.* 1988).

The subunits adjacent to the α subunit can influence the characteristics of the ligand binding site. The two α subunits of Torpedo and muscle AChR have different binding affinities and kinetics for nicotinic antagonists: it has been suggested that the γ subunit adjacent to the α subunit could be involved in a high affinity site, whereas the δ subunit adjacent to the other α subunit contributes to the low affinity site. In neuronal AChRs, β-subunits are very important in determining the pharmacology of the AChR subtype, for example $\alpha3\beta2$ is highly sensitive to DMPP and is blocked by n-bungarotoxin, and the $\alpha3\beta4$ is highly sensitive to cytisine and is not blocked by n-bungarotoxin (Luetje and Patrick 1991). The region of the β-subunits that contributes to the binding site also resides in the N-terminal domain, which also contains the AA sequences involved in subunit recognition and the correct packaging of AChRs.

A number of compounds can modulate the function of AChR by binding to allosteric sites other than the ACh binding site; the best examples are physostigmine and corticosteroids.

Channel

The five AChR subunits delimit a channel that is wide on the extracellular side, narrow in the inner part of the plasmamembrane, and widens again at the mouth of the cytoplasmic site (Fig. 1). It is conceivable that the wide part of the channel on the extracellular side is important to concentrate cations; the narrow part is responsible for the ion selectivity that occurs by dimension and charge.



NEURONAL AChRs

		AGONIST POTENCY	BLOCK BY αBGTX	BLOCK BY nBGTX
SUBTYPES EXPRESSED IN OOCYTES:	α2 β2	Nic > DMPP = Ach > Cyt	-	+
	α2 β4	Cyt > Nic > Ach > DMPP	-	+
	α3 β2	DMPP = Ach > Nic > Cyt	-	+++
	α3 β4	Cyt > Nic = Ach = DMPP	-	-
	α4 β2	Nic = Ach > DMPP > Cyt	-	++
	α4 β4	Cyt > Nic > Ach > DMPP	-	?
	α7	Nic > Cy t> Ach > Carb	+	
	α8	Ach = Nic	+	

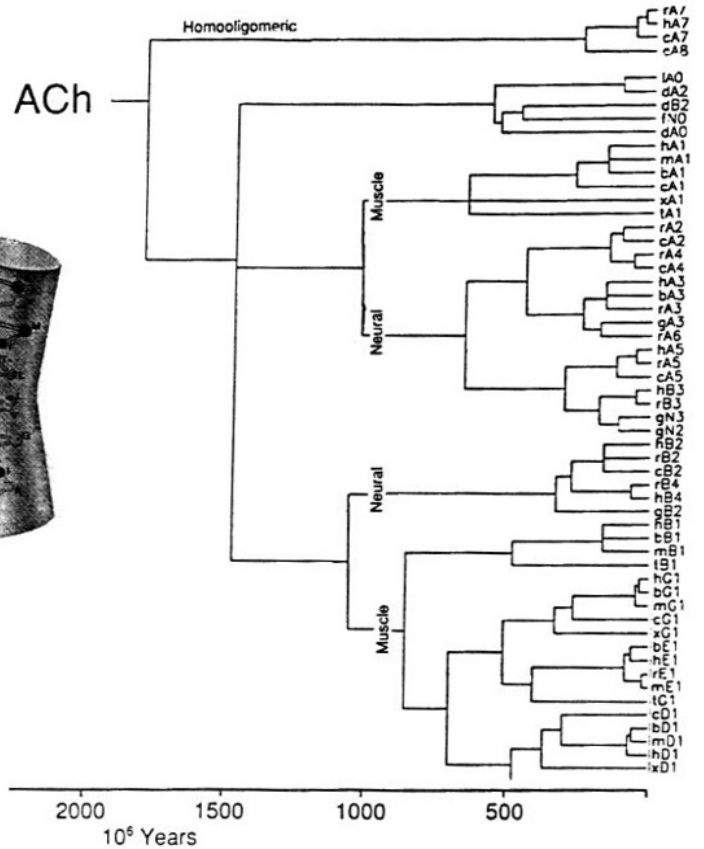


Figure 1. On the left, a schematic view of a closed nicotinic receptor (A) whose aperture is controlled by the binding of acetylcholine (B). The receptor is formed of five subunits whose M2 transmembrane regions delimit the channel (D). The inset shows the enlargement of the domain of two M2 regions that control the permeability of the channel, the charged rings and the leucine ring are also illustrated.

On the right, an evolutionary tree of nicotinic receptors, built on the basis of amino acid sequences of the cloned subunits of both muscular and neuronal receptors of different animal species, shows the different origin of the subunits composing muscle, neuronal, and homomeric receptors.

The table shows the potency of agonists and the effect of toxins on different subtypes of neuronal AChRs. Muscle and neuronal AChRs are more permeable to Na than to Ca ions, whereas the homomeric α7 and α8 subtypes are highly permeable to Ca^{2+} ions (see for details Luetje and Patrick 1991; Changeux 1993; Elogoyen *et al.* 1994; Gotti *et al.* 1994; Zhang *et al.* 1994; Ortells and Lunt 1995).

Crosslinking and mutation experiments have provided convincing evidence that the wall of the channel is formed by the M2 segments of the five subunits, although the M1 segment can also partially contribute to the wall of the channel. The M2 segment has an α-helical structure arranged in such a way that charged amino acids line the channels; in particular, they form at least three negatively charged rings that are responsible for drawing positive ions through the channel. In the central part of the narrow region, a leucine ring is probably the most important mechanism for controlling the aperture and selectivity of the channel (see Fig. 1). Using high resolution electron microscopy and quick freezing techniques, Unwin has recently been able to show part of the molecular mechanism that controls channel opening. First, ACh triggers distinct and localized disturbances of the binding sites of the two α subunits; second, these disturbances are communicated through small rotations of the subunits to the structure inside the membrane; and third, the M2 helices transmit the rotations to the gate-forming parts, drawing them apart from the central axis and thus opening the channel (Unwin 1995).

Assembly and location

The function of AChRs is best achieved when they are located in the proper site of the postsynaptic plasmamembrane. This is particularly clear in the neuromuscular junction, but experimental evidence suggests that this also happens in neuronal synapses. The proper location of AChRs is reached and maintained through strong interactions with the cytoskeleton that are mediated by specific proteins (43 K in the neuromuscular junction). The number of AChRs at the synapse is not fixed and can be modulated by pre- and postsynaptic factors, which mainly affect AChR turnover. The intracellular assembly of such a complex molecule with different subunits, and the correct selection of the subunits in the AChR subtypes are processes that are still not completely understood.

Pathology

In Myasthenia Gravis, an autoimmune disease characterized by the progressive failure of the functions of neuromuscular junctions, AChRs are the target of autoantibodies that induce a decrease in muscle AChRs by accelereting their degradation rate (Albuqureque and Eldefrawi 1983). α-Bungarotoxin binding to AChR has been exploited for a specific antibody assay, which is very useful for the clinical assessment of patients. In other diseases of the neuromuscular junction, it is suggested that mutations of AChR may be involved.

Tools

Labelled toxins, synthetic peptides mimicking defined regions of the receptor, monoclonal antibodies specific for single epitopes (Tzartos *et al.* 1995), and several oligonucleotide probes are now available for the study of AChRs.

References

Albuquerque, E. X. and Eldefrawi, A. T. (1983). *Myasthenia gravis*, Chapman and Hall, London.

Changeux, J. P. (1993). Chemical signaling in the brain. *Scientific American*, **58**, 62.

Devillers-Thiéry, A., Galzi, J. L., Eiselé, J. L., Bertrand, S., Bertrand, D., and Changeux, J. P. (1993). Functional architecture of the nicotinic acetylcholine receptor: a prototype of ligand-gated channel. *J. Membr. Biol.*, **136**, 97–112.

Elgoyen, A. B., Johnson, D. S., Boulter, J., Veltor, D. E., and Heinemann, S. (1994). α9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell*, **79**, 705–15.

Gotti, C., Frigerio, F., Bolognesi, M., Longhi, R., Racchetti, G., and Clementi, F. (1988). Nicotinic acetylcholine receptor: a structural model for α subunit peptide 188–201, the putative binding site for cholinergic agents. *FEBS Lett.*, **228**, 118–22.

Gotti, C., Hanke, W., Maury, K., Moretti, M., Ballivet, M., Clementi, F., *et al.* (1994). Pharmacology and biophysical properties of α7 and α7–α8 α-bungarotoxin receptor subtypes immunopurified from the chick optic lobe. *Eur. J. Neurosci.*, **6**, 1281–91.

Luetje, Ch. W. and Patrick, J. (1991). Both α- and β-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. *J. Neurosci.*, **11**, 837–45.

Ortells, M. and Lunt, G. G. (1995). Evolutionary history of the ligand-gated ion channel superfamily of receptors. *Trends Neurosci.*, **18**, 121–7.

Ramirez-Latorre, J., Yu, C., Qu, X., Perin, F., Karlin, A., and Role, L. (1996). Functional contributions of α5 subunit to neuronal acetylcholine receptor channels. *Nature*, **380**, 347–51.

Stroud, R. M., McCarthy, M. P., and Shuster, M. (1990) Nicotinic acetylcholine receptor superfamily of ligand gated ion channels. *Biochemistry*, **29**, 11009–23.

Tzartos, S. J., Kouvatsou, R., and Tzartos, E. (1995). Monoclonal antibodies as site-specific probes for the AChR δ subunit tyrosine and serine phosphorylationa sites. *Eur. J. Biochem.*, **228**, 463–72.

Unwin, N. (1995). Acetylcholine receptor channel imaged in the ioen state. *Nature*, **373**, 37–43.

Zhang, Z., Vijayaraghavan, S., and Berg, S. (1994). Neuronal acetylcholine receptors that bind α-bungarotoxin with high affinity functions ligand-gated ion channel. *Neuron*, **12**, 167–77.

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α-Bungarotoxin (*Bungarus multicinctus*)

α-Bungarotoxin (α-Bgtx) is an 8 kDa protein present in the venom of the banded krait Bungarus multicinctus. *By binding with high affinity and specificity to the postsynaptic muscle nicotinic acetylcholine receptors (AChRs) and to some subtypes of neuronal AChRs, it prevents their activation by the acetylcholine (ACh) neurotransmitter.*

α-Bgtx is a single polypeptide containing 74 amino acids cross-linked by five disulfide bridges (Fig. 1). Its complete amino acid sequence was reported in 1972 (Mebs *et al.* 1972) and is homologous to that of other α-toxins obtained from cobra and sea snakes; in fact on the basis of their amino acid sequences, more than 80 αneurotoxins (or postsynaptic neurotoxins) have been identified (Endo and Tamiya 1987, 1991). These αneurotoxins have been classified into two distinct subclasses: the 'short' toxins that all have four disulfide bridges and 60–62 amino acids, and the 'long' toxins with five disulfide bridges and 66–74 amino acids. In Fig. 1, the amino acids that are highly conserved in the long toxins are circled while those invariant among all αneurotoxins are enclosed by a square.

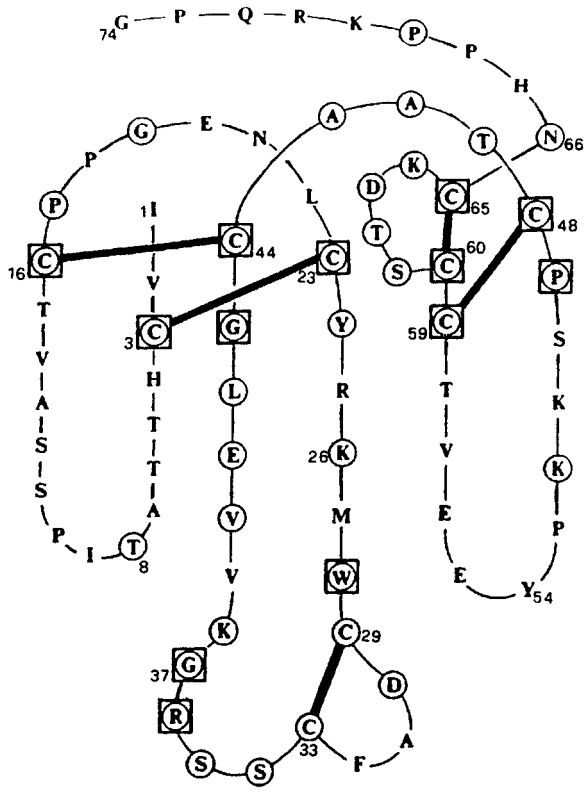

Figure 1. Schematic representation of the α-bungarotoxin structure. The residues that are highly conserved among long neurotoxins are circled, those invariant among all neurotoxins are enclosed by a square (from Love and Stroud 1986).

The X-ray crystal structure of the α-Bgtx has been described (Love and Stroud 1986) and two-dimensional NMR experiments have further refined our knowledge of its structure (Basus *et al.* 1988). This toxin has a three-loop structure and apart, from the COOH-terminal tail, it is a relatively flat molecule with the three main extended loops roughly in one plane and covering an area of about 40 × 30 Å with a depth of about 20 Å. In α-Bgtx, the clustering of the four disulfide bridges common to all αneurotoxins at one end of the molecule preserves its three-loop structure and allows considerable flexibility to the loops which contain many functionally important amino acids.

Numerous studies have established a number of residues that are important for the binding of α-Bgtx to muscle AChRs. These include all of the cysteines (with the exception of the extra disulfide 29–33), Trp 28, Arg 36, and Gly 37. The modification of a single amino acid does not eliminate the activity of the toxin but, as more modifications are made to critical residues, there is less binding affinity. This is consistent with the hypothesis that multi-point interactions take place between the toxin and the AChRs (Stroud *et al.* 1990; Endo and Tamiya 1991).

Purification and sources

α-Bungarotoxin is isolated from *Bungarus multicinctus* venom by means of sequential chromatography (Gotti *et al.* 1985). α-Bgtx can also be purchased from Sigma. The purity of the toxin at each stage of the toxin purification, as well as that of any purchased toxin, should be checked by means of SDS–PAGE.

The expression of a synthetic gene of α-Bgtx in *Escherichia coli* has recently been described (Rosenthal *et al.* 1994). The purified recombinant α-Bgtx is capable of binding AChRs with an affinity that is only 1.7 times less than that from that of authentic α-Bgtx (Rosenthal *et al.* 1994).

Toxicity

α-Bgtx represents 10 per cent of the dry weight of the venom and toxicity experiments involving the i.v administration of total venom have revealed that the LD_{50} in mouse, rabbit, and guinea pig is, respectively, 0.07, 0.2, and 0.005 mg/kg (Chang 1979).

The toxicity of the purified toxin has been tested by using nerve–muscle preparations obtained from various animal species, including mouse, frog, and rat. At a concentration of 0.1–1 μg/ml, the toxin blocks transmission between muscle and nerve (Chang 1979).

The use of the toxin is not dangerous but it should always be handled using gloves.

Use in cell biology and pharmacology

Due to its high affinity to the muscle AChRs (K_d 10^{-12}–10^{-9} M) α-Bgtx has been used as a ligand to identify, localize, quantify, and isolate AChRs present in detergent-solubilized Torpedo electric organs and mammalian muscle. These functionally reconstituted purified AChRs made it possible to obtain the partial amino acid sequence of the AChRs subunits, which subsequently were used as probes for cloning of the complete cDNA and genomic sequences of the muscle AChRs subunits (Stroud *et al.* 1990).

Using different approaches, a number of groups have mapped the major α-Bgtx binding site on the α-subunit of mammalian and Torpedo AChRs, which resides between residues 173–204 of the α-subunit (Stroud *et al.* 1990).

In addition to skeletal muscle AChRs, receptors that bind ^{125}I-α-Bgtx with nicotinic pharmacology are also present in the central and peripheral nervous system of vertebrates. By cloning and functionally expressing the α7, α8, and α9 subunits in oocytes (Sargent 1993; Elgoyhen *et al.* 1994), reconstituting affinity purified α-Bgtx receptors in artificial lipid bilayers (Gotti *et al.* 1991), and making electrophysiological recordings from neurons (Zhang *et al.* 1994) it has recently been clearly demonstrated that these neuronal α-Bgtx receptors are also ligand-gated cation channels.

References

Basus, V. J., Billeter, M., Love, R., Stroud, R., and Kuntz, I. (1988). Structural studies of α-bungarotoxin. 1. Sequence-specific ^{1}H NMR resonance assignments. *Biochemistry*, **27**, 2763–71.

Chang, C. C. (1979). The action of snake venoms on nerve and muscle. In *Snake venoms*, Handb. Exp. Pharm. Vol 52 (ed. C.-Y. Lee), pp. 309–76, Springer-Verlag, Berlin, Heidelberg, New York.

Elgoyhen, A. B., Johnson, D. S., Boulter, J., Vetter, D., and Heinemann, S. (1994). α9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell*, **79**, 705–15.

Endo, T. and Tamiya, N. (1987). Current view of the structure–function relationship of postsynaptic neurotoxins from snake venoms. *Pharmacol. Ther.*, **34**, 403–51.

Endo, T. and Tamiya, N. (1991). Structure–function relationships of postinaptic neurotoxins from snake venoms. In *Snake toxins* (ed. A. L. Harvey), pp. 165–222, Pergamon Press, New York.

Gotti, C., Omini, F., Berti, F., and Clementi, F. (1985). Isolation of a polypeptide from the venom of *Bungarus multicinctus* that binds to ganglia and blocks the ganglionic transmisssion in mammals. *Neuroscience*, **15**, 563–75.

Gotti, C., Esparis Ogando, A., Hanke, W., Sclue, R., Moretti, M., and Clementi, F. (1991). Purification and characterization of an α-bungarotoxin receptor that forms a functional nicotinic channel. *Proc. Natl. Acad. Sci. USA*, **88**, 3258–62.

Love, R. and Stroud, R. (1986). The crystal structure of α-bungarotoxin at 2. 5 Å resolution: relation to solution structure and binding to acetylcholine reeptor. *Protein Eng.*, **1**, 37–46.

Mebs, D., Narita, K., Iwanaga, S., Samejima, Y., and Lee, C.-Y. (1972). Purification, properties and amino acid sequences of α-bungarotoxin from the venom of *Bungarus multicinctus*. *Hoppe-Seyler's Z. Physiol. Chem.*, **353**, 243–62.

Rosenthal, J., Hsu, S., Schneider, D., Gentile, L., Messier, N., Vaslet, C. *et al.* (1994). Functional expression and site-directed mutagenesis of a synthetic gene for α-bungarotoxin. *J. Biol. Chem.*, **269**, 11178–85.

Sargent, P. (1993). The diversity of neuronal nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.*, **16**, 403–43.

Stroud, R. M., McCarthy, M. P., and Shuster, M. (1990). Nicotinic acetylcholine receptor superfamily of ligand gated ion channels. *Biochemistry*, **29**, 11009–23.

Zhang, Z., Vijayaraghavan, S., and Berg, D. K. (1994). Neuronal acetylcholine that bind α-bungarotoxin with high affinity function as ligand-gated ion channel. *Neuron*, **12**, 167–77.

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α-Cobratoxin (*Naja kaouthia*)

α-Cobratoxin is the major toxic component of the venom of the Asian snake Naja kaouthia. *It is a long-chain toxin of 71 amino acids including five disulfides. It binds to nicotinic acetylcholine receptors from various tissues with high affinity, causing flaccid paralysis and hence respiratory failure.*

The cobras (*Elapidae*) form a group of snakes that are wide-spread in Africa and Asia (Harding and Welsh 1980). Their venoms contain various types of toxins to subdue prey. These include toxins, called α-neurotoxins or curaremimetic toxins, that recognize the nicotinic acetylcholine receptors (AChRs), causing flaccid paralysis and hence respiratory failure. They are basic proteins classified as long-chain toxins with 66–72 amino acids and 4/5 disulfides, and short-chain toxins with 60–62 amino acids and four disulfides. The snake *Naja kaouthia*, previously named *Naja naja kaouthia* or *Naja naja siamensis*, is largely distributed in India, Bangladesh, Nepal, Burma, Thailand, Vietnam, and south-west China. Its main curaremimetic toxin is α-cobratoxin, initially called *siamensis* toxin or toxin *siamensis* 3 (Karlsson *et al.* 1971). It is a long-chain toxin of 71 amino acids which includes five disulfides (Endo and Tamiya 1991) and which binds to AChRs from *Torpedo marmorata* with a K_d value equal to 60 pM (Charpentier *et al.* 1990). The three-dimensional structure of α-cobratoxin has been elucidated by X-ray crystallography (Walkinshaw *et al.* 1980) and NMR spectroscopy (Le Goas *et al.* 1992), revealing the presence of three adjacent loops rich in β-sheet protruding from a small globular core with the four disulfides that are commonly shared by long-chain and short-chain toxins. As compared to short-chain toxins, α-cobratoxin has nine additional residues in its C-terminal region and a fifth disulfide at the tip of its central loop. Some of the residues by which α-cobratoxin recognizes AChR were identified on the basis of chemical modifications (Lobel *et al.* 1985; Johnson *et al.* 1990; and reviewed in Endo and Tamiya 1991). These include Lys-23, Trp-25, Arg-33, and Lys-49 which respectively correspond to Lys-27, Trp-29, Arg-33, and Lys-47 in erabutoxins. The complete AChR binding site of α-cobratoxin remains to be established. Various attempts were made to identify residues of AChR that are recognized by α-cobratoxin, using coated receptor fragments (Fulachier *et al.* 1994 and references quoted therein). In particular, α-subunit fragments 128–142 and 185–199 specifically bind the radioactive toxin. Furthermore, Lys-49 of the toxin is apparently involved in recognition of the fragment 128–142. Finally, immunological properties of α-cobratoxin as well as the molecular mechanisms associated with the neutralization of the toxin by toxin-specific monoclonal antibodies have been investigated (Ménez 1991).

The amino acid sequence of α-cobratoxin (Number in the Swiss Prot Databank PO1391 NXL1_NAJKA) is:

IRCFITPDITSKDCPNGHVCYTKTWCDAFCSIRGKRVDL
GCAATCPTVKTGVDIQCCSTDNCNPFPTRKRP

The three-dimensional structure of the toxin is shown in Fig. 1.

Purification

α-Cobratoxin is readily purified from venom on cation-exchange chromatography (Karlsson *et al.* 1971). The venom (*N. kaouthia* or *N. n. siamensis*) can be purchased from Latoxan (A.P. 1724, O550, Rosans, France) and

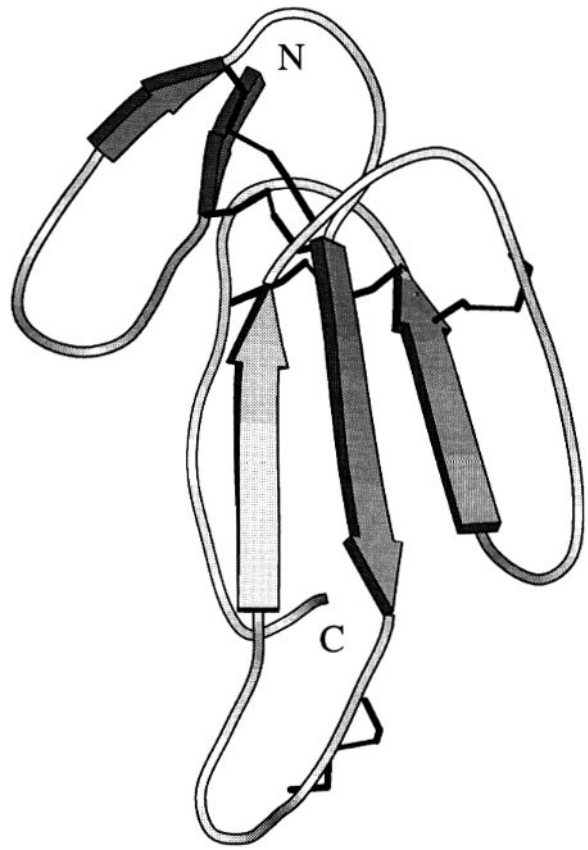

Figure 1. Three-dimensional structure of α-cobratoxin.

numerous serpentariums. A purified toxin can be purchased from Sigma. Due to a confusion in the nomenclature of toxins, users are recommended to proceed to simple but appropriate controls regarding the amino acid sequence of available purified toxins.

Toxicity

Upon intraveinous injection, the LD_{50} of α-cobratoxin is 0.1 μg/g mouse (Karlsson *et al.* 1971).

Use in cell biology

α-Cobratoxin, like other long-chain curaremimetic toxins constitutes an important tool for studying competitive inhibition of AChR from peripheral tissues and also of some AChRs from neuronal origin, like the homopentameric α7 receptor from chicken brain (unpublished). Little is know of the aspects dealing with biosynthesis and regulation of the genes that encode the toxin.

References

Charpentier, I., Pillet, L., Karlsson, E., Couderc, J., and Ménez, A. (1990). Recognition of the acetylcholine receptor binding site of a long-chain neurotoxin by toxin-specific monoclonal antibodies. *J. Mol. Recognition*, **3**, 74–81.

Endo, T. and Tamiya, N. (1991). Current view on the structure–function relationships of postsynaptic neurotoxins from snake venoms. In *Snake toxins* (ed. A. L. Harvey), pp. 403–51, Pergamon Press, New York.

Fulachier, M.-H., Mourier, G., Cotton, J., Servent, D., and Ménez, A. (1994). Interaction of protein ligands with receptor fragments. On the residues of curaremimetic toxins that recognize fragments 128–142 and 185–199 of the α-subunit of the nicotinic acetylcholine receptor. *FEBS Lett.*, **338**, 331–8.

Harding, K. A. and Welsh, K. R. G. (1980). *Venomous snakes of the world. A check list*, Pergamon Press, New York.

Johnson, D. A., Cushman, R., and Malekzadeh, R. (1990). Orientation of cobra α-toxin on the nicotinic acetylcholine receptor. Fluorescence studies. *J. Biol. Chem.*, **265**, 7360–8.

Karlsson, E., Arnberg, H., and Eaker, D. (1971). Isolation of the principal neurotoxins of two *Naja naja* subspecies. *Eur. J. Biochem.*, **21**, 1–16.

Le Goas, R., Laplante, S. R., Mikou, A., Delsuc, M.-A., Guittet, E., Robin, M., *et al.* (1992). α-Cobratoxin: Proton NMR assignments and solution structure. *Biochemistry*, **31**. 4867–75.

Lobel, P., Kao, P. N., Birken, S., and Karlin, A. (1985). Binding of a curaremimetic toxin from cobra venom to the nicotinic acetylcholine receptor. Interactions of six biotinyltoxin derivatives with receptor and avidin. *J. Biol. Chem.*, **260**, 10605–12.

Ménez, A. (1991). Immunology of snake toxins. In *Snake toxins* (ed. A. L. Harvey), pp. 35–78, Pergamon Press, New York.

Walkinshaw, M. D., Saenger, W., and Maelicke, A. (1980). Three-dimensional structure of the 'long' neurotoxin from cobra venom. *Proc. Natl. Acad. Sci. USA*, **77**, 2400–4.

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Erabutoxins (*Laticauda semifasciata*)

Erabutoxins are single chain proteins of 62 residues, including four disulfides, which adopt a 'three finger fold'. They specifically bind with high affinity to various nicotinic acetylcholine receptors including those from fish and rodents. They recognize their targets by a homogeneous 'toxic surface' comprising at least 10 residues.

Venomous sea snakes (*Hydrophiidae*) live in tropical waters from the western coast of America to the eastern coast of Africa, with a greatest species diversity in waters of southeastern Asia and Northern Australia. Venoms help sea snakes to subdue their prey, essentially fish. Most toxic components are small curaremimetic proteins which bind to nicotinic acetylcholine receptors (AChR) with high affinity (K_d values are in picomolar range), producing flaccid paralysis and causing death by respiratory failure. Snake curaremimetic toxins, also called α neurotoxins, are classified as long-chain toxins with 66–74 residues and 4/5 disulfides and short-chain toxins with 60–62 residues and four disulfides (Endo and Tamiya 1991), with no immunological crossreactivity (Ménez 1991). Erabutoxins a, b, and c (Ea, Eb, Ec) were isolated from venom of *Laticauda semifasciata*, collected in Japanese waters, by Tamiya and coworkers (Tamiya 1975). They are basic (pI = 9.5) short-chain isotoxins (62 residues), whose three-dimensional structures, as elucidated by X-ray crystallography (Low 1979) and NMR spectroscopy (Hatanaka *et al.* 1994), consist of three adjacent loops forming a flat β-pleated sheet which emerge from a globular core containing the four conserved disulfides. Cloning experiments revealed that each specific mRNA

encodes a single copy of pre-erabutoxin (Ducancel *et al.* 1991). Genomic data on erabutoxins are available (Lajeunesse *et al.* 1994). As judged from a mutational analysis, the receptor binding site of Ea is composed of at least ten residues located on the concave face of the toxin (Pillet *et al.* 1993; Trémeau *et al.* 1995). A comprehensive bibliography on venomous sea snakes and their toxins is available (Culotta and Pickwell 1993).

The amino acid sequence of Ea (Accession number in the Swiss Prot databank PO1435 NXS1_LATSE) is:

RICFNHQSSQPQTTKTCSPGESSCYNKQWSDFRGT
IIERGCGCPTVKPGIKLSCCESEVCNN

Figure 1 shows the folding of erabutoxin backbone and the residues that have been identified as being critical for the toxin function, on the basis of a mutational analysis.

Purification and sources

Erabutoxins are currently purified from crude venom by cation exchange chromatography (Tamiya 1975). Recombinant erabutoxins are purified on RP-HPLC (μbondapack) (Pillet *et al.* 1993; Trémeau *et al.* 1995). Erabutoxins can be purchased from Sigma (USA) and from Latoxan (A.P. 1724, 0550, Rosans, France).

Toxicity

Upon intramuscular injection to 20 g mouse, erabutoxins display LD_{50} values of 0.13–0.15 μg/g mouse (Tamiya 1975). Eb kills Japanese frogs, tortoise, chick, or mouse with LD_{50} values of 0.1–0.3 μg/g mouse whereas 300 μg/g fail to kill mangoose or snakes, presumably due to critical amino acid substitutions in AChRs of resistant animals (Barchan *et al.* 1992; Krienkamp *et al.* 1994; Keller *et al.* 1995).

Use in cell biology

Like other curaremimetic toxins, erabutoxins recognize AChRs from various sources, including *Torpedo marmorata* to which they bind with high affinity (K_d = 70 pM). They compete with agonists (acetylcholine) and antagonists (curare) for binding to AChR and produce nondepolarizing neuromuscular block (Tamiya 1975). Erabutoxins stimulated pioneering studies aiming at localizing AChRs in various tissues and at isolating them (Endo and Tamiya 1991; Tamiya 1975). Recently, 36 individual positions of Ea were individually mutated by genetic means. Functional analysis of the mutants showed that the site by which Ea binds to AChR from *T. marmorata* is composed of at least 10 residues. Some

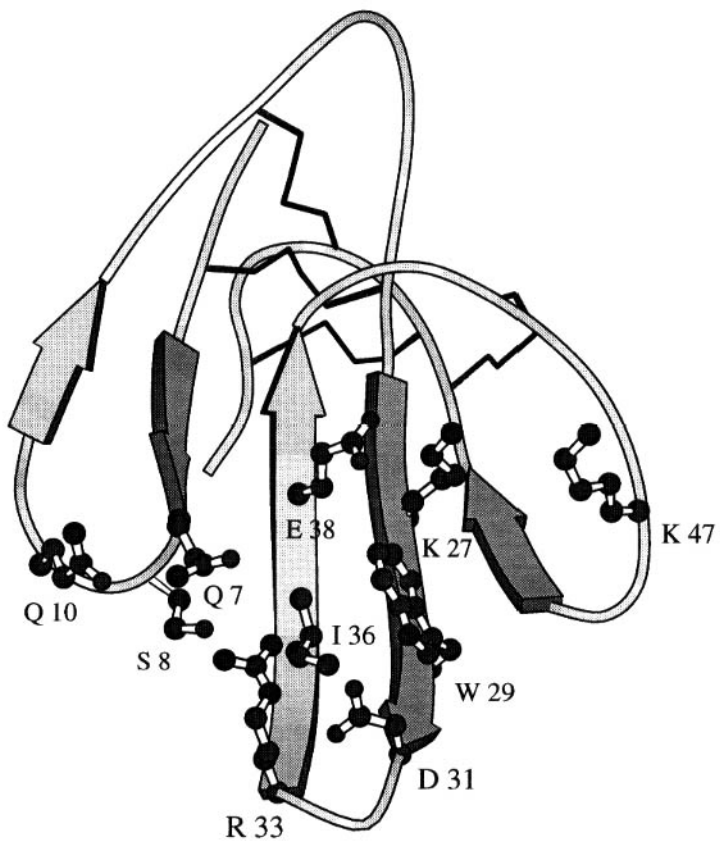

Figure 1. Schematic representation of erabutoxin

of these are common to most curaremimetic toxins whereas others are variable. Functionally variable residues might increase the selectivity of Ea for AChR of sea snake prey (Pillet *et al.* 1993; Trémeau *et al.* 1995).

References

Barchan, D., Kachalsky, S., Neumann, D., Vogel, Z., Ovadia, M., Kochva, E., *et al.* (1992). How the mongoose can fight the snake. The binding site of the mongoose acetylcholine receptor. *Proc. Natl. Acad. Sci. USA*, **89**, 7717–21.

Culotta, W. A. and Pickwell, G. V. (1993). *The venomous sea snakes. A comprehensive bibliography*, Krieger publishing Cie, Malabar, Florida.

Ducancel, F., Bouchier, C., Tamiya, T., Boulain, J.-C., and Ménez, A. (1991). Cloning and expression of cDNAs encoding snake toxins. In *Snake toxins* (ed. A. L. Harvey), pp. 385–414, Pergamon Press, New York.

Endo, T. and Tamiya, N. (1991). Current view on the structure–function relationships of postsynaptic neurotoxins from snake venoms. In *Snake toxins* (ed. A. L. Harvey), pp. 403–51, Pergamon Press, New York.

Hatanaka, H., Oka, M., Kohda, D., Tate, S., Suda, A., Tamiya, N., *et al.* (1994). Tertiary structure of erabutoxin b in aqueous solution as elucidated by two-dimensional nuclear magnetic resonance. *J. Mol. Biol.*, **240**, 155–66.

Keller, S. H., Krienkamp, H. J., Kawanishi, C., and Taylor, P. (1995). Molecular determinants conferring α-toxin resistance in recombinant DNA-derived acetylcholine receptors. *J. Biol. Chem.*, **270**, 4165–71.

Krienkamp, H. J., Sine, S. M., Maeda, R. K., and Taylor, P. (1994). Glycosylation sites selectivity interfere with α-toxin binding to the nicotinic acetylcholine receptor. *J. Biol. Chem.*, **269**, 8108–14.

Lajeunesse, E., Ducancel, F., Gillet, D., Trémeau, O., Drevet, P., Boulain, J.-C., *et al.* (1994). Molecular biology of sea-snake toxins: Cloning, expression of cDNAs and analysis of gene organization. In *Sea snake toxinology* (ed. P. Gopalakrishnakone), pp. 66–92, Singapore University Press, Kent Ridge.

Low, B. W. (1979). The three-dimensional structure of postsynaptic snake toxins: consideration of structure and function. In *Snake venoms* (ed. C-Y Lee), pp. 213–57S, Springer-Verlag, Berlin, Heidelberg, New York.

Ménez, A. (1991). Immunology of snake toxins. In *Snake toxins* (ed. A. L. Harvey), pp. 35–78, Pergamon Press, New York.

Pillet, L., Trémeau, O., Ducancel, F., Drevet, P., Zinn-Justin, S., Pinkasfeld, S., *et al.* (1993). Genetic engineering of snake toxins. Role of invariant residues in the structural and functional properties of a curaremimetic toxin, as probed by site-directed mutagenesis. *J. Biol. Chem.*, **268**, 909–16.

Tamiya, N. (1975). Sea snake venoms and toxins. In *The biology of sea snakes* (ed. W. A. Dunson), pp. 385–415, University Park Press, Baltimore, London, Tokyo.

Trémeau, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulain, J.-C., *et al.* (1995). Genetic engineering of snake toxins. The functional site of erabutoxin a, as delineated by site-directed mutagenesis includes variant residues. *J. Biol. Chem.*, **270**, 9362–9.

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Toxin α (*'Naja nigricollis'*)

Toxin α from venom of the African spitting cobra originally designed as 'Naja nigricollis', is a small protein of 61 amino acids including four disulfides and which adopts a 'three finger fold'. Toxin α binds to nicotinic acetylcholine receptors (AChRs) from various tissues with high affinity and great specificity. Its receptor binding site is anticipated to involve at least 10 residues, like erabutoxins. Immunological properties of toxin α have been extensively studied at the molecular level.

The cobra snakes of the species *Naja* (*Elapidae*) produce potent venoms with which they subdue their prey. Among the most toxic components are the curaremimetic toxins which provoke flaccid paralysis and death, as a result of respiratory failure. Detected by Boquet and coworkers (1966), Eaker and Porath showed, in 1967, that toxin α possesses a single chain of 61 amino acids with four disulfides. Toxin α is currently isolated from venom of an African spitting cobra originally named *Naja nigricollis* (Boquet *et al.* 1966). Further taxonomical studies suggested that the snake should be named *Naja mossambica pallida* (Fryklund and Eaker 1975) and more recently *Naja pallida* (B. Hughes, personal communication). Toxin α was radioactively labelled by replacing a proton of histidine 31 by an atom of tritium, using a procedure based on catalytic deshalogenation (Ménez *et al.* 1971). In pioneering studies Changeux and coworkers used [^{3}H]-labelled toxin α to localize and isolate the nicotinic acetylcholine receptor [AChR] from electric organs of electric fish (Changeux 1990). The toxin binds to AChR specifically and with high affinity (K_d = 20 pM). As judged from NMR and modelling studies (Zinn-Justin *et al.* 1992), the polypeptide chain of toxin α adopts a 'three finger fold' similar to that adopted by erabutoxins (see erabu-

toxins p. 195). The AChR binding site of toxin α includes multiple residues (Faure *et al.* 1983), presumably similar to those identified for erabutoxins (Trémeau *et al.* 1995). Toxin α is immunogenic in mice, rabbits, goats, and horse (Boquet 1979). It elicits neutralizing antisera which not only prevent binding of the toxin to AChR but also increase the dissociation rate of the toxin–AChR complex (Boulain and Ménez 1982). Antibodies that accelerate the dissociation kinetics of the toxin–AChR complex are anticipated to form a ternary complex with AChR-bound toxins (Ménez 1991). The epitopes associated with the capacity of toxin α to stimulate T cells in Balb/C mice are localized between residues 24 and 41 (Léonetti *et al.* 1990; Maillère *et al.* 1993). Injection of the cyclic fragment 24–41 in a free state, elicits antibodies that neutralize the toxin.

The amino acid sequence of toxin α (Accession number in the Swiss Prot Databank: PO1426 NXS1_NAJPA) is:

LECHNQQSSQPPTTKTCPGETNCYKKVWRDHRGTI
IERGCGCPTVKPGIKLNCCTTDKCNN

The folding of toxin α is similar to that of erabutoxins.

Purification and sources

Venom of '*Naja nigricollis*' can be obtained at the Institut Pasteur or Sigma. The venom is filtered through Sephadex G50 and the major fraction is submitted to cation-exchange chromatography to which a gradient of salt is applied (Changeux 1990). Pure toxin α can be purchased from Latoxan (Rosans, 0550, France).

Toxicity

Intravenous and subcutaneous injections of toxin α in mice are, respectively, characterized by LD_{100} of 0.09 μg/g and LD_{50} of 0.036 μg/g body weight.

Use in cell biology

Toxin α recognizes nicotinic acetylcholine receptors from various tissues, including from electric organs from *Torpedo marmorata*, with high affinity (K_d = 20 pM). The [^{3}H]-labelled toxin was extensively used in biochemical and histological studies of AChRs (Changeux 1990). It is also used in immunological studies which include a sensitive solution radio-immunoassay of toxin α (Boulain and Ménez 1982). Toxin α was submitted to chemical modifications at various individual residues, including Tyr, Trp, and Lys residues (Boulain and Ménez 1982). The molecular environment of toxin α bound to the high and low affinity sites of AChR was investigated using toxin derivatives having a photoactivatable group at single residues (Chatrenet *et al.* 1990). Finally, toxin α is now produced in high amounts either recombinantly in *E. coli* or by stepwise solid-phase synthesis (unpublished data).

References

Boquet, P. (1979). Immunological properties of snake venoms. In *Snake toxins*, (ed. A. L. Harvey), pp. 751–824, Pergamon Press, New York.

Boquet, P., Izard, Y., Jouannet, M., and Meaume, J. (1966). Etude de deux antigènes toxiques du venin de *Naja nigricollis*. *C. R. Acad Sci. [D] (Paris)*, **262**, 1134–7.

Boulain, J.-C. and Ménez, A. (1982). Neurotoxin-specific immunoglobulins accelerate dissociation of the neurotoxin–acetylcholine receptor complex. *Science*, **217**, 732–3.

Changeux, J.-P. (1990). Functional architecture and dynamics of the nicotinic acetylcholine receptor: An allosteric ligand-gated ion channel. *Fidia Research Foundation Neuroscience Award Lectures*, **4**, pp. 21–168, Raven Press, New York.

Chatrenet, B., Trémeau, O., Bontems, F., Goeldner, M. P., Hirth, C. G., and Ménez, A. (1990). Topography of toxin–acetylcholine receptor complexes by using photoactivatable toxin derivatives. *Proc. Natl. Acad. Sci. USA*, **87**, 3378–82.

Eaker, D. and Porath, J. (1967). The amino acid sequence of a neurotoxin from *Naja nigricollis* venom. *Jap. J. Microbiol.*, **11**, 353–5.

Faure, G., Boulain, J.-C., Bouet, F., Montenay-Garestier, T., Fromageot, P., and Ménez, A. (1983). Role of indole and amino groups in the structure of *Naja nigricollis* toxin α. *Biochemistry*, **22**, 2068–76.

Fryklund, L. and Eaker, D. (1975) The complete covalent structure of a cardiotoxin from the venom of *Naja nigricollis* (African black-necked spitting cobra). *Biochemistry*, **14**, 2865–71.

Léonetti, M., Pillet, L., Maillère, B., Lamthanh, H., Frachon, P., Couderc, J., *et al.* (1990). Immunization with a peptide having both T-cell and conformationally restricted B-cell epitopes elicits neutralizing antisera against a snake neurotoxin. *J. Immunol.*, **145**, 4214–21.

Maillère, B., Cotton, J., Mourier, G., Léonetti, M., Leroy, S., and Ménez, A. (1993). Role of thiols in the presentation of a snake toxin to murine T cells. *J. Immunol.*, **150**, 5270–80.

Ménez, A. (1991). Immunology of snake toxins. In *Snake toxins* (ed. A. L. Harvey), pp. 35–78, Pergamon Press, New York.

Ménez, A., Morgat, J.-L., Fromageot, P., Ronseray, A. M., Boquet, P., and Changeux, J.-P. (1971). Tritium labelling of the α-neurotoxin of *Naja nigricollis*. *FEBS Lett.*, **17**, 333–5.

Trémeau, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulain, J.-C., *et al.* (1995). Genetic engineering of snake toxins. The functional site of erabutoxin a, as delineated by site-directed mutagenesis includes variant residues. *J. Biol. Chem.*, **270**, 9362–9.

Zinn-Justin, S., Roumestand, C., Gilquin, B., Bontems, F., Ménez, A., and Toma, F. (1992). Three-dimensional solution structure of a curaremimetic toxin from *Naja nigricollis* venom. *Biochemistry*, **31**, 11335–47.

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κ-Bungarotoxin *(Bungarus multicinctus)*

κ-Bungarotoxin (κ-Bgt) is a 7.3 kDa protein present in the venom of the banded krait, Bungarus multicinctus. *It is a potent antagonist at a subset of neuronal nicotinic acetylcholine receptors (AChRs) which contain one or more a3 or a4 subunits, and is much less effective at muscle AChRs. The major use is for functional and pharmacological studies of various neuronal AChRs, and for localization of these receptors in brain and autonomic ganglia.*

κ-Bgt is a 66 amino acid protein that shares considerable sequence homology with α-Bgt (Grant and Chiappinelli 1985; Fig. 1). It is the prototype of a family of neurotoxins that includes κ2-Bgt and κ3-Bgt (from *Bungarus multicinctus*; Chiappinelli *et al.* 1990) and κ-flavitoxin (from *Bungarus flaviceps*; Chiappinelli *et al.* 1987). All κ-neurotoxins exist as noncovalently bound dimers, which distinguishes them from the monomeric α-neurotoxins (Chiappinelli and Wolf 1989). Solution structure of the κ-Bgt dimer has been described using two-dimensional ^{1}H NMR spectroscopy (Oswald *et al.* 1991) and circular dichroism (Fiordalisi *et al.* 1994*a*). The crystal structure at 2.3-A resolution is closely related to that of the NMR study and predicts 10 β-strands in the dimer, including an extended six-stranded antiparallel β-sheet shared between the subunits, with residues Phe 49 and Leu 57 forming additional van der Waals interactions across the dimer interface (Dewan *et al.* 1994; Fig. 2). The crucial residue Arg 34 is at the tip of the central loop for each subunit.

An artificial gene for κ-Bgt has been expressed in *Escherichia coli* (Fiordalisi *et al.* 1991). About 5 per cent of the recombinant protein is correctly folded, dimeric, and biologically active with potency comparable to venom-derived κ-Bgt. Single-point recombinant mutants demonstrate that replacing the invariant Arg 34 with Ala 34, or Pro 36 with Lys 36 (but not with Ala 36) greatly reduces potency at both chick ciliary ganglion and skeletal muscle AChRs (Fiordalisi *et al.* 1994*a,b*). Replacing Gln 26 with Trp 26 (invariant in α-neurotoxins) has no effect on potency against neuronal receptors but enhances binding to muscle AChRs.

κ-Bgt has also been called Bungarotoxin 3.1, Toxin F, and neuronal bungarotoxin (Loring 1993).

Purification and sources

Purification of κ-Bgt from crude venom of *Bungarus multicinctus* is by sequential cation exchange columns, with

```
                        10          20        30        40        50        60
                        ↓           ↓         ↓         ↓         ↓         ↓
   κ-Neurotoxins
                                          ◇       # ▼           ★       ★
κ-Bgt     RTCL---ISPSSTPQT---CPNGQDICFLKAQCDKFCSIRGPVIEQGCVATCPQFRSNYRSLLCCTTDNCNH

κ2-Bgt    KTCL---KTPSSTPQT---CPQGQDICFLKVSCEQFCPIRGPVIEQGCAATCPEFRSNDRSLLCCTTDNCNH

κ3-Bgt    RTCL---ISPSSTPQT---CPNGQDICFRKAQCDNFCHSRGPVIEQGCVATCPQFRSNYRSLLCCRTDNCNH

κ-Fvt     RTCL---ISPSSTSQT---CPKGQDICFTKAFCDRWCSSRGPVIEQGCAATCPEFTSRYKSLLCCTTDNCNH

                                  \------- loop 2 --------/
   α-Neurotoxins

α-Bgt     IVCHTTATSPIS-AVT---CPPGENLCYRKMWCDAFCSSRGKVVELGCAATCPSKKPYEEVT-CCSTDKCNPHPKQRPG

Toxin 3   IRCF---ITPDITSKD---CPNG-HVCYTKTWCDAFCSIRGKRVDLGCAATCPTVKTGVDIQ-CCSTDNCNPFPTRKRP

L.s.III   RECY---LNPHD-TQT---CPSGQEICYVKSWCNAWCSSRGKVLEFGCAATCPSVNTGTEIK-CCSADKCNTYP
```

Figure 1. Amino acid sequence homologies between κ-neurotoxins and α-neurotoxins. Numbering at top refers to residues in κ-Bgt. Underlined residues are important invariants in both families, and the position of the central loop (loop 2) containing several residues crucial for binding to AChRs is indicated. Highlighted residues include Arg 34 (#), Pro 36 (▼), and Gln 26 (◇) that have been modified in recombinant studies. Phe 49 and Leu 57 (★) are invariant in κ-neurotoxins and contribute to dimer formation in κ-Bgt. See Chiappinelli (1993) for sources of amino acid sequences.

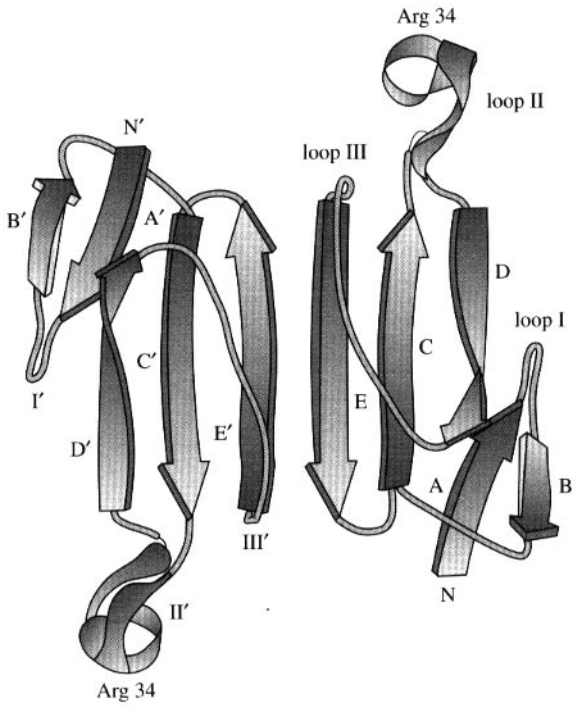

Figure 2. Diagram of the κ-Bgt dimer based on its crystal structure at 2.3 Å resolution. The six-stranded antiparallel β-sheet consists of the β-strands D–C–E–E′–C′–D′. The N-terminus (N) is indicated for each subunit, as are the three main loops (I–III). The approximate positions of the crucial Arg 34 residues are indicated for each subunit. These are separated from each other by 44 Å. (Reprinted in modified form with permission from Dewan *et al.* 1994. Copyright 1994 American Chemical Society.)

cation exchange HPLC or preparative isoelectric focusing as a final step (Chiappinelli 1983; Loring *et al.* 1986; Chiappinelli and Wolf 1989). Yields are small, and typically represent 2–5 per cent of total crude venom protein. Particularly troublesome is contamination with small amounts of co-purifying α-Bgt, which can produce erroneous results when examining α-Bgt-sensitive receptors (Fiordalisi *et al.* 1994*b*). Crude venom is available from a number of suppliers, but is becoming scarce due to the over-harvesting of the snake in Taiwan. Taiwanese snakes produce only κ-Bgt, whereas some snakes from mainland China produce κ2-Bgt and κ3-Bgt only (Chiappinelli *et al.* 1990). Recombinant κ-Bgt has been described (Fiordalisi *et al.* 1991). There is no commercial source for κ-Bgt, and supplies of the purified toxin worldwide are extremely limited. It is hoped that yields of recombinant κ-Bgt can be increased in the near future to provide a stable supply.

Toxicity

There is no available data on the toxicity of κ-Bgt after injection into animals. It should be handled with care.

Use in cell biology

AChRs are integral membrane proteins found on neurons and vertebrate muscle cells. These pentameric ligand-gated channels respond to acetylcholine with the brief opening of a cationic channel that is permeable to Na^+, K^+, and Ca^{2+} (Chiappinelli 1993). Receptors in muscle consist of four subunits (two α1 subunits in combination with β1 and δ plus either γ or ε). α-Neurotoxins bind with sub-nanomolar affinity to sites overlapping the two agonist recognition sites on the muscle receptors, while the structurally related κ-neurotoxins antagonize muscle receptors only at much higher concentrations (IC_{50} = 10 μM for recombinant κ-Bgt; Fiordalisi *et al.* 1994*b*). In contrast, κ-Bgt blocks certain neuronal nicotinic receptors in autonomic ganglia and central nervous system at 10–100 nM, while α-Bgt is without effect on these receptors (Chiappinelli 1993; Loring 1993). Neuronal AChRs consist of one or more α subunits (α2 through α9 are known) combined in homomeric (α7, α8, α9) or heteromeric (with β2–β4) pentamers. Heterologous expression studies indicate that κ-Bgt is most potent against α3/β2 receptors, while α4/β2 receptors are blocked at higher concentrations, and α2/β2 and α3/β4 receptors are not sensitive to κ-Bgt (Luetje *et al.* 1990; Wheeler *et al.* 1993). Studies with native AChRs confirm that κ-Bgt is potent at α3-containing receptors in the chick ciliary ganglion and other autonomic ganglia, as well as similar receptors in retina (Chiappinelli 1993; Loring 1993). The toxin is somewhat less potent at inhibiting presumed α4/β2 presynaptic receptors in the rat striatum, and does not block receptors exhibiting a high affinity for nicotine in the chick lateral spiriform nucleus (Chiappinelli 1993; Loring 1993). κ-Bgt appears to exhibit some low affinity binding to many subtypes of AChRs, including muscle receptors, but this effect is rapidly reversible in contrast to the prolonged action of the toxin (several hours) at ganglionic receptors (Papke *et al.* 1993). While vertebrate AChRs can readily be divided into κ-Bgt-sensitive (some but not all combinations of α3- and α4-containing AChRs) and α-Bgt-sensitive (α1, α7, α8, and α9) categories, invertebrate neuronal AChRs are blocked by both toxins at submicromolar concentrations (Loring 1993).

Both functional and binding studies indicate that κ-Bgt is a competitive antagonist at AChRs. The sites of interaction between κ-Bgt and the α3 subunit have been examined using synthetic α3 peptide fragments (McLane *et al.* 1993). κ-Bgt binds to two regions of this subunit, one near the N-terminus and the other near two vicinal cysteines that are found in all α subunits. This second site is known to be important for α-Bgt and agonist binding to muscle AChR. Studies with heterologously expressed chimeric AChRs confirm that the region of the α3 subunit near the vicinal cysteines determines whether κ-Bgt will block the receptor, but indicate that binding of the toxin to the site near the N-terminus of the subunit may not be critical for inhibition of the receptor (Luetje *et al.* 1993). Other chimeric studies demonstrate that the N-terminus region of the β subunit is important in determining potency of κ-Bgt, so that the binding site for κ-Bgt likely extends across at least two subunits of the AChR (Papke

et al. 1993; Wheeler *et al.* 1993). Since the distance between the two agonist binding sites on the AChR is similar to the 44 Å separating the two crucial Arg 34 residues in the κ-Bgt dimer, it has been proposed that κ-Bgt can bind simultaneously to both agonist sites on AChRs (Dewan *et al.* 1994).

Localization of κ-Bgt-sensitive receptors in autonomic ganglia and the central nervous system has been done using radioiodinated toxin (Loring and Zigmond 1987; Chiappinelli 1993; Loring 1993). A photoaffinity derivative of the toxin has also been shown to bind to ganglionic AChRs (Halvorsen and Berg 1987). Antibodies against κ-Bgt have been prepared (Fiordalisi *et al.* 1991).

References

Chiappinelli, V. A. (1983). Kappa-bungarotoxin: a probe for the neuronal nicotinic receptor in the avian ciliary ganglion. *Brain Res.*, **277**, 9–22.

Chiappinelli, V. A. (1993). Neurotoxins acting on acetylcholine receptors. In *Natural and synthetic neurotoxins* (ed. A. L. Harvey), pp. 65–128, Academic Press, London.

Chiappinelli, V. A. and Wolf, K. M. (1989). Kappa-neurotoxins: heterodimer formation between different neuronal nicotinic receptor antagonists. *Biochemistry*, **28**, 8543–7.

Chiappinelli, V. A., Wolf, K. M., DeBin, J. A., and Holt, I. L (1987). Kappa-flavitoxin: isolation of a new neuronal nicotinic receptor antagonist that is structurally related to kappa-bungarotoxin. *Brain Res.*, **402**, 21–9.

Chiappinelli, V. A., Wolf, K. M., Grant, G. A., and Chen, S.-J. (1990). Kappa$_2$-bungarotoxin and kappa$_3$-bungarotoxin: two new neuronal nicotinic receptor antagonists isolated from the venom of *Bungarus multicinctus*. *Brain Res.*, **509**, 237–48.

Dewan, J. C., Grant, G. A., and Sacchettini, J. C. (1994). Crystal structure of κ-bungarotoxin at 2. 3–A resolution. *Biochemistry*, **33**, 13147–54.

Fiordalisi, J. J., Fetter, C. H., TenHarmsel, A., Gigowski, R., Chiappinelli, V. A., and Grant, G. A. (1991). Synthesis and expression in *Escherichia coli* of a gene for kappa-bungarotoxin. *Biochemistry*, **30**, 10337–43.

Fiordalisi, J. J., Al-Rabiee, R., Chiappinelli, V. A., and Grant, G. A. (1994*a*). Site-directed mutagenesis of kappa-bungarotoxin: Implications for neuronal receptor specificity. *Biochemistry*, **33**, 3872–7.

Fiordalisi, J. J., Al-Rabiee, R., Chiappinelli, V. A., and Grant, G. A. (1994*b*). Affinity of native kappa-bungarotoxin and site-directed mutants for the muscle nicotinic acetylcholine receptor. *Biochemistry*, **33**, 12962–7.

Grant, G. A. and Chiappinelli, V. A. (1985). Kappa-bungarotoxin: complete amino acid sequence of a neuronal nicotinic receptor probe. *Biochemistry*, **24**, 1532–7.

Halvorsen, S. W. and Berg, D. K. (1987). Affinity labeling of neuronal acetylcholine receptor subunits with an α-neurotoxin that blocks receptor function. *J. Neurosci.*, **7**, 2547–55.

Loring, R. H. (1993). The molecular basis of curaremimetic snake neurotoxin specificity for neuronal nicotinic receptor subtypes. *J. Toxicol.-Toxin Reviews*, **12**, 105–53.

Loring, R. H. and Zigmond, R. E. (1987). Ultrastructural distribution of ^{125}I-toxin F binding sites on chick ciliary neurons: synaptic localization of a toxin that blocks ganglionic nicotinic receptors. *J. Neurosci.*, **7**, 2153–62.

Loring, R. H., Andrews, D., Lane, W., and Zigmond, R. E. (1986). Amino acid sequence of toxin F, a snake venom toxin that blocks neuronal nicotinic receptors. *Brain Res.*, **385**, 30–7.

Luetje, C. W., Wada, K., Rogers, S., Abramson, S. N., Tsuji, K., Heinemann, S., *et al.* (1990). Neurotoxins distinguish between different neuronal nicotinic acetylcholine receptor subunit combinations. *J. Neurochem.*, **55**, 632–40.

Luetje, C. W., Piattoni, M. and Patrick, J. (1993). Mapping of ligand binding sites of neuronal nicotinic acetylcholine receptors using chimeric α subunits. *Mol. Pharmacol.*, **44**, 657–66.

McLane, K. E., Weaver, W. R., Lei, S., Chiappinelli, V. A., and Conti-Tronconi, B. (1993). Homologous kappa-neurotoxins exhibit residue-specific interactions with the α3 subunit of the nicotinic acetylcholine receptor: a comparison of the structural requirements for kappa-bungarotoxin and kappa-flavitoxin binding. *Biochemistry*, **32**, 6988–94.

Oswald, R. E., Sutcliffe, M. J., Bamberger, M., Loring, R. H., Braswell, E., and Dobson, C. M. (1991). Solution structure of neuronal bungarotoxin determined by two-dimensional NMR spectroscopy: sequence-specific assignments, secondary structure, and dimer formation. *Biochemistry*, **30**, 4901–9.

Papke, R. L., Duvoisin, R. M., and Heinemann, S. F. (1993). The amino terminal half of the nicotinic β subunit extracellular domain regulates the kinetics of inhibition by neuronal-bungarotoxin. *Proc. R. Soc. London, Ser. B*, **252**, 141–7.

Wheeler, S. V., Chad, J. E., and Foreman, R. (1993). Residues 1 to 80 of the N-terminal domain of the β subunit confer neuronal bungarotoxin sensitivity and agonist selectivity on neuronal nicotinic receptors. *FEBS Lett.*, **332**, 139–42.

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α-Conotoxins (*Conus* spp.)

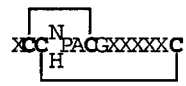
The α-conotoxins are small, disulfide-rich peptides (most are 13–18 amino acids in length, with two disulfide bonds), which are competitive inhibitors of nicotinic acetylcholine receptors; this family of neurotoxins has been found in the venoms of all species of Conus *examined so far. The α-conotoxins first characterized were found to be specific inhibitors of the nicotinic acetylcholine receptor at the postsynaptic terminus of the neuromuscular junction (examples are α-conotoxins G_I, M_I, and S_I). More recent work has revealed that some α-conotoxins preferentially inhibit neuronal subtypes of nicotinic acetylcholine receptors (i.e., α-conotoxin IM_I).*

The α-conotoxins are probably the largest family of paralytic neurotoxins found in the venoms of cone snails (genus *Conus*), a very successful group of predatory molluscs comprising *c.* 500 species (Olivera *et al.* 1985; Myers *et al.* 1991). The first neurotoxin purified from *Conus* venoms was an α-conotoxin (α-conotoxin GI from *Conus geographus* venom); this peptide has 13 amino acids and two disulfide bonds (Gray *et al.* 1981). α-Conotoxins found in Indo-Pacific fish-hunting cone snails are among the major peptidic components used to paralyse fish prey, and have the consensus sequence shown in Fig. 1. Even the unusual α-conotoxin, S_{II}, which has three disulfide bonds, fits the consensus sequence (Ramilo *et al.* 1992).

However, there are clearly α-conotoxins which diverge from this group. α-Conotoxin E_T, a peptide from the Atlantic fish-hunting species *Conus ermineus* is an example (Martinez *et al.* 1995). In addition, several α-conotoxins from non-fish-hunting *Conus* species have been characterized. One of these peptides, α-conotoxin ImI, is of particular interest, not only because it was the first α-conotoxin from a worm-hunting *Conus* species, but because it preferentially inhibits mammalian neuronal nAChRs (vs. the muscle subtype) (McIntosh *et al.* 1994; Johnson *et al.* 1995).

Another family of peptides, the αA-conotoxins, appear to be independently evolved from the α-conotoxins, but target to the same ligand site on the nicotinic acetylcholine receptor. The first peptide of this type, αA-conotoxin P_{IVA} was purified from the Panamic fish-hunting species, *Conus purpurascens* (Hopkins *et al.* 1995).

Indopacific Fish-hunting Species

Conus geographus	α-conotoxin GI	ECC-NPACGRHYSC*
Conus magus	α-conotoxin MI	GRCC-HPACGKNYSC*
Conus striatus	α-conotoxin SI	ICC-NPACGPKYSC*
	α-conotoxin SIA	YCC-HPACGKNFDC*
	α-conotoxin SII	GCCCNPACGPNYGCGTSCS^
	Consensus	

Other Fish-hunters

C. ermineus	α-conotoxin EI	RDOCCYHPTCNMSNPQICC*

Non-fish-hunting *Conus*

Conus imperialis	α-conotoxin ImI	GCCSDPRCAWRC*
Conus pennaceus	α-conotoxin PnIA	GCCSLPPCAANNPDYC*

αA-Conotoxin Family

*Conus purpurascens*α	A-conotoxin PIVA	GCCGSYONAACHOCSCKDROSYCGQ*

Figure 1. α-Conotoxins.

Purification

α-Conotoxins historically have been isolated from acid extracts of dissected venom ducts from cone snails. More recently, a venom milking procedure has been used for some species (Hopkins *et al.* 1995; Martinez *et al.* 1995). Venom purification is accomplished via reversed phase HPLC (McIntosh *et al.* 1994).

Toxicity

Toxicity is tested by intraperitoneal and intracerebral injection into mice and rats. With intraperitoneal injection, death time is directly proportional to the amount of toxin injected. The specific activities of α-conotoxin E_I, G_I, and M_I are 0.36, 1.5, and 4.0 units/nmol toxin respectively (Gray *et al.* 1983; Martinez *et al.* 1995) where the unit of activity is defined as the quantity of material needed to kill a 20 g mouse in 20 min (Cruz *et al.* 1978).

Use in cell biology and neuroscience

The nicotinic acetylcholine receptor (nAChR) is a heteropentamer which has multiple molecular forms. In muscle, the nAChR is comprised of $(\alpha 1)2\beta\gamma\delta$ and is known to contain two ligand binding sites. In neuronal tissue the nAChR is made up of a combination of α and β subunits with each α/β combination forming a pharmacologically distinct receptor subtype. α-Conotoxins are used to selectively block different molecular forms of the nAChR (Johnson *et al.* 1995). In addition, α-conotoxins are used to distinguish between the agonist binding sites near the α/γ and α/δ subunit interface in muscle nAChRs. α-Conotoxins discriminate between the binding sites by up to four orders of magnitude with some conotoxins preferentially binding the α/γ site and other toxins targeting the α/δ site (Groebe *et al.* 1995; Hann *et al.* 1994; Kreienkamp *et al.* 1994; Utkin *et al.* 1994; Martinez *et al.* 1995).

References

Cruz, L. J., Gray, W. R., and Olivera, B. M. (1978). Purification and properties of a myotoxin from *Conus geographus* venom. *Arch. Biochem. Biophys.*, **190**, 539–48.

Gray, W. R., Luque, A., Olivera, B. M., Barrett, J., and Cruz, L. J. (1981). Peptide toxins from *Conus geographus* venom. *J. Biol. Chem.*, **256**, 4734–40.

Gray, W. R., Rivier, J. E., Galyean, R., Cruz, L. J., and Olivera, B. M. (1983). Conotoxin MI. Disulfide bonding and conformational states. *J. Biol. Chem.*, **258**, 12247–51.

Groebe, D. R., Dumm, J. M., Levitan, E. S.,and Abramson, S. N. (1995). Alpha-conotoxins selectively inhibit one of the two acetylcholine binding sites of nicotinic receptors. *Mol. Pharmacol.*, **48**(1), 105–11.

Hann, R. M., Pagan, O. R., and Eterovic, V. A. (1994). The alpha-conotoxins GI and MI distinguish between the nicotinic acetylcholine receptor agonist sites while SI does not. *Biochemistry*, **33**, 14058–63.

Hopkins, C., Grilley, M., Miller, C., Shon, K.-J., Cruz, L. J., Gray, W. R., *et al.* (1995). A new family of *Conus* peptides targeted to the nicotinic acetylcholine receptor. *J. Biol. Chem.*, **270**, 22361–7.

Johnson, D. S., Martinez, J., Elgoyhen, A. B., Heinemann, S. S., and McIntosh, J. M. (1995). Alpha-conotoxin ImI exhibits subtype-specific nioctinic acetylcholine receptor blockade: preferential inhibition of homomeric alpha 7 and alpha 9 receptors. *Mol. Pharmacol.*, **48**, 194–9.

Kreienkamp, H.-J., Sine, S. M., Maeda, R. K., and Taylor, P. (1994). Glycosylation sites selectively interfere with alpha-toxin binding to the nicotinic acetylcholine receptor. *J. Biol. Chem.*, **269**, 8108–14.

McIntosh, J. M., Yoshikami, D., Mahe, E., Nielsen, D. B., Rivier, J. E., Gray, W. R., *et al.* (1994). A nicotinic acetylcholine receptor ligand of unique specificity, alpha-conotoxin ImI. *J. Biol. Chem.*, **269**, 16733–9.

Martinez, J. S., Olivera, B. M., Gray, W. R., Craig, A. G., Groebe, D. R., Abramson, S. N., *et al.* (1995). Alpha-conotoxin EI, a new nicotonic acetylcholine receptor antagonist with novel selectivity. *Biochemistry*, **34**, 14519–26.

Myers, R. A., Zafaralla, G. C., Gray, W. R., Abbott, J., Cruz, L. J., and Olivera, B. M. (1991). Alpha-conotoxins, small peptide probes of nicotonic acetylcholine receptors. *Biochemistry*, **30**, 9370–7.

Olivera, B. M., Gray, W. R., Zeikus, R., McIntosh, J. M., Varga, J., Rivier, J., *et al.* (1985). Peptide nerutoxins from fish-hunting cone snails. *Science*, **230**, 1338–43.

Ramilo, C. A., Zafaralla, G. C., Nadasdi, L., Hammerland, L. G., Yoshikami, D., Gray, W. R., *et al.* (1992). Novel alpha-omega and omega-conotoxins from *Conus striatus* venom. *Biochemistry*, **31**, 9919–26.

Utkin, Y. N., Kobayashi, F. H., Hucho, F., and Tsetlin, V. I. (1994). Relationship between the binding sites for an alpha-conotoxin and snake venom neurotoxins in the nicotinic acetylcholine receptor from *Torpedo californica*. *Toxicon*, **32**, 1153–7.

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Snake toxins against muscarinic acetylcholine receptors

*The mamba (*Dendroaspis *sp.) muscarinic toxins consist of a single peptide chain of 64 to 66 amino acid residues and contain four disulfide bonds. They bind to muscarinic acetylcholine receptors, often with a remarkably high degree of selectivity for one or two subtypes.*

Muscarinic acetylcholine receptors participate in many physiological functions, such as reduction of heart rate, contraction of smooth muscles, glandular secretion, transmitter release and cognitive functions like learning and memory. There are five subtypes of receptors (m1–m5) and one organ or even a cell can express several subtypes. One exception is heart muscle which has only m2. Muscarinic receptors belong to a group of G-protein coupled receptors that consist of a single peptide chain which forms four extracellular, seven transmembrane and four intracellular domains (reviews: Hulme *et al.* 1990; Caulfield 1993: Wess 1993).

According to a model of muscarinic receptors the transmembrane parts are tightly packed and form a deep cleft with an invariant aspartic acid residue in the third transmembrane region. This residue is situated 15Å from the entrance of the cleft, next to a conserved hydrophobic pocket (Trumpp-Kallmeyer *et al.* 1992).

Acetylcholine binding was lost when the invariant Asp was changed to Asn by site-directed muta-genesis.

Antibodies against the various subtypes have been used to study their distribution in different organs, but other types of specific ligands better suited for functional studies are rare. A large number of low molecular weight ligands are known, but they bind to all five subtypes, often with slightly higher affinity for one or two of them. One of the more selective is pirenzepine, with six times higher affinity for m1 (K_i = 6 nM) than for m4 receptors and 14–35 times higher than for the other three subtypes (Dörje *et al.* 1991). However, this is often not sufficient for studies of m1 receptors especially in the presence of large amounts of m4 receptors.

Mambas, African snakes of genus *Dendroaspis*, have so called muscarinic toxins (Adem *et al.* 1988) that bind to muscarinic receptors. They consist of 64–66 amino acids and four disulfides, and several of them have been sequenced (Fig. 1).

Some of the toxins are the most selective ligands for m1 or m4 receptors known to date. m1-Toxin from *D. angusticeps* (Eastern green mamba) has about

```
                1         10        20        30        40        50        60     66
                .    .    .    .    .    .    .    .    .    .    .    .    .    .
Toxin   MT1     LTCVTSKSIFGITTENCPDGQNLCFKKWYYIVPRYSDITWGCAATCPKPTNVRETIRCCETDKCNE
                ****     *   * * ** *** ***   *           ** ****         * ** *****
        MT4     LTCVTSKSIFGITTENCPDGQNLCFKKWYYIVPRYSDITWGCAATCPKPTNVRETIHCCETDKCNE
                ****     *   * * ** *** ***   *           ** ****         * ** *****
        MT2     LTCVTTKSIGGVTTEDCPAGQNVCFKRWHYVTPKNYDIIKGCAATCPKVDNN-DPIRCCGTDKCND
                ****     *   * * ** *** ***   *           ** ****         * ** *****
        MT5     LTCVTSKSIFGITTEDCPDGQNLCFKRRHYVVPKIYDITRGCVATCPKPENY-DSIHCCKTDKCNE
                ****     *   * * ** *** ***   *           ** ****         * ** *****
  m1-toxin      LTCVKSNSIWFPTSEDCPDGQNLCFKRHWYISPRMYDFTRGCAATCPKAE-YRDVINCCGTDKCN
                ****     *   * * ** *** ***   *           ** ****         * ** *****
        MT3     LTCVTKNTIFGITTENCPAGQNLCFKRWHYVIPRYTEITRGCAATCPIPENY-DSIHCCKTDKCNE
                ****     *   * * ** *** ***   *           ** ****         * ** *****
        MTα     LTCVTSKSIFGITTENCPDGQNLCFKKWYYLNHRYSDITWGCAATCPKPTNVRETIHCCETDKCNE
                ****     *   * * ** *** ***   *           ** ****         * ** *****
        MTß     LTCVTSKSIFGITTEDCPDGQNLCFKRRHYVVPKIYDITRGCVATCPIPENY-DSIHCCKTDKCNE
                .    .    .    .    .    .    .    .    .    .    .    .    .    .
                1         10        20        30        40        50        60     66
```

Figure 1. Amino acid sequences of muscarinic toxins from the green mamba *Dendroaspis angusticeps* (MT1–5 and m1-toxin) and from the black mamba *D. polylepis* (MTα and MTβ). Asterisks indicate invariant amino acids. Sequences are from: MT1, Jolkkonen *et al.* (1995); MT2, Ducancel *et al.* (1991); Karlsson *et al.* (1991); MT3, Jolkkonen *et al.* (1994); m1-toxin, Max *et al.* (1993); MT4, Vandermeers *et al.* (1995); MT5, unpublished MTα and MTβ , (Jolkkonen *et al.* (1995). A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, Y = Tyr, V = Val, W = Trp.

100 times higher affinity for m1 than for m4 receptors (Fig. 11, Max *et al.* 1993). MT7 (muscarinic toxin 7) from the same venom is even more selective with a K_i = 0.2 nM for m1 and no or very low affinity for other subtypes (Table 1). The two toxins have a very similar amino acid composition, m1-toxin has one His and MT7 has instead one Glu or Gln. m1-Toxin seems to have about the same affinity as MT7 for m1 receptors. Toxin MT3 from *D. angusticeps* has high selectivity for m4 receptors; K_i = 2 nM for m4 and 80 nM for m1 and no or very low (K_i > 1 μM) affinity for m2, m3, and m5 (Jolkkonen *et al.* 1994) (Table 1).

Small changes in a sequence can change the specificity. MTβ and MT5 differ only in amino acid 48, Ile in MTβ and Lys in MT5. MTβ binds to m3, m4, and m5 with affinities (K_i) between 140 and 350 nM, while MT5 binds to m1 (K_i = 180 nM) and m4 (K_i = 520 nM). MTα and MT4 differ only in the sequence 31 – 33 which is Leu–Asn–His in MTα (Fig. 1) and Ile–Val–Pro in MT5. MTα has high affinity (K_i between 3 and 44 nM) for all subtypes and is the only toxin with high affinity for m3 and m5. MT4 binds only to m1 (K_i = 62 nM) and to m4 (K_i = 87 nM), but not to m2, m3, or m5. A point mutation with no effect on the specificity is Arg/His 57 in MT1/MT4.

Iodination can also change the specificity. ^{125}I-MT1 bound only to cloned m1 receptors (Waelbroeck, personal communication). Autoradiography with ^{125}I-MT1 and ^{125}I-MT2 showed that the toxins bind to the same or slightly smaller area in rat brain than ^{3}H-pirenzepine. A low concentration (2.5–5 nM) of pirenzepine was used to ensure binding predominantly to m1 receptors.

Table 1 Affinities (apparent K_i-values in nM) of muscarinic toxins for cloned human receptors expressed in Chinese hamster ovary cells

Toxin	Receptor				
	m1	m2	m3	m4	m5
D. angusticeps					
MT1	28	> 1000	> 1000	49	> 1000
MT2[a]	1500	> 2000	> 2000	750	> 2000
MT3	80	> 1000	> 1000	2	> 1000
MT4	62	> 1000	> 1000	87	> 1000
MT5	180	> 1000	> 1000	620	> 1000
MT7	0.2	> 2000	> 2000	> 2000	> 2000
m1-toxin	High[b]				
D. polylepis					
MTα	23	44	4	5	8
MTβ	> 1000	> 2000	140	120	350

[a] MT2 is an unstable molecule. This probably explains the low affinity of the toxin preparation assayed. Using an apparently fully active toxin, a K_d of 14 nM was obtained for the binding of ^{125}I-MT2 to synaptosomal membranes from bovine cerebral cortex (Jerusalinsky *et al.* 1992).

[b] About 100 times higher affinity for m1 than for m4 receptors and no binding to m2, m3, and m5. m1-toxin and MT7 probably have about the same affinity for m1.

Iodination (chloramine T method) was carried out for a short time (30–60 s) using a high excess of toxin and only the most reactive tyrosine should have been labelled (both toxins lack histidine). Since iodination had the same

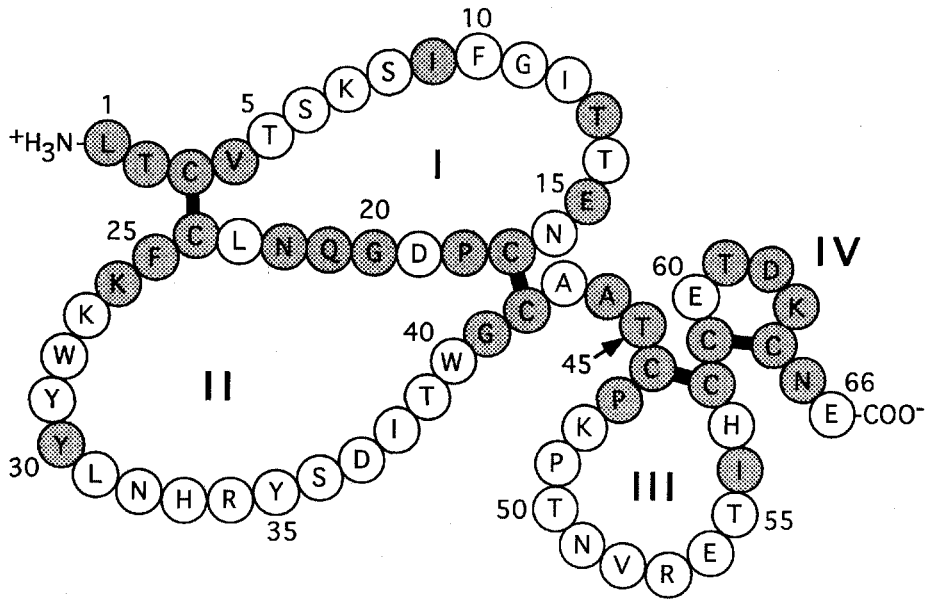

Figure 2. The sequence of the nonselective toxin MTα of *D. polylepis* (black mamba). MTα has high affinity for all subtypes of receptors. Amino acids invariant in muscarinic toxins are indicated by darker shade. The disulfide pairing is assumed to be the same as for toxin MT2, whose three-dimensional structure is known (Ségalas *et al.* 1995). Loop I, Cys 3 to Cys 24; II, Cys 24 to Cys 42; III, Cys 45 to Cys 58; and IV, Cys 59 to Cys 64.

effect on both toxins, probably the same tyrosine was labelled and MT1 and MT2 have only the invariant Tyr 30 in common (Table 1). This tyrosine should be very reactive, since it has a very exposed position (Ségalas *et al.* 1995).

Mutations or modifications that change the specificity are found in loop II (Fig. 2), LNH/IVP (31–33) in MTα/MT4 and the probable iodination site Tyr 30 in MT1 and MT2 (all tyrosines are also in loop II), or in loop III, Ile/Lys 48 in MTβ/MT5. The active site of these toxins should, therefore, include parts of these loops.

MTα binds with high affinity to all subtypes and probably to structurally similar sites on the various receptors. But the more selective toxins must recognize sites which are not identical in all subtypes. The active sites of toxins with different specificity must also be different, and if all toxins have their active sites in the same parts of the molecules, then these parts must be variable. 13 of 17 amino acids in the second loop and 8 of 11 in the third loop are variable.

Muscarinic toxins are homologous to a large number of other snake toxins, e.g. α-neurotoxins (block nicotinic acetylcholine receptors), cardiotoxins/cytotoxins (increase permeability of membranes), and fasciculins (inhibit acetylcholinesterase). The three-dimensional structure has been elucidated for several of these toxins, including MT2 (Ségalas *et al.* 1995). All have a three-finger structure, i.e. the loops I, II and III are extended like the three middle fingers of a hand with disulfides in the palm of the hand (review: Le Du *et al.* 1992; Rees and Bilwes 1993). Loop II in MT2 is very protruding with a narrow tip where Lys 34 is located. This position can also be occupied by arginine. Ségalas *et al.* (1995) suggested that loop II penetrates into the cleft of the muscarinic receptors and Lys 34 binds to the invariant Asp. The toxins have a cluster of hydrophobic amino acids near Lys/Arg 34 which may facilitate the penetration of the cationic group into the hydrophobic cleft and themselves bind to the hydrophobic pocket close to the invariant Asp. Many low molecular weight ligands are cationic molecules with a hydrophobic moiety. Because of their small size they will probably interact only with amino acids in the transmembrane regions of the receptors. With the exception of the first transmembrane region, which has only nine conserved amino acids out of 24, the transmembrane regions are rather similar in all subtypes (Caulfield 1993). This may be one reason why low molecular weight ligands have poor selectivity.

The better selectivity of the snake toxins probably depends on two factors. Firstly, they discriminate better between the differences in the clefts of the various subtypes. The amino acids 31–33 responsible for the different selectivity of MTα and MT4 are close to the cationic group at position 34, and the different selectivity should depend on different interactions of these three residues with amino acids in the cleft.

Secondly, the toxins should also interact with the extracellular domains, which are variable both in length and composition. If loop II penetrates into the cleft, loops I and III should remain outside and be able to interact with the extracellular parts of the receptors. The mutation Ile/Lys 48 in MTβ/MT5 that changes the specificity of the two toxins might depend on different interactions with an extracellular domain.

Purification

The toxins were isolated by gel filtration on Sephadex G-50, chromatography on the cation exchangers Bio-Rex 70 (carboxyl as ion-exchange group) and Sulphopropyl-Sephadex C-25, and for m1-toxin, reversed-phase HPLC instead of ion-exchange. So far toxins have been isolated only from two mambas, *D. angusticeps* (Eastern green mamba) and *D. polylepis* (black mamba), but similar toxins are also present in *D. viridis* (Western green mamba). The toxin content is variable, m1-toxin and MT7 are present in very low amounts (< 0.1 mg/g venom) while MT2 is the most abundant (up to 6–7 mg/g). The content of the other toxins is usually 0.5–2 mg/g.

Toxicity

The lethal doses are not known, but a toxin CM-3 from *D. polylepis* very similar to MTβ was nonlethal to mice at 49.5 μg/g body weight (Joubert 1985). The two toxins differ only at positions 62 (Glu in CM-3, Asp in MTβ) and 66 (Asn in CM-3 and Glu in MTβ). CM-3 is probably a muscarinic toxin, but its affinity for muscarinic acetylcholine receptors has not been assayed.

Use in cell biology

Specific toxins should be very valuable tools for studying subtypes of muscarinic receptors. So far, they have been used only sporadically. MT1 and MT2 act as agonists (review: Jerusalinsky and Harvey 1994) and m1-toxin is an antagonist (Max *et al.* 1993). ^{125}I-MT1 and ^{125}I-MT3 have been used to quantify m1 and m4 receptors in various regions of rat brain (Adem, personal communication).

References

Adem, A., Åsblom, A., Johansson, G., Mbugua, P. M., and Karlsson, E. (1988). Toxins from the venom of the green mamba *Dendroaspis angusticeps* that inhibit the binding of quinuclidinyl benzilate to muscarinic acetylcholine receptors. *Biochim. Biophys. Acta*, **968**, 340–5.

Caulfield, M. P. (1993). Muscarinic receptors – characterization, coupling and function. *Pharmacol Ther.*, *58*, 319–79.

Dörje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschler, E., and Brann, M. R. (1991). Antagonist binding profiles of five cloned muscarinic receptor subtypes. *J. Pharm. Exp. Ther.*, **256**, 727–33.

Ducancel, F., Rowan, E. G., Cassart, E., Harvey, A. L., Ménez, A., and Boulain, J. C. (1991). Amino acid sequence of a muscarinic toxin deduced from the cDNA sequence. *Toxicon*, **29**, 516–20.

Hulme, E. C., Birdsall, N. J. M., and Buckley, N. J. (1990). Muscarinic receptor subtypes. *Annu.* Rev. *Pharmacol. Toxicol.*, **30**, 633–73.

Jerusalinsky, D. and Harvey, A. L. (1994). Toxins from mamba venoms: small proteins with selectivity for different subtypes of muscarinic receptors. *Trends Pharmacol. Sci.*, **15**, 424–30.
Jerusalinsky, D., Cerveñansky, C., Peña, C., Raskovsky, S., and Dajas, F. (1992). Two polypeptides from *Dendroaspis angusticeps* venom selectively inhibit the binding of central muscarinic cholinergic receptor ligands. *Neurochem. Int.*, **20**, 237–46.
Jolkkonen, M., van Giersbergen, P. L. M., Hellman, U., Wernstedt, C., and Karlsson, E. (1994). A toxin from the green mamba *Dendroaspis angusticeps*: amino acid sequence and selectivity for muscarinic m4 receptors. *FEBS Lett.*, **352**, 91–4.
Jolkkonen, M., van Giersbergen, P. L. M., Hellman, U., Wernstedt, C., Oras, A., Satyapan, N., Adem, A., and Karlsson, E. (1995). Muscarinic toxins from the black mamba *Dendroaspis polylepsis*. *Eur. J. Biochem.*, **234**, 579–85.
Joubert, F. (1985). The amino acid sequence of protein CM-3 from *Dendroaspis polylepis* (black mamba). *Int. J. Biochem.*, **17**, 695–700.
Karlsson, E., Risinger, C., Jolkkonen, M., Wernstedt, C., and Adem, A. (1991). Amino acid sequence of a snake venom toxin that binds to the muscarinic acetylcholine receptor. *Toxicon*, **29**, 521–6.
Le Du, M. H., Marchot, P., Bougis, P., and Fontecilla-Camps, J. (1992). 1.9 Å resolution of fasciculin 1, an anticholinesterase toxin from the green mamba snake venom. J. *Biol. Chem.*, **267**, 22122–30.
Max, S. I., Liang, J. S., and Potter, L. T. (1993). Purification and properties of m1-toxin, a specific antagonist of m1 muscarinic receptors. *J. Neurosci.*, **13**, 4293–300.
Rees, B., and Bilwes, A. (1993). Three-dimensional structures of neurotoxins and cardiotoxins. *Chem. Res. Toxicol.*, **6**, 385–406.
Ségalas, I., Roumestand, C., Zinn-Justin, S., Gilquin, B., Ménez, R., Ménez, A., *et al.* (1995). Solution structure of a green mamba toxin that activates muscarinic acetylcholine receptors, as studied by nuclear magnetic resonance and molecular modeling. *Biochemistry*, **34**, 1248–60.
Trumpp-Kallmeyer, S., Hoflack, J., Bruinvels, A., and Hibert, M. (1992). Modelling of G-protein coupled receptors: Application to dopamine, adrenaline, serotonin, acetylcholine and opsin receptors. *J. Med. Chem.*, **35**, 3448–62.
Vandermeers, A., Vandermeers-Piret, M. C., Rathé, J., Waelbroeck, M., Jolkkonen, M., Oras, A., *et al.* (1995). Purification of a new muscarinic toxin (MT4) from the venom of the green mamba (*Dendroaspis angusticeps*). *Toxicon*, **33**, 1171–9.
Wess, J. (1993). Mutational analysis of muscarinic acetylcholine receptors: Structural basis for ligand/receptor/G-protein interaction. *Life Sci.* **53**, 1447–63.

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11

Ryanodine receptor Ca^{2+} channel toxins

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Introduction

- *Molecular and structural properties of the ryanodine receptor Ca^{2+} channel*
- *Regulation of the ryanodine receptor Ca^{2+} channel*
- *Topology of RYR regulatory binding sites*
- *Molecular pathology of the ryanodine receptor Ca^{2+} channel*
- *Pharmacological tools and peptide toxins*

Molecular and structural properties of the ryanodine receptor Ca^{2+} channel

Ca^{2+} release from intracellular stores plays an important role in the regulation of numerous cellular functions ranging from muscle contraction to cell division and neurotransmitter release. The intracellular Ca^{2+} stores of excitable and nonexcitable cells utilize one or two of the intracellular Ca^{2+} channels to release Ca^{2+} upon cell stimulation, namely the ryanodine and the inositol 1,4,5-trisphosphate receptors (Berridge 1993).

The mammalian ryanodine receptor (RYR) is a large tetrameric oligomer made up of four subunits of 560 kDa, which migrate as a single protein in SDS-polyacrylamide gel electrophoresis (Pessah *et al.* 1986; Imagawa *et al.* 1987; Lai *et al.* 1988). The purified RYR complex also contains the FK-506 binding protein, a protein of 12 kDa, which is tightly associated to the RYR promoter with a stoichiometric ratio of 1:1 (Jayaraman *et al.* 1992). Experimental evidence has demonstrated the existence of three distinct isoforms which are preferentially expressed in skeletal muscle, heart, and brain, respectively. Comparison of the primary structures of the skeletal, cardiac, and brain ryanodine receptors has established an overall homology at the amino acid level of approximately 60 per cent (Takeshima *et al.* 1989; Nakai *et al.* 1990; Otsu *et al.* 1990; Zorzato *et al.* 1990; Hakamata *et al.* 1992). Analysis of the primary sequence of the RYR led to the identification of potential domain boundaries (Fig. 1): the channel forming portion comprises transmembrane segments which are resctricted to the last 1000 COOH-terminal residues, while the remainig 4000 NH_2-terminal residues form the cytoplasmic portion of the RYR. The three-dimensional architucture of the RYR (approx. 3 nm resolution) has been deduced from image reconstruction analysis of negatively stained and frozen hydrated RYR complexes (Radermacher *et al.* 1994). According to such a three-dimensional model, the size of the hydrophobic portion (basal plate) of the ryanodine receptor is about 120 × 120 × 70 Å. The basal plate presumably contains the transmembrane portion of the RYR and it may accomodate up to 100 helices of 11 Å in diameter. This is consistent with the RYR model which predicts up to 12 transmembrane segments per RYR protomer (Zorzato *et al.* 1990). The cytoplasmic portion

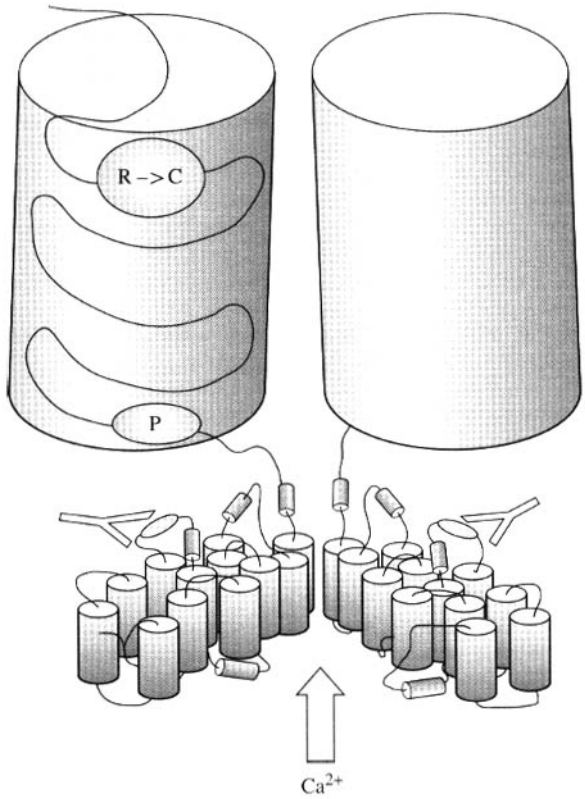

Figure 1. Model of the ryanodine receptor Ca^{2+} release channel. The cartoon depicts two protomers side by side (P, phosphorylation site; R → C, mutation 615, associated with malignant hyperthermia). The transmembrane segments are represented by the bottom cylinders; the smaller central cylinders represent the calmodulin binding domains while the top large cylinders represent the large cytoplasmic NH_2-terminal portion. The figure also shows the region recognized by antibodies which affect Ca^{2+}-induced Ca^{2+} release. The arrow indicates the flow of Ca^{2+} from the lumen of the sarco(endo)plasmic reticulum to the cytoplasm.

results from the assembly of 10 domains which are formed by each of the four subunits (Radermacher *et al.* 1994). It has been estimated that over 50 per cent of the space delimited by the RYR cytoplasmic assembly is accessible to solvent. Such a peculiar three-dimensional conformation may facilitate the interaction of RYR agonists with potential binding sites distributed within the 4000 amino acid long sequence encompassing the RYR's cytoplasmic domain.

Regulation of the ryanodine receptor Ca^{2+} channel

Regulation of the mammalian Ca^{2+} channels is achieved by different mechanisms, depending on the RYR isform. The skeletal muscle RYR is under the control of (i) transverse tubule depolarization (Rios and Pizzaro 1991), and (ii) several putative physiological ligands including Ca^{2+}, ATP, Mg^{2+}, calmodulin, and cADP-ribose (for review see Coronado *et al.* 1994; Meissner 1994). The cardiac and brain RYR isoforms are mainly regulated by a mechanism involving the latter ligands. Because of the importance of Ca^{2+} in cell physiology, much attention has been devoted to the investigation of the Ca^{2+}-dependent regulation of the RYR Ca^{2+} channels. It has been clearly demostrated that the RYR contains two classes of Ca^{2+} binding sites having high (μM) and low (mM) affinity, which are involved in the activation and inhibition of channel activity, respectively. It should be mentioned that physiological concentration of both ATP and Mg^{2+} dramatically increase the cooperativity of Ca^{2+}-dependent activation of the RYR. In the presence of the latter modulators, Ca^{2+} activation of the skeletal muscle RYR channel occurs within a narrow range of Ca^{2+} concentrations (1 to 10 μM) (Meissner 1986), the optimal activating Ca^{2+} concentration being 10 μM.

Physiological regulation of the RYR Ca^{2+} channel also involves phosphorylation by cAMP-, cGMP, and calmodulin-dependent protein kinase (Witcher *et al.* 1992). Phosphorylation of Ser residue 2809 of the cardiac RYR by calmodulin-dependent kinase removes the inhibitory effect of calmodulin, as demonstrated by single-channel currents of cardiac RYR reconstituted in planar lipid bilayers (Witcher *et al.* 1991). In contrast, phosphorylation of the skeletal RYR in correspondence with the homologous Ser residue (2843), causes different effects. Some data point to a phosphorylation-dependent inactivation of the skeletal RYR (Wang and Best 1992), while others demonstrate an activating effect of cAMP-dependent phosphorylation of the RYR on single channel currents (Hain *et al.* 1994). Taken together, these experimental results clearly indicate that phosphorylation modulates the activity of the members of the RYR Ca^{2+} channel family, however, its exact mode of action remains to be determined.

Calmodulin is an intracellular Ca^{2+} transducer which regulates the activity of a variety of structurally distinct proteins (O'Neil and DeGrado 1990), including the members of the RYR Ca^{2+} channel family. Calmodulin has been shown to interact directly with the channel and down-regulate the Ca^{2+}-dependent activity of the channel (Smith *et al.* 1989; McPherson and Campbell 1993). The effect of calmodulin on RYR Ca^{2+} channel activity was recently investigated in more detail (Tripathy *et al.* 1995). These studies show that calmodulin can act both as an activator and as an inhibitor of the skeletal muscle RYR Ca^{2+} channel, depending upon the free Ca^{2+} concentration: it activates the RYR channel at submicromolar [Ca^{2+}], while inhibition occurs at μM Ca^{2+}.

Topology of RYR regulatory binding sites

Though the putative binding regions of RYR modulators was predicted from the analysis of the deduced primary sequence, several experiments were designed to identify and characterize the binding region of such modulators. Using anti-RYR Ab it was shown that a region (residues 4425–4621) within the myoplasmic loop #2, between transmembrane segments M4 and M5 is involved in the Ca^{2+}-dependent activation of the skeletal muscle RYR (Fill *et al.* 1991; Treves *et al.* 1993). An amino acid sequence located in the myoplasmic loop #2 was also shown to be involved in the phenomenon of Ca^{2+}-induced Ca^{2+} release (Chen *et al.* 1993). Upon Ca^{2+} stimulation, the anti-RYR Ab decreased the open probability of the Ca^{2+} channel, while it did not affect the activation of the RYR by ATP (Fill *et al.* 1991), indicating that the ATP-activatory site is functionally separated from the Ca^{2+}-activatory site, and that the latter site is located next to the junctional sarcoplasmic reticulum membrane at the level of, or closely associated with, the putative myoplasmic loop #2. The region of the myoplasmic loop #2 implicated in the Ca^{2+}-dependent regulation of RYR also encompasses a calmodulin binding site (CaM#3, residues 4540–4557; Menegazzi *et al.* 1994). Other skeletal muscle RYR calmodulin binding sites defined by residues 3042–3057 (CaM#1) and 3617–3634 (CaM#2; Menegazzi *et al.* 1994) were also identified. Of interest, the calmodulin binding sites present in the skeletal muscle RYR were also found to be present in the homologous sequences of both cardiac and brain RYRs (Guerrini *et al.* 1995). As to other Ca^{2+}-release modulators such as ATP, Mg^{2+}, and cADP-ribose, data has yet to be obtained.

Molecular pathology of the ryanodine receptor Ca^{2+} channel

Malignant hyperthermia (MH) is a potentially lethal autosomal dominant disorder which causes muscle rigidity, rapid increase of body temperature (approx. 1 °C every 10 min) in response to inhalational anesthetics such as halothane enflurane (Mickelson and Louis 1996). Skeletal muscle RYRs from MH-susceptible pigs (the animal model for human MH), display significant differences in the Ca^{2+}-dependency of [^{3}H]-ryanodine binding and kinetics of single channel-activity when compared to those of MH-normal pigs (Mickelson *et al.* 1988). Such functional alterations are caused by an Arg to Cys mutation at

position 615 in the primary structure of the skeletal muscle RYR promoter (Treves *et al.* 1994). In humans MH phenotype displays both allelic and nonallelic heterogeneity (Sudbrak *et al.* 1995).

Pharmacological tools and peptide toxin

The RYR Ca^{2+} channels are the target of many structurally unrelated drugs and peptides toxins (for review see Palade *et al.* 1989; Coronado *et al.* 1994). Needless to say that the most popular drug used to study the Ca^{2+}-release channel is ryanodine. However, though ryanodine is a specific agonist, its use to monitor the Ca^{2+}-release process has been hampered by its slow binding kinetics (several minutes). Recently, peptide toxins were purified to homogeneity from the scorpion venom of *Bothus hottentota* and *Pandidius imperator*, and used as specific agents to probe Ca^{2+} release via RYR (Valdivia *et al.* 1991, 1992). In particular, the venom of *Pandidius imperator* contains two peptides having a molecular weight of 8700 and 10500 Da which interfere with the gating of skeletal and cardiac RYR. The smaller toxin ($IpTx_a$) peptide appears to be an isoform specific activator of the RYR, since nM concentrations stimulate [^{3}H]-ryanodine binding and increase the open channel probability of the skeletal muscle RYR alone. While the larger 10.5 kDa toxin peptide ($IpTx_i$) does not appear to be isoform specific; it causes (i) inhibition of [^{3}H]-ryanodine binding and (ii) the decrease of the open probability of cardiac and skeletal muscle RYR (K_i of 10 nM). The $IpTx_i$ has been used to block Ca^{2+} release via RYR in Indo1 loaded ventricular myocyte. Data obtained on the effect of the 5–8 kDa toxin purified from *Bothus hottentota* venom, demonstrate that it increases ryanodine binding to both the skeletal and cardiac RYR in a Ca^{2+}-dependent manner.

References

Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature*, **361**, 315–25.

Chen, S. R. W., Zhang, L., and MacLennan, D. H. (1993). Characterization of a Ca^{2+} binding and regulatory site in the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.*, **267**, 23318–26.

Coronado, R., Morrissette, J., Sukhareva, V., and Vaughan, D. M. (1994). Structure and function of ryanodine receptors. *Am. J. Physiol.*, **266**, C1485–504.

Fill, M., Meja-Alvarez, R., Zorzato, F., Volpe, P., and Stefani, E. (1991). Antibodies as probes for ligand gating of single sarcoplasmic reticulum Ca^{2+}-release channels. *Biochem. J.*, **273**, 449–57.

Guerrrini, R., Menegazzi, P., Anacardio, R., Marastoni, M., Tomatis, R., Zorzato, F., *et al.* (1995). Calmodulin binding sites of the skeletal, cardiac, and brain ryanodine Ca^{2+} channels modulation by the catalytic subunit of cAMP-dependent protein kinase? *Biochemistry*, **34**, 5120–9.

Hain, J., Nath, S., Mayrleitner, M., Fleisher, S., and Schindler, H. (1994). Phosphorylation modulates the function of the calcium release channel of sarcoplasmic reticulum from skeletal muscle. *Biophys. J.*, **67**, 1823–33.

Hakamata, Y., Nakai, J., Takeshima, H., and Imoto, K. (1992). Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain. *FEBS Lett.*, **312**, 229–35.

Imagawa, T., Smith, J. S., Coronado, R., and Campbell, K. (1987). Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+} permeable pore of the calcium release channel. *J. Biol. Chem.*, **262**, 16636–43.

Jayaraman, T., Brillantes, A. M., Timmerman, A. P., Fleisher, S., Erdjument-Bromgel, H., Tempst, P., *et al.* (1992). FK506 binding protein associated with the calcium release channel (ryanodine receptor). *J. Biol. Chem.*, **267**, 9474–7.

Lai, F. A., Erickson, H. P., Rosseau, E., Liu, Q. Y., and Meissner, G. (1988). Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature*, **331**, 315–19.

McPherson, P. S. and Campbell, K. P. (1993). Characterization of the major brain from of the ryanodine receptor/Ca^{2+} release channel. *J. Biol. Chem.*, **268**, 19785–90.

Meissner, G. (1986). Evidence of a role for calmodulin in the regulation of calcium release from skeletal muscle sarcoplasmic reticulum. *Biochemistry*, **25**, 244–51.

Meissner, G. (1994). Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. *Ann. Rev. Physiol.*, **56**, 485–508.

Menegazzi, P., Larini, F., Treves, S., Guerrini, R., Quadroni, M., and Zorzato, F. (1994). Identification and characterization of three calmodulin binding sites of the skeletal muscle ryanodine receptor. *Biochemistry*, **33**, 9078–84.

Mickelson, J. R., Gallant, E. M., Litterer, L. A., Jonhson, K. M., Rempel, W. E., and Louis, C. H. (1988). Abnormal sarcoplasmic reticulum ryanodine receptor in malignant hyperthermia. *J. Biol. Chem.*, **263**, 9310–15.

Mickelson, J. R. and Louis, C. F. (1996). Malignant hyperthermia: excitation–contraction coupling, Ca^{2+} release channel, and cell Ca^{2+} regulation. *Physiol. Rev.* **76**, 537–92.

Nakai, J., Imagawa, T., Hakamata, Y., Shigekawa, M., Takeshima, H., and Numa, S. (1990). Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calcium release channel. *FEBS Lett.*, **271**, 169–77.

O'Neil, K. T. and DeGrado, W. F. (1990). How calmodulin binds its targets: sequence independent recognition of amphiphilic alpha-helices. *Trends Biochem. Sci.*, **15**, 59–64.

Otsu, K., Willard, H. F., Khanna, V. J., Zorzato, F., Green, N. M., and MacLennan, D. H. (1990). Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. *J. Biol. Chem.*, **265**, 13472–83.

Palade, P., Dettbarn, C., Brunder, D., Stein, P., and Hals, G. (1989). Pharmacology of calcium release from sarcoplasmic reticulum. *J. Bioenerg. Biomembr.*, **21**, 295–320.

Pessah, I. N., Francini, A. O., Scales, D. J., Waterhouse, A. L., and Casida, J. E. (1986). Calcium–ryanodine receptor complex. Solubilization and partial characterization from skeletal muscle junctional sarcoplasmic reticulum vesicles. *J. Biol. Chem.*, **261**, 8643–8.

Radermacher, M., Rao, V., Grassucci, R., Frank, J., Timmeran, A. P., Fleisher, S., *et al.* (1994). Cryo-electron microscopy and three-dimensional reconstruction of the calcium release channel/ryanodine receptor from skeletal muscle. *J. Cell Biol.*, **127**, 411–23.

Rios, E. and Pizarro, G. (1991). Voltage sensor of excitation–contraction coupling in skeleted muscle. *Physiol. Rev.*, **71**, 849–908.

Smith, J. S., Rousseau, E., and Meissner, G. (1989). Calmodulin modulation of single sarcoplasmic reticulum Ca^{2+} release channels from cardiac and skeletal muscle. *Circ. Res.*, **64**, 352–9.

Sudbrak, R., Procaccio, V., Klausnitzer, M., Curran, J. L., Monsieurs, K., VanBroeckhoven, C., *et al.* (1995). Mapping of a further malignant hyperthermia susceptibility locus to chromosome 3q13. 1. *Am. J. Genet.*, **56**, 684–91.

Takeshima, H., Nishimura, S., Matsumoto, H., Ishida, K., Kangawa, N., Minamino, H., *et al.* (1989). Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature*, **339**, 439–45.

Treves, S., Chiozzi, P., and Zorzato, F. (1993). Identification of the domain recognized by anti-(ryanodine receptor) antibodies which affect $Ca^{(2+)}$-induced Ca^{2+} release. *Biochem. J.*, **291**, 757–63.

Treves, S., Larini, F., Menegazzi, P., Steinbeg, T. H., Koval, M., Vilsen, B., *et al.* (1994). Alteration of intracellular Ca^{2+} transients in COS-7 cells transfected with the cDNA encoding skeletal-muscle ryanodine receptor carrying a mutation associated with malignant hyperthermia. *Biochem. J.*, **301**, 661–5.

Tripathy, A., Xu, I. L., Mann, G., and Meissner, G. (1995). Calmodulin activation and inhibition of sketetal muscle Ca^{2+} release channel (ryanodine receptor). *Biophys. J.*, 106–19.

Valdivia, H. H., Fuentes, O., El-Hayek, R., Morrissette, J., and Coronado, R. (1991). Activation of the ryanodine receptor Ca^{2+} release channel of sarcoplasmic reticulum by a novel scorpion venom. *J. Biol. Chem.*, **266**, 19135–8.

Valdivia, H. H., Kirby, M. S., Lederer, W. J., and Coronado, R. (1992). Scorpion toxins targeted against the sarcoplasmic reticulum $Ca^{(2+)}$-release channel of skeletal and cardiac muscle. *Proc. Natl. Acd. Sci. USA*, **89**, 12185–9.

Wang, J. and Best, P. (1992). Inactivation of the sarcoplasmic reticulum calcium channel by protein kinase. *Nature*, **359**, 739–41.

Witcher, D. R., Kovacs, R. J., Schulman, H., Cefali, D. C., and Jones L. R. (1991). Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity. *J. Biol. Chem.*, **206**, 11144–52.

Witcher, D. R., Strifler, B. A., and Jones, L. R. (1992). Cardiac-specific phosphorylation site for multifunctional Ca^{2+}/calmodulin-dependent protein kinase is conserved in the brain ryanodine receptor. *J. Biol. Chem.* **267**, 4963–7.

Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N. M., Lai, F. A., *et al.* (1990). Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.*, **265**, 2244–56.

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Helothermine (*Heloderma horridum horridum*)

Helothermine, a 25.5 kDa toxin purified from the venom of Heloderma horridum horridum causes lethargy, partial paralysis ,and lowering of body temperature in mice. Previous studies showed no effect on voltage-dependent Na^+, K^+, or Ca^{2+} channels. Recent results suggest that physiological effects may be due to inhibition of the ryanodine sensitive Ca^{2+}-release channel and other Ca^{2+} channels of non-P type.

Helothermine (HLTX) is a toxin isolated from the venom of the Mexican beaded lizard *Heloderma horridum horridum* (Mochca-Morales *et al.* 1990). It is synthesized as a single chain polypeptide precursor of 242 amino acid residues including a signal peptide of 19 residues (Morrissette *et al.* 1995). One of its remarkable characteristics (mature toxin of 223 residues and a mol. wt of 25.5 kDa) is the high content of cysteine residues (16, with 10 of them located within the last 54 carboxy-terminal residues). It has an isoelectric point (pI) of 6.8, and among the observed effects in mice injected with this toxin are: lethargy, partial paralysis of rear limbs, and lowering of body temperature (Mochca-Morales *et al.* 1990). This hypothermic effect gave us the idea to call it helothermine, from Heloderma and hypothermic.

No structural similarities have been detected between compiled Na^+, K^+, or Ca^{2+} channel specific toxins (Adams and Swanson 1994) and helothermine (Morrissette *et al.* 1995). However, it showed an important homology with a family of cysteine-rich secretory proteins (CRISP) isolated from human and mouse testis, with which it shares its 16 cysteine residues, suggesting, a common pattern of disulfide bridges (Morrissette *et al.* 1995). It has been proposed that HLTX could be constituted by two domains: (1) an amino-terminus domain with 6 out of 16 cysteine residues and (2) a carboxy-terminus domain containing the other 10 cysteine residues included in the last 54 amino acid residues.

■ Purification and sources

Helothermine (HLTX) is isolated from the venom of the Mexican beaded lizard *Heloderma horridum horridum* (Mochca-Morales *et al.* 1990) by gel filtration (Sephadex G-75) and ion exchage chromatography into both DEAE-cellulose and CM-cellulose. Specific questions can be addressed to Dr Lourival D. Possani (see authors' address at the end of this entry). Helothermine can easily be purified from commercially available venom of *Heloderma horridum* by the procedure described.

```
        10         20         30         40         50         60
EASPKLPGLMTSNPDQQTEITDKHNNLRRIVEPTASNMLKMTWSNKIAQNAQRSANQCTLEHTSK
   70         80         90        100        110        120        130
EERTIDGVECGENLFFSSAPYTWSYAIQNWFDERKYFRFNYGPTAQNVMIGHYTQVVWYRSYELG
       140        150        160        170        180        190
CAIAYCPDQPTYKYYQVCQYCPGGNIRSRKYTPYSIGPPCGDCPDACDNGLCTNPCKQNDVYNNC
  200        210        220
PDLKKQVGCGHPIMKDCMATCKCLTEIK
```

Figure 1. Amino acid sequence of helothermine. Based on the distribution of cysteine residues, the sequence has been divided arbitrarily into two putative domains: the amino-terminus domain (residues 1–161; shown in regular face characters) containing 6 of the total 16 cysteine residues (shown in bold), and the carboxy-terminus domain (residues 162–223; in bold face characters) with 10 cysteine residues (bold and underlined) clustered within this domain.

Toxicity

Toxicity was tested by intraperitoneal injection of different amounts of toxin. Mouse LD_{50} was between 1.25–2.50 μg/g (Mochca-Morales *et al.* 1990). From the data reported, this LD_{50} can be estimated as 1.9 μg/g.

Use in cell biology

HLTX inhibits ryanodine binding to cardiac and skeletal sarcoplasmic reticulum from rabbit (Morrissette *et al.* 1994, 1995). It blocks cardiac and skeletal ryanodine receptor channels (Morrissette *et al.* 1994, 1995) incorporated into planar bilayers (Coronado *et al.* 1992) and blocks Ca^{2+}-induced Ca^{2+} release activated by photolysis of nitr-5 in saponin-permeabilized trabeculae from rat ventricle (Morrissette *et al.* 1995). HLTX also inhibits Ca^{2+}-channels (distinct from P type) (Nobile *et al.* 1994*a*) and K^+ currents in cerebellar granule cells of new born rats (Nobile *et al.* 1994*b*). These data suggest that HLTX could be used as a molecular probe to study Ca^{2+}-channels (ryanodine sensitive and non-P type Ca^{2+}-channels), and K^+ channels of the type present in cerebellar granule cells. Due to the apparent dual specificity of HLTX, it is currently being investigated if the effects observed are mediated by another protein, or if there is a close relationship between these two types of channels.

Genes

The cDNA encoding HLTX was isolated using a specific oligonucleotide as a probe from a venom gland library (Morrissette *et al.* 1995). The characterized cDNA consists of 1104 nucleotides (GenBank accession number U13619), containing an open reading frame for a 242 amino acid precursor including a signal peptide of 19 residues (223 residues for mature toxin). A cDNA clone from a similar preparation was also isolated, but screened by means of polyclonal antibodies against pure native toxin, and essentially the same sequence was found except that the clone was truncated at the 5′ region. It contained only the sequence encoding the last seven residues of the signal peptide, and ends exactly at the same point as the full length cDNA (L. Pardo, B. Becerril, and L.D. Possani, unpublished results).

References

Adams, M. E. and Swanson, G. (1994). Neurotoxins. Trends Neurosci., **17**, (supplement), 1–28.

Coronado, R., Kawano, S., Lee, J. C., Valdivia, C., and Valdivia, H. H. (1992). Planar bilayer recording of ryanodine receptors of sarcoplasmic reticulum. *Meth. Enzymol.*, **207**, 699–707.

Mochca-Morales, J., Martin, B. M., and Possani, L. D. (1990). Isolation and characterization of helothermine a novel toxin from *Heloderma horridum horridum* (Mexican beaded lizard) venom. *Toxicon*, **28**, 299–309.

Morrissette, J., El-Hayek, R., Possani, L. D., and Coronado, R. (1994). *Biophys. J.*, **66**, A415 (abstract 330).

Morrissette, J., Kratzschmar, J., Haendler, B., El-Hayek, R., Mochca-Morales, J., Martin, B. M., *et al.* (1995). Primary structure and properties of helothermine, a peptide toxin that blocks ryanodine receptors. *Biophys. J.*, **68**, 2280–8.

Nobile, M., Possani, L. D., Spadavecchia, L., and Prestipino, G. (1994*a*). *Biophys. J.*, **66**, A422 (abstract 371).

Nobile, M., Magnelli, V., Lagostena, L., Mochca-Morales, J., Possani, L. D., and Prestipino, G. (1994*b*). The toxin helothermine affects potassium currents in new born rat cerebellar granule cells. *J. Membr. Biol.*, **139**, 49–55.

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Presynaptic toxins

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Introduction

The synapse is a privileged site of communication of messages within the animal body. Synapses show different anatomical specializations depending on the type of cells involved: the main difference is between nerve–nerve and nerve–muscle synapses. The majority of synapses of vertebrates are present within the central nervous system. They are well protected from the rest of the body by anatomical barriers and are not easily reachable by toxins. Animal movement and behaviour strictly depends on the correct and timely function of central synapses and peripheral. The latter type of synapses are rapidly and easily accessible to a variety of molecules that enter body fluids. On this basis, it is not surprising that hundreds of protein toxins have been elaborated by many different animal species to block the transmission of nerve impulses to muscles. This result can be obtained by acting on the nerve terminal, i.e. presynaptically, or on the muscle cells, i.e. post-synaptically. This section focuses on those toxins blocking synaptic functions by binding to the presynaptic membrane. Several toxins acting on channels of the presynaptic membrane have been considered in previous parts. Apart from pardaxin, the mechanism of action of the toxins grouped here is unknown at the molecular level. This limits, but does not prevent, their use in cell biology. In fact, their cellular effects are well documented and hence the consequences of their application to cells are well defined. As such, these toxins are already widely used, and it can be anticipated that the uncovering of their mode of action will provide novel information on synapse function, thus widening their usefulness.

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β-Bungarotoxin (*Bungarus multicinctus*)

β-Bungarotoxin is a two-chain phospholipase A_2-neurotoxin from snake venom (Bungarus multicinctus) *acting at the presynaptic site of motor nerve terminals by blocking transmitter release. It binds with high affinity to a subtype of voltage-dependent K^+ channels competing with other neurotoxins such as dendrotoxins.*

β-Bungarotoxin (β-Bgt) is a presynaptically acting phospholipase A_2 (PLA_2) -neurotoxin present in the venom of the snake *Bungarus multicinctus,* the Chinese banded krait. It consists of two polypeptide chains, A- and B-chain, crosslinked by one interchain disulfide bond. The A-chain is structurally homologous to PLA_2 enzymes (13.5 kDa, 120 amino acid residues, sequence accession number to the GenEMBL databank: X53406, the nucleotide sequence encoding the A-chain has been elucidated by Danse *et al.* 1990). The B-chain (7 kDa, 60 amino acid residues, sequence accession number: X53407) has close sequence homology with trypsin inhibitors (Kunitz-type) and dendrotoxins from mamba *(Dendroaspis* sp.) venoms. Cleavage of the interchain disulfide bond leads to the complete inactivation of lethal toxicity as well as of PLA_2 enzymatic activity. Recombination of the two chains (A and B) fails to restore the biological properties (Tzeng 1993). β-Bgt exists in various isotoxinforms (more than 16; Chu *et al.* 1995); β_1-Bgt seems to be the most active.

At motor nerve terminals β-Bgt induces a triphasic change in neurotransmitter release, which varies with animal models and experimental conditions: an initial reduction in meep frequency, e.g. decrease in transmitter release, is followed by a phase of an increase and, finally, by a complete inhibition of release (Harris 1991; Hawgood and Bon 1991). Although PLA_2-activity has been supposed to be related to the neuromuscular blockade by the toxin, the specific and effective binding to a membrane receptor (or acceptor), most probably through the molecule's B-chain, is essential for its neurotoxic effect. Studies on brain synaptosomes using ^{125}I-labelled β-Bgt revealed that the toxin binds with high affinity to neuron-specific proteins in the plasma membrane of the

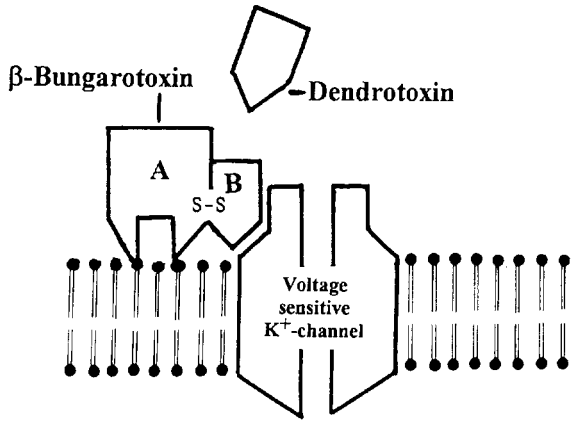

Figure 1. Mechanism of action of β-bungarotoxin. The toxin's B-chain binds to a site of a subtype of voltage-sensitive K^+ channels, where it competes with dendrotoxin. Site-directed hydrolysis of membrane phospholipids by PLA_2 (A-chain) may disturb membrane permeability affecting transmitter release.

synaptosomes. A subtype of voltage-dependent K^+ channels, which also binds dendrotoxins, charybdotoxin, and the mast-cell degranulating (MCD) peptide, serves as membrane receptor. However, inhibition of dendrotoxin-binding by β-Bgt is noncompetitive. Site directed hydrolysis of membrane phospholipids may lead to disturbance of the exocytotic release sites followed by degenerative processes at the nerve terminal membrane ('omega'-lesions). On the other hand, β-Bgt is not myotoxic like other PLA_2-neurotoxins (Harris 1991).

Purification and sources

β-Bgt and its isotoxins are isolated from the crude venom of *Bungarus multicinctus* by ion exchange chromatography on CM-Sephadex C-25 (Kondo *et al.* 1978, 1982) or by gel filtration followed by various steps of HPLC ion exchange chromatography for separating the various isotoxins (Chu *et al.* 1995). β-Bgt can be purchased from Calbiochem, La Jolla, CA and from Sigma Chemical Co., St.Louis, MO, USA.

Toxicity

Mouse LD_{50} (i.p. injection) values of the major five β-Bgt isotoxins vary from 0.019 mg/kg for β_1-Bgt to 0.13 mg/kg for β_5-Bgt. Toxicity does not correlate with the PLA_2 enzymatic activity of the toxins (Rosenberg 1990). The use of the toxins does not require exceptional precautions.

Use in cell biology

A voltage-dependent K^+ channel provides a binding site for β-Bgt (Rehm 1991; Tzeng 1993). Identification, solubilization, and purification of the receptor from rat brain have been accomplished using affinity chromatography on β-Bgt and dendrotoxin gels. The purified receptor proteins have been reconstituted into functional K^+ channels. Among other toxins (dendrotoxins, MCD-peptide, charybdotoxin) β-Bgt proved to be a valuable tool to distinguish and characterize K^+ channel subtypes, which exhibit diverse kinetic and conductance properties. Using recombinant DNA-techniques shaker K^+-channels have been expressed, which are sensitive to dendrotoxin or charybdotoxin, however, the expression of β-Bgt-sensitive channels has still to be performed.

By light-microscopic autoradiography differences in the distribution of β-Bgt- and of dendrotoxin-binding sites in the rat central nervous system have been demonstrated (Pelchen-Matthews and Dolly 1989). Both toxins are enriched in the grey matter and synapse-rich areas of hippocampus and the cerebellum. β-Bgt competes with the majority of dendrotoxin-binding sites in the grey, but less efficaciously in the white matter. Applying these and similar techniques the toxin may be useful to localize K^+ channel sites in tissues.

References

Chu, C. C., Li, S. H., and Chen, Y. H. (1995). Resolution of isotoxins in the β-bungarotoxin family. *J.Chromatogr.*, A **694**, 492–7.

Danse, J. M., Toussaint, J. L., and Kempf, J. (1990). Nucleotide sequence encoding β-bungarotoxin A_2-chain from the venom glands of *Bungarus multicinctus*. *Nucleic Acid Res.*,**18**, 4609.

Harris, J. B. (1991). Phospholipases in snake venoms and their effects on nerve and muscle. In *Snake toxins* (ed. A. L. Harvey), pp. 91–129, Pergamon Press, Oxford.

Hawgood, B. and Bon, C. (1991). Snake venom presynaptic toxins. In Handbook of natural toxins, Vol. 5, Reptile venoms and toxins (ed. A. T. Tu) pp. 3–52, Marcel Dekker, New York.

Kondo, K., Narita, K., and Lee, C. Y. (1978) Amino acid sequences of the two polypeptide chains in β-bungarotoxin from the venom of *Bungarus multicinctus*. *J. Biochem.*, **83**, 101–15.

Kondo, K., Toda, H., Narita, K., and Lee, C. Y. (1982). Amino acid sequences of three β-bungarotoxins (β3-, β4- and β5-bungarotoxins) from *Bungarus multicinctus* venom. Amino acid substitutions in the A-chain. *J. Biochem.*, **91**, 1531–48.

Pelchen-Matthews, A. and Dolly, J. O. (1989). Distribution in the rat central nervous system of acceptor sub-types for dendrotoxin, a K^+ channel probe. *Neuroscience*, **29**, 347–61.

Rehm, H. (1991). Molecular aspects of neuronal voltage-dependent K^+ channels. *Eur. J. Biochem.*, **202**, 701–13.

Rosenberg, P. (1990). Phospholipases. In *Handbook of toxinology* (ed. W. T. Shier and D. Mebs), pp. 67–277, Marcel Dekker, New York.

Tzeng, M. C. (1993). Interaction of presynaptically toxic phospholipases A2 with membrane receptors and other binding sites. *J. Toxicol.–Toxin Rev.*, **12**, 1–62.

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Rattlesnake venom neurotoxins: crotoxin-related proteins (crotoxin from *Crotalus durissus terrificus*, Mojave toxin from *C. s. scutulatus*, concolor toxin from *C. v. concolor*, vergrandis toxin from *C. vegrandis*, and canebrake toxin from *C. h. atricaudatus*)

Crotoxin-related proteins from Crotalus *venoms are heterodimeric phospholipase A_2 neurotoxins. The primary cause of death after systemic intoxication by crotoxin-related proteins is respiratory failure. The proteins appear to bind to specific protein receptors on presynaptic membranes, alter release of neurotransmitters from synaptosomes, and inhibit the release of acetylcholine from peripheral cholinergic synapses. They also inhibit the postsynaptic response of acetylcholine receptor and exhibit myotoxic effects on muscle tissues.*

Only two of the neurotoxins from rattlesnake venoms, crotoxin and Mojave toxin, have been characterized to any appreciable extent. These two toxins have very similar structures, comparable activities, and are immunologically crossreactive. Briefly, the toxins are noncovalent heterodimeric protein complexes with isoelectric points in the pH range of 5 to 6 and molecular masses near 24 kDa. The complexes can be dissociated into distinct separable subunits (Rübsamen *et al.* 1971; Cate and Bieber 1978): one a basic phospholipase, the other an acidic protein without any known biological activity in the absence of the basic subunit. The sequences for both subunits of crotoxin (Aird *et al.* 1986; 1990*a*) and of Mojave toxin (Aird *et al.* 1990*b*; Bieber *et al.* 1990*a*) have been published. The data indicate that the two distinct subunits are related to one another and that both show sequence similarity to secretory phospholipases A_2. A high degree of sequence similarity is also apparent when the sequences of subunits from *C. d. terrificus* and *C. s. scutulatus* are compared to one another.

Variants or isoforms have been isolated in the case of crotoxin (Faure and Bon 1988). Two isoforms of the basic subunit and four acidic subunit isoforms have been purified and sequenced (Faure and Bon 1988; Faure *et al.* 1991). As shown in Fig. 1, the acidic subunit consists of three distinct polypeptide chains (α, β, and γ) which correspond to three different regions of a phospholipase A_2-like precursor, whose the cDNA has been cloned (EMBL Data bank accession number: CA X12606) (Bouchier *et al.* 1991). The polypeptide sequences of mature CA isoforms indicate that they are generated by different post-translational processings of the precursor (Faure *et al.* 1991). Two cDNAs encoding isoforms of crotoxin basic subunit have also been cloned (EMBL data bank accession number: CB1 X12603 and CB2 X16100), indicating that the isoforms of the basic subunit result from different mRNAs (Bouchier *et al.* 1991). Genomic sequences encoding the acidic and basic subunits of Mojave toxin have been isolated and similar observations have been made (John *et al.* 1994).

Purification and sources

Purification and partial characterization of native toxins and the individual subunits have been described on several occasions (Cate and Bieber 1978; Faure and Bon 1988; Aird *et al.* 1990*a*; Faure *et al.* 1991). Typically, purification is accomplished by a variety of chromatography procedures. The purity is usually assessed by gel electrophoresis, isoelectric focusing, and/or high performance liquid chromatography. Mojave toxin can be purchased from CALBIOCHEM (San Diego, California, USA).

Toxicity

The lethal potency of crotoxin related proteins is usually determined on mice. It is defined as the toxin quantity which causes the death of 50 per cent of the injected mice (LD_{50}). Mice (18–20 g) are injected intravenously or subcutaneously (0.2 ml or 0.1 ml of the tested solution per 20 g body weight, respectively) with toxin doses that differ by a factor of 1.414 ($\sqrt{2}$). The LD_{50} value and its fiducial limits are calculated by the statistical method of Sperman-Kärber, as recommended by the World Health

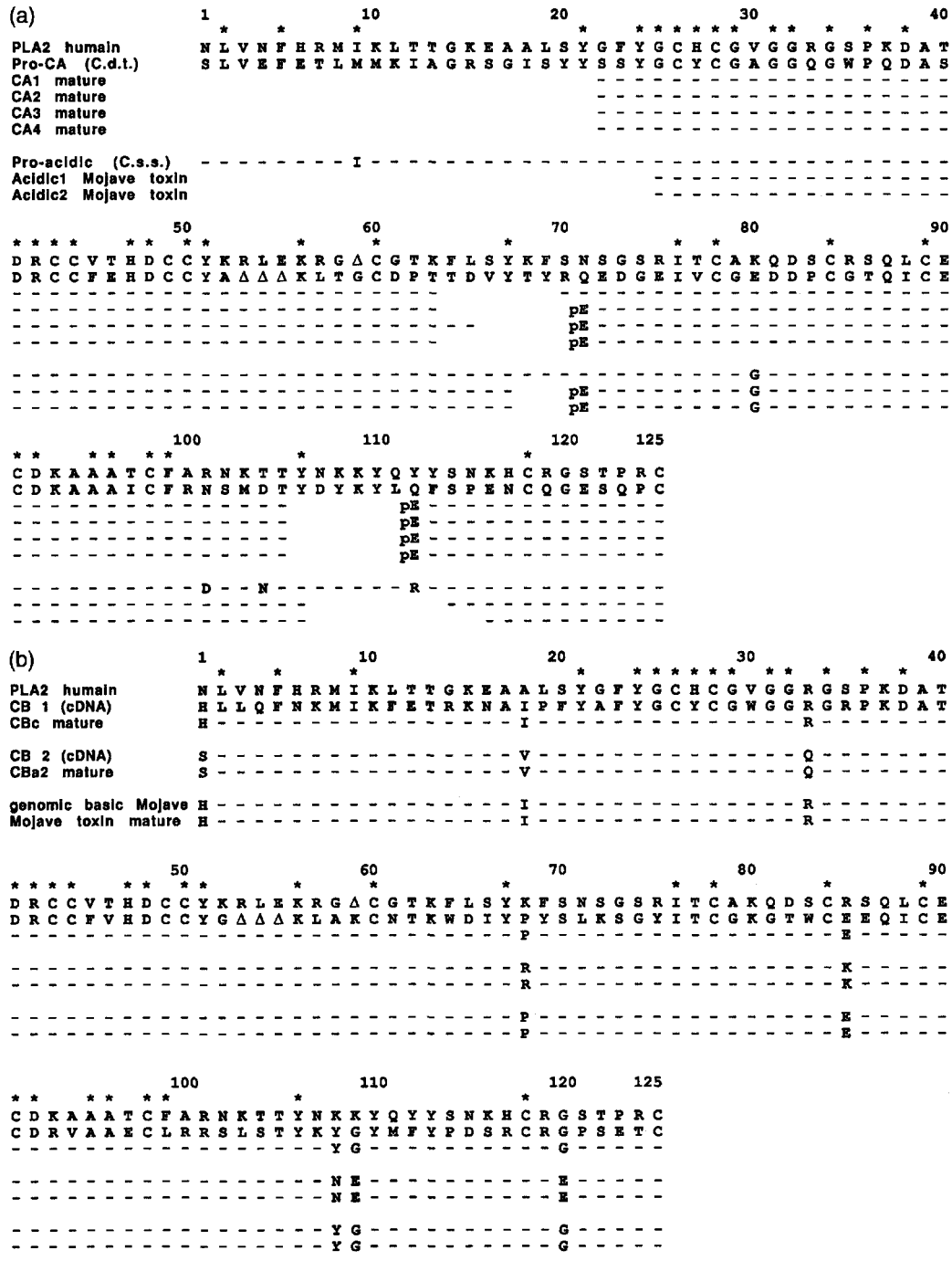

Figure 1. (a) Sequence alignment of human phospholipase A_2 (PLA_2) (Kramer *et al.* 1989), Pro-CA from *Crotalus durissus terrificus* (C.d.t.) (Bouchier *et al.* 1991), mature CA isoforms (Faure *et al.* 1991), pro-acidic subunit from *Crotalus s. scutulatus* (C.s.s.) (John *et al.* 1994), and mature acidic Mojave isoforms (Bieber *et al.* 1990*a*).
(b) Sequence alignment of human phospholipase A_2 (Kramer *et al.* 1989), CB1 and CB2 cDNAs (Bouchier *et al.* 1991), mature CBa_2 and CBc isoforms (Faure *et al.* 1994), genomic basic Mojave (John *et al.* 1994), and mature basic Mojave toxin (Aird *et al.* 1990*b*). The amino acid residues which are absent in the mature proteins are indicated by a blank; ( ) indicates deletion; –, indicates identical amino acid. The amino acid residues conserved in all phospholipase A_2 are indicated by asterisks.

Organization (1981). In the absence of acidic subunit, there is no significant difference in toxicity between the various basic subunit isoforms (i.v. LD_{50} = 540 ± 100 μg/kg). In the presence of acidic subunit isoforms, however, two classes of crotoxin complexes can be distinguished: class 1 (i.v. LD_{50} = 93 ± 20 μg/kg) and class 2 (i.v. LD_{50} = 435 ± 65 μg/kg) (Faure *et al.* 1993). Crotoxin toxicity varies in different animal species (Hawgood and Bon 1991). Crotoxin is considered nontoxic by ingestion.

Use in cell biology

Physiological studies showed that crotoxin and related proteins block neuromuscular transmission of skeletal muscle, acting primarily at the presynaptic level altering the transmitter release, but inhibiting also the postsynaptic response of muscular receptors to acetylcholine. In addition, crotoxin related neurotoxins possess significant myotoxic activity (Hawgood and Bon 1991). Electrophysiological studies showed that crotoxin, along with several other neurotoxic phospholipases, significantly reduce K^+ current of motor nerve terminals (Harvey *et al.* 1992), while studies with isolated brain synapotosomes indicated that Mojave toxin alters uptake and release of several neurotransmitters (Bieber *et al.* 1990*b*) and may interfer with a Ca^{2+} channel (Valdes *et al.* 1989). Native Mojave toxin and its basic subunit prevented the fusion of myoblasts to myotubes and destroyed existing myotubes without altering the myoblast proliferation, whereas the acidic subunit alone had no effect (Bieber 1992). The presence of high affinity specific binding sites for crotoxin, Mojave toxin, and several other neurotoxic phospholipases from snake venoms has been demonstrated on guinea pig brain synaptosomes (Degn *et al.* 1991). The interaction of crotoxin with biological membranes and phospholipid vesicles resulted in the dissociation of the native toxin complex. The acidic subunit was released into solution while the basic subunit remained bound (Bon *et al.* 1979; Radvanyi *et al.* 1989). The kinetics of binding of radiolabelled crotoxin to presynaptic membranes suggests the acidic subunit is involved in the formation of a transient complex between native toxin and its specific receptor site which will dissociate, leaving the basic subunit at the specific binding site (Délot and Bon 1993). Negatively charged phospholipids may play a role in the design of the target for these toxins, but membrane proteins are likely to be involved in high affinity binding (Radvanyi *et al.* 1989; Degn *et al.* 1991; Délot and Bon 1993; Križaj *et al.* 1996). The specific presynaptic receptor for crotoxin and related proteins remains, however, to be purified.

References

Aird, S. D, Kaiser I. I., Lewis, R. V., and Kruggel, W. G. (1986). A complete amino acid sequence for the basic subunit of crotoxin. *Arch. Biochem. Biophys.*, **249**, 296–300.

Aird, S. D., Yates, J. R. III, Marino, P. A., Shabanowitz, J., Hunt, D. F., and Kaiser, I. I. (1990a). The amino acid sequence of the acidic subunit β-chain of crotoxin. *Biochim. Biophys. Acta*, **1040**, 217–24.

Aird, S. D., Kruggel, W. G., and Kaiser, I. I. (1990*b*). Amino acid sequence of the basic subunit of Mojave toxin from the venom of the Mojave rattlesnake (*Crotalus scutulatus scutulatus*). *Toxicon*, **28**, 669–74.

Bieber, A. L. (1992). Recent studies of Mojave toxin and its constitutent subunits on cells in culture. In Neuroreceptors, Ion Channels and the Brain (ed. N. Kawai, T. Nakajima, and E. Barnard), pp. 43–53, Elsevier, .

Bieber, A. L., Becker, R. R., McParland, R., Hunt, D. F., Shabanowitz, J.,Yates, J. R. III, *et al.* (1990a). The complete sequence of the acidic subunit from Mojave toxin determined by Edman degradation and mass spectrometry. *Biochim. Biophys. Acta*, **1037**, 413–21.

Bieber, A. L., Mills, J. P., Ziolkowski, Jr, C., and Harris, J. (1990*b*). Rattlesnake neurotoxins: Biochemical and biological aspects. *J. Toxicol. – Toxin Rev.*, **9**, 285–306.

Bon, C., Changeux, J. P., Jeng, T. W., and Fraenkel-Conrat, H. (1979). Postsynaptic effects of crotoxin and of its isolated subunits. *Eur. J. Biochem.*, **99**, 471–81.

Bouchier, C., Boulain, J. C., Bon, C., and Ménez, A. (1991). Analysis of cDNAs encoding the two subunits of crotoxin, a phospholipase A_2 neurotoxin from rattlesnake venom: the acidic non enzymatic subunit derives from a phospholipase A_2-like precursor. *Biochim. Biophys. Acta*, **1088**, 401–8.

Cate, R. L. and Bieber, A. L. (1978). Purification and characterization of Mojave (*Crotalus scutulatus scutulatus*) toxin and its subunits. *Arch. Biochem. Biophys.*, **189**, 397–408.

Degn, L. L. Seebart, C. C., and Kaiser, I. I. (1991). Specific binding of crotoxin to brain synaptosomes and synaptosomal membranes. *Toxicon*, **29**, 973–88.

Délot, E. and Bon, C. (1993). Model for the interaction of crotoxin, a phospholipase A_2 neurotoxin, with presynaptic membranes. *Biochemistry*, **32**, 10708–13.

Faure, G. and Bon, C. (1988). Crotoxin, a phospholipase A_2 neurotoxin from the South American rattlesnake, *Crotalus durissus terrificus*: purification of several isoforms and comparison of their molecular structure and of their biological activities. *Biochemistry*, **27**, 730–8.

Faure, G., Guillaume, J. L., Camoin, L., and Bon, C. (1991). Multiplicity of acidic subunit isoforms of crotoxin, the phospholipase A_2 neurotoxin from *Crotalus durissus terrificus* venom, results from posttranslational modifications. *Biochemistry*, **30**, 8074–83.

Faure, G., Harvey, A. L., Thomson, E., Saliou, B., Radvanyi, F., and Bon, C. (1993). Comparison of crotoxin isoforms reveals that stability of the complex plays a major role in its pharmacological action. *Eur. J. Biochem.*, **214**, 491–6.

Faure, G., Choumet, V., Bouchier, C., Camoin, L., Guillaume, J. L., Monegier, B., *et al.* (1994). The origin of the diversity of crotoxin isoforms in the venom of *Crotalus durissus terrificus*. *Eur. J. Biochem.*, **223**, 161–4.

Harvey, A. L., Anderson, A. J., and Rowan, E. G. (1992). Potassium channel-blocking toxins from snake venoms and neuromuscular transmission. *Meth. Neurosci.*, **8**, 396–407.

Hawgood, B. and Bon, C. (1991). Snake venom presynaptic toxins. In *Handbook of natural toxins, reptile and amphibian venoms*, Vol. 5, *Reptile and amphibian venoms* (ed. A. T. Tu), pp. 3–52, Marcel Dekker, New York.

John, T. R., Smith, L. A., and Kaiser, I. I. (1994). Genomic sequences encoding the acidic and basic subunits of Mojave toxin: unusually high sequence identity on non-coding regions. *Gene*, **139**, 229–34.

Kramer, R. M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E. P., *et al.* (1989). Structure and properties of human non-pancreatic phospholipase A_2. *J. Biol. Chem.*, **264**, 5768–75.

Križaj, I. Faure, G., Gubenšek, F. and Bon, C. (1996). Re-examination of crotoxin-membrane interactions. *Toxicon*, **34**, 1003–9.
Radvanyi, F., Saliou, B., Lembezat, M. P., and Bon, C. (1989). Binding of crotoxin, a presynaptic phospholipase A_2 neurotoxin, to negatively charged phospholipid vesicles. *J. Neurochem.*, **53**, 1252–9.
Rübsamen, K., Breithaupt, H., and Habermann, E. (1971). *Biochemistry*, and pharmacology of the crotoxin complex. I. Subfractionation and recombination of the crotoxin complex. *Naunyn. Schmiedeberg's Arch. Pharmacol.*, **270**, 274–88.
Valdes, J. J., Thompson, R. G., Wolff, V. L., Menking, D. E., Rael, E. D., and Chambers, J. P. (1989). Inhibition of calcium channel dihydropyridine receptor binding by purified Mojave toxin. *Neurotoxicol. and Teratol.*, **11**, 129–33.
World Health Organization Bulletin (1981). Offset publication no. 58, pp. 1, 5–27.

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Ammodytoxins (*Vipera ammodytes ammodytes*)

*Western sand viper (*Vipera ammodytes ammodytes*) venom contains considerable amounts of phospholipase A_2-related toxins, among them ammodytoxins A, B, and C, which exhibit presynaptic neurotoxicity, and ammodytin L, which is myotoxic. Ammodytoxins act specifically at peripheral nerve endings in motor end plates, and all these four bind to acceptors in bovine and* Torpedo *synaptosomal membranes. Their binding sites overlap to different extent with binding sites of some other phospholipase A_2-based presynaptic neurotoxins and related non-neurotoxic proteins. The acceptor protein has been partially characterized and is proposed to be a subunit of one of the subtypes of the K^+ channels.*

Ammodytoxins (atx) A, B, and C (PIR accession numbers A00768, A25806, and S06853) and ammodytin (atn) L (PIR, S19570) are group II secretory phospholipases A_2 (PLA_2), having 122 amino acids and molecular weights around 13 800 Da. The protein (Ritonja and Gubenšek 1985; Ritonja *et al.* 1986; Križaj *et al.* 1989) and cDNA sequences (Pungerčar *et al.* 1991) of atx A, B, and C (EMBL accession numbers: X53471, X52241, and X15138) exhibit over 97 and 99 per cent indentity, respectively. Atn L (EMBL accession number: X53036) being less similar to atx A, exhibits only 74 per cent identity on the protein and 90 per cent identity on the DNA level (Križaj *et al.* 1991; Pungerčar *et al.* 1991). It is enzymatically inactive since Asp 49, essential for binding of Ca^{2+}, is substituted by Ser. An additional mutation (G32N) in the highly conserved Ca^{2+} binding loop might also interfere with the binding of Ca^{2+}. The low enzymatic activity of this protein reported earlier (Thouin *et al.* 1982) was apparently a consequence of the presence of small amounts of other venom PLA_2s. The lethality of atx A, B, and C varies due to mutations Y115H, R119M, and N119Y in atx B (Ritonja *et al.* 1986) and F124I and K12SE in atx C (Križaj *et al.* 1989), underlining the importance of these residues for presynaptic neurotoxicity. An antibody which binds to the surface region between Phel06 and Tyr113 (common numbering system as in Renetseder *et al.* 1985) completely blocks the toxicity, indicating that this part of the molecule must be exposed to exert its full toxicity (Čurin-Šerbec *et al.* 1991; Gubenšek and Čurin-Šerbec 1994). Atx A is one of the most lethal single chain presynaptically active PLA_2s (Thouin *et al.* 1982; Lee *et al.* 1984).

All four isotoxins bind specifically to bovine and *Torpedo marmorata* synaptic membranes, although with different affinities. In the equilibrium binding of radioiodinated atx A and C to bovine brain synaptosomal membranes the following binding parameters were obtained: for atx A, K_d = 4.1 nM, B_{max} = 6.7 pmol/mg membr. prot. and for atx C, K_d = 6 nM, B_{max} = 5.7 pmole/mg membr. prot. (Križaj *et al.* 1994, 1995). In addition to the high-affinity binding sites, low-affinity sites having dissociation constants about two orders of magnitude higher were also observed but not further characterized.

Atx high-affinity binding sites in bovine brains are proteins but certain lipid structures are probably also involved in the specific binding (Križaj *et al.* 1994, 1995). A model of interaction of atx with the neuronal membrane, shown in Fig. 1, was proposed (Križaj, Ph.D. Thesis, University of Ljubljana, 1994). Binding studies using radioiodinated atx C performed on *Torpedo marmorata* synaptosomal membranes (Križaj *et al.* manuscript in

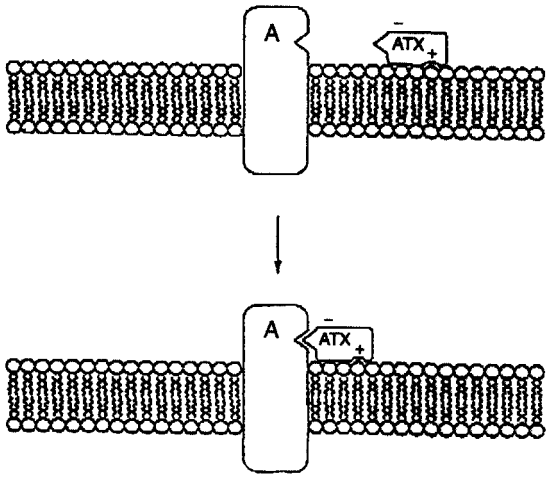

Figure 1. Model of ammodytoxin interaction with presynaptic membrane indicating high- (A) and low-affinity binding sites Note that membrane surface is negatively charged.

preparation) confimed the proposed model. The low-affinity binding site was common to all PLA_2 while the high-affinity binding site was specific only for neurotoxic PLA_2.

Specific binding and crosslinking of atx to bovine brain synaptosomal membranes was strongly inhibited by CB, a basic subunit of crotoxin from *Crotalus durissus terrificus*. *Vipera berus berus* PLA_2 was a weaker inhibitor, while nontoxic PLA_2, atn I_2, and myotoxic atn L, both from the *V. ammodytes* venom, were poor inhibitors. α-DTX, a presynaptic toxin from green mamba venom *(Dendroaspis angusticeps)*, β-BuTX from *Bungarus multicinctus* venom, and crotoxin acidic subunit, CA, did not compete with the atx C binding. Tetraethylammonium chloride ($\leqslant$ 10 mM) and 4-aminopyridine ($\leqslant$ 10 mM), both K^+ channel blockers, were also without effect on the specific binding of ^{125}I-atx C to synaptic membranes. The influence of atx A on the perineural waveform measured on mouse triangularis sterni preparations, suggested action on K^+ currents (Križaj *et al.* 1995). Specific binding of atx A or atx C to bovine synaptic membranes was not affected by K^+ and Na^+ ions but was very sensitive to divalent ions. Specific binding was high only in the presence of Ca^{2+}. Slight conformational changes, which were noticed by CD measurements upon substitution of Ca^{2+} for Sr^{2+} in the molecule of atx C, could affect the binding (Križaj *et al.* 1994).

On the other hand, in *Torpedo marmorata* synaptosomal membranes, high specific binding was observed when Sr^{2+} and EGTA were present in the bind buffer. All PLA_2 tested, except β-BuTX, were able to inhibit atx C-specific binding to the low-affinity binding site, whereas atx C high-affinity binding was inhibited to different extents, only by neurotoxic PLA_2s and, surprisingly, by myotoxic atn L. Atx A, CB, and atn L inhibited high-affinity binding completely. Notexin *(Notechis scutulatus scutulatus)*, OS_2 *(Oxyuranus scutellatus scutellatus)*, and agkistrodotoxin *(Agkistrodon blomhoffii brevicaudus)* were partial inhibitors, while crotoxin did not inhibit atx C high-affinity binding at all. In the reverse inhibition assay, atx C did not inhibit ^{125}I-crotoxin crosslinking to *Torpedo* membranes, which means that atx and crotoxin do not share high-affinity binding sites in *Torpedo* neuronal preparation (Križaj *et al.*, manuscript in preparation).

Purification and sources

Atx A, B, C, and atn L were purified using CM cellulose and Sephadex G-100 chromatography as described (Ritonja *et al.* 1978). Currently, FPLC on mono S column seems the method of choice. RP HPLC can also be used although the losses due to irreversible adsorption are not unexpected. The isoelectric points of atx A, B, C, and atn L are 10.2, 10.0, 9.5, and about 10.5, respectively (Lee *et al.* 1984). The content of atx can vary considerably in venoms from different locations. Atx is also available from Latoxan, Rosans, France.

Toxicity

Lethalities (LD_{50}) of presynaptically neurotoxic atx A, B, and C (previously designated as venom fractions 'k2', 'kl', and 'j'), determined by i.v. injections in white mice are: 0.021, 0.58, and 0.36 mg/kg (Thouin *et al.* 1982; Lee *et al.* 1984). The myotoxic atn L (Thouin *et al.* 1982; Križaj *et al.* 1991) is considerably less toxic, with LD_{50} = 3.6 mg/kg. The deleterious effect of atn L on muscles (Thouin *et al.* 1982) was confirmed by studies of liposome content leakage, which is suggested to be responsible for its myotoxicity (Rufini *et al.* 1992). This myotoxin can presumably covalently bind fatty acids by an apparently autocatalytic mechanism (Pedersen *et al.* 1995). The toxicity of atx A could be completely blocked by site-specific antibodies raised against peptide L2 Phel06–Tyr113 and partially by antibodies against peptides L1 (Tyr113–Pro122) and by one of the monoclonal antibodies L3 (Thr70–Glu78) indicating the importance of these surface regions for the toxicity of atx A (Gubenšek and Čurin-Šerbec 1994). These antibodies were also able to protect mice against the lethal potency of crotoxin basic subunit CB, whereas in the case of crotoxin the onset of the lethal effect was only delayed (Čurin-Šerbec *et al.* 1994).

Use in cell biology

Ammodytoxins could be used in studies of synaptosomal acceptors, presumably K^+ channel subunit, in combination with other presynaptically toxic PLA_2 toxins of similar or different specificities. Atn L promises, due to its moderate binding affinity, to be a suitable ligand for affinity chromatography of PLA_2 neuronal acceptors.

Genes

Complete gene structures are available for atx C (EMBL accession number X76731) (Kordiš and Gubenšek, 1996) and atn L (EMBL accession number X84017) (Kordiš and Gubenšek, manuscript in preparation). In the proximal part of the promoter region of both genes, a number of putative binding sites for transcription factors were detected. Many of them occupy identical positions in both genes, the functional analysis was, however, not performed. The role of highly conserved intron sequences, also observed in other known venom PLA_2 genes, remains obscure. The fourth intron of both acres contains an artiodactyla ART-2 retroposon (Kordiš and Gubenšek 1995), which may serve as a convenient evolutionary marker.

References

Čurin-Šerbec, V., Novak, Dj., Babnik, I., Turk, D., and Gubenšek, F. (1991). Immunological studies of the toxic site ammodytoxin A. *FEBS Lett.*, **280**, 175–8.

Čurin-Šerbec, V., Delot, E., Faure, G., Saliou, B., Gubenšek, F., Bon, C., *et al.* (1994). Antipeptide antibodies directed to the C-terminal part of amrnodytoxin A react with the PLA_2 subunit of crotoxin and neutralize its pharmacological activity. *Toxicon*, **32**, 1237–42.

Gubenšek, F. and Čurin-Šerbec, V. (1994) Immunological approach to the study of the structure–function relationship in neurotoxic phospholipase A_2 from *Vipera ammodytes* venom. In *Supramolecular structure and function* (ed. G. Pifat), pp. 123–32, Balaban and Ordentlich, Rehovoth.

Kordiš, D. and Gubenšek, F. (1995). Horizontal transfer of a SINE between vertebrate classes. *Nature Genetics*, **10**, 131–2.

Kordiš, D. and Gubenšek, F. (1996). Ammodytoxin C gene helps to elucidate the irregular structure of crotalinae group II phospholipase A_2 genes. *Eur. J. Biochem.*, **240**, 83–90.

Križaj, I., Turk, D., Ritonja, A., and Gubenšek, F. (1989). Primary structure of arnmodytoxin C further reveals the toxic site of ammodytoxin. *Biochim. Biophys. Acta*, **999**, 198–202.

Križaj, L., Bieber, A. L., Ritonja, A., and Gubenšek, F. (1991). The primary structure of ammodytin L, a myotoxin, phospholipase A_2 homologue from *Vipera ammodytes* venom. *Eur. J. Biochem.*, **202**, 1165–8.

Križaj, L., Dolly, J. O., and Gubenšek, F. (1994). Identification of the neuronal acceptor in bovine cortex for ammodytoxin C, a presynaptically neurotoxic pbospholipase A_2. *Biochemistry*, **33**, 13919–45.

Križaj, I., Rowan, E. G., and Gubenšek, F. (1995). Ammodytoxin A acceptor in bovine brain synaptic membranes. *Toxicon*, **33**, 437–49.

Lee, C. Y., Tsai, M. C., Chen, Y. M., Ritonja, A., and Gubenšek, F. (1984). Mode of neuromuscular blocking action of toxic phospholipase A_2 from *Vlipera ammodytes* venom. *Arch. Int. Pharmacodyn. Therap.*, **268**, 313–24.

Pedersen, I. Z., Lomonte, B., Massoud, R., Gubenšek, F., Gutierrez, I. M., and Rufini, S. (1995). Autocatalytic acylation of phospholipase-like myotoxins. *Biochemistry*, **34**, 4670–5.

Pungerčar, J., Kordiš., D., Štrukelj, B., Liang, N. S., and Gubenšek, F. (1991). Cloning and nucleotide sequence of a cDNA encoding ammodytoxin A, the most toxic phospholipase A_2 from the venom of long-nosed viper (*Vipera ammodytes*). *Toxicon*, **29**, 269–73.

Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J., and Sigler, P. B. (1985). A comparison of the crystal structure of phospholipase A_2 from bovine pancreas and *Crotalus atrox* venom. *J. Biol. Chem.*, **260**, 11627–34.

Ritonja, A. and Gubenšek, V. (1985), Ammodytoxin A, a highly lethal phospholipase A_2 from *Vipera ammodytes* venom. *Biochim. Biophys. Acta*, **828**, 306–12.

Ritonja, A., Ferlan, I., and Gubenšek, F. (1978). Initial studies of the primary structure of lethal phospholipase A from *Vipera ammodytes* venom. *Period. Biol.*, **80** (Suppl. 1), 37–43.

Ritonja, A., Machleidt, W., Turk, V., and Gubenšek, F. (1986). Amino acid sequence of ammodytoxin B partially reveals the location of the site of toxicity of ammodytoxins. *Biol. Chem. Hoppe-Seyler*, **367**, 919–23.

Rufini, S., Cesaroni, P., Desideri, A, Farias, R., Gubenšek, F., Gutierrez, J. M., *et al.* (1992). Calcium ion independent membrane leakage induced by phospholipase myotoxins. *Biochemistry*, **31**, 12424–50.

Thouin, L. G., Ritonja, A., Gubenšek, F., and Russell, F. E. (1982). Neuromuscular and lethal effects of phospholipase A from *Vipera ammodytes* venom. *Toxicon*, **20**, 1051–8.

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Notexins (*Notechis scutatus scutatus*)

Notexins Np and Ns are two phospholipases A_2 (PLA_2s) isoforms which, in addition to their enzymatic activity, possess both myotoxic and presynaptic neurotoxic activities. The relationship between all these activities as well as the putative target(s) of notexins remain to be identified.

The tiger snake *Notechis scutatus scutatus* is the world's fourth most venomous snake. Its venom contains curaremimetic neurotoxins and several PLA_2s homologues which display various activities. Among the latter, notexins Np and Ns (Karlsson *et al.* 1972; Chwetzoff *et al.* 1990) which are frequently mixed in current preparations called 'notexin', are the most potent homologues. They are basic monomeric enzymatically active PLA_2s which differ from each other by one residue (Halper and Eaker 1975; Chwetzoff *et al.* 1990). As judged from their primary (Halper and Eaker 1975; Chwetzoff *et al.* 1990) (see below) and tertiary (Westerlund *et al.* 1992) structures (Fig. 1), they belong to the PLA_2s group I.

Notexins inhibit the release of acetylcholine, causing paralysis of skeletal muscles (Harris 1991). Injection in mice of notexins (10 μg) causes, in phrenic nerve–diaphragm preparations, a decrease of the frequency but not of the amplitude of miniature end plate potentials (mepp), which is accompanied by a reduction of evoked transmitter release (Harris 1991). This is not due to a lack of depolarization propagation since addition of K^+ does not increase mepp frequency. Notexins also block neurogenic contractions in guinea pig vas deferens preparations by reducing the amplitude of evoked but not of spontaneous excitatory junctional potentials.

Regarding their myotoxic activity, notexins are more potent toward rats and chickens than toward mice. They cause skeletal muscle degeneration but leave the basal lamina intact so that subsequent muscle fibre regeneration can proceed. The mechanisms by which notexins exert their toxic activities are still unknown. Chemical modifications of notexins suggest a dissociation of enzymatic activity and lethal toxicity (Mollier *et al.* 1989; Rosenberg *et al.* 1989; Yang and Chang 1991).

The amino acid sequence of notexin Np (Accession number to the SwissProt databank: PA22_NOTSC) is:

NLVQFSYLIQCANHGKRPTWHYMDYGCYCGAGGSG
TPVDELDRCCKIHDDCYDEAGKKGCFPKMSAYDYYCGENG
PYCRNIKKKCLRFVCDCDVEAAFCFAKAPYNNAN
WNIDTKKRCQ

Notexin Ns has an Arg in position 16 instead of a Lys as in Np.

The venom of *Notechis scutatus scutatus* contains additional PLA_2s that are similar to notexins in terms of structure. These are notechis II-5 (PA23_NOTSC) which is neurotoxic and myotoxic (Harris 1991), notechis 11-2 (PA20_NOTSC) which is only myotoxic (Bouchier *et al.* 1991), and notechis II-1 (PA21_NOTSC) which has neither enzymatic nor toxic activities (Harris 1991).

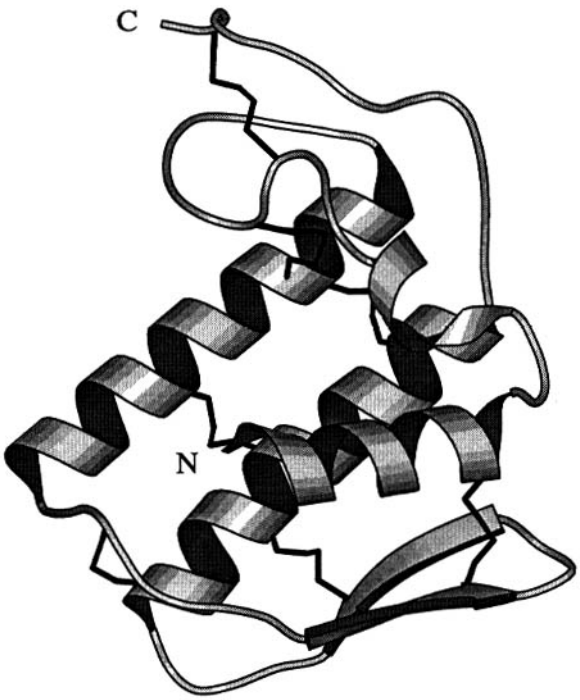

Figure 1. Schematic representation of notexin.

Purification

'Notexin' was first purified by a gel filtration step followed by cation-exchange chromatography (Karlsson *et al.* 1972). An additional RP-HPLC step further resolved 'notexin' in the two isoforms Np and Ns (Chwetzoff *et al.* 1990). They represent 6 per cent of the total protein content of the venom.

Toxicity

Intraveinous injection of notexins induces death by causing respiratory paralysis. LD_{50} values of Np and Ns are equal to 0.34 ± 0.02 μg per 20 ± 2 μg female BALB/c mouse (Chwetzoff *et al.* 1990).

Use in cell biology

Notexins are inducers of muscle regeneration for fundamental studies (Grubb *et al.* 1991) or transplantation of myoblasts (Huard *et al.* 1994; Kinoshita *et al.* 1994). *Notechis scutatus scutatus* venom also increases the yield of proliferating muscle cells from biopsies of normal and distrophic dogs (Dux *et al.* 1993).

References

Bouchier, C., Boyot, P., Tesson, F., Trémeau, O., Bouet, F., Hogdson, D., *et al.* (1991). Notechis 11'2, a non toxic phospholipase A_2 from the venom of *Notechis scutatus scutatus*. *Eur. J. Biochem.*, **202**, 493–500.

Chwetzoff, S., Mollier, P., Bouet, F., Rowan, E. G., Harvey, A. L., and Ménez, A. (1990). On the purification of notexin. *FEBS Lett.*, **261**, 226–30.

Dux, L., Cooper, B. J., Sewry, C. A., and Dubowitz, V. (1993) *Notechis scutatus* venom increases the yield of proliferating muscle cells from biopsies of normal and dystrophic canine muscle. A possible source for myoblast transfer studies. *Neuromuscul. Disord.*, **3**, 23–9.

Grubb, B. D., Harris, J. B., and Schofield, I. S. (1991). Neuromuscular transmission at newly formed neuromuscular junctions in the regenerating *soleus* muscle of the rat. *J. Physiol. Lond.*, **441**, 405–21.

Halper, J. and Eaker, D. (1975). Amino acid sequence of a presynaptic neurotoxin from the venom of *Notechis scutatus* (Australian tiger snake). *J. Biol. Chem.*, **250**, 6990–7.

Harris, J. B. (1991). Phospholipases in snake venoms and their effects on nerve and muscle. In *Snake toxins*, pp. 91–129, Pergamon Press, New York.

Huard, J., Acsadi, G., Jani, A., Massie, B., and Karpati, G. (1994). Gene transfer into skeletal muscles by isogenic myoblasts. *Hum. Gene Ther.*, **5**, 949–58.

Karlsson, E., Eaker, D., and Ryden, L. (1972). Purification of a presynaptic neurotoxin from the venom of the Australian tiger snake *Notechis scutatus scutatus*. *Toxicon*, **10**, 405–13.

Kinoshita, I., Vilquin, J. T., Guerette, B., Asselin, I., Roy, R., and Tremblay, J. P. (1994). Very efficient myoblast allotransplantation in mice under FK506 immunosuppression. *Muscle-Nerve*, **17**, 1407–15.

Mollier, P., Chwetzoff, S., Bouet, F., Harvey, A. L., and Ménez, A. (1989). Tryptophan 110, a residue involved in the toxic activity but not in the enzymatic activity of notexin. *Eur. J. Biochem.*, **185**, 263–70.

Rosenberg, P., Ghassemi, A., Condrea, E., Dhillon, D., and Yang, C-C. (1989). Do chemical modifications dissociate between the enzymatic and pharmacological activities of β-bungarotoxin and notexin? *Toxicon*, **27**, 137–59.

Westerlund, B., Nordlund, P., Uhlin, U., Eaker, D., and Eklund, H. (1992). The three-dimensional structure of notexin, a presynaptic neurotoxic phospholipase A_2 at 2.0 Å resolution. *FEBS Lett.*, **301**, 159–64.

Yang, C.-C. and Chang, L.-S. (1991). Dissociation of lethal toxicity and enzymatic activity of notexin from *Notechis scutatus scutatus* (Australian tiger snake) venom by modification of tyrosine residues. *Biochem. J.*, **280**, 739–44.

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Textilotoxin *(Pseudonaja textilis textilis)*

Textilotoxin is a protein neurotoxin of M_r 70 551 found in the venom of the Australian Eastern or common brown snake, Pseudonaja textilis textilis. *It is the most lethal and structurally the most complex of all known snake venom toxins. Textilotoxin produces a presynaptic blockade of neuromuscular transmission involving a disruption of the regulatory mechanism that controls acetylcholine release.*

Textilotoxin is the main neurotoxin responsible for the high lethality of the venom of *P. textilis textilis*. This elapid snake was the cause of nine snake bite fatalities in Australia in the period 1981 to 1991 (Sutherland 1992). The toxin possesses phospholipase A_2 (PLA_2) activity, like other β-neurotoxins from elapid, crotalid, and viperid snakes. Studies on the mechanism of action using electromyography in the mouse demonstrated a use-dependent rate of development of neuromuscular blockade (Lloyd *et al.* 1991), in which the development of such blockade *in vivo* was accelerated by increasing rates of nerve stimulation. The neuromuscular blockade in the mouse phrenic-hemidiaphragm nerve muscle preparation revealed a triphasic nature, and 'coated omega figures' were present in the presynaptic plasmalemma following incubation with textilotoxin (Nicholson *et al.* 1992). This was held to be an indication

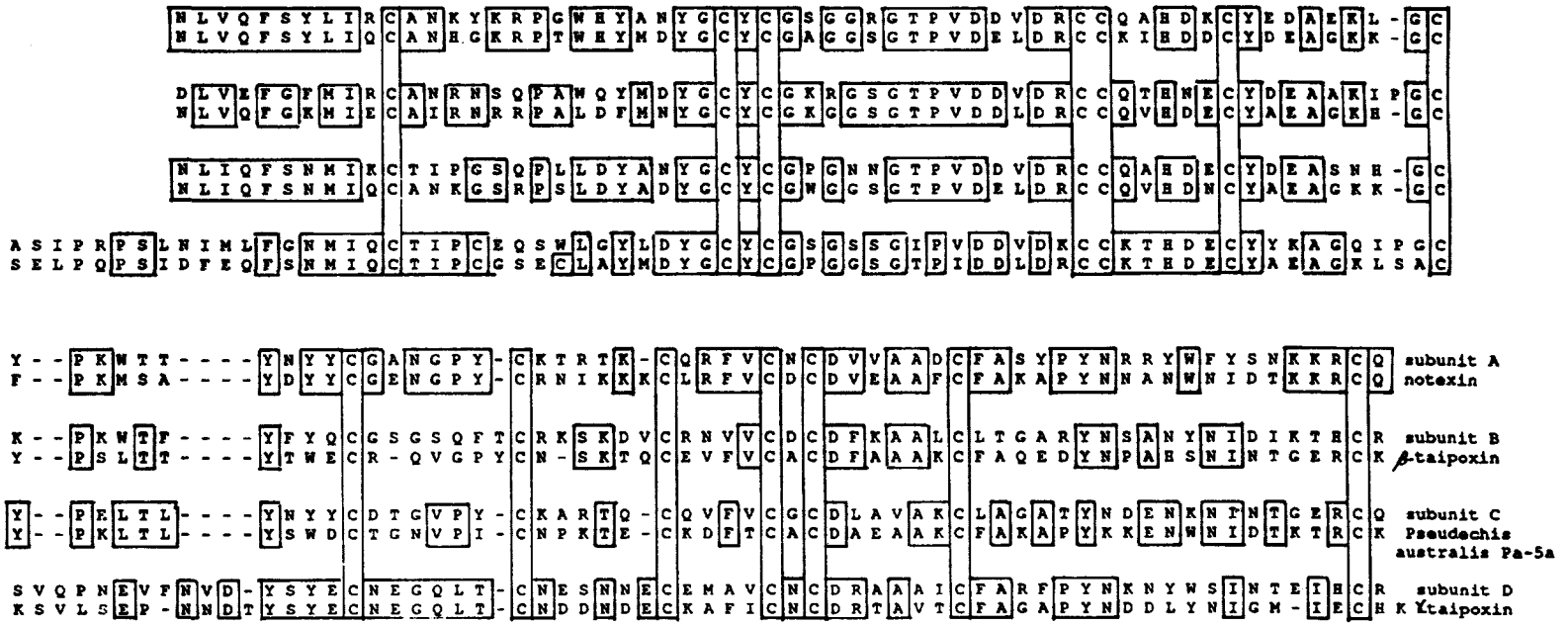

Figure 1. Alignment at the Cys residues of each textilotoxin subunit with the snake venom PLA_2 with which it has considerable homology. Identical residues in each pair of sequences are boxed.

that the toxin prevented acetylcholine release by inhibiting vesicle recycling, in a manner analogous to that proposed for β-neurotoxins (Hamilton *et al.* 1980).

Textilotoxin is composed of 623 amino acid residues arranged in five subunits (subunit A, 118 residues; subunit B, 121 residues; subunit C, 118 residues; subunit D, two identical chains of 133 residues each, which are covalently linked) (Pearson *et al.* 1991*a*; 1993). Subunit A is a basic polypeptide (pI 9.2) and has the highest PLA_2 activity of any of the subunits. However, the PLA_2 activity of textilotoxin itself is much less than that of subunit A (Tyler *et al.* 1987), presumably due to the masking of the PLA_2 active site in A by the other subunits in the toxin complex. All subunits contain the putative PLA_2 active site. The complete amino acid sequences of all of the subunits have been determined (Pearson *et al.* 1993). These show considerable homology with each other and with other snake venom PLA_2 β-neurotoxins, as shown in Fig. 1. Modification of histidine residues in textilotoxin with *p*-bromophenacyl bromide occurred preferentially in subunit A, indicating that A is exposed on the surface of the complex (Tyler *et al.*, unpublished data).

Purification and sources

P. textilis textilis venom may be purchased from Sigma Chemical Company. Textilotoxin was isolated from crude venom by repeated gel filtration chromatography (Tyler *et al.* 1987). The subunits of textilotoxin were readily separated and purified by reverse phase HPLC (Tyler *et al.* 1987; Pearson *et al.* 1993).

Toxicity

The venom of *P. textilis textilis* is lethal to humans (Sutherland 1992) under conditions of snake bite. Textilotoxin is potentially lethal to humans by injection. LD_{50} in mice is 1 μg/kg by intraperitoneal injection (Tyler *et al.* 1987) and 0.6 μg/kg by intravenous injection (Coulter *et al.* 1979). This is the highest lethality of any known snake venom toxin. Subunit A was the only subunit lethal to mice, but at doses 1000-fold greater than for textilotoxin (Pearson *et al.* 1991*b*). Separation of textilotoxin into its subunits by chromatography was reversible, and reformed toxin had the same M_r and lethality in mice as the native toxin (Tyler *et al.* 1987). All subunits were necessary for maximum lethality. Only combinations which contained three or more subunits were lethal to mice, and both A and D were required (Tyler *et al.* 1987). Great care should be exercised to avoid accidental injection by textilotoxin. An antivenom is available from the Commonwealth Serum Laboratories, Melbourne.

Use in cell biology

The actions of textilotoxin at the amphibian neuromuscular junction were confirmed to be presynaptic in nature, leading to a triphasic alteration of acetylcholine release from the terminal, and an eventual complete neuromuscular blockade. An analysis of miniature end-plate potential (MEPP) amplitude showed no change in the post-synaptic sensitivity, but demonstrated clear disruptions in the presynaptic control of MEPP amplitude with the appearance of increasing numbers of large amplitude and 'giant' MEPPs. These features are characteristic of the actions of other β-neurotoxins. These included the triphasic nature of the development of neuromuscular blockade, the irreversible nature of the blockade after a critical binding period, and the many changes in MEPPs release characteristics (Wilson *et al.* 1995). Textilotoxin, with its complex molecular architecture and high potency, has potential for use in investigating the mechanisms of presynaptic neuromuscular blockade, including the relationship between PLA_2 activity and toxicity. Its subunits may act as protective chaperones, assisting in transporting subunit A to the site of activity at the nerve synapses and, thus, enabling more efficient blocking of the release of acetylcholine than happens with a single chain PLA_2 toxin (Pearson *et al.* 1993).

References

Coulter, A. R., Broad, A. J., and Sutherland, S. K. (1979). Isolation and properties of a high molecular weight neurotoxin from the Eastern brown snake (*Pseudonaja textilis*). In *Neurotoxins, fundamental and clinical advances* (ed. I. Chubb and L. Geffen), p. 260, Adelaide University Union Press, Adelaide.

Hamilton, R. C., Broad, A. J., and Sutherland, S. K. (1980). Effects of Australian Eastern brown snake (*Pseudonaja textilis*) venom on the ultrastructure of nerve terminals on the rat diaphragm. *Neurosci. Lett.*, **19**, 45–50.

Lloyd, D. R., Nicholson, G. M., Spence, I., Connor, M., Tyler, M. I., and Howden, M. E. H. (1991). Frequency-dependent neuromuscular blockade by textilotoxin *in vivo*. *Toxicon*, **29**, 1266–9.

Nicholson, G. M., Spence, I., Lloyd, D. R., Wilson, H. I., Harrison, B. M., Tyler, M. I., *et al.* (1992). Presynaptic actions of textilotoxin, a neurotoxin from the Australian common brown snake (*Pseudonaja textilis*). In *Recent advances in toxinology research* (Proceedings of 10th World Congress on Animal, Plant and Microbial Toxins) (ed. P. Gopalakrishnakone and C. K. Tan), pp. 540–8, National University of Singapore Press, Singapore.

Pearson, J. A., Tyler, M. I., Retson, K. V., and Howden, M. E. H. (1991*a*). Studies on the subunit structure of textilotoxin, a potent presynaptic neurotoxin from the venom of the Australian common brown snake (*Pseudonaja textilis*). 2. The amino acid sequence and toxicity studies of subunit D. *Biochim. Biophys. Acta*, **1077**, 147–50.

Pearson, J. A., Tyler, M. I., and Howden, M. E. H. (1991*b*). Immunological relationships between the subunits of textilotoxin and rabbit antisera raised against textilotoxin and some snake venoms. *Toxicon*, **29**, 375–8.

Pearson, J. A., Tyler, M. I., Retson, K. V., and Howden, M. E. H. (1993). Studies on the subunit structure of textilotoxin, a potent presynaptic neurotoxin from the venom of the Australian common brown snake (*Psuedonaja textilis*). 3. The complete amino-acid sequences of all the subunits. *Biochim. Biophys. Acta*, **1161**, 223–9.

Sutherland, S. K. (1992). Deaths from snake bite in Australia, 1981–1991. *Med. J. Aust.*, **157**, 740–6.

Tyler, M. I., Barnett, D., Nicholson, P., Spence, I., and Howden, M. E. H. (1987). Studies on the subunit structure of

textilotoxin, a potent neurotoxin from the venom of the Australian common brown snake (*Pseudonaja textilis*). *Biochim. Biophys. Acta*, **915**, 210–16.
Tyler, M. I., Pearson, J. A., Retson, K. V., Comis, A., and Howden, M. E. H., unpublished data.
Wilson, H. I., Nicholson, G. M., Tyler, M. I., and Howden, M. E. H. (1995). Induction of giant miniature end-plate potentials during blockade of neuromuscular transmission by textilotoxin. *Naunyn-Scheideberg's Arch. Pharmacol.*, **352**, 79–87.

■ *J. A. Pearson, D. Barnett, A. Comis, M. Connor, B. M. Harrison, D. R. Lloyd, G. M. Nicholson, P. Nicholson, K. V. Retson, I. Spence, M. I. Tyler, H. I. Wilson, and M. E. H. Howden:*
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Taipoxin

Taipoxin, isolated from the crude venom of the Australian taipan Oxyuranus scutellatus scutellatus, *is a complex of three subunits each of which is an homologue of mammalian pancreatic phospholipase A_2. The complex consists of a 1:1:1 molar ratio of the sub-units. It has hydrolytic activity, it blocks the release of transmitter from the nerve terminals of the motor nerve of skeletal muscle, and it is a potent myotoxin.*

Taipoxin is a presynaptically active, myotoxic phospholipase A_2. It is a complex of three polypeptide subunits noncovalently assembled in a 1:1:1 molar ratio. The subunits can be dissociated in media of high ionic strength and low pH or in 6 M guanidine at neutral pH. Dissociation in guanidine is readily reversible. The three subunits are designated, respectively, α, β, and γ (Fohlman *et al.* 1976, 1979).

The α-subunit, is strongly basic (pI ~ 10), consists of 120 amino acid residues, and is homologous with mammalian pancreatic phospholipase A_2. It is crosslinked by seven disulfide bridges. It has phospholipase A_2 activity, and is myotoxic and neurotoxic (LD_{50} ~ 300 μg kg^{-1} i.p. mouse).

The β-subunit is neutral, and also consists of 120 amino acid residues crosslinked with seven disulfide bridges. It is neither hydrolytic nor toxic. Two isoforms of the β-subunit are known, differing slightly in primary composition.

The γ-subunit is acidic (pI ~ 2.5) and consists of 135 amino acid residues crosslinked by eight disulfide bridges. It also contains a large carbohydrate moiety with four sialic acid residues. The γ-subunit is hydrolytic but nontoxic.

The complex of three subunits is much more toxic than its component parts (LD_{50} of the complex ~ 2 μg kg^{-1} i.p. mouse), an enhancement probably due to the interaction (as yet undefined in molecular terms) between the α and β subunits (see Fig. 1 for example).

The toxicity of taipoxin derives primarily from its ability to block neuromuscular transmission at the neuromuscular junction by inhibiting transmitter release from the nerve terminals (Kamenskaya and Thesleff 1974). There may be several phases to this primary neurotoxicity – initial inhibition of transmitter release, followed by temporary enhancement, and then progressive failure – but the precise pattern of events varies with the nature of the neuromuscular preparation and the species from which the neuromuscular preparation is derived (Harris 1991; Hawgood and Bon 1991). Hydrolytic activity is an essential requirement for the expression of toxicity, but its precise role is not yet known. Taipoxin is also a potent myotoxin, destroying mammalian skeletal muscle rapidly

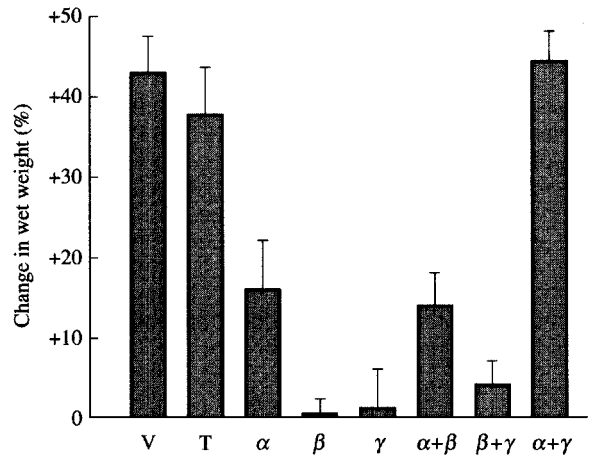

Figure 1. Oedema, leading to an increase in wet weight, is characteristically associated with muscle damage caused by the toxic phospholipases A_2. This figure illustrates the change in wet weight in the solcus muscles of rats inoculated with the venom (V) of the taipan or with taipoxin (T; dose equivalent to that found in the dose of venom used), or with individual doses or combinations of the subunits α-, β-, and γ-taipoxin. Each point is the mean ± s.e.m. of six experiments (Harris and Maltin, unpublished).

and completely (Harris and Maltin 1982). In terms of molecular structure taipoxin is rather more complex than most other toxic phospholipases A_2 derived from snake venoms, but toxins composed of multiple (i.e. > 2) homologous subunits have been identified in a few other Australian elapids (Su *et al.* 1983). Such toxins appear to be unique to the Australian elapids, since none have been found in the venoms of either Asian or African elapids or in viperid or crotalid venoms.

There is good evidence from biochemical and immunological studies on structurally related toxic phospholipases A_2 that distinct regions on these toxins are responsible for hydrolytic, neurotoxic, and myotoxic activity respectively, but they have not yet been identified (see Tzeng (1993) and Rosenberg (1986) for detailed discussions of this topic).

The molecular mechanism of action of the toxin, with respect to both its neurotoxicity and its myotoxicity is not known. Work on related toxins suggest that neurotoxicity involves a number of events, not necessarily directly related, including changes in membrane fluidity resulting from hydrolysis of membrane lipids and the release of lysophosphatides and free fatty acids; loss of ionic homeostasis; inhibition of a slowly activating K^+ channel in the nerve terminal; inhibition of transmitter synthesis; inhibition of synaptic vesicle recycling and filling; destruction of synaptic vesicles (see Harris and Maltin 1982; Harris 1991). It has been suggested on numerous occasions that taipoxin and related toxins are internalized at the motor nerve terminal, but there is, as yet, no definitive evidence in favour of the suggestion.

Myotoxicity probably results from the direct disruption of the plasma membrane (Dixon and Harris 1995).

Purification and sources

Taipoxin was originally isolated from the crude venom of *Oxyuranus scutellatus scutellatus* by gel filtration of a solution of 0.1 g ml^{-1} in 0.1 M sodium acetate on Sephadex G-75 equilibrated with 0.1 M ammonium acetate. Fractions were eluted, screened for toxicity, and lyophilized twice. Further purification utilized column zone electrophoresis (Fohlman *et al.* 1976, 1979). The toxin is not widely available, but can be obtained in limited quantity from Venom Supplies, P.O. Box 547, Tanunda, South Australia 4352.

Toxicity

Toxicity is typically defined by the LD_{50}, defined as the dose causing 50 per cent mortality in mice 24 h after intraperitoneal injection. The LD_{50} should ideally be calculated using the techniques defined by the WHO to ensure statistical accuracy and comparison between sets of data (WHO 1981). The toxic phospholipases A_2 should he handled with great care. The eyes are particularly sensitive and any contact of toxin with the eyes requires immediate medical care. Workers should note that small polypeptides are potent antigens.

Use in cell biology

The cellular receptor on neuronal and muscle cells is not yet known. Neurotoxicity may require the uptake and internalization of the toxin, but the possible mechanisms of uptake remain unknown. Less complex toxic phospholipases A_2 (e.g. notexin, crotoxin) tend to be used to study synaptic function. Taipoxin has been used to study some aspects of muscle degeneration and regeneration (Maltin *et al.* 1983).

References

Dixon, R. and Harris, J. B. (1995). The early expression of mytoxicity and localization of the binding sites of notexin in the soleus muscle of the rat. In *Advances in experimental biology and medicine* Vol. 391 (ed. B. R. Singh and A. L. Tu), pp. 233–30, Plenum Press, New York.

Fohlman J., Eaker, D., Karlsson, E., and Thesleff, S. (1976). Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the Australian snake taipan (*Oxyuranus s. scutellatus*). Isolation, characterization, quaternary structure and pharmacological properties. *Eur. J. Biochem.*, **681**, 457–69.

Fohlman, J., Eaker, D., Dowdall, M. J., Lullman-Rauch, R., Sjodin, I., and Leander, S. (1979). Chemical modification of taipoxin and the consequences for phospholipase activity, pathophysiology, and inhibition of high-affinity choline uptake. *Eur. J. Biochem.*, **94**, 531–40.

Harris, J. B. (1991). Phospholipases in snake venoms and their effects on nerve and muscle. In *Snake toxins* (ed. A. L. Harvey), pp. 91–124, Pergamon, Oxford.

Harris, J. B. and Maltin, C. A. (1982). Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, *Oxyuranus scutellatus. Br. J. Pharmacol.*, **76**, 61–75.

Hawgood, B. and Bon, C. (1991). Snake venom presynaptic toxins. In *Handbook of natural toxins*, Vol. 5 (ed. A. T. Tu), pp. 3–52, Marcel Dekker, New York.

Kamenskaya, M. A. and Thesleff, S. (1974). The neuromuscular blocking action of an isolated toxin from the *Oxyuranus scutellatus. Acta Physiol. Scand.*, **90**, 716–24.

Maltin, C., Harris, J. B., and Cullen, M. J. (1983). Regeneration of mammalian skeletal muscle following the injection of the snake venom toxin, taipoxin. *Cell Tiss. Res.*, **232**, 565–77.

Rosenberg, P. (1986). The relationship between enzymatic activity and pharmacological properties of phospholipases in natural poisons. In *Natural toxins* (ed. J. B. Harris), pp. 129–74, Oxford University Press, Oxford.

Su, M. J., Coulter, A. R., Sutherland, S. K., and Chang, C. C. (1983). The presynaptic neuromuscular blocking effect and phospholipase A_2 activity of texilotoxin, a potent toxin isolated from the venom of the Australian brown snake. *Toxicon*, **21**, 143–51.

Tzeng, M. C. (1993). Interaction of presynaptically active toxic phospholipases A_2 with membrane receptors and other binding sites. *J. Toxicol.–Toxin Rev.*, **12**, 1–62.

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α-Latrotoxin (black widow spider)

α-Latrotoxin (α-LTx), the major high M_r *protein toxin secreted by the salivary glands of* Latrodectus *spiders (the black widows), binds to membrane protein receptors specifically expressed at all vertebrate synaptic terminals, and triggers massive quantal neurotransmitter release up to exhaustion of synaptic vesicles.*

α-LTx is a single chain, slightly acidic (pI 5.2–5.5), non-glycosylated protein composed of 1401 amino acids (Kiyatkin *et al.* 1990). The structure includes an N-terminal region of ~500 amino acids free of repeats, whereas the remaining region is of high intrinsic homology with short repeats occurring up to 19 times. α-LTx is homologous to α-latroinsectotoxin, and not to other proteins, including Ca^{2+} channels of any type (GenBank accession number: X55009). The site of receptor binding has not been identified. α-LTx causes massive stimulation of transmitter release from all investigated neurons (Rosenthal and Meldolesi 1989). Other cell types are insensitive to the toxin (Scheer and Meldolesi 1985; Rosenthal and Meldolesi 1989) with the exception of nerve cell lines (e.g. PC12). Sensitivity is due to expression of neuron-specific receptors localized in the presynaptic membrane of central and peripheral synapses (Malgaroli *et al.* 1989). The receptors included high M_r (160–200 kDa) glycopeptides which have been identified as neurexin Ia (Petrenko 1993; Petrenko *et al.* 1993), i.e. the members of a large, neuron-specific protein family (63 forms due to alternative splicing), characterized by a voluminous extracellular domain, a single membrane spanning region, and a short (40 amino acid) cytoplasmic sequence (Ushkaryov *et al.* 1992; Ullrich *et al.* 1995). Neurexin, however, cannot be the only receptor because its α-LTx binding is strictly Ca^{2+} dependent (Davietov *et al.* 1995), whereas considerable binding (Meldolesi *et al.* 1983) and important effects of the toxin (see below) take place in Ca^{2+}-free media. These events appear to be due to another, recently identified receptor named synaptophilin (Davletov *et al.* 1996).

Purification and sources

α-LTx has been isolated from the venom or, most often, from salivary gland homogenates of the European spider, *Latrodectus mactans tredecimguttatus*, collected in the wild, as breeding has not been successful so far. Decades ago these spiders were widespread, whereas now they are found only in restricted areas (Sardinian islands as well as areas in Bosnia, Israel desert, and Uzbekistan among others). Whether and to what extent the presynaptic stimulatory neurotoxins produced by other *Latrodectus* subspecies (*L. mactans mactans*, present in the USA, and *L. geometricus*, which is cosmotropic) differ from α-LTx is still unclear. A high degree of purification of α-LTx from other *L. tredecimguttatus* venom components (some of which are also toxins, see in this volume the entry on α-latroinsectotoxin, p. 235) can be obtained by simple procedures of column chromatography (Frontali *et al.* 1976) or, more quickly, by HPLC (Grebinozhko and Nikolaenko 1987). Electrophoresis has revealed coexistence in purified preparations of a small (70 amino acids) protein homologous to hyperglycemic hormones, which, however, has no role in neurotoxicity (Gasparini *et al.* 1994). Until recently no commercial source of α-LTx was available, whereas now the toxin can be purchased from Alomone, Jerusalem. When kept at –80 °C (neutral pH) α-LTx is stable indefinitely.

Toxicity

Data are available only for the crude homogenate of venom glands. For *L. tredecimguttatus* the LD_{50} is 0.15 μg protein/kg body weight (mouse) (Hurlburt and Ceccarelli 1979). The clinical picture (lactrodectism) is characterized by acute pain; muscle twitches, fibrillations, cramps, and even tetanus; anxiety, mental excitation, sweating, oliguria, sinus bradycardia, and hypertension (Maretic 1983). The hypertension and arrhythmias, caused by massive release of catecholamines (both adrenaline and noradrenaline) from ganglia and adrenal glands, may evolve into collapse and pulmonary oedema.

Use in cell biology

The mechanisms of action of α-LTx are not completely understood, yet this toxin represents a valuable tool for cell biologists interested in gaining information on synaptic quantal release. α-LTx causes massive release of classical neurotransmitters (Hurlbut and Ceccarelli 1979), whereas release of peptides, contained within large dense core vesicles, seems to be less affected (Matteoli *et al.* 1988). Among the synaptic mechanisms underlying these effects the following have been demonstrated:

(1) stimulation of synaptic vesicle exocytosis, even in the absence of external Ca^{2+} (Hurlbut *et al.* 1990);

(2) presynaptic Ca^{2+} rise (Nicholls *et al.* 1982);

(3) plasma membrane depolarization (Nicholls *et al.* 1982);

(4) impairment of synaptic vesicle retrieval, better revealed in the absence of external Ca^{2+}.

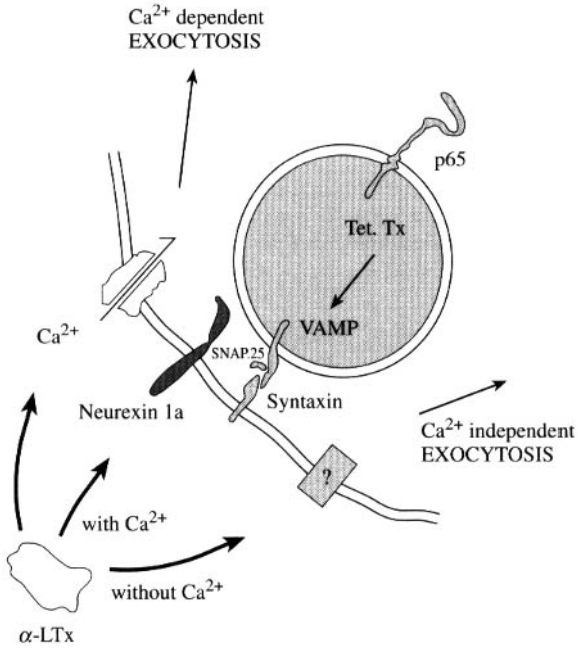

Figure 1. Schematic view of the interaction of α-LTx with its receptors located at the presynaptic active region. Neurexin 1a has been recognized as the receptor component responsible for Ca^{2+}-dependent binding of the α-LTx. The receptor complex however includes an additional, component, synaptophilin (?), that may account for Ca^{2+}-independent α-LTx binding and the ensuing initiation of transmitter release. Other presynaptic membrane proteins which are supposed to interact, directly or indirectly, with the α-LTx receptors have also been included in this scheme: synaptotagmin (P65), synaptobrevin (VAMP), syntaxin, and SNAP-25.

The combination of increased exocytosis with block of retrieval leads to synaptic vesicle depletion (Ceccarelli and Hurlbut 1980). The Ca^{2+}-independent exocytosis triggered by α-LTx points towards an action downstream Ca^{2+} entry, at one of the late post-docking stages of secretion, which, however, remains to be identified (see Fig. 1). The latter view is strengthened by the finding that tetanus toxin, an agent known to cleave the vSNARE protein, synaptobrevin (otherwise referred to as VAMP, see entry p. 100 is known to suppress α-LTx-induced transmitter release (Dreyer *et al.* 1987).

References

Ceccarelli, B. and Hurlbut, W. P. (1980). Ca^{2+} dependent recycling of synaptic vesicles at the frog neuromuscular junction. *J. Cell. Biol.*, **87**, 297–303.

Davletov, B. A., Krasnoperov, V., Petrenko, A. G., and Sudhof, T. C. (1995). High affinity binding of α-latrotoxin to recombinant neurexin 1-α. *J. Biol. Chem.*, **270**, 23903–5.

Davletov, B. A., Shamotienko, O. G., Lelianova, V. G., Grishin, E. V., and Ushkaryov, V. A. (1996). Isolation and biochemical characterization of a Ca^{2+}-independent α-latratoxin-binding protein. *J. Biol. Chem.*, **271**, 23239–45.

Dreyer, F., Rosenberg, F., Becker, C., Bigalke, H., and Penner, R. (1987). Differential effects of various secretagogues on quantal transmitter release from mouse motor nerve terminals treated with botulinum A and tetanus toxin. *Arch. Pharmacol.*, **335**, 1–7.

Frontali, N., Ceccarelli, B., Gorio, A., Mauro, A., Siekevitz, P., Tzeng, M. C., *et al.* (1976). Purification from the black widow spider venom of a protein factor causing the depletion of synaptic vesicles at neuromuscular junction. *J. Cell. Biol.*, **68**, 462–79.

Gasparini, S., Kiyatkin, N., Drevet, P., Boulain, J., Tacnet, F., Ripoche, P., *et al.* (1994). The low molecular weight protein which co-purifies with α-latrotoxin is structurally related to crustacean hyperglicemic hormones. *J. Biol. Chem.*, **269**, 19803–9.

Grebinozhko, E. L. and Nikolaenko, A. N. (1987). Isolation of α-latrotoxin using HPLC chromatography. *Ukrainskii Biok. Zhurnal*, **59**, 93–7.

Hurlbut, W. P. and Ceccarelli, B. (1979). Use of black widow spider venom to study the release of neurotransmitters. *Adv. Cytopharmacol.*, **3**, 87–115.

Hurlbut, W. P., Iezzi, N., Fesce, R., and Ceccarelli, B. (1990). Correlation between quantal secretion and vesicle loss at the frog neuromuscular junction. *J. Physiol. London*, **425**, 501–26.

Kiyatkin, N. I., Dulubova, I. A., Chekhovskaya, I. N., and Grishin E. V. (1990). Cloning and structure of cDNA encoding α-latrotoxin from black vidow spider venom. *FEBS Lett.*, **270**, 127–31.

Malgaroli, A., DeCamilli, P., and Meldolesi, J. (1989). Distribution of α-latrotoxin receptor in the rat brain by quantitative autoradiography: comparison with the nerve terminal protein, synapsin I. *Neuroscience*, **32**, 393–404.

Maretic, Z. (1983). Latrodectism: variations in clinical manifestations provoked by latrodectus species of spiders. *Toxicon*, **21**, 457–66.

Matteoli, M., Haimann, C., Torri-Tarelli, F., Polak, J. M., Ceccarelli, B., and DeCamilli, P. (1988). Differential effects of α-latrotoxin on exocytosis from small synaptic vesicles and large dense-core vesicles containing calcitonin gene-related peptide at the frog neuromuscular junction. *Proc. Natl. Acad. Sci. USA*, **85**, 7366–70.

Meldolesi, J., Madeddu, L., Torda, M., Gatti, G., and Niutta, E. (1983). The effect of α-latrotoxin on the neurosecretory PC12 cell line: studies on toxin binding and stimulation of transmitter release. *Neuroscience*, **3**, 997–1009.

Nicholls, D. G., Rugolo, M., Scott, I. G., and Meldolesi, J. (1982). α-Latrotoxin of black widow spider venom depolarizes the plasma membrane, induces massive Ca^{2+} influx and stimulates transmitter release in guinea pig brain synaptosomes. *Proc. Natl. Acad. Sci. USA*, **79**, 7924–8.

Petrenko, A. G. (1993). α-Latrotoxin receptor. Implications in nerve terminal function. *FEBS Lett.*, **325**, 81–5.

Petrenko, A. G., Lazaryeva, V. D., Geppert, M., Tarasyuk, T. A., Moomaw, C., Khokhlatchev, A. V., *et al.* (1993). Polypeptide composition of the α-latrotoxin receptor. *J. Biol. Chem.*, **268**, 1860–7.

Rosenthal, L. and Meldolesi, J. (1989). Alfpha-latrotoxin and related toxins. *Pharmac. Ther.*, **42**, 115–34.

Scheer, H. and Meldolesi, J. (1985). Purification of the putative α-latrotoxin receptor from bovine synaptosomal membranes in an active binding form. *EMBO J.*, **4**, 323–7.

Ullrich, B., Ushkaryov, Y. A., and Sudhof, T. C. (1995). Cartography of neurexins: more than 1000 isoforms generated by alternative splicing and expressed in distinct subsets of neurons. *Neuron*, **14**, 497–507.

Ushkaryov, Y. A., Petrenko, A. G., Geppert, M., and Sudhof, T. C. (1992). Neurexins: synaptic cell surface proteins related to the α-LTx receptor and laminin. *Science*, **257**, 50–6.

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α-Latroinsectotoxin *(Latrodectus mactans tredecimguttatus)*

α-Latroinsectotoxin is a high molecular weight (~120 kDa) protein purified from black widow spider venom and is a major insect-specific neurotoxin of the venom. It binds with high affinity to presynaptic receptor of insect nerve endings and causes massive and exhausting release of neurotransmitters resulting in blockade of synapse transmission.

α-Latroinsectotoxin (α-LIT) is a most potent and abundant IS-specific neurotoxin found in black widow spider venom (Krasnoperov *et al.* 1992). It is synthesized as a precursor (1411 amino acid residues, GenBank EMBL Z14086) and processing is required both in N- and C-termini to produce mature toxin (Kiyatkin *et al.* 1993). α-LIT shares similar protein organization with vertebrate-specific α-latrotoxin (Kiyatkin *et al.* 1990) and δ-latroinsectotoxin (Dulubova *et al.* 1996). As presented in Fig. 1, the N-terminal part of α-LIT contains two predicted membrane-spanning segments which are conserved among other studied latrotoxins. A most striking feature of α-LIT is an abundance of Ank repeats (Michaely and Bennett 1992) that entirely make up the central part of the toxin molecule. The C-terminal part of the repeated domain contains unusual clustering of seven cysteine residues, and is found to be the most divergent region in latrotoxins (Grishin 1994). The C-terminal domain of α-LIT precursor, which is cleaved during maturation of the toxin, contains multiple copies of microbodies targeting signal (Dehoop and Ab 1992) and is likely to be responsible for the protein transport.

At nanomolar concentrations α-LIT causes a substantial increase in frequency of spontaneous quantal transmitter release from the motor nerve endings of blowfly (Magazanik *et al.* 1992). Radioactively labelled α-LIT binds almost irreversibly ($K_d \simeq 0.38$ nM) to insect membrane preparations. α-LIT also forms cation-selective channels in artificial lipid bilayers (Shatursky *et al.* 1995). These effects strongly resemble those of α-latrotoxin. Thus, α-LIT can be considered an insect-specific analogue of α-latrotoxin.

■ Purification and sources

α-LIT is purified to homogeneity from total venom or venom glands of black widow spider by three rounds of

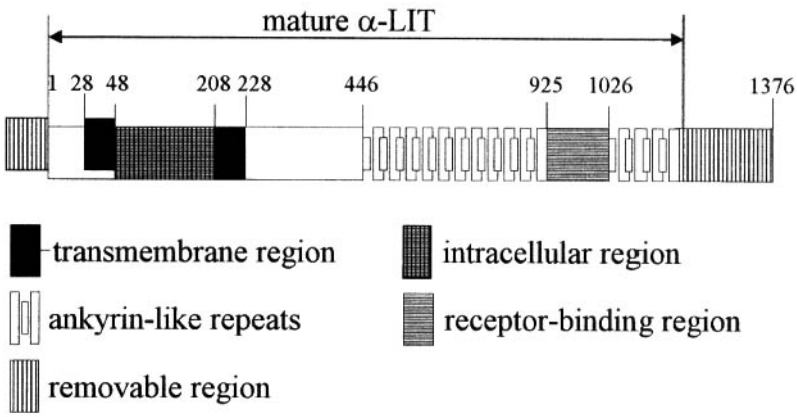

Figure 1. Schematic representation of the α-LIT molecule.

column chromatography (Kovalevskaia *et al.* 1990; Krasnoperov *et al.* 1990). α-LIT can be purchased from Latoxan. Commercial preparations of the toxin should be tested for toxicity towards insects and for purity by SDS-PAGE.

Toxicity

Toxicity towards insects can be measured by injection of varying doses of the toxin into perinotum of different species larvae. *Galleria mellonella* larvae LD_{50} is 15 μg/kg (Krasnoperov *et al.* 1990). α-LIT is shown to be toxic to different insect species, e.g. *Musca domestica* larvae and *Periplaneta americana*. The toxin is not active after intraperitonal injections in mice even at 5 mg/kg. High insectospecificity of α-LIT suggests that work with the toxin is safe although reasonable precautions are required because of allergenic properties of latrotoxins.

Use in cell biology

The presynaptic receptor of α-LIT is not identified. Studies of the α-latrotoxin receptor reveal the functional importance of a new class of synapse-specific proteins, the neurexins (Petrenko, 1993). Characterization of the α-LIT receptor, which is likely to be an insect analogue of neurexins, will provide a useful model to study their role in maintenance and development of the nervous system. Of great interest is the nature of the tight and specific coupling of α-LIT to its receptor. This interaction can be mediated by Ank repeats that form a prominent motif of protein recognition. α-LIT can also be used as a selective marker of the active zone in insect presynaptic membranes.

References

Dehoop, M. J. and Ab, G. (1992). Import of proteins into peroxisomes and other microbodies. *Biochem. J.*, **286**, 657–69.

Dulubova, I. E., Krasnoperov, V. G., Khvotchev, M. V., Pluzhnikov, K. A., Volkova, T. M., Grishin, E. V., Vais, H., Bell, D. R., and Usherwood, P. N. R. (1996). Cloning and structure of δ-latroinsectotoxin, a novel insect-specific member of the latrotoxin family. Functional expression requires C-terminal truncation. *J. Biol. Chem.* **271**, 7535–43.

Grishin, E. (1994). Spider neurotoxins and their neuronal receptors. *Pure Appl. Chem.*, **66**, 783–90.

Kiyatkin, N., Dulubova, I. E., Chekhovskaya, I. A., and Grishin, E. V. (1990). Cloning and structure of cDNA encoding alpha-latrotoxin from black widow spider venom. *FEBS Lett.*, **270**, 127–31.

Kiyatkin, N., Dulubova, I., and Grishin, E. (1993). Cloning and structural analysis of alpha-latroinsectotoxin cDNA. Abundance of ankyrin-like repeats. *Eur. J. Biochem.*, **213**, 121–7.

Kovalevskaia, G. I., Pashkov, V. N., Bulgakov, O. V., Fedorova, I. M., and Magazanik, L. G. (1990). Identification and isolation of the protein insect toxin (alpha-latroinsectotoxin) from venom of the spider *Latrodectus mactans tredecimguttatus*. *Bioorgan. Khimiya*, **16**, 1013–18.

Krasnoperov, V. G., Shamotienko, O. G., and Grishin, E. V. (1990). Isolation and properties of insect-specific neurotoxins from venoms of the spider *Lactodectus mactans tredecimguttatus*. *Bioorgan. Khimiya*, **16**, 1138–40.

Krasnoperov, V. G., Shamotienko, O. G., and Grishin, E. V. (1992). Isolation and properties of insect and crustacean-specific neurotoxins from the venom of the black widow spider (*Latrodectus mactans tredecimguttatus*). *J. Natural Toxins*, **1**, 17–23.

Magazanik, L. G., Fedorova, I. M., Kovalevskaya, G. I., Pashkov, V. N., and Bulgakov, O. V. (1992). Selective presynaptic insectotoxin (alpha-latroinsectotoxin) isolated from black widow spider venom. *Neuroscience*, **46**, 181–8.

Michaely, P. and Bennett, V. (1992). The ANK repeat: a ubiquitous motif involved in macromolecular recognition. *Trends Cell Biol.*, **2**, 127–9.

Petrenko, A. G. (1993). alpha-Latrotoxin receptor. Implications in nerve terminal function. *FEBS Lett.*, **325**, 81–5.

Shatursky, O. Y., Pashkov, V. N., Bulgakov, O. V., and Grishin, E. V. (1995). Interaction of alpha-latroinsectotoxin from *Latrodectus mactans* venom with bilayer lipid membranes. *Biochim. Biophys. Acta–Biomembranes*, **1233**, 14–20.

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Pardaxin (*Pardachirus marmoratus*)

Pardaxin, *a toxin isolated from the gland's secretion of the red sea flatfish* Pardachirus marmoratus, *is used as a pharmacological tool to investigate mechanisms of neurotransmitter release. In different neuronal preparations pardaxin induced massive release of neurotransmitters by both calcium-dependent and calcium-independent signal transduction pathways. Pardaxin forms voltage-dependent pores which are involved in pardaxin-induced elevations in intracellular calcium and calcium-dependent exocytosis. Calcium-independent, pardaxin-induced neurotransmitter release does not involve intracellular calcium stores, protein kinase C activation, or pertussis-toxin sensitive G proteins but is attributed to certain, not yet identified arachidonic acid and/or lipooxygenase(s) metabolite(s). This specific and complex mechanism of action makes pardaxin an attractive ionophore tool in cell biology for the investigation of the fine regulation of neurotransmitter release, in particular, and exocytosis, in general.*

Pardaxin is an acidic, amphipathic, and hydrophobic polypeptide, of a 3500 daltons molecular weight, composed of 33 common amino acids (Fig. 1). The toxin is secreted together with aminoglycosteroides (Lazarovici *et al.* 1986) into the sea water causing toxicity and/or repellency of marine organisms (Lazarovici *et al.* 1990). Pardaxin has a neurotoxic excitatory action on neurons expressed in a massive exocytosis of a variety of neurotransmitters: acetylcholine at the neuromuscular junction (Renner *et al.* 1987) and *Torpedo* electric organ synaptosomes (Arribas *et al.* 1993); 5-HT and norepinephrine from rat cortical brain slices (Wang and Friedman 1986); dopamine from chromaffin cells (Lazarovici and Lelkes 1992) and PC12 cells (Bloch-Shilderman *et al.* 1995); glutamate from rat brain slices (Lazarovici, unpublished data) etc. This excitatory effect might explain pardaxin toxicity to marine organisms and provide pardaxin as a new pharmacological tool to study the process of neurotransmitter exocytosis. Pardaxin has been sequenced and synthesized (Loew *et al.* 1985) and shown to be biologically active, similar to the native toxin (Shai *et al.* 1988). In lyposomes and planar bilayers, pardaxin formed voltage-dependent pores (Loew *et al.* 1985; Lazarovici *et al.* 1988). These pores behave as hydrated pores for permeant cations and show only a modest selectivity for charge (Shi *et al.* 1995). Modelling of pardaxin pores suggests they are composed of a cylinder of eight parallel monomers in which eight stranded β-barrels are surrounded by eight amphipathic α-helices (Fig. 1 and Lazarovici *et al.* (1992). Charge considerations predict that this cylinder will be inserted into the membrane only if the potential on the exoplasmic face of the membrane is positive (Lazarovici *et al.* 1992).

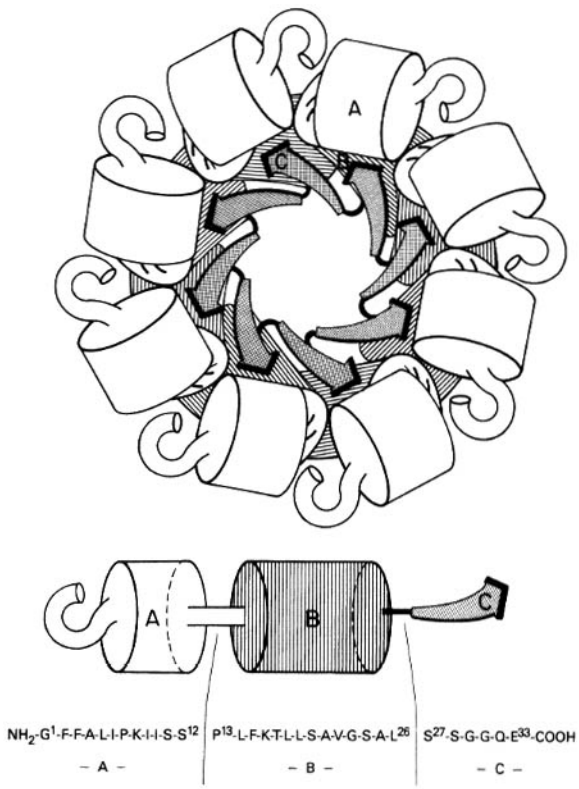

Figure 1. A schematic representation of pardaxin pore based on computer graphic models (Shi *et al.* 1995). Eight parallel pardaxin monomers form the channel (top). The primary and secondary structure of an individual pardaxin monomer is presented (bottom). A, α-helix hydrophilic residues organization; B, amphipathic residues α-helix; C, β-barrel organization.

Purification and sources

Pardaxin is isolated from the fish secretion by several successive steps of liquid chromatography including: gel permeation on Sephadex G-25, ion-exchange on QAE-Sephadex, chromatofocusing on PBE-94 gel, and reverse

phase high pressure liquid chromatography on Spherisorb ODSz columns (Lazarovici *et al.* 1986). During this procedure an additional pardaxin isoform is obtained. The major concern of this purification protocol is the complete separation between pardaxin polypeptides and interacting sterols (Lazarovici *et al.* 1986). The pure toxin is thermolabile and self aggregates in aqueous buffers. Pardaxin is stored as a lyophilized powder. The toxin is not commercially available and is provided upon request from the author or Prof. E. Zlotkin (The Institute of Life Sciences, Hebrew University, Jerusalem, Israel), Prof. Tachibana (Department of Chemistry, University of Tokyo, Japan), or Dr I. Shai (Department of Membrane Research, Weizmann Institute of Science, Rehovot, Israel).

Toxicity

Pardaxin is toxic to marine organisms (10^{-6}–10^{-8} M) but not toxic (up to 10 mg/kg body weight) to mice or rats (Primor and Lazarovici 1981) upon i.v. injection. Pardaxin is toxic only upon direct injection into the brain (1 mg/kg body weight). *In vitro*, pardaxin is toxic to cell cultures and cellular preparations at relative high concentration (10^{-3}–10^{-5} M), therefore this toxin is not dangerous to humans and does not require special safety protection. The toxin is inactivated by 0.1 N HCl or 0.1% glutaraldehyde or heating.

Use in cell biology

Pardaxin is an useful toxin in neurosciences to investigate neuronal excitability and neurotransmitter release. Pardaxin may be used as a voltage-dependent ionophore to affect intracellular ionic composition in studies aimed to clarify the relationships between neuronal depolarization and ion-mediated signal transduction pathways (Lazarovici and Lelkes 1992). Since pardaxin-induced exocytosis of neurotransmitter involved both calcium-dependent and calcium-independent mechanisms (Lazarovici 1994), pardaxin may be used in neurochemical studies to investigate calcium-dependent and calcium-independent enzymes/proteins of the exocytosis secretory machinery of the cell (Arribas *et al.* 1993). In biophysical studies, pardaxin pores characterization may provide clues towards a better understanding of the structure and function of eukaryotic cells' ionic channels (Nikodijevic *et al.* 1992). Pardaxin, selectively activates the arachidonic acid cascade of the cells providing a pharmacological tool to search and develop specific inhibitors of PLA_2, cyclooxygenase, and lypooxygenase (Bloch-Shilderman *et al.* 1995). The ionophore properties of pardaxin may be exploited for transient permeabilization of cells in order to introduce cytotoxic drugs such as taxol (Dr I. Ringel, personal communication) and other molecules. Finally, the pore-forming property of pardaxin may be considered in designing a variety of chemical toxins to be used in cancer research.

References

Arribas, M., Blasi, J., Lazarovici, P., and Marsal, J. (1993). Calcium-dependent and independent acetylcholine release from electric organ synaptosomes by pardaxin: Evidence of a biphasic action of an excitatory neurotoxin. *J. Neurochem.*, **60**, 552–8.

Bloch-Shilderman, E., Abu-Raya, S., Rasouly, D., Furman, O., Trembovler, V., Shavit, D., *et al.* (1996). The role of calcium, protein kinase C, Pertussis toxin substrates and eicosanoids on pardaxin-induced dopamine release from pheochromocytoma PC12 cell. In *Biochemical aspects of marine pharmacology* (ed. P. Lazarovici, M. Spira, and E. Zlotkin), pp. 158–74. Alaken Press. Fort Collins, Co., USA.

Lazarovici, P. (1994). Challenging catecholamine exocytosis with pardaxin, an excitatory ionophore fish toxin. *J. Toxicol Toxin Rev.*, **13**, 45–63.

Lazarovici, P. and Lelkes, P. I. (1992). Pardaxin induces exocytosis in bovine adrenal medullary chromatin cells independent of calcium. *J. Pharmacol. Exp. Therap.*, **263**, 1317–26.

Lazarovici, P., Primor, N., and Loew, L. M. (1986). Purification and pore form activity of two hydrophobic polypeptides from the secretion of the red sea Moses sole (*Pardachirus marmoratus*). *J. Biol. Chem.*, **261**, 16704–13.

Lazarovici, P., Primor, N., Caratsch, C. G., Munz, K., Lelkes P. I., Loew, L. M., *et al.* (1988) Action on artificial and neuronal membranes of pardaxin, a new presynaptic excitatory polypeptide neurotoxin with ionophore activity. In *Neurotoxins in neurochemistry* (ed. J. O. Dolly), pp. 219–70, Ellis Horwood, Chichester.

Lazarovici, P., Primor, N., Gennaro, J., Fox, J., Shai, Y., Lelkes, P. I., *et al.* (1990). Origin, chemistry and mechanisms of action of a repellent, presynaptic excitatory, ionophore polypeptide. In *Marine toxins: Origin, structure and molecular pharmacology* (ed. S. Hall and G. Strichartz), pp. 347–65, American Chemical Society, Washington, DC.

Lazarovici, P., Edwards, C., Raghunathan, G., and Guy, H. R. (1992). Secondary structure, permeability and molecular modeling of pardaxin pores. *J. Natural Toxins*, **1**, 1–15.

Loew, L. M., Benson, L., Lazarovici, P., and Rosenberg, I. (1985). Fluorometric analysis of transferable membrane pores. *Biochemistry*, **24**, 2101–4.

Nikodijevic, B., Nikodijevic, D., and Lazarovici, P. (1992). Pardaxin-stimulated calcium uptake in PC12 cells is blocked by cadmium and is not mediated by L-type calcium channels. *J. Basic Clin. Physiol. Pharmacol.*, **3**, 359–70.

Primor, N. and Lazarovici, P. (1981). *Pardachirus marmoratus* (Red Sea flatfish) secretion and its isolated toxic fraction pardaxin: the relationship between hemolysis and ATPase inhibition. *Toxicon*, **19**, 573–8.

Renner, P., Caratsch, C. G., Waser, P. G., Lazarovici, P., and Primor, N. (1987). Presynaptic effects of the pardaxins, polypeptides isolated from the gland secretion of the flatfish *Pardachirus marmoratus*. *Neuroscience*, **23**, 319–25.

Shai, Y., Fox, J., Caratsch, C. G., Shih, Y. L., Edwards, C., and Lazarovici, P. (1988). Sequencing and synthesis of pardaxin, a polypeptide from the red sea moses sole with ionophore activity. *FEBS Lett.*, **242**, 161–6.

Shi, Y., Eduards, C., and Lazarovici, P. (1995). Ion selectivity of the channels formed by pardaxin, an ionophore, in bilayer membranes. *Natural Toxins*, **3**, 151–5.

T. Wang, H. Y. and Friedman, E. (1986). Increased 5-hydroxtryptamine and norepinephrine release from rat brain slices by the red sea flatfish toxin pardaxin. *J. Neurochem.*, **47**, 656–8.

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Palytoxin (corals of the spp. *Palythoa*)

Palytoxin, a linear peptide composed of a large polyhydroxy ω-amino acid and dehydro-β-alanine amidated with aminopropanol, is the most potent animal toxin. It binds with high affinity to Na^+, K^+-ATPase in an ouabain-sensitive manner and converts the pump into a channel permselective for alkali ions. Palytoxin serves as a tool to assess the function of the pump and its localization.

Palytoxin (MW about 2670) occurs in several *Palythoa* species. It is unknown whether they produce the toxin or acquire it from symbiotic organisms. The heat-resistant toxin is also found in crabs and fish, which possibly take it up with their food. Its unique structure (Cha *et al.* 1982) has been synthesized (Armstrong *et al.* 1989) and its stereochemistry (Moore *et al.* 1982) determined (Fig.1). A long hydrophobic stretch spanning C_{21} and C_{40} markedly

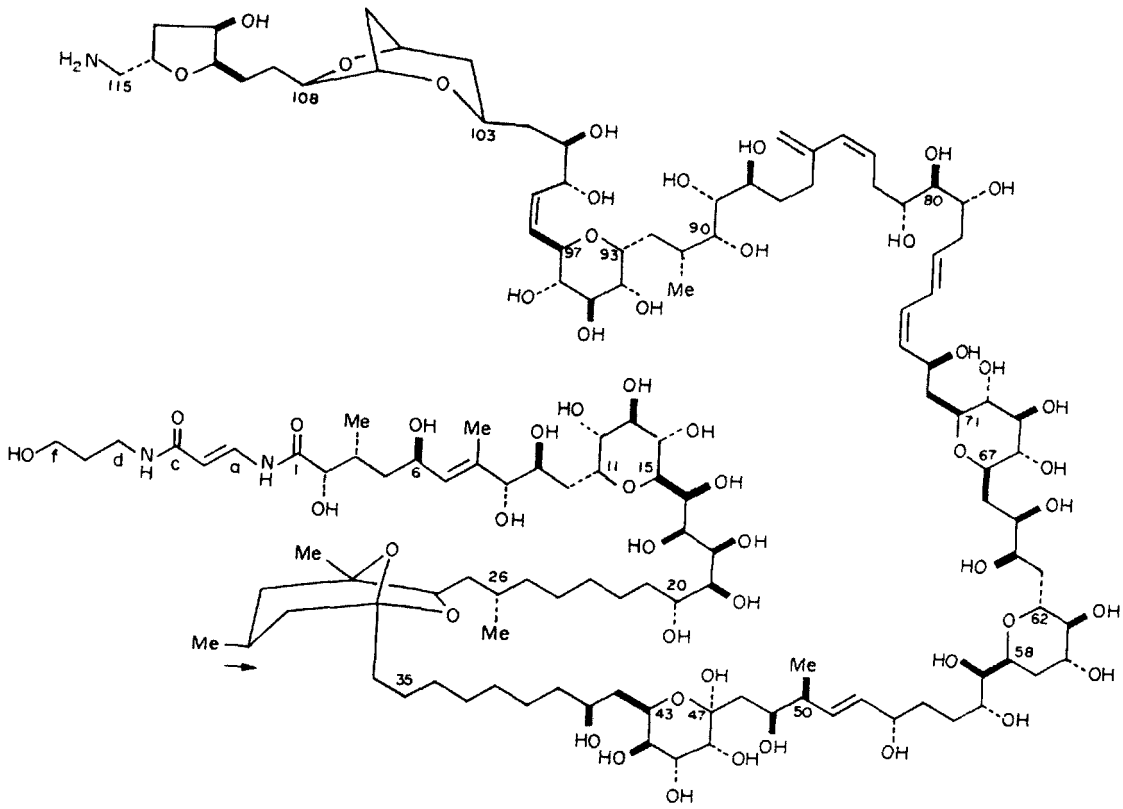

Figure 1. The structure of palytoxin (Cha *et al.* 1982)

contrasts with the otherwise hydrophilic structure, and may be responsible for the moderate surface activity. Substitutions at the N- or C-terminus cause dramatic losses in potency (Tosteson *et al.* 1995). Specific iodination is useful for radioimmunoassays (Levine *et al.* 1988) only; however, some pharmacological activity survives shotgun iodination (Böttinger *et al.* 1986).

Palytoxin acts through Na^+, K^+-ATPase (Habermann 1989). This is the only enzyme equipped with an ouabain receptor. The binding sites for ouabain and for palytoxin overlap, however the kinetics of the respective interactions differ considerably. For instance, the affinity of the rat enzyme is low to ouabain, but in the picomolar range to palytoxin. Binding of palytoxin is promoted by Ca^{2+}, whereas binding of ouabain is promoted by Mg^{2+} and P_i. Both ouabain and palytoxin (Ishida *et al.* 1983; Böttinger and Habermann 1984; Tosteson *et al.* 1991) inhibit the hydrolysis of ATP. However, whereas ouabain arrests the pump in the closed state, palytoxin keeps it in the open state. Thus the pump becomes a receptor-operated channel with bidirectional permeability for alkali cations, which are normally transported unidirectionally through and by the enzyme. Excitable cells respond to their ensuing depolarization by voltage-dependent Ca^{2+}-entry, leading to contraction or exocytosis. Acute death due to palytoxin in rodents is accompanied by massive hyperkalemia and by pulmonary vasoconstriction (Habermann 1989). The actions of palytoxin and ouabain are compared in Fig. 2.

Yeast is devoid of Na^+, K^+-ATPase. Its natural resistance to palytoxin is overcome by simultaneous transformation with vectors for the α and β subunits of the Na^{2+} pump, whereas single subunits are insufficient (Scheiner-Bobis *et al.* 1994). The palytoxin channels are small 10 ps) and display typical on/off kinetics under patch clamp conditions. As whole cell permeabilities in erythrocytes, the single-channel activities are inhibited by ouabain and vanadate but promoted by ATP. Palytoxin channels can be formed in lipid bilayers incorporated with kidney ATPase. Under appropriate conditions such channels can be observed in Na^+, K^+-ATPase in the absence of palytoxin (Kim *et al.* 1995).

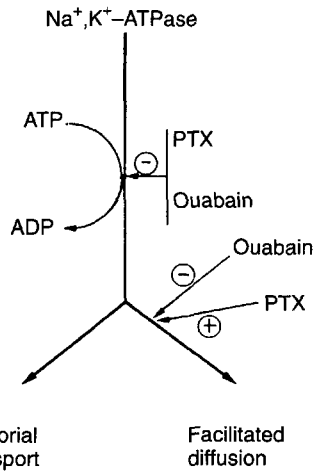

Figure 2. Convergent and divergent actions of palytoxin and ouabain on Na^+, K^+-ATPase (from Habermann 1989). Both palytoxin (PTX) and ouabain inhibit the enzyme, but facilitated diffusion through the enzyme is inhibited by ouabain and promoted by palytoxin.

Purification and sources

Palytoxin (Fig.1), together with some derivatives (Hirata *et al.* 1979), can be extracted from frozen palythoa tuberculosa (Hirata *et al.* 1979) or from lyophilized *P. caribaeorum* (Béress *et al.* 1983) with ethanol and purified by column chromatography. It can be purchased from L. Beress, Institute of Toxicology, University of Kiel, Germany, and from some chemical companies, e.g. Sigma. Purity can be controlled by thin layer chromatography.

Toxicity and biological activity

In vivo toxicity in laboratory mammals is between 25 (rabbits) and 450 (mouse) ng/kg upon parenteral application. However, *in vitro* comparison with a reference sample for K^+ release from human erythrocytes (Böttinger *et al.* 1986; Habermann 1989; Tosteson *et al.* 1991, 1995) is elegant, precise, and saves animals. The test can be rendered very specific by inclusion of ouabain (for references see Habermann 1989). A radioimmunoassay is available too (Levine *et al.* 1988).

Although oral compared to parenteral toxicity is lower, palytoxin is a potentially lethal food poison for man and domestic animals. Aerosolization, for instance by homogenization of corals, and preparative work in general, requires special precautions as severe mucosal damages resembling influenza have occurred in such work. Palytoxin should be kept in solution, if possible. Residues may be destroyed by heating in alkaline solution.

Use in cell biology

The sodium pump is the specific and in our view unique target of palytoxin. Therefore, the toxin can be used for analysing the function of genuine and the genetically modified pumps. Biochemical and electrophysiological signals may be registered. Target systems from rats should be avoided, if possible, because the rat sodium pump displays a high affinity to palytoxin but not to ouabain, which prevents the use of the cardenolide as a specific palytoxin inhibitor.

Promotion of neurotransmitter, particularly catecholamine release (see Habermann 1989) is a secondary phenomenon, resulting from the primary sodium entry through the palytoxin pores which then triggers the opening of voltage-dependent Ca^{2+} channels. Thus palytoxin is not a specific presynaptic toxin. Palytoxin can also be used for localization of the sodium pump and its rele-

vance, for instance in basolateral vs. apical regions of polar cell layers (Mullin *et al.* 1991).

References

Armstrong, R. W., Beaù, J. M., Cheon, S. H., Christ, W. J., Fujoka, H., and Horn, W. H. (1989). Total synthesis of palytoxin carboxylic acid and palytoxin amide. *J. Am. Chem. Soc.*, **111**, 7530–3.

Béress, L., Zwick, J., Kolkenbrock, H. J., Kaul, P. N., and Wassermann, O. (1983). A method for the isolation of the carribean palytoxin (C-PTX) from the coelenterate (zooanthid) *Palythoa caribaeorum*. *Toxicon*, **21**, 285–90.

Böttinger, H. and Habermann, E. (1984). Palytoxin binds to and inhibits kidney and erythrocyte Na^+,K^+-ATPase. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **325**, 85–7.

Böttinger, H., Béress, L., and Habermann, E. (1986). Involvement of Na^+, K^+-ATPase in binding and actions of palytoxin on human erythrocytes. *Biochim. Biophys. Acta*, **861**, 165–76.

Cha, J. K., Christ, W. J., Finan, J. M., Fuijoka, M., Kishi, Y., Klein, L. L., *et al.* (1982). Stereochemistry of palytoxin. 4. Complete structure. *J. Am. Chem. Soc.*, **104**, 7369.

Habermann, E. (1989). Palytoxin acts through Na^+, K^+-ATPase. *Toxicon*, **27**, 1171–87.

Hirata, Y., Uemura, D., Ueda, K., and Takano, S. (1979). Several compounds from palythoa tuberculosa (coelenterata). *Pure Appl. Chem.* **51**, 1875–83.

Ishida, A., Takagi, K. Takahashi, M., Satake, N., and Shibata, S. (1983). Palytoxin isolated from marine coelenterates. The inhibitory action on (Na, K) ATPase. *J. Biol. Chem.*, **258**, 7900–2.

Kim, S. Y., Marx, K. A., and Wu, C. H. (1995). Involvement of Na,K-ATPase in the induction of ion channels by palytoxin. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **351**, 542–54.

Levine, L., Fujiki, H., Gjäka, H. B., and Van Vunakis, H. (1988). A radioimmunoassay for palytoxin. *Toxicon*, **26**, 1115–21.

Moore, R. E., Bartolini, G. J., and Bachi, J. (1982). Absolute stereochemistry of palytoxin. *J. Am. Chem. Soc.*, **104**, 3776–9.

Mullin, J. M., Snock, K. V., and McGinn, M. T. (1991). Effects of apical vs. basolateral palytoxin on LLC-PK1 renal epithelia. *Am. J. Physiol.*, **260**, C1201–11.

Scheiner-Bobis, G., Meyer zu Heringdorf, D., Christ, M., and Habermann, E. (1994). Palytoxin induces K^+ efflux from yeast cells expressing the mammalian sodium pump. *Mol. Pharmacol.*, **45**, 1132–6.

Tosteson, M. T., Halperin, J. A., Kishi, Y., and Tosteson, D. C. (1991). Palytoxin induces an increase in the cation conductance of red cells. *J. Gen. Physiol.*, **98**, 969–85.

Tosteson, M., Scriven, R. L., Bharadway, A. K., Kishi, Y., and Tosteson, D. C. (1995). Interaction of palytoxin with red cells: Structure function studies. *Toxicon*, **33**, 1799–807.

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Equinatoxins (*Actinia equina* L., sea anemone)

Equinatoxins I, II, and III are 19 kDa, basic polypeptides isolated from the sea anemone Actinia equina *L. They form cation-selective pores ≈1.1 nm in diameter in artificial lipid and cell membranes. Sphingomyelin and some other ceramide lipids are acceptor molecules which promote toxin insertion into the lipid phase.*

Equinatoxins I, II, and III (EqTx) are lethal and cytolytic 19 kDa polypeptides (Maček and Lebez 1988) belonging to a larger family of pore-forming toxins from sea anemones (*Actiniaria*) (see reviews Kem 1988; Turk 1991; Maček 1992). The most abundant isoform, equinatoxin II (synonym: equinatoxin; Ferlan and Lebez 1974), is a single chain, 179 amino acids long, without cysteine (Belmonte *et al.* 1994). EqTx II is nearly identical to tenebrosin-C from *A. tenebrosa* (Simpson *et al.* 1990; Norton *et al.* 1992) (sequences in Swiss Prot Databank: P17723 TENC_ACTTEX; Genbank: U41661).

Cytolytic activity of EqTx II is enhanced by Ca^{2+} and a pH above 7 (Maček and Lebez 1981, 1988; Belmonte *et al.* 1993), and it is antagonized by lipoproteins (Turk *et al.* 1989; Batista *et al.* 1990), sphingomyelin, and ganglioside GM_1 (Belmonte *et al.* 1994; Maček *et al.* 1994). The activity has been ascribed to formation of pores in natural and artificial lipid membranes (Zorec *et al.* 1990; Belmonte *et al.* 1993). No particular membrane receptor has been identified but low-affinity acceptors with a ceramide moiety have been proven (Belmonte *et al.* 1993, 1994; Maček *et al.* 1994). Toxin monomer insertion and oligomerization of 3–4 molecules in a lipid bilayer has been suggested to build up an approx. 1.1 nm wide cation-selective pore readily permeable by monovalent cations, Ca^{2+}, water, and neutral solutes (Zorec *et al.* 1990; Belmonte *et al.* 1993). The amphiphilic N-terminus segment, analogous to melittin and virus fusion peptides (Belmonte *et al.* 1994), and an $\alpha\alpha$-hairpin have been predicted to anchor the toxin monomer into a lipid bilayer

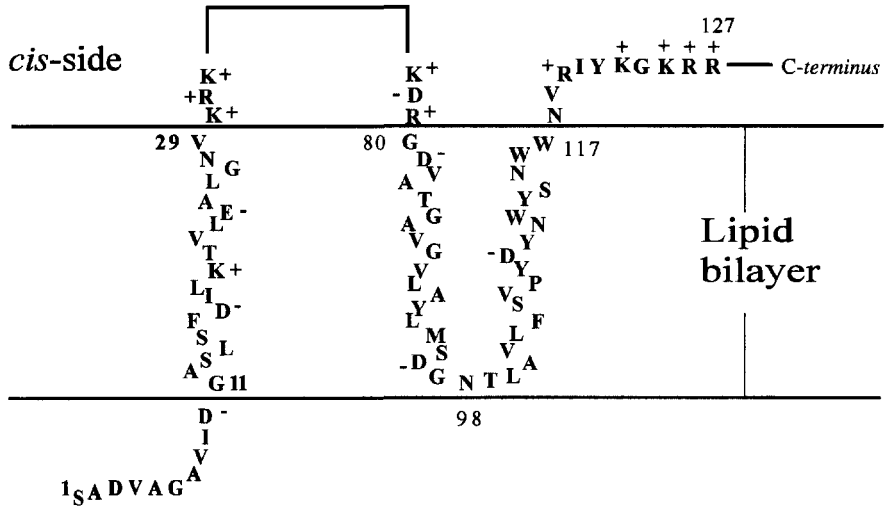

Figure 1. Putative model of EqTx II insertion into a lipid bilayer from the *cis*-side. Three or four already inserted molecules may form a pore.

(see model in Fig. 1), and to form pore walls upon monomer association.

Purification and sources

EqTx II is isolated from fresh animal exudates, while whole body macerates contain additionally EqTx I and III. Toxins are purified by cold acetone precipitation (50–80 per cent fraction), Sephadex G-50 size-exclusion permeation, and CM-cellulose ion exchange chromatography (Maček and Lebez 1988). The purity of EqTx II and III is over 97 per cent. EqTx I should be repurified on a CM-Sephadex column at a higher pH or by a reversed phase HPLC. Native and recombinant toxins are available from P. Maček, University of Ljubljana.

Toxicity

Acute toxicity was determined in rats (Ferlan and Lebez 1974) and mice (Maček and Lebez 1988) by intravenous injection. Mouse LD_{50}s for EqTx I, II, and III are 23, 35, and 83 μg/kg, respectively. An 'all-or-none' lethal effect is characteristic. Animals die within 5–10 min but survivors do not suffer from any evident symptoms. No chronic toxicity has been observed. An intraperitoneal LD_{50} is ~100x larger. No precautions by operators are needed for toxin handling.

Use in cell biology

In mammals, EqTx II is highly cardiotoxic, producing severe coronary vasospasm and disappearance of EKG QRS-waves (Budihna *et al.* 1992), positive and negative inotropy, and fibrillation (Ho *et al.* 1987). EqTx II, 10^{-11}–10^{-12} M, is cytotoxic to isolated guinea pig cardiocytes (Maček *et al.* 1994), and cytolytic to a variety of cells (Maček 1992; Maček *et al.* 1994). At sublytic concentrations, i.e. $\leq$1 nM, the toxin induces platelet aggregation (Teng *et al.* 1988), leukocyte degranulation (Bunc *et al.* 1994), and it inhibits synaptosomal GABA and choline uptake as was also reported for a *Heteractis magnifica* cytolysin (Khoo *et al.* 1995).

There have been attempts to use EqTx II, and *Stichodactyla helianthus* cytolysin III as a toxic part of a mitotoxin (Pederzolli *et al.* 1995) or immunotoxin (Avila *et al.* 1988). Long-lived pores in cell membranes produced by EqTxs may make these toxins useful as cell permeabilizing agents. Still, some activities of EqTx at sublytic concentrations and their underlying mechanisms, are not fully understood.

References

Avila, A. D., Mateo de Acosta, C., and Lage, A. (1988). A new immunotoxin built by linking a hemolytic toxin to a monoclonal antibody specific for immature T lymphocytes. *Int. J. Cancer*, **142**, 568–71.

Batista, U., Maček, P., and Sedmak, B. (1990). The cytotoxic and cytolytic activity of equinatoxin II from the sea anemone *Actinia equina*. *Cell Biol. Int. Rep.*, **14**, 1013–24.

Belmonte, G., Pederzolli, C., Maček, P., and Menestrina, G. (1993). Pore formation by the sea anemone cytolysin equinatoxin II in red blood cells and model lipid membranes. *J. Membrane Biol.*, **131**, 11–22.

Belmonte, G., Menestrina, G., Pederzolli, C., Križaj, I., Gubensek, F., Turk, T., *et al.* (1994). Primary and secondary structure of a pore-forming toxin from the sea anemone, *Actinia equina* L., and its association with lipid vesicles. *Biochim. Biophys. Acta*, **1192**, 197–204.

Budihna, M. V., Maček, P., and Šuput, D. (1992). Some possible mechanisms involved in the cardiotoxicity of equinatoxin II. In

Recent advances in toxinology research (ed. P. Gopalakrishnakone and C. K. Tan), pp. 402–7, National University of Singapore, Singapore.

Bunc, M., Frangeš, R., Horvat, I., Turk, T., and Šuput, D. (1994). Effects of equinatoxins *in vivo*. Possible role of degranulation of thrombocytes and granulocytes. *Ann. N. Y. Acad. Sci.*, **710**, 162–7.

Ferlan, I. and Lebez, D. (1974). Equinatoxin, a lethal protein from *Actinia equina*. – I. Purification and characterization. *Toxicon*, **12**, 57–61.

Ho, C. L., Ko, J. L., Lue, H. M., Lee, C. Y., and Ferlan, I. (1987). Effects of equinatoxin on the guinea-pig atrium. *Toxicon*, **25**, 659–64.

Kem, W. R. (1988). Sea anemone toxins: Structure and function. In *The biology of nematocysts* (ed. D. A. Hessinger and H. M. Lenhoff), pp. 375–405, Academic Press, New York.

Khoo, H. E., Lim, J. P. C., and Tan, C. H. (1995). Effect of sea anemone (*H. magnifica* and *A. equina*) cytolysins on synaptosomal uptake of GABA and choline. *Toxicon*, in press.

Maček, P. (1992). Polypeptide cytolytic toxins from sea anemones (*Actiniaria*). *FEMS Microbiol. Immunol.*, **105**, 121–30.

Maček, P. and Lebez, D. (1981). Kinetics of hemolysis induced by equinatoxin, a cytolytic toxin from the sea anemone *Actinia equina*. Effect of some ions and pH. *Toxicon*, **19**, 233–40.

Maček, P. and Lebez, D. (1988). Isolation and characterization of three lethal and hemolytic toxins from the sea anemone *Actinia equina* L. *Toxicon*, **26**, 441–51.

Maček, P., Belmonte, G., Pederzolli, C., and Menestrina, G. (1994). Mechanism of action of equinatoxin II, a cytolysin from the sea anemone *Actinia equina* L. belonging to the family of actinoporins. *Toxicology*, **87**, 205–27.

Norton, R. S., Maček, P., Reid, G. E., and Simpson, R. J. (1992). Relationship between the cytolysins tenebrosin-C from *Actinia tenebrosa* and equinatoxin II from *Actinia equina*. *Toxicon*, **30**, 13–23.

Pederzolli, C., Belmonte, G., Dalla Sera, M., Maček, P., and Menestrina, G. (1995). Biochemical and cytotoxic properties of conjugates of transferrin with equinatoxin II, a cytolysin from a sea anemone. *Bioconjugate Chem.*, **6**, 166–73.

Simpson, R. J., Reid, G. E., Moritz, R. L., Morton, C., and Norton, R. S. (1990). Complete amino acid sequence of tenebrosin-C, a cardiac stimulatory and haemolytic protein from the sea anemone *Actinia tenebrosa*. *Eur. J. Biochem.*, **190**, 319–28.

Teng, C. M., Lee, L. G., Lee, C. Y., and Ferlan, I. (1988). Platelet aggregation induced by equinatoxin. *Thromb. Res.*, **52**, 401–11.

Turk, T. (1991). Cytolytic toxins from sea anemones. *J. Toxicol.–Toxin Rev.*, **10**, 223–62.

Turk, T., Maček, P., and Gubenŏek, F. (1989). Chemical modification of equinatoxin II, a lethal and cytolytic toxin from the sea anemone *Actinia equina* L. *Toxicon*, **27**, 375–84.

Zorec, R., Tester, M., Maček, P., and Mason, W. T. (1990). Cytotoxicity of equinatoxin II from the sea anemone *Actinia equina* involves ion channel formation and an increase in intracellular calcium activity. *J. Membrane Biol.*, **118**, 243–9.

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13

Glutamate receptor targeted toxins

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Introduction

In the central nervous system L-glutamate is the major excitatory neurotransmitter utilized by neurons. Glutamate acts as a neurotransmitter through different receptors, which can be divided into two major categories: ionotropic and metabotropic. The ionotropic receptors are ligand-gated ion channels, whereas the metabotropic receptors are coupled to second messenger systems through the G-proteins (Nakanishi 1992).

Ionotropic receptors are further subdivided into three receptor subtypes based on different agonist affinity, namely *N*-methyl-D-aspartate (NMDA), (α-amino-3-hydroxy-5-methyl-4–isoxazolepropionic acid (AMPA), and kainate (Monaghan *et al.* 1989).

The NMDA receptor–channel complex is highly permeable to Ca^{2+} as well as to Na^{+} and K^{+}. These receptors have several characteristic features, including modulation by glycine on a strychnine-insensitive site, polyamine activation, and Zn^{2+} inhibition (Hollmann and Heinemann 1994). Similarly to nicotinic receptors, they are supposed to be pentameric transmembrane proteins composed of two different types of homologous subunits named NMDAR1 and NMDAR2A-D. All NMDA subunits are postulated to possess four transmembrane segments following a large NH_2-terminal domain. These subunits would be arranged around a central cation-permeating pore. To date, the molar stoichiometry in which the NMDA receptor subunits are assembled is unknown.

For AMPA and kainate receptors a large number of subunits have also been cloned and described (Sommer and Seeburg 1992). All subunits have approximately 900 amino acid residues, and share a common transmembrane topology and channel architecture with other members of ligand-gated ion channels, with four putative membrane-spanning segments such as the NMDA subunits. The synthesis of the AMPA subunits GluR1 to GluR4 (also termed GluRA–D) involves alternative splicing mechanisms, thereby generating two versions of each subunit differing in a 38 amino acid residue sequence. These derivatives have been named flip and flop, they display different regional expression patterns in the brain and different pharmacological profiles (Monyer *et al.* 1991). Further diversity in AMPA receptor subunits is generated by a post-transcriptional event called RNA editing (Sommer *et al.* 1991). This mechanism influences the permeability characteristics of the AMPA receptors.

At present, five subunits GluR 5 to 7 and KA1–KA2 displaying kainate binding activity have been cloned, and the pharmacological properties have been described (Sommer and Seeburg 1992). Alternative splicing and RNA editing occurs also among kainate receptor subunits, however, in contrast to AMPA subunits which are expressed in only the edited version, both the edited and unedited versions of GluR5 and 6 are expressed.

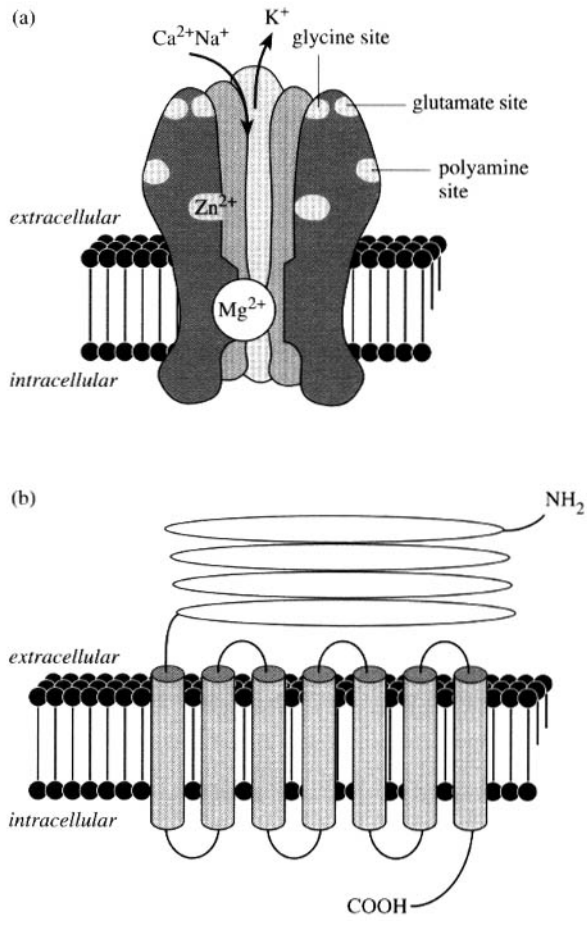

Figure 1. (a) Schematic representation of the NMDA receptor. (b) Schematic representation of the mGlu receptor.

Molecular cloning has revealed the existence of eight subtypes of metabotropic glutamate receptor mGluRs, which are larger than the other members of the G-protein coupled receptor superfamily, in that they show a common structural architecture with a large extracellular NH_2-terminal domain preceding the seven transmembrane segments (Pin and Duvoisin 1995). These receptors are largely present in the central nervous system, although each shows a particular pattern of distribution.

Glutamate ionotropic receptor activation increases membrane permeability to both monovalent ions and Ca^{2+}, through opening of the receptor channel. These receptors mediate fast synaptic events as well as persistent changes in membrane potential (e.g. long-term

potentiation). For NMDA receptors the opening probability under physiological conditions depends upon membrane potential. The NMDA-gated channel is, in fact, blocked by Mg^{2+} at resting membrane potential. The activation of non-NMDA subtypes, by changing the membrane potential, removes the Mg^{2+} blockade.

AMPA-kainate receptors generally conduct monovalent cations, however, glutamate in certain neuronal cells can trigger the activation of Ca^{2+}-permeable AMPA-kainate receptors. The different ionic selectivity depends on the combination of different subunits, and in particular on the presence of the GluR2 subunit. This subunit contains an arginine residue within the second transmembrane segment, whereas the other three subunits carry a glutamine residue at the corresponding position. This difference confers a very weak Ca^{2+} permeability thus determining the channel conductance and Ca^{2+} permeation (Verdoorn *et al.* 1991).

Activities

Ionotropic glutamate receptors are widely distributed both in the central and in the peripheral nervous system and are involved in different brain functions requiring synaptic activity and plasticity. In particular, they appear to play a major role during central nervous system development, in cognitive functions, and in the integration between sensory transmission and motor outputs (Hollmann and Heinemann 1994). The AMPA-kainate receptors evoke fast, voltage-independent synaptic responses, and in turn promote the activation of voltage-dependent NMDA receptors (Nakanishi 1992). The mGluR subtypes, on the other hand, exert long-lasting actions through the activation and inhibition of intracellular signals. The physiological role of mGluRs is not well understood but recent evidence has underlined many potential regulatory actions in the central nervous system (Conquet *et al.* 1994; Pin and Duvoisin 1995).

Some neuropathological conditions are characterized by an over-activation of glutamate receptors, namely ionotropic, leading to excitotoxicity and permanent changes in neuronal structure and function.

Genes

Complete coding sequences are available for glutamate receptors in rat and most of them also in human and mouse. Accession numbers for rat NMDARs are: NMDAR1 (Emm U08259/U08260/U08261/U08262/U08263/U08264/U08265/U08266/U0S267/U08268); NMDAR2A (Emm M91561); NMDAR2B (Emm M91562); NMDAR2C (Emm M91563); NNDAR2D (Emm D13213).

Accession numbers for rat AMPA-kainate receptors are: GluR2 (Emm. M85035); GluR3 (Emm. M85036); GluR4 (Emm. M85037); GluR5 (Emm. M83560–M83561); GluR6 (Emm. ZI 1548); GluR7 (Emm. M83552); KA1 (Emm. X59996); KA2 (Emm. ZI 1581).

Accession numbers for rat mGluRs are: mGluR1 (Emm M61099); mGiuR2 (Emm. M92075); mGluR3 (Emm .M92076); mGluR4 (Emm M92077); mGluR5 (Emm. D1089); mGluR (Emm. D13963); mGluR7 (Emm. U06832); mGluRS (GB U17252).

The murine NMDA receptor subunit genes NMDARI, NMDAR2A, NMDAR2B, NMDAR2C, and NMDAR2D have been assigned to chromosomes 3, 10, 4, 10, and 1, respectively. The metabotropic receptor subtype genes mGluRI, mGluR2, mGluR3, mGluR4, mGluR5, and mGluR6 have been mapped and assigned to chromosomes 1, 8, 4, 20, 1 and 10, respectively (Kuramoto *et al.* 1994).

References

Conquet, F., Bashir, Z. I., Davies, C.H., Daniel, H., Ferraguti, F., Bordi, F., *et al.* (1994). Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1 [see comments]. *Nature*, **372**, 237–43.

Hollmann, M. and Heinemann, S. (1994). Cloned glutamate receptors. *Ann. Rev. Neurosci.*, **17**, 31–108.

Kuramoto, T., Maihara, T., Masu, M., Nakanishi, S., and Serikawa, T. (1994). Gene mapping of NMDA receptors and metabotropic glutamate receptors in the rat (*Rattus norvegicus*). *Genomics*, **195**, 358–61.

Monaghan, D. T., Bridges, R. J., and Cotman, C. W. (1989). *Ann. Rev. Pharmacol. Toxicol.*, **29**, 365.

Monyer, H., Seeburg, P., and Wisden, W. (1991). Glutamate-operated channels: developmentally early and mature forms arise by alternative splicing. *Neuron*, **6**, 799–810.

Nakanishi, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. *Science*, **258**, 597–603.

Pin, J. P. and Duvoisin, R. (1995). The metabotropic glutamate receptors: strucutre and functions. *Neuropharmacology*, **34**, 1–26.

Sommer, B. and Seeburg, P. (1992). Glutamate receptor channels: novel properties and new clones. *TIPS*, **13**, 291–6.

Sommer, B., Kohler, M., Sprengel, R., and Seeburg, P. (1991). RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell*, **67**, 11–19.

Verdoorn, T. A., Bumashev, N., Monyer, H., Seeburg, P., and Sakmann, B. (1991). Structural deteminants of ion flow through recombinant glutamate receptor channels. *Science*, **252**, 1715–18.

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Conantokins (*Conus* spp.)

The conantokins are a class of 17–21 AA peptides which have been isolated from fish-hunting cone snails. They are the only natural peptides known to inhibit glutamate receptors of the N-methyl-D-aspartate (NMDA) class. Unlike most other peptides found in Conus venoms, the conantokins do not have multiple disulfide bonds, but 4–5 residues of the unusual post-translationally modified amino acid γ-carboxyglutamate, which is derived from glutamate via a vitamin K-dependent carboxylation reaction. An unusual feature of these peptides is that the in vivo symptoms they induce in mammals following central administration are developmentally dependent. A sleep-like state is induced in mice under two weeks of age, but in mice over three weeks of age, a hyperactivity characterized by climbing and running from corner to corner is found. It is not yet certain whether these age-dependent effects are the result of differential subunit composition of the receptor.

The conantokins are peptides from the venom of fish-hunting cone snails which interact with and inhibit the *N*-methyl-D-aspartate (NMDA) receptor, a subset of the glutamate receptor family (Olivera *et al.* 1990). Two conantokins have been described (Table 1), conantokin-G from *Conus geographus* venom (Rivier *et al.* 1987) and conantokin-T from *Conus tulipa* venom (Haack *et al.* 1990). The sequences of the two peptides are highly divergent from one another, but certain sequence features are conserved, notably four out of the five γ-carboxyglutamate residues. In contrast to other *Conus* venom peptides which contain two to three internal disulfide bridges that are believed to contribute to their structural rigidity, the conantokins contain few or no cysteine residues. It has been suggested that the γ-carboxyglutamate groups assume a structural role in the absence of disulfide bridges. In the presence of calcium ions, the carboxyl groups of γ-carboxyglutamate chelate the metal ion, resulting in stabilization of an α-helical structure for the peptide (Myers *et al.* 1990*a*, *b*). The interpretation of an α-helical structure for conantokin-G has recently been disputed, however (Chandler *et al.* 1993).

There is substantial evidence from different experimental models that conantokins interact selectively with NMDA receptors:

1. Conantokin-G specifically inhibits NMDA receptors expressed in *Xenopus* oocytes following injection of mouse brain mRNA (Hammerland *et al.* 1992).
2. The peptide blocks the NMDA-induced increase in intracellular calcium in fura2-loaded cerebellar granule cells in culture (Haack *et al.* 1990).
3. Conantokin-G antagonizes NMDA-elicited increases in cerebellar cGMP (Mena *et al.* 1990).

In each of these cases, conantokin did not affect kainate or quisqualate-evoked responses.

Several lines of evidence suggest that the conantokins may have subtype specificity for NMDA receptors. First, NMDA receptors from different sources have widely varying affinities for the same conantokin. Second, the symptomatologies induced by conantokins are unusual in that they are developmentally dependent in mammals. Both conantokin-G and conantokin-T elicit a sleep-like state in mice under two weeks of age, but a hyperactive climbing syndrome in mice over three weeks of age. Since the spectrum of NMDA receptor subunits expressed changes during this time period, the difference in the symptomatology elicited may be due to the appearance and disappearance of conantokin high-affinity target subtypes (Haack *et al.* 1993*a*).

The physiological target of conantokins is unknown at present. However, the involvement of the NMDA receptor complex in diverse CNS functions including excitatory neurotransmission, induction of long-term potentiation in learning and memory, and mediation of post-hypoxic stress reactions suggest a number of possible neuroregulatory roles (Mena *et al.* 1990; Hammerland *et al.* 1992; Haack *et al.* 1993*b*; Scatton 1993).

Purification and chemical synthesis

The conantokins have been purified from *Conus geographus* and *Conus tulipa* venoms by standard high

Table 1 Conantokins

Conus species	Peptide	Structure[a]
C. geographus	conantokin-G	GEγγLQγNQγLIRγKSN*
C. tulipa	conantokin-T	GEγγYQKMLγNLRγAEVKKNA*

[a] γ = γ-carboxyglutamate, also abbreviated Gla; * = C-terminal amide.

performance liquid chromatography (HPLC) techniques. In contrast to the paralytic conotoxins which tend to be highly polymorphic, there appears to be only one major conantokin found in each venom. The peptides available for use in cell biology and neuroscience are synthetic. Chemical synthesis of these peptides has special requirements because of the presence of the γ-carboxyglutamate residues; however, successful chemical synthesis is routine for both conantokin-G and conantokin-T (Rivier *et al.* 1987).

Potency

Intracerebroventricular administration of either native or synthetic conantokin-G induced sleep in 10-day-old mice at doses between 4 and 600 pmoles per gram body weight, with the duration of sleep ranging from 1–15 hours in proportion to the dose. Animals older than two weeks exhibited hyperactivity at the low dose and 'sleepy climbing' activity at the higher end of the range (Rivier *et al.* 1987). In *in vitro* assays, 1 μM conantokin-G was able to antagonize NMDA-mediated currents in *Xenopus* oocytes and NMDA-evoked intracellular calcium release; the IC50 for antagonism of NMDA-stimulated cerebellar cGMP was 171 nM (Mena *et al.* 1990).

Uses in cell biology

Since the conantokins are the only known peptides which target NMDA receptors, they have unusual potential to serve as both functional and biochemical probes for these important complexes in the nervous system. In addition, the presence of γ-carboxyglutamate residues has led to a number of hypotheses about how these ligands might be structurally constrained, and be able to specifically target particular NMDA receptor subtypes with high affinity.

It is known that the mammalian NMDA receptor is composed of a heteromeric assembly of subunits consisting of NMDAR 1A/B and NMDAR 2A/B/C/D elements. These and other accessory proteins can directly or allosterically modulate the conductance of the integral ion channel. Known modulators include PCP, glycine, zinc, and polyamines (Scatton 1993). The precise mechanism of conantokin interaction with the receptor complex is not known. Studies of NMDA currents in mouse brain mRNA-injected *Xenopus* oocytes are consistent with a component of competitive inhibition at the NMDA binding site (Hammerland *et al.* 1992). Other studies have provided evidence of non-competitive allosteric inhibition at the polyamine binding site (Skolnick *et al.* 1992; Chandler *et al.* 1993).

References

Chandler, P., Pennington, M., Maccecchini, M. L., Nasheda, N. T., and Skolnick, P. (1993). Polyamine-like actions of peptides derived from conantokin-G, an *N*-methyl-D-aspartate (NMDA) antagonist. *J. Biol. Chem.*, **268**, 17173–8.

Haack, J. A., Rivier, J., Parks, T. N., Mena, E. E., Cruz, L. J., and Olivera, B. M. (1990). Conantokin-T. A gamma-carboxyglutamate containing peptide with *N*-methyl-D-asparate antagonist activity. *J. Biol. Chem.*, **265**, 6025–9.

Haack, J. A., Parks, T. N., and Olivera, B. M. (1993*a*). Conantokin-G antagonism of the NMDA receptor subtype expressed in cultured cerebellar granule cells. *Neurosci. Lett.*, **163**, 63–6.

Haack, J., Kinser, P., Yoshikami, D., and Olivera, B. M. (1993*b*). Biotinylated derivatives of omega-conotoxins GVIA and MVIID: probes for neuronal calcium channels. *Neuropharmacology*, **32**, 1151–9.

Hammerland, L. G., Olivera, B. M., and Yoshikami, D. (1992). Conantokin-G selectively inhibits N-methyl-D-aspartate-induced currents in *Xenopus* oocytes injected with mouse brain mRNA. *Eur. J. Pharmacol.*, **226**, 239–44.

Mena, E. E., Gullak, M. F., Pagnozzi, M. J., Richter, K. E., Rivier, J., Cruz, L. J., *et al.* (1990). Conantokin-G: a novel peptide antagonist to the *N*-methyl-D-aspartic acid (NMDA) receptor. *Neurosci. Lett.*, **118**, 241–4.

Myers, R. A., McIntosh, J. M., Imperial, J., Williams, R. W., Oas, T., Haack, J. A., *et al.* (1990*a*). Peptides from *Conus* venoms which affect Ca^{++} entry into neurons. *J. Toxin–Toxin Rev.*, **9**, 179–202.

Myers, R. A., River, J., and Olivera, B. M. (1990*b*). Conantokins: Peptide probes of the NMDA receptor. *Soc. Neurosci.* Abstr., 16, 958.

Olivera, B. M., Rivier, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., *et al.* (1990). Diversity of *Conus* neuropeptides. *Science*, **249**, 257–63.

Rivier, J., Galyean, R., Simon, L., Cruz, L. J., Olivera, B. M., and Gray, W. R. (1987). Total synthesis and further characterization of the gamma-carboxyglutamate-containing 'sleeper' peptide from *Conus geographus* venom. *Biochemistry*, **26**, 8508–12.

Scatton, B. (1993). The NMDA receptor complex. *Fundam. Clin. Pharmacol.*, **7**, 389–400.

Skolnick, P., Boje, K., Miller, R., Pennington, M., and Maccecchini, M.-L. (1992). Noncompetitive inhibition of *N*-methyl-D-asparate by conantokin-G: evidence for an allosteric interaction at polyamine sites. *J. Neurochem.*, **59**, 1516–21.

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