

MINI-REVIEW

Use of Toxins to Study Potassium Channels

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Abstract

Potassium channels comprise groups of diverse proteins which can be distinguished according to each member's biophysical properties. Some types of K^+ channels are blocked with high affinity by specific peptidyl toxins. Three toxins, charybdotoxin, iberiotoxin, and noxiustoxin, which display a high degree of homology in their primary amino acid sequences, have been purified to homogeneity from scorpion venom. While charybdotoxin and noxiustoxin are known to inhibit more than one class of channel (i.e., several Ca^{2+} -activated and voltage-dependent K^+ channels), iberiotoxin appears to be a selective blocker of the high-conductance, Ca^{2+} -activated K^+ channel that is present in muscle and neuroendocrine tissue. A distinct class of small-conductance Ca^{2+} -activated K^+ channel is blocked by two other toxins, apamin and leiurotoxin-1, that share no sequence homology with each other. A family of homologous toxins, the dendrotoxins, have been purified from venom of various related species of snakes. These toxins inhibit several inactivating voltage-dependent K^+ channels. Although molecular biology approaches have been employed to identify and characterize several species of voltage-gated K^+ channels, toxins directed against a particular channel can still be useful in defining the physiological role of that channel in a particular tissue. In addition, for those K^+ channels which are not yet successfully probed by molecular biology techniques, toxins can be used as biochemical tools with which to purify the target protein of interest.

Key Words: Potassium channels; venoms; charybdotoxin; noxiustoxin; iberiotoxin; apamin; leiurotoxin I; dendrotoxin; membrane vesicles; peptides.

Introduction

Ion channels are integral membrane proteins that perform the remarkable task of allowing transmembrane movement of ions through aqueous-pores

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with unusually high selectivity. Conformational changes within these proteins which control ion conduction are regulated by membrane potential and/or ligand binding. With the introduction of patch-clamp techniques, an extensive characterization of different ion channels has been accomplished. These studies provide insight into the biophysical properties of individual channel proteins, allowing their classification into different categories based on ion selectivity, single-channel conductance, kinetics of activation and inactivation, and dependence on membrane potential and/or ligand interactions. Although biophysical methods have furnished unique tools with which to study ion channels, our conceptual understanding of how these proteins function at the molecular level is limited. Nonetheless, some progress has been made in this area with the purification and genomic cloning of certain channel proteins. Purification of some channels (e.g., Na⁺ and Ca²⁺ channels, the acetylcholine receptor) has been made possible because specific high-affinity probes exist for the protein of interest. These ligands, which are either peptidyl toxins (e.g., as in the case with Na⁺ channels or the acetylcholine receptor) or small organic molecules (e.g., Ca²⁺ entry blockers that interact with L-type Ca²⁺ channels), have allowed not only purification to homogeneity and subsequent cloning of the target channel, but also the extensive biochemical characterization of these proteins. In addition, such ligands have been used to define the physiological and pharmacological role of specific channels in intact tissues.

K⁺ channels comprise a family of different proteins which are all selectively permeable to K⁺. Although well defined biophysically, the biochemical and pharmacological characterization of these channels is incomplete because of lack of appropriate high-affinity ligands. However, two important approaches employed during the last few years have started to provide information about the structure and function of several types of K⁺ channels. One such approach is the discovery of high-affinity toxins which interact with selected K⁺ channels. The other is the molecular cloning of one type of K⁺ channel, the product of the "Shaker" gene from *Drosophila*, followed by subsequent cloning of other K⁺ channels that share homology with Shaker through low-stringency hybridization techniques. Although the purpose of this review is to describe the properties of toxins that interact with K⁺ channels, it is obvious that the combined application of biochemical, molecular biological, and biophysical techniques is necessary to gain a better understanding of how K⁺ channels function.

Classes of Toxin-Sensitive Potassium Channels

As a first approximation, K⁺ channels can be subdivided into four general categories: (a) voltage-gated channels; (b) Ca²⁺-activated channels;

(c) receptor-coupled channels; and (d) ATP-sensitive channels. Some representatives of the first two channel types are blocked with high affinity by specific toxins. Although no toxin inhibitors have been identified for members of categories (c) and (d), antidiabetic sulfonylurea drugs are potent inhibitors of ATP-dependent K^+ channels present in pancreatic β -cells. They also inhibit similar channels found in cardiac and smooth muscle tissue, but with lower affinity. [3H]glybenclamide, a member of this class of drugs, has recently been used to identify and purify a protein from brain that may correspond to one subtype of these channels (Bernardi *et al.*, 1988). In the following sections, only those K^+ channels which are inhibited by specific toxins will be discussed.

Voltage-gated K^+ channels are a heterogeneous group of proteins that can be subdivided into noninactivating, or "delayed," and rapidly inactivating, or "transient," outward current carriers based on their biophysical properties (Hille, 1984). The delayed rectifier K^+ channel of squid axon was originally described by Hodgkin and Huxley (1952) in their studies on currents underlying the genesis of action potentials. In this work, the voltage-dependent K^+ channel was considered to be noninactivating. However, delayed rectifying channels differ greatly in their inactivation kinetics among various tissues, and are found in most nerve and muscle cells. Their primary function is to repolarize the cell membrane during the late phase of the action potential.

The transient, rapidly inactivating ("A-type") K^+ channel has been observed in many cell types (Rogawski, 1985). This channel rapidly activates and then becomes inactivated upon membrane depolarization. It is thought to regulate the firing frequency of neurons (Connor and Stevens, 1971). More information about this type of K^+ channel is available because of the successful cloning and functional expression of the *Drosophila* Shaker gene (Tempel *et al.*, 1987; Timpe *et al.*, 1988). Mutations at the Shaker locus affect rapidly inactivating K^+ currents in *Drosophila*, and mRNA transcribed from Shaker cDNA clones induces functional A-type K^+ channels when expressed in frog oocytes (Timpe *et al.*, 1988) or mammalian cells (Leonard *et al.*, 1989). The Shaker locus is a complex transcriptional unit producing transcripts with different 5' and/or 3' ends joined to a constant central region that shares homology with Na^+ (Noda *et al.*, 1986) and Ca^{2+} (Tanabe *et al.*, 1987) channels. This suggests that alternative splicing may be responsible for the multiplicity of A-type K^+ channels found (Papazian *et al.*, 1987; Kamb *et al.*, 1988; Pongs *et al.*, 1988; Schwarz *et al.*, 1988). Consequently, when different Shaker channel proteins are expressed in *Xenopus* oocytes, a variety of A-type currents are observed which differ in their kinetic properties (Iverson *et al.*, 1988; Timpe *et al.*, 1988). Using the Shaker gene, homologous genes have been identified in genomic libraries from mouse and rat using

low-stringency hybridization protocols (Tempel *et al.*, 1988; Christie *et al.*, 1989; McKinnon, 1989; Stuhmer *et al.*, 1989). The primary structures deduced for the mammalian proteins suggest a high degree of homology with those of *Drosophila*. Expression of these mammalian proteins in *Xenopus* oocytes yields voltage-activated K^+ channels with different voltage dependences, channel conductances, and pharmacological properties (Stuhmer *et al.*, 1989). One of these proteins, the product of the RCK1 gene, inactivates very slowly during a prolonged depolarization step, more closely resembling the classical delayed rectifier channel than the A-type channel. This finding suggests that there may be considerable structural similarity between these distinct classes of channel proteins. A related *Drosophila* K^+ channel gene, "Shab," also exhibits alternative splicing of a primary transcript, giving rise to two different proteins (Butler *et al.*, 1989). Recently, a novel K^+ channel gene was isolated by expression cloning from rat brain using *Xenopus* oocytes (Frech *et al.*, 1989). This gene, drK1, encodes a type of delayed rectifier K^+ channel, and structurally, it is more closely related to the *Drosophila* Shab gene than to the Shaker gene.

Ca^{2+} -activated K^+ channels ($P_{K,Ca}$) are found in a variety of electrically excitable and nonexcitable cells (Schwartz and Passow, 1983; Petersen and Maruyama, 1984; Blatz and Magleby, 1987). Channel conductance, calcium sensitivity, voltage dependence, and pharmacological properties have all been used to distinguish between different channels in this family. Since channel conductances range from a few to several hundred picosiemens (pS), $P_{K,Ca}$ channels have been subdivided according to this property.

High-conductance $P_{K,Ca}$, also known as BK or maxi-K channels because of their large conductance (130–300 pS), are the best studied $P_{K,Ca}$. These channels are widely distributed in neurons, striated and smooth muscle, endocrine and exocrine glands, kidney tubules, and the choroid plexus (Latorre *et al.*, 1989). They provide a pathway for cell repolarization subsequent to membrane depolarization. Consequently, they have been implicated in regulation of neuro-endocrine secretion, in control of muscle contractility, and in a variety of other cellular processes, such as K^+ secretion by the cortical collecting tubule of kidney. $P_{K,Ca}$ are activated by both cytoplasmic Ca^{2+} and depolarizing voltage. In the absence of other divalent cations, binding of at least 2–4 Ca^{2+} are required for channel opening. However, in the presence of millimolar internal Mg^{2+} , the Hill coefficient for Ca^{2+} increases to 6 with an increase in the apparent affinity of the channel for this ion. Increasing concentrations of internal Ca^{2+} cause a shift in the voltage dependence of activation of $P_{K,Ca}$ along the voltage axis toward more negative potentials. A paradoxical property of these channels is their large conductance and high cation selectivity. High conductance can be explained by postulating that the channel is a multi-ion pore which contains fixed negative

charges in its mouth. These channels have recently been a major focus of attention since they are blocked by low nanomolar concentrations of the scorpion toxin, charybdotoxin (ChTX), making this peptide a useful tool with which to study the biochemistry, pharmacology, and physiology of $P_{K,Ca}$.

Small-conductance Ca^{2+} -activated K^+ channels, also known as SK channels, are in general very sensitive to internal Ca^{2+} , and can be divided into two different categories: voltage-dependent SK channels, such as those present in *Aplysia* and *Helix* neurons, and voltage-independent channels that are found in muscle, olfactory neurons, and erythrocytes. The SK channel in rat skeletal muscle displays a conductance of 12 pS and possesses the properties necessary to account for the after-hyperpolarization in myotubes (Blatz and Magleby, 1986). This channel is blocked by low concentrations of the bee venom peptide, apamin. Although apamin does not block high-conductance $P_{K,Ca}$, ChTX cross-reacts with some SK channels. For example, ChTX inhibits the 35-pS SK channel present in *Aplysia* neurons (Hermann and Erxleben, 1987), and Ca^{2+} -activated K^+ fluxes in red blood cells, most likely by affecting the 40-pS SK channel (Wolff *et al.*, 1988). However, neither of these channels are sensitive to apamin.

Unlike voltage-gated K^+ channels, limited structural information is available for $P_{K,Ca}$ channels. This may suggest that although some pharmacological characteristics are similar for both classes of channels, significant differences in primary structure must exist. If molecular biology approaches are not successful in clarifying this issue, then biochemical techniques must be used to purify these proteins to homogeneity, and to define their molecular properties.

Charybdotoxin, Iberitoxin and Noxiustoxin: Three Homologous Peptides Directed against K^+ Channels

Charybdotoxin

Venom of the scorpion *Leiurus quinquestriatus* has been used in biochemical studies to probe Na^+ channels since it contains several peptide toxins that modify the gating kinetics of these channels (Caterall, 1980). It has recently been noted that this venom is also a rich source of peptide toxins which inhibit the activity of several different types of K^+ channels. The first K^+ channel toxin identified in *Leiurus quinquestriatus* var *hebraeus* venom, and perhaps the most extensively studied member of this series, was named charybdotoxin by Miller *et al.* (1985). This group found that crude scorpion venom reversibly blocked the large-conductance $P_{K,Ca}$ present in mammalian skeletal muscle when added to the membrane surface opposite from that at which Ca^{2+} activates the channel (i.e., at the external side). ChTX block of

channel activity is characterized by silent periods, during which time no activity is observed, separated by "bursts" of typical channel activity. Within such a burst, channel gating kinetics, as well as the unitary conductance, are identical to those observed under control conditions. Silent periods of activity have been interpreted as time intervals during which a molecule of toxin is bound to the channel and ion conduction is blocked. Since binding of this toxin is a reversible reaction, when toxin dissociates from the channel, normal behavior is observed because kinetics of gating are faster than those of toxin association. ChTX reversibly binds to the channel through a bimolecular reaction as demonstrated by the fact that average blocked time is independent of toxin concentration, while average time in the active state is inversely proportional to toxin concentration (Smith *et al.*, 1986).

Purification of ChTX to homogeneity has been achieved in a number of different laboratories using combinations of ion-exchange and reversed-phase chromatographies (Gimenez-Gallego *et al.*, 1988; Valdivia *et al.*, 1988; Luchesi *et al.*, 1989; Strong *et al.*, 1989; Schweitz *et al.*, 1989a). The complete amino acid sequence of ChTX has also been elucidated (Gimenez-Gallego *et al.*, 1988), and later confirmed (Luchesi *et al.*, 1989; Strong *et al.*, 1989; Schweitz *et al.*, 1989a). ChTX is a 37 amino acid peptide that contains six cysteine residues (Fig. 1). Its N-terminal amino group is blocked in the form of pyroglutamine, but treatment of toxin with pyroglutamate aminopeptidase cleaves this residue allowing the unblocked peptide to be sequenced by automated Edman degradation. A striking characteristic of ChTX is its high content of positively charged residues (i.e., 4 Lys, 3 Arg, and His), which gives this peptide a net charge of +5. These residues play an important role in the mechanism by which the toxin blocks K^+ channel activity (see below). A partial sequence of ChTX corresponding to the carboxyl-terminal portion of the peptide has assigned one more residue, a Ser, at the C-terminus (Valdivia *et al.*, 1988). In this investigation, ChTX was purified from venom of *Leiurus quinquestriatus* var *quinquestriatus*, whereas *Leiurus quinquestriatus* var *hebraeus* venom was used for the other studies. This difference could account for the small discrepancy observed at the C-terminus of the peptides.

The chemical synthesis of ChTX has been accomplished using solid-phase Fmoc pentafluorophenyl ester methodology (Sugg *et al.*, 1990a). The

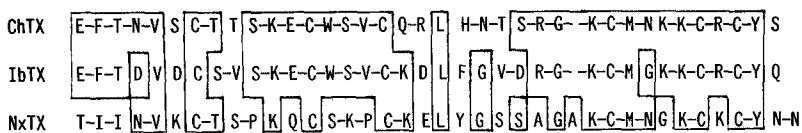


Fig. 1. Amino acid sequences of charbydotoxin (ChTX), iberiotoxin (IbTX), and noxiustoxin (NxTX). Standard one-letter notation is used to depict the amino acids. Homologies are highlighted in boxes.

reduced synthetic peptide can be dioxidized in a facile manner to yield biologically active material which is indistinguishable from native toxin. Enzymatic digestion of either native or synthetic oxidized ChTX results in identical peptide fragmentation patterns. When the amino acid content of these fragments are analyzed, data from both species are equivalent, indicating that both are folded in an identical fashion. Analysis of these peptides allows the assignment of the disulfide bonds in ChTX as: Cys⁷-Cys²⁸, Cys¹³-Cys³³, and Cys¹⁷-Cys³⁵. This disulfide pattern differs from one previously postulated by Gimenez-Gallego *et al.* (1988) that was based on sequence homologies between ChTX and α -bungarotoxin, whose three-dimensional X-ray structure is known. The current results predict a model for ChTX in which the C-terminus is buried within the molecule. This is consistent with the poor reactivity of the carboxyl group at the C-terminal residue of ChTX, and also with the difficulties encountered in iodinating Tyr³⁶, the residue penultimate from the C-terminus.

Since the discovery and purification of ChTX, a number of investigators have studied the effects of this toxin on different types of K⁺ channels. It is well known that ChTX does not affect Na⁺ or Ca²⁺ channels (Miller *et al.*, 1985; Hermann and Erxleben, 1987; Gimenez-Gallego *et al.*, 1988; Sands *et al.*, 1989). However, its selectivity as an inhibitor of high-conductance P_{K,Ca} has been questioned (Schweitz *et al.*, 1989b), and there is accumulating evidence that ChTX blocks several different types of K⁺ channels (see below). Nevertheless, the ability of ChTX to block P_{K,Ca} in different tissues has been widely explored. This toxin inhibits with nanomolar potency the high-conductance channel in skeletal muscle (Anderson *et al.*, 1988; Valdivia *et al.*, 1988), cultured kidney epithelia (Guggino *et al.*, 1987), rat anterior pituitary (GH₃) cells (Gimenez-Gallego *et al.*, 1988), bovine aortic smooth muscle (Vazquez *et al.*, 1989), and rat brain (Reinhart *et al.*, 1989). In this last study, four types of P_{K,Ca} were found after incorporation of synaptic plasma membrane vesicles into planar lipid bilayers. These exhibit unitary conductances of 242, 236, 135, and 76 pS in symmetrical KCl media. One of these channels, with a conductance of 236 pS, is unique in that it is not blocked by ChTX, and it also displays slow gating kinetics. At present, this channel is the only high-conductance P_{K,Ca} that is not sensitive to inhibition by ChTX. In addition, the toxin has been used to study the pharmacological properties of high-conductance P_{K,Ca} in vascular (Winqvist *et al.*, 1989) and airway (Jones *et al.*, 1990) smooth muscle. In portal vein, ChTX causes contraction by blocking a repolarization pathway in this spontaneously firing tissue. In trachea, basal tone is increased, perhaps because P_{K,Ca} controls resting membrane potential in airway smooth muscle.

The mechanism by which ChTX blocks P_{K,Ca} has been the subject of detailed studies using channels incorporated into planar lipid bilayers from

skeletal muscle t-tubule membranes (Anderson *et al.*, 1988; MacKinnon and Miller, 1988). By monitoring ChTX block of channel activity to determine the association and dissociation rates of toxin, it was noted that the interaction of ChTX with the channel protein senses voltage, as well as the channel's conformational state. ChTX can bind to both open and closed $P_{K,Ca}$, but the affinity for the open state is slightly higher. This is a consequence of a 7-fold faster association rate of toxin for the open channel. The dissociation rate of ChTX is the same for both channel conformations. Membrane depolarization enhances the dissociation rate e -fold per 28 mV, but toxin association rates are voltage-independent if the channel's open probability is maintained constant as voltage is varied. It was also found that increasing ionic strength of the external medium by varying salt concentration from 20 to 300 mM reduces the association rate of toxin by two orders of magnitude, with little effect on dissociation kinetics. These data suggest that a region of negatively charged residues exist near the ChTX receptor in the channel. The observed voltage dependence of ChTX binding appears related to voltage effects on K^+ entering from the internal medium, and binding at a site located along the ion conduction pathway to destabilize ChTX at its receptor, rather than direct action of voltage on the toxin. As internal K^+ is raised, the dissociation rate of ChTX increases, and as the membrane is depolarized, internal K^+ more effectively accelerates toxin dissociation. Rb^+ , another permeant ion, also accelerates toxin dissociation when applied at the inner surface of the channel, while impermeant ions such as Li^+ , Na^+ , Cs^+ , and arginine do not. These results suggest that ChTX binds at the external pore of the channel and blocks ion conduction by direct occlusion.

Support for this conclusion also comes from experiments in which the effect of externally applied tetraethylammonium ion (TEA) was studied on ChTX blocking kinetics (Miller, 1988). TEA affects ChTX on-rates in exact proportion to its blocking of single-channel currents, but has no effect on the off-rate of toxin, suggesting that TEA and ChTX interact at a common site on the channel. Since the TEA site is known to be located on the external side of the conduction pathway (Villarroel *et al.*, 1988), these results support the hypothesis that the ChTX receptor is in the exterior mouth of the channel. The idea that negatively charged residues in this region influence ChTX binding was further explored in experiments where residues at the external pore were chemically modified with the carboxyl-group selective reagent trimethyloxonium ion (TMO). Alkylation of the channel by this agent lowers single-channel conductance, shifts the voltage activation curve to more depolarized potentials, and greatly reduces the affinity of ChTX (MacKinnon and Miller, 1989a). ChTX bound to the channel during exposure to the methylating reagent prevents TMO-induced reduction of single-channel conductance. Moreover, TMO modification not only reduces the association rate

of ChTX, but also greatly reduces its ionic strength dependence (MacKinnon *et al.*, 1989). This suggests that surface charges around the pore may cause a locally increased concentration of toxin.

In addition to high-conductance Ca^{2+} -activated K^+ channels, other $\text{P}_{\text{K,Ca}}$ have been shown to be inhibitable by ChTX. In neurons from *Aplysia californica*, ChTX has no effect on a transient K^+ channel, or a delayed rectifying K^+ channel, but it inhibits a 35-pS $\text{P}_{\text{K,Ca}}$ with a K_i of 30 nM at $V_h = -30$ mV (Hermann and Erxleben, 1987). In this system, block by ChTX is voltage-dependent, with the K_i increasing e -fold for 50–70 mV of depolarization, indicating that the toxin is sensitive to ca. 35% of the membrane electric field. ChTX also suppresses a membrane leakage conductance, and a resting K^+ conductance activated by internal Ca^{2+} , in this preparation. In electrically active pacemaker cells from *Aplysia*, ChTX increases the number of action potentials per burst, and prolongs the duration of the last action potential in the burst, suggesting that $\text{P}_{\text{K,Ca}}$ contributes to repolarization during firing.

In bullfrog ganglion B neurons, ChTX prolongs action potential duration, consistent with blockade of the voltage- and calcium-dependent K^+ channels known to control membrane repolarization in these cells (Goh and Pennefather, 1987). In addition, ChTX also reduces an apamin-sensitive, Ca^{2+} -dependent, after-hyperpolarizing K^+ current. However, in a later study it was shown that inhibition of this apamin-sensitive channel was not due to ChTX, but to contamination of the ChTX preparation used by another peptide present in *Leiurus quinquestriatus hebraeus* venom which specifically blocks that channel (Chicchi *et al.*, 1988; Pennefather *et al.*, 1989). These findings bespeak the importance of ascertaining whether a given toxin preparation is homogeneous. Several laboratories have reported that crude *Leiurus quinquestriatus* venom, as well as purified ChTX, inhibit Ca^{2+} -activated K^+ efflux from human erythrocytes mediated by the “Gardos” channel (Abia *et al.*, 1986; Castle and Strong, 1986; Wolff *et al.*, 1988; Lucchesi *et al.*, 1989). In the latter investigation, ChTX blocked Ca^{2+} -activated K^+ efflux in a partial fashion (ca. 70%), and displayed potent inhibitory activity (IC_{50} of 0.8 nM).

Voltage-activated K^+ channels are the major ion conduction pathway present in human T lymphocytes, murine T lymphocytes, thymocytes, and related cell lines (Lewis and Cahalan, 1988a). In murine lymphoid cells, three distinct types of K^+ channels have been characterized. These channels differ in their voltage dependences, inactivation and closing kinetics, and sensitivity to inhibition by agents such as TEA. The most commonly found channel in either human or murine T cells has been termed n type (Cahalan *et al.*, 1985; Deutsch *et al.*, 1986; DeCoursey *et al.*, 1987a; Lewis and Cahalan, 1988b). A second class of K^+ channel, called n' because of similar but not identical properties to n type channels, is found in murine thymocytes (Lewis and

Cahalan, 1988a,b). A third category of K^+ channel, i-type, is present in abnormally proliferating T cells from mice homozygous for the *lpr* mutation (Chandy *et al.*, 1986; DeCoursey *et al.*, 1987a; Lewis and Cahalan, 1988b). None of these channels are activated by intracellular Ca^{2+} . However, ChTX blocks both *n* and *n'* types of K^+ channels in human and murine T lymphocytes. In human quiescent peripheral blood T cells of both the helper ($CD4^+$) and suppressor ($CD8^+$) phenotype, ChTX blocks voltage-dependent K^+ channels with a K_d of ca. 0.3 nM (Price *et al.*, 1988). A similar kind of K^+ channel is present in the Jurkat human leukemia cell line. In this preparation, ChTX reversibly inhibits K^+ currents with a K_i of ca. 1 nM (Sands *et al.*, 1989). Calculated rate constants for ChTX association and dissociation are $5.6 \times 10^7 M^{-1} s^{-1}$ and $0.1 s^{-1}$, respectively. In murine thymocytes, *n* type channels are also blocked by ChTX, and similar sensitivities to toxin appear characteristic of these channels in double negative, double positive, and helper phenotypes, as well as in rat thymocytes. In lymphocytes, ChTX can bind to either closed or open channels, and at comparable ionic strengths, toxin association and dissociation kinetics are more rapid for the lymphocyte K^+ channel than for $P_{K,Ca}$. In analogy with the profile observed for high-conductance $P_{K,Ca}$ block, inhibition of lymphocyte K^+ channels by ChTX is sensitive to the ionic strength of the medium (Price *et al.*, 1989). However, cleavage of the two N-terminal amino acids by α -chymotrypsin does not alter toxin affinity for the lymphocyte channel, but abolishes its activity as an inhibitor of $P_{K,Ca}$ from skeletal muscle.

Because ChTX inhibits the T lymphocyte K^+ channel at low concentrations, it is a useful tool with which to investigate the physiological role of these channels. Two lines of evidence suggest that T cell K^+ channels are involved in controlling proliferation, as well as underlie volume regulation. First, an increased current density precedes DNA synthesis following mitogen stimulation (Deutsch *et al.*, 1986, Lee *et al.*, 1986, DeCoursey *et al.*, 1987b), and K^+ channel-dependent volume regulation correlates with changes in K^+ conductance (Lee *et al.*, 1988). Second, agents that inhibit K^+ conductance also inhibit volume regulation and cell proliferation (Chandy *et al.*, 1984; Deutsch *et al.*, 1986; Lee *et al.*, 1986, 1988). ChTX inhibits volume regulation in T lymphocytes (Grinstein *et al.*, 1988), and, in addition, blocks lymphocyte mitogenesis (Price *et al.*, 1989). In this system, toxin inhibits mitogen- and antigen-stimulated proliferation with a K_i of 0.5 nM. Inhibition of mitogenesis can be overcome selectively by addition of exogenous interleukin 2. Endogenous levels of interleukin 2 in culture supernatants of stimulated cells are decreased in the presence of ChTX, suggesting that the voltage-gated K^+ channel is somehow involved in interleukin 2 synthesis and/or secretion. In an independent report, ChTX was also shown to block phytohemagglutinin-induced activation of human peripheral blood lymphocytes (Bono *et al.*, 1989).

Perhaps, one of the most striking findings for ChTX is the observation that Shaker K^+ channels, expressed in either *Xenopus* oocytes or mammalian cells, are sensitive to inhibition by toxin (MacKinnon *et al.*, 1988; Leonard *et al.*, 1989; Zagotta *et al.*, 1989). Channels with biophysical properties similar to the Shaker channel are present in several other preparations, but are not inhibited by ChTX (Gimenez-Gallego *et al.*, 1988). In addition, ChTX does not block the A-type K^+ channel in *Drosophila* (Zagotta *et al.*, 1989). One possibility to account for these findings, given that all preparations of ChTX tested are homogeneous, is that different post-translational processing mechanisms exist between *Drosophila* and vertebrate cells. In *Xenopus* oocytes injected with the *Drosophila* Shaker clone H4, transient K^+ currents are expressed which are reversibly suppressed by ChTX with a K_i of 3.6 nM (MacKinnon *et al.*, 1988). Toxin does not alter the time course of K^+ channel activation or inactivation, and it binds to the closed channel since full block is observed within the first few milliseconds of the activating pulse. In addition, the effect of ionic strength on inhibition of Shaker H4 channels is identical to that seen for the large-conductance $P_{K,Ca}$, suggesting that both channels contain negatively charged residues at or close to the ChTX receptor.

The primary amino acid sequence of proteins coded by the Shaker gene is known (Pongs *et al.*, 1988; Schwarz *et al.*, 1988), and a model for the secondary structure of this class of K^+ channel has been proposed based on homologies with the voltage-dependent Na^+ channel. (Catterall, 1988). With this model in hand and the finding that interaction between ChTX and the channel relies strongly on electrostatic attraction, site-directed mutagenesis has been employed to search for residues involved in ChTX binding (MacKinnon and Miller, 1989b). Using this strategy, acidic residues located in putative external loops were replaced with neutral amino acids, and the effects of these alterations on ChTX block of K^+ currents was determined. Four mutations in the first two outer loops have no effect on ChTX inhibition. However, a residue in the third external loop, Glu⁴²², is associated with a dramatic effect on ChTX action when changed to a neutral Gln or a positive Lys. Although these mutations have no effect on the characteristics of the K^+ current, ChTX blocking activity is lowered by a factor of 3.5 or 12, depending on whether Gln or Lys is substituted at position 422. Replacement of Glu⁴²² by Asp had no effect on toxin binding. These results suggest that Glu⁴²² plays a role in the electrostatic interaction between toxin and channel, although it is not part of the ChTX receptor, but rather is presumably located at a distance of 17 Å from the putative binding site.

With the use of molecular biological techniques, several cDNA clones have been isolated from rat brain which code for a variety of K^+ channels when examined in an expression system (Baumann *et al.*, 1988; Stuhmer *et al.*, 1988; Tempel *et al.*, 1988; Christie *et al.*, 1989; Frech *et al.*, 1989;

McKinnon, 1989; Stuhmer *et al.*, 1989; Koren *et al.*, 1990). These cDNA clones have been identified by either cross-hybridization with probes based on the *Drosophila* Shaker gene, or by expression cloning in *Xenopus* oocytes. DrK1 codes for a selective K⁺ channel with properties of a delayed rectifier, and is not sensitive to ChTX inhibition (Frech *et al.*, 1989). Four other cDNA clones (RCK1, RCK3, RCK4, and RCK5) express K⁺ channels in *Xenopus* oocytes with dissimilar biophysical and pharmacological properties. Interestingly, two of these clones, RCK3 and RCK5, display high-affinity block by ChTX, while RCK1 is less sensitive, and RCK4 has little sensitivity to toxin at all (Stuhmer *et al.*, 1989). A comparison of the sequences of these four clones suggests that the number of acidic amino acid residues in the S3–S4 bend region may correlate with the affinity of the RCK channels for ChTX. Although sequences in this region are quite variable among RCK proteins, the ChTX insensitivity of RCK4 channels could be due to the replacement of acidic amino acids by glutamine residues immediately adjacent to the carboxy-terminal end of proposed transmembrane segment S3. A clone identical to RCK1, RBK1, has been isolated from rat hippocampus and shown to express a K⁺ channel that is also sensitive to ChTX (Christie *et al.*, 1989).

The finding that some of the expressed rat brain K⁺ channels are ChTX-sensitive is not surprising since there is biochemical precedence for this observation. Using ⁸⁶Rb⁺ fluxes from rat brain synaptosomes as an assay of K⁺ channel activity, it was shown that ChTX blocks both Ca²⁺-activated and Ca²⁺-independent, voltage-gated K⁺ channels (Schneider *et al.*, 1989; Sorensen *et al.*, 1989). The voltage-gated K⁺ channel in this preparation is a rapidly inactivating, A-type, K⁺ channel that is sensitive to inhibition by α -dendrotoxin (α Da-TX, see below); Blaustein *et al.*, 1988). By direct electrophysiological measurements, it has also been shown that ChTX blocks a voltage-activated K⁺ channel in rat dorsal root ganglion cells, which is Ca²⁺-insensitive (Schweitz *et al.*, 1989b), but α -DaTX-sensitive. Moreover, binding studies employing the neuronal K⁺ channel inhibitors [¹²⁵I] α DaTX, [¹²⁵I] β -dendrotoxin, and [¹²⁵I]-mast cell degranulating peptide have identified high-affinity receptors in brain for each of these agents, the binding of which is inhibited by ChTX through allosteric mechanisms (Sorensen *et al.*, 1988, 1989; Schweitz *et al.*, 1989a, b).

In summary, ChTX is not a selective agent because of its cross-reactivity with several different types of K⁺ channels. Given this situation, can ChTX still be useful as a probe of specific K⁺ channels? Traditionally, selective high-affinity probes have been required to develop the biochemistry of ion channels and aid in their purification. Using this approach, voltage-dependent Na⁺ and Ca²⁺ channels have been purified, and partial sequence information obtained from these preparations were useful in constructing cDNA probes that allowed cloning of the full-length protein. In the K⁺ channel field,

molecular biology approaches seem to have yielded faster results than traditional biochemical methodologies. However, not all K^+ channels of interest have been identified using cloning strategies. One such channel is the high conductance $P_{K,Ca}$. The fact that low-stringency hybridization protocols using dissimilar K^+ channel cDNA probes have failed to detect this channel suggests that its primary sequence is unique, despite sharing some common properties with other K^+ channels, such as sensitivity to ChTX. Therefore, a more successful approach might be to use ChTX to purify this class of K^+ channels to homogeneity, and then use sequence information obtained from the pure protein to clone and express $P_{K,Ca}$.

ChTX contains a single Tyr residue penultimate from its C-terminus that is a potential site for labelling of the molecule to high specific activity with ^{125}I . Using Iodo-Gen or lactoperoxidase/glucose oxidase protocols, ChTX can be iodinated, and the resulting species separated by reversed-phase chromatography (Luchessi *et al.*, 1989; Vazquez *et al.*, 1989). In one study employing Iodo-Gen methodology, monoiodinated toxin was prepared and characterized (Vazquez *et al.*, 1989). Sequence analysis confirms that a single iodine is incorporated into the Tyr residue of the peptide. Monoiodotyrosine-ChTX ($[^{127}I]$ ChTX) reversibly blocks high-conductance $P_{K,Ca}$ in isolated outside-out patches of cultured bovine aortic smooth muscle cells by a mechanism identical to that of native toxin. However, the potency of $[^{127}I]$ ChTX is reduced ca. 10-fold when compared to native toxin, and block only occurs when the patch is bathed on low ionic strength media. Although these characteristics could make it difficult to study toxin binding in intact cells, because of ionic strength considerations, they do not prevent binding studies using isolated membrane vesicles. Smooth muscle is a rich source of $P_{K,Ca}$, and highly purified preparations of sarcolemmal membrane vesicles can be derived from either bovine aortic (Slaughter *et al.*, 1989) or tracheal smooth muscle (Slaughter *et al.*, 1987). These preparations are clearly of sarcolemmal origin, and ChTX-sensitive $P_{K,Ca}$ have been observed after incorporation of membranes into planar lipid bilayers. When bovine aortic sarcolemmal vesicles are incubated with $[^{125}I]$ ChTX, there is time- and concentration-dependent association of toxin. Saturation experiments indicate that $[^{125}I]$ ChTX binds to a single class of sites which display a K_d of 100 pM and a B_{max} of 0.5 pmol/mg protein, under defined conditions (Vazquez *et al.*, 1989). The B_{max} of detergent-permeabilized vesicles is slightly higher (Fig. 2). Toxin binding is a freely reversible bimolecular reaction (Fig. 3), and displays characteristics similar to those found for the functional interaction of ChTX with the channel. Thus, binding is highly sensitive to ionic strength, and is blocked by a number of metal ions that are known to bind with high affinity to sites along the ion conduction pathway of the channel (Fig. 4). In addition, TEA blocks ChTX binding by an apparently competitive

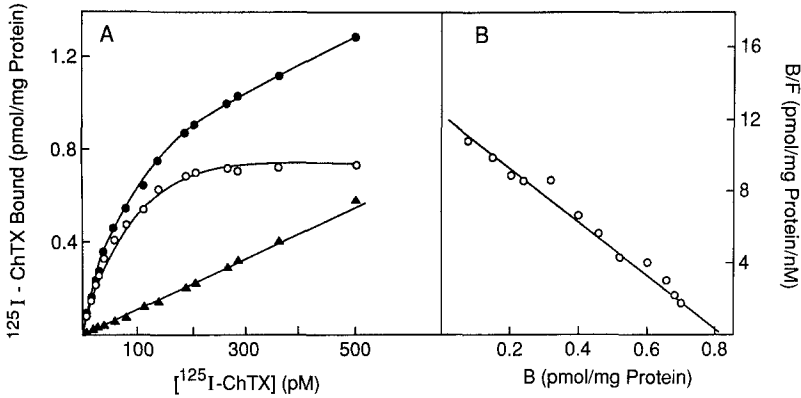


Fig. 2. Binding of $[^{125}\text{I}]\text{ChTX}$ to bovine aortic sarcolemmal membrane vesicles. (A) Sarcolemmal membrane vesicles were incubated with increasing concentrations of $[^{125}\text{I}]\text{ChTX}$ in a medium consisting of 20 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin, and 0.1% digitonin. Incubations were carried out at room temperature for 60 min. At the end of the incubation period, samples were diluted with 4 ml of ice-cold 100 mM NaCl and 20 mM Hepes-Tris, pH 7.4, filtered through GF/C glass fiber filters which have been presoaked in 0.3% polyethylenimine, and washed twice with ice-cold media. Total binding (\bullet); nonspecific binding determined in the presence of 10 nM ChTX (\blacktriangle); specific binding defined as the difference between total and nonspecific binding (\circ). (B) Specific binding data from (A) is presented in the form of a Scatchard representation. $K_d = 68$ pM; $B_{\text{max}} = 0.81$ pmol/mg protein.

mechanism with a K_i of $150 \mu\text{M}$, a value identical to that necessary for inhibition of $P_{\text{K,Ca}}$. The effect of TEA is specific since other quaternary ammonium ions, such as tetrabutylammonium ion, have no effect on toxin binding at concentrations up to 5 mM. Therefore, binding sites identified for $[^{125}\text{I}]\text{ChTX}$ in this preparation display the properties expected for an interaction with $P_{\text{K,Ca}}$. An identical class of high-affinity receptors has been discovered in purified sarcolemmal preparations derived from bovine tracheal smooth muscle (Slaughter *et al.*, 1988). In addition, high-affinity ChTX binding sites with slightly different properties are found in membranes made from porcine uterine smooth muscle (E. Stefani and M. L. Garcia, unpublished data) and rabbit skeletal muscle t-tubule membranes (S. Fleischer and M. L. Garcia, unpublished data).

$[^{125}\text{I}]\text{ChTX}$ binding sites from aortic sarcolemma have been solubilized in functional form using digitonin, and partially purified through a combination of conventional chromatographic techniques (Vazquez *et al.*, 1988). Purification of the ChTX receptor to homogeneity is necessary before protein sequence information can be obtained for constructing cDNA probes to clone the protein. In addition, the purified receptor must be incorporated into artificial bilayers to demonstrate whether or not it constitutes the basic components necessary to form a functional K^+ channel. Although protein

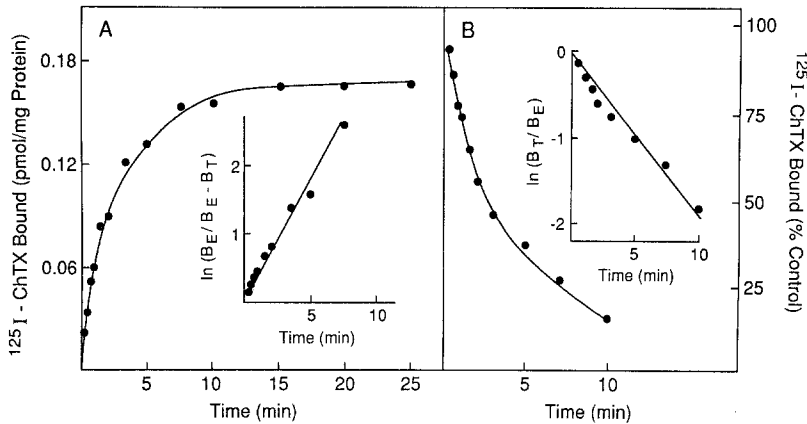


Fig. 3. Association and dissociation kinetics of [125 I]ChTX in bovine aortic sarcolemmal membrane vesicles. (A) Association kinetics. Sarcolemmal membrane vesicles were incubated with 30-pM [125 I]ChTX for different periods of time at room temperature in the same media as in Fig. 2. Inset: semilogarithmic representation of the pseudo-first order association reaction. $k_{\text{obs}} = 0.38 \text{ min}^{-1}$; k_1 calculated according to the equation $k_1 = k_{\text{obs}} \times (B_{\text{eq}}/[L] \times B_{\text{max}}) = 4.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. (B) Dissociation kinetics. After incubating with [125 I]ChTX for 60 min, dissociation of bound toxin was initiated by addition of 10 nM ChTX. Inset: semilogarithmic representation of the first-order dissociation reaction: $k_{-1} = 0.2 \text{ min}^{-1}$; $K_d = k_{-1}/k_1 = 76 \text{ pM}$.

purification, especially that of channel proteins, is a difficult task given the small quantities of protein present in the starting material, recent progress in our laboratory indicates that a nearly homogeneous receptor preparation can be obtained on a routine basis. This receptor preparation displays all the properties that are found for interaction of toxin with intact membranes, and, therefore, is believed to represent the high-conductance $P_{\text{K,Ca}}$, since this is the only K^+ channel in bovine aortic smooth muscle cells that is sensitive to ChTX (J. Reuben and M. L. Garcia, unpublished data).

A distinct class of high-affinity ChTX receptor which displays much different biochemical and pharmacological properties has been identified in rat brain synaptic plasma membrane vesicles (Garcia *et al.*, 1988; Schweitz *et al.*, 1989a; Vazquez *et al.*, 1990), as well as in intact human T-lymphocytes (Deutsch *et al.*, 1990; R. Slaughter and S. Lin, personal communication), Jurkat leukemia cells, and plasma membranes derived from this cell line (R. Slaughter and S. Lin, personal communication). In human T-lymphocytes, [125 I]ChTX binds to a single class of sites which display a density that correlates closely with the number of ChTX-sensitive, voltage-dependent K^+ channels measured in that preparation by voltage-clamp protocols. Other properties of the binding reaction (e.g., ionic strength and metal ion dependences, sensitivity to noxiustoxin and α -DaTX, insensitivity to iberiotoxin, see below) suggest that the binding sites for [125 I]ChTX in human T-lymphocytes

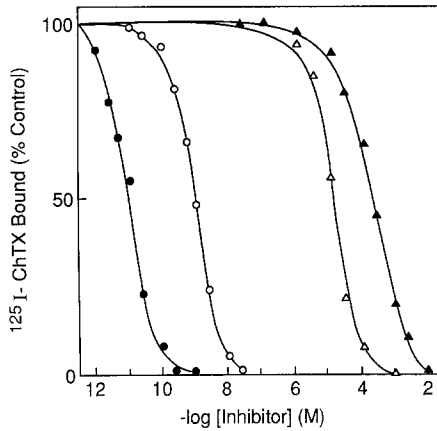


Fig. 4 Effect of $P_{K,Ca}$ modulators on [125 I]ChTX binding to bovine aortic sarcolemmal membrane vesicles. Sarcolemmal membrane vesicles were incubated with 30 pM [125 I]ChTX in the absence or presence of increasing concentrations of either ChTX (●), IbTX (○), BaCl₂ (△), or TEA (▲) at room temperature until equilibrium was achieved. Inhibition of binding was assessed relative to an untreated control.

represent the *n* type voltage-dependent K^+ channel. Similarly, the ChTX site in rat brain appears to be functionally associated with an RCK3 or RCK5-like protein.

Therefore, by using [125 I]ChTX and appropriate vesicle systems, high-affinity binding sites coupled to single types of ChTX-sensitive K^+ channels can be studied in isolation. Although our laboratory has been successful in preparing radiolabelled toxin that is biologically active, reports from other laboratories have indicated problems in accomplishing this. In work by Luchessi *et al.* (1989), the monoiodotyrosine derivative of ChTX was prepared and analyzed for its ability to inhibit high-conductance $P_{K,Ca}$ of skeletal muscle t-tubule membranes incorporated into lipid bilayers. It was found that iodinated ChTX was ca. 10^3 -fold weaker than native ChTX as a blocker of $P_{K,Ca}$, which could be accounted for by alterations in both the association and dissociation rate constant of modified toxin. Binding studies with [125 I]ChTX indicate the presence of a low-affinity site which does not correspond with $P_{K,Ca}$ monitored in bilayers. Another laboratory also reported failure in making a biologically active derivative of ChTX (Harvey *et al.*, 1989), while yet another group has made iodinated toxin that has diminished potency in binding experiments (Schewitz *et al.*, 1989a). The reason for such discrepancies is presently unclear.

Iberiotoxin

The observation that ChTX is not a selective K^+ channel probe has spurred research efforts to find a specific modulator of $P_{K,Ca}$. Using [125 I]ChTX

binding to bovine aortic sarcolemma, a variety of scorpion venoms were tested for their ability to modulate the binding reaction. Several scorpion venoms were noted to possess inhibitory activity, and venom of the scorpion *Buthus tamulus* was further fractionated in order to isolate and identify its active component(s) (Galvez *et al.*, 1990). When an extract of crude venom is loaded onto a MonoS cation-exchange FPLC column equilibrated at pH 9.0, and then eluted with a linear gradient of NaCl, ChTX binding inhibitory activity is found in two well-separated fractions. Further fractionation of the less basic material by reversed-phase chromatography leads to the purification of a homogeneous peptide that upon SDS-PAGE migrates as a 4.3-kDa protein. This peptide has been named iberitoxin (IbTX), and determination of its primary sequence reveals 70% homology with ChTX (Galvez *et al.*, 1990; Fig. 1). Like ChTX, the N-terminus of IbTX is blocked in the form of a pyroglutamic acid residue, and it also contains 6 Cys. IbTX, however, is a much less basic peptide than ChTX since it contains four more acidic amino acids. In single-channel recordings of $P_{K,Ca}$ incorporated into planar lipid bilayers from either bovine aortic sarcolemmal membranes (O. McManus, unpublished data) or rabbit skeletal muscle t-tubules membranes (R. Latorre, unpublished data), IbTX reversibly inhibits channel activity by causing much longer silent periods than those observed in the presence of ChTX. However, IbTX is a noncompetitive inhibitor of [125 I]ChTX binding in smooth muscle, suggesting that it interacts at a unique site on the channel.

Perhaps the most interesting feature of IbTX is its high selectivity for $P_{K,Ca}$. In voltage-clamp experiments using GH₃ cells, IbTX affects neither Na⁺, Ca²⁺, nor A-type K⁺ currents, although it completely abolishes currents due to $P_{K,Ca}$. Other voltage-dependent, ChTX-sensitive K⁺ channels are also unaffected by IbTX. These include *n*-type K⁺ channels in human T-lymphocytes, and channels expressed in *Xenopus* oocytes after injecting Shaker H4 or other brain K⁺ channel clones. In addition, IbTX has no effect whatsoever on [125 I]ChTX binding to either brain synaptic membrane vesicles (Vazquez *et al.*, 1990), human T-lymphocytes, Jurkat cells, or Jurkat plasma membrane vesicles (R. Slaughter, unpublished data), consistent with the observation that ChTX binding in these systems is associated with a voltage-dependent, but IbTX-insensitive, K⁺ channel. To date, the high-conductance $P_{K,Ca}$ is the only target sensitive to this toxin, making IbTX a unique tool with which to investigate the physiological role of $P_{K,Ca}$ in different tissues. Unfortunately, attempts to radiolabel IbTX have yielded adducts that display no biological activity. Since IbTX has been synthesized by solid-phase peptide chemistry and oxidized to produce material identical to native toxin (Sugg *et al.*, 1990b), a synthetic approach may be more appropriate for obtaining a radiolabelled derivative of the toxin in biologically active form. It is noteworthy that *n*-type K⁺ channels in Jurkat cells are blocked by crude

Buthus tamulus venom (Sands *et al.*, 1989). However, the inhibitory activity of this K^+ channel is associated with the second more basic fraction from ion-exchange chromatography that blocks ChTX binding in smooth muscle. Further fractionation of this material by reversed-phase chromatography has led to the purification of a homogeneous peptide that produces inhibition of *n*-type K^+ channels in human T-lymphocytes (M. L. Garcia and C. Deutsch, unpublished data).

To determine those characteristics of the ChTX and IbTX structures which are responsible for each respective type of inhibitory activity, hybrid molecules were synthesized in which the N-terminus 19 amino acid residues of one peptide were joined to the C-terminus of the other, and vice versa (Sugg *et al.*, 1990b). Evaluation by [125 I]ChTX binding in vascular smooth muscle and brain, coupled with single-channel recording of $P_{K,Ca}$ in aortic smooth muscle, indicates that IbTX₁₋₁₉ChTX₂₀₋₃₇ is ChTX-like, while ChTX₁₋₁₉IbTX₂₀₋₃₇ displays IbTX-like behavior. Thus, the c-terminus of each peptide dictates receptor specificity. Moreover, residues 21-24 of IbTX appear to be critical for an interaction at the IbTX site, while both N- and C-termini are involved in high-affinity binding to the ChTX receptor in brain and smooth muscle.

Noxiustoxin

A third peptide displaying significant primary sequence homology with both ChTX and IbTX is noxiustoxin (NxTX), a 39 amino acid peptide isolated from venom of the scorpion *Centruroides noxius* (Fig. 1). In squid axon preparations, it has been demonstrated that this toxin specifically depresses peak permeability due to K^+ channels without affecting their voltage-dependent opening or closing kinetics (Carbone *et al.*, 1982). This effect of toxin is selective for K^+ currents, K^+ channel block is fully reversible, and the reaction can be described by a bimolecular binding scheme at a single binding site with a K_d of 390 nM. NxTX causes increased [3 H]GABA release and also blocks $^{86}Rb^+$ efflux from perfused mouse brain synaptosomes (Sitges *et al.*, 1986), consistent with the finding that toxin affects K^+ permeability. Subsequently, it was shown that lower concentrations of NxTX block K^+ currents in a voltage-independent manner, but, at concentrations above 1.5 μ M, inhibition by NxTX becomes voltage-dependent, and could be partially removed by repetitive pulsing with strong depolarizations (Carbone *et al.*, 1987). However, long repolarizations, and more negative holding potentials, favor channel block. Using axons from the squid *Loligo vulgaris* and a NxTX affinity column, four polypeptides have been isolated after membrane solubilization (Prestipino *et al.*, 1989). Three of these polypeptides (molecular weights of 66, 160, and 220 kDa) were identified as NxTX binding

sites by crosslinking with [125 I]NxTX to the affinity-purified preparation. When this preparation was reconstituted into liposomes and fused with planar lipid bilayers, single channel currents were observed with three distinct conductances. The most frequent of these was 11 pS, while the other two displayed conductances of 22 and 32 pS, respectively. The reversal potential, voltage dependence, and sensitivity to NxTX and TEA suggest that the purified preparation contains the squid axon delayed rectifier K^+ channel. Delayed rectifier K^+ currents exist in many other tissues, and recently several K^+ channel clones have been expressed in *Xenopus* oocytes that display properties typical of the delayed rectifier (Stuhmer *et al.*, 1988; Frech *et al.*, 1989; Yokogama *et al.*, 1989). However, it is not known whether any of these currents are sensitive to NxTX. Therefore, it remains to be determined if NxTX is a probe for a variety of delayed rectifier K^+ channels, or is selective for the squid axon channel.

It seems reasonable to consider NxTX as another peptide like ChTX, in terms of its limited specificity as a K^+ channel blocker. NxTX suppresses $P_{K,Ca}$ activity from skeletal muscle t-tubule membranes incorporated into planar lipid bilayers (Valdivia *et al.*, 1988). NxTX inhibits this system with a K_d of 450 nM by driving the channel into brief blocked states. However, in bovine aortic smooth muscles cells, NxTX has no effect on $P_{K,Ca}$ at concentrations up to 1 μ M. Consistent with this, NxTX does not affect [125 I]ChTX binding to bovine aortic sarcolemmal membrane vesicles (Vazquez *et al.*, 1989). Based on these studies, NxTX is not a high-affinity probe for $P_{K,Ca}$. However, in Jurkat cells, NxTX blocks *n*-type K^+ channels with a K_d of 0.2 nM (Sands *et al.*, 1989). Consistent with this is the observation that toxin also blocks [125 I]ChTX binding to human T-lymphocytes, Jurkat cells, and membranes derived from this cell line with high affinity (R. Slaughter, unpublished data). In addition, NxTX is a potent inhibitor of [125 I]ChTX binding to brain membranes (Vazquez *et al.*, 1990). These data suggest that the high-affinity target for NxTX is neither the delayed rectifier K^+ channel nor $P_{K,Ca}$, but rather the ChTX-sensitive, voltage-dependent K^+ channel. This hypothesis can be tested with several of the K^+ channel clones that are currently available. A different toxin isolated from venom of the scorpion *Tityus serrulatus* has been shown to affect K^+ conductance in squid axons similar to NxTX (Possani *et al.*, 1982; Carbone *et al.*, 1987). The active component of this venom appears to have approximately the same molecular weight and similar amino acid sequence as NxTX.

Other Less Well Characterized Toxins

The search for high-affinity probes of K^+ channels is at an early stage, and several different venoms have been identified to contain ChTX-like

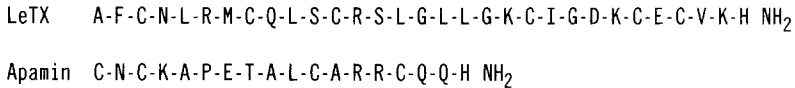


Fig. 5 Amino acid sequences of leiurototoxin I (LeTX) and apamin. Standard one letter notation is used to depict the amino acids.

activity (Moczydlowski *et al.*, 1988; Galvez *et al.*, 1990). Moreover, venom of the scorpion *Leiurus quinquestriatus* is a rich source for a number of different K⁺ channel blockers. In rat brain synaptosomes, this venom blocks a Ca²⁺-insensitive component of ⁸⁶Rb⁺ efflux (Blaustein *et al.*, 1988), and the peptides causing this activity have been isolated (Sorensen *et al.*, 1989). At least five components of the venom, one of which is ChTX, were identified. No sequence information regarding the structure of these peptides is available at the present time. Venom of *Leiurus quinquestriatus* has also been shown to inhibit ⁸⁶Rb⁺ efflux from rat portal vein induced by the putative K⁺ channel agonist BRL 34915 (Quast and Cook, 1988). This ⁸⁶Rb⁺ efflux pathway is thought to represent activity through an ATP-dependent K⁺ channel. The active component of the venom has been purified, although its composition is not known (Strong *et al.*, 1989). A distinct K⁺ channel inhibitory activity has been described in venom of the scorpion *Pandinus imperator* (Pappone and Cahalan, 1987; Pappone and Lucero, 1988). In GH₃ cells, this venom decreased A-type K⁺ currents, and shifted the voltage dependence of activation to more positive potentials. Blocking activity was found to be irreversible and could be mimicked by zinc at concentrations ≤ 0.2 mM.

Apamin and Leiurotoxin I: Distinct Toxin Inhibitors of Small-Conductance P_{K,Ca}

Apamin is a neurotoxin isolated from venom of the honeybee *Apis mellifera*. It is an 18 amino acid polypeptide containing two disulfide bridges (Fig. 5) that causes hyperactivity, convulsions, and death in mice at doses greater than 1 mg/kg body weight (Habermann, 1972). It is the only polypeptide neurotoxin known to cross the blood-brain barrier. In addition to its action in the central nervous system, apamin has effects on adrenal cortex, skeletal muscle, liver, and gastrointestinal smooth muscle (Habermann, 1984). A structure-activity analysis indicates that Arg¹³ and Arg¹⁴ are important residues in the action of the toxin (Vincent *et al.*, 1975). Chemical modifications elsewhere in the sequence may decrease toxicity of the polypeptide, but do not suppress its biological activity. Thus, modifications at the α-amino group of the N-terminal Cys, the ε-amino group of Lys⁴, the γ-carboxyl group of Glu⁷ or the imidazole group of His¹⁸ yield peptides that

are decreased in relative toxicity only 2- to 3-fold. The solid phase synthesis of apamin and related analogs has been successfully completed (Cosland and Merrifield, 1977; Granier *et al.*, 1978). These studies confirm that the two adjacent arginine residues at positions 13 and 14 are essential for toxin activity. Apamin has not yet been crystallized. However, spectroscopic studies using NMR techniques indicate that the toxin is a highly ordered structure with an α -helical core of residues 9–15 and flanking regions consisting of β -type turns (Bystrov *et al.*, 1980; Wemmer and Kallenbach, 1983).

The inhibitory action of apamin against $P_{K,Ca}$ was suspected from experiments where this toxin was found to inhibit K^+ efflux from guinea pig hepatocytes (Banks *et al.*, 1979) and taenia coli (Maas *et al.*, 1980) induced by either ATP or noradrenaline. A direct demonstration of the action of apamin was obtained using voltage clamp techniques with neuroblastoma cells (Hugues *et al.*, 1982a), cultured rat myotubes (Hugues *et al.*, 1982b), and bullfrog sympathetic ganglion cells (Pennefather *et al.*, 1985). At concentrations of 100 nM or less, apamin blocks a slow after-hyperpolarization following the firing of action potentials in these cells. Voltage clamp studies demonstrate that this phenomenon is due to inhibition of a Ca^{2+} -activated K^+ current which is insensitive to TEA, and which does not display a voltage dependence. High-conductance $P_{K,Ca}$ is not a major contributor to the after-hyperpolarization in non-innervated skeletal muscle or neuronal cells because apamin does not block this channel, and a well-known inhibitor of these channels, TEA, does not reduce the after-hyperpolarization. The identity of the after-hyperpolarization K^+ current in cultured rat muscle cells appears related to a small-conductance channel of 10–14 pS (Blatz and Magleby, 1986). These channels are blocked by apamin, but not by 5 mM external TEA, and are highly sensitive to Ca^{2+} at negative membrane potentials associated with the after-hyperpolarization response.

Radiolabelled monoiodoapamin can be prepared at high specific activity by incorporation of iodine into the imidazole ring of His¹⁸, the C-terminal residue of the toxin (Hugues *et al.*, 1982c; Seagar *et al.*, 1984). Iodinated apamin is about 2-fold less active than native toxin as measured in experiments where either the mean lethal dose was determined by intracisternal injection in mice, or the inhibition of neurotensin-induced relaxation of guinea-pig proximal colon was monitored (Hugues *et al.*, 1982c). [¹²⁵I] Apamin has been used to identify specific high-affinity receptors for this toxin in different tissues including brain synaptosomes (Hugues *et al.*, 1982c), cultured rat embryonic neurons (Seagar *et al.*, 1984), neuroblastoma cells (Hugues *et al.*, 1982a), cultured rat muscle cell membranes (Hugues *et al.*, 1982b), smooth muscle tissue (Hugues *et al.*, 1982d), and hepatocytes (Cook and Haylett, 1985). In these studies, high-affinity binding sites were observed with K_d 's in the range of 10–400 pM. However, the maximum density of

binding sites is very low, with B_{\max} values between 1–30 fmol/mg protein. As a comparison, rat brain synaptosomes contain 125 times less apamin-sensitive K^+ channels than tetrodotoxin-sensitive Na^+ channels (Hugues *et al.*, 1982c). Higher levels of apamin receptors have been found in undifferentiated pheochromocytoma cells (Schmid-Antomarchi *et al.*, 1986). These cells express receptor levels that are at least 50-fold greater than those found in other cell types, making these cells a potential source for purification of the apamin receptor.

The interaction of apamin with its receptor displays several interesting features. Dissociation of toxin from its binding site is slow. Dissociation rate constants of $1.5\text{--}4 \times 10^{-4} \text{ s}^{-1}$ have been determined in various nerve and muscle preparations (Hugues *et al.*, 1981a, c). Nontoxic analogs of apamin do not block the interaction of apamin, and binding of [^{125}I]apamin is highly sensitive to cations. In rat neurons, binding increases 3-fold as extracellular K^+ is raised from 0.1 to 10 mM, at constant ionic strength (Seagar *et al.*, 1984). This effect is due to an increase in the maximum number of binding sites, without any effect on K_d . In rat brain synaptosomes, concentrations of K^+ and Rb^+ between 10 μM and 5 mM are able to increase the binding of apamin to its receptor by a factor of 1.8, while other cations such as Li^+ , Na^+ , and guanidinium completely inhibit binding in the 1–100 mM range (Hugues *et al.*, 1982c). In this preparation, the stimulatory effect of K^+ is due to a decrease in the K_d of toxin, rather than an effect on B_{\max} . The effects of cations on [^{125}I]apamin binding have been interpreted to indicate the existence of two different ion binding sites: one site is specific for K^+ and Rb^+ , but distinct from the apamin receptor, and binding to this site at a K_d of ca. 500 μM results in an increased affinity for toxin; the second site likely corresponds to the location where the two essential Arg residues of apamin bind since molecules which contain guanidinium groups (e.g., amiloride, neurotensin, which has two contiguous arginine residues like apamin, or guanidium itself) possess higher affinity for the apamin receptor than metal ions or other positively charged molecules (Hugues *et al.*, 1982a, c). Interestingly, the apamin receptor in rat muscle is only expressed in noninnervated cells (Schmid-Antomarchi *et al.*, 1985). In normal adult rat muscle, binding of apamin is not detectable, and the action potential in this preparation is insensitive to apamin. After denervation, binding sites for apamin, and an apamin-sensitive, after-hyperpolarization current, both appear in a time-dependent fashion.

Data concerning the structure of the apamin-sensitive channel have been obtained from studies with membrane vesicles. The molecular weight of the intact receptor was initially determined by employing radiation inactivation techniques. A molecular weight of 250 kDa has been estimated using lyophilized rat brain synaptic membranes (Schmid-Antomarchi *et al.*,

1984). However, with frozen rat brain membranes, the target size of the functional binding site appears to be 84–115 kDa (Seagar *et al.*, 1986). Other attempts to identify the component(s) that constitute the apamin receptor have also yielded conflicting results. Covalent incorporation of [¹²⁵I]apamin into rat brain membranes with the bifunctional crosslinking reagent disuccinimidyl suberate has identified a single polypeptide of 30 kDa (Hugues *et al.*, 1982e; Schmid-Antomarchi *et al.*, 1984). In another study, photoreactive apamin derivatives were prepared with aryl azide groups located at two different positions within the molecule (Seagar *et al.*, 1986). The Lys⁴ derivative labelled polypeptides of 33 and 22 kDa in both cultured neurons and synaptic plasma membranes, whereas the Cys¹ derivative labelled only an 86 kDa polypeptide, or 86 and 59 kDa components, in these two preparations, respectively. These results suggest that the apamin binding site may be located at the frontier between three or more putative K⁺ channel subunits which are not equally accessible to the different receptor-associated photoprobes. Further studies have shown that the 59 kDa component is found in cultured rat astrocytes, but not in neurons, indicating that glial cells may be responsible for the component of that molecular weight which was labelled in brain membranes (Seagar *et al.*, 1987a). In a recent report, both photoreactive derivatives of apamin and disuccinimidyl suberate crosslinking procedures were used to identify apamin receptor components in rat brain membranes and pheochromocytoma cell membranes (Auguste *et al.*, 1989). Results of these experiments indicate that a variety of polypeptides are labelled with the different apamin derivatives. One major component has a molecular mass of ca. 30 kDa, but other polypeptides of 45, 58, and 86 kDa were also identified. Data obtained with PC12 cell membranes are not identical to brain, consistent perhaps with differences observed in the affinity of apamin for its receptor in these two systems.

The apamin receptor has been solubilized in functional form from rat brain synaptic membranes using sodium cholate (Seagar *et al.*, 1987b). [¹²⁵I]apamin binding was stimulated in the solubilized preparation by K⁺ with an ED₅₀ of 0.6 mM, suggesting that the regulatory K⁺ site is associated with the soluble complex. Analysis of the covalently labelled apamin receptor/sodium cholate complex by density gradient centrifugation indicates an apparent sedimentation coefficient of 20S, which corresponds to a molecular weight of ca. 700 kDa, although as much as 50% of this mass may be contributed by detergent. Assuming a molecular weight of 250–350 kDa, a homogeneous preparation should contain 3–4 nmol receptor/mg protein. Given the amount of receptor present in most apamin-sensitive tissues, isolation of the pure protein will require purification by a factor of 200,000 to 400,000-fold to reach homogeneity. To achieve this goal, use of affinity columns containing either apamin or anti-receptor antibodies may be required.

Venom of the scorpion *Leiurus quinquestriatus* var. *hebraeus*, was found to contain an apamin binding inhibitory activity (Castle and Strong, 1986). It also blocks an apamin-sensitive K^+ channel in guinea pig hepatocytes (Abia *et al.*, 1986; Castle and Strong, 1986). The agent responsible for this activity has been purified to homogeneity and shown to consist of a 3.4 kDa peptide (Fig. 5) with little structural homology to apamin (Chicchi *et al.*, 1988). However, the peptide has some homology with other scorpion toxins such as ChTX, N α TX, and neurotoxin P2. This peptide, termed leiurotoxin I, completely inhibits [125 I]apamin binding to rat brain synaptic plasma membranes with a K_i of 75 pM, which is 10-fold less potent than apamin, and acts by an apparently noncompetitive mechanism. Like apamin, leiurotoxin I blocks epinephrine-induced relaxation of guinea pig taenia coli, but has no effect on the rate, or force of contraction, of guinea pig atrial or rabbit portal vein preparations. In addition, leiurotoxin I blocks the apamin-sensitive, Ca^{2+} -dependent, after-hyperpolarization K^+ current in bullfrog ganglion B cells (Pennefather *et al.*, 1989). Therefore, leiurotoxin I represents a new structural class of inhibitors directed against the small-conductance $P_{K,Ca}$.

Recently, another laboratory has reported the independent purification of leiurotoxin I, to which they assigned the name, scyllatoxin (Auguste *et al.*, 1990). Primary amino acid sequence determination reveals that both toxins are identical. Scyllatoxin and two analogs have been synthesized by solid-phase methodologies. One of these analogs, in which a Tyr residue is introduced at position 2 to replace Phe, displays activity equivalent to native toxin, and has been used to obtain a radiolabelled derivative for binding studies. [125 I](Tyr 2)scyllatoxin binds to a single class of sites in rat brain membranes with a K_d of 80 pM. In these studies, apamin and scyllatoxin were shown to be competitive. Moreover, as found for the interaction of apamin, [125 I]scyllatoxin binding is activated by low K^+ concentrations, inhibited at high K^+ levels, and blocked by Ca^{2+} , Na^+ , and guanidinium ions. In addition, in crosslinking experiments with scyllatoxin, two polypeptides of 27 and 57 kDa were identified. Thus, these observations are particularly interesting since they indicate that there may be a common binding site for two toxins which share no sequence homology. It will be important to determine the three-dimensional structure of these toxins in order to rationalize these findings.

Dendrotoxins: a Family of Snake Toxins Directed against K^+ Channels

Neurotransmitter release is stimulated at peripheral (Harvey and Karlsson, 1982), and central (Docherty *et al.*, 1983; Dolly *et al.*, 1984) neurons by a group of homologous polypeptides derived from snake venoms. These neurotoxins, which are now known to modulate K^+ channel activity, have been identified in crude venom from several related species of snakes:

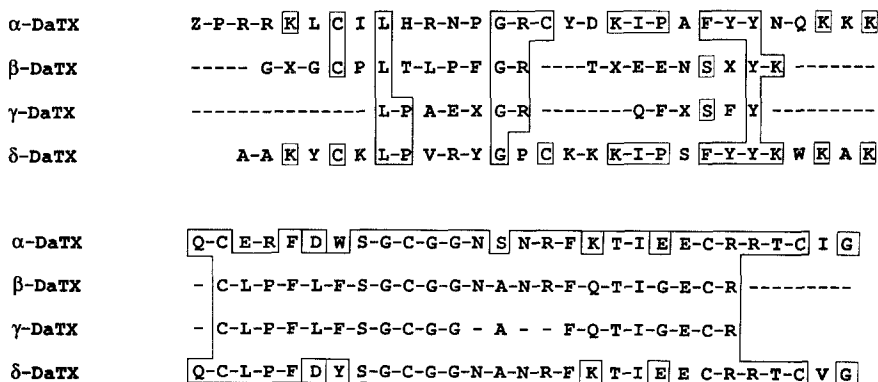


Fig. 6. Amino acid sequences of α , β , γ - and δ -dendrotoxin (DaTX). In this representation, (x) indicates an unidentified residue, while (–) indicates that no sequence is available in this region. Homologies are highlighted in boxes.

two types of the green mamba, *Dendroaspis angusticeps* and *Dendroaspis viridis*; and a species of black mamba, *Dendroaspis polylepis* (Harvey and Anderson, 1985). α -Dendrotoxin, α -DaTX, is a basic 7 kDa polypeptide isolated from *Dendroaspis angusticeps* which contains 59 amino acids, and is structurally similar to toxin I, or DaTX, isolated from *Dendroaspis polylepis*. In addition, three other polypeptide toxins have been isolated from *Dendroaspis angusticeps* venom. δ -DaTX contains 57 amino acids and has marked sequence homology with α -DaTX in the C-terminal region: 25 or 30 amino acids in this segment of the peptide are identical. There is also considerable homology in the N-terminal region: 8 of 15 amino acids between positions 12 and 26 are identical. β and γ -DaTX have been partially sequenced. Their sequences from position 30 through 52 are identical. In addition, their primary structures are very similar to that of α - and δ -DaTX in the C-terminal region where 20 of 23 amino acids are conserved in all four dendrotoxins (Fig. 6). Few structural similarities exist between these snake toxins and scorpion K^+ channel toxins. However, α -DaTX shows considerable homology with the smaller β -subunit of β -bungarotoxin (β -BTX), an inhibitory presynaptic neurotoxin from *Bungarus multicinctus*.

Dendrotoxins are highly neurotoxic when injected intracerebrally into rats (Mehraban *et al.*, 1985). Electrophysiological records in hippocampal slices from guinea pig or rat demonstrate that α -DaTX is a potent convulsant, causing enhancement of cell excitability, with a consequent potentiation of transmitter release (Docherty *et al.*, 1983; Dolly *et al.*, 1984). The electrophysiological basis for the central convulsive action of α -DaTX was examined using intracellular recording of CA_1 neurons from hippocampal slices (Halliwell *et al.*, 1986). In the presence of 50 nM α -DaTX, depolarizing currents elicited repetitive firing of Ca^{2+} -action potentials. α -DaTX caused

little change in the responses to hyperpolarizing current or in resting membrane potential. Voltage-clamp experiments indicate that the toxin does not directly affect a Ca^{2+} -activated K^+ conductance, a noninactivating, voltage-dependent K^+ conductance, inactivating or noninactivating Ca^{2+} conductances, or an inwardly rectifying K^+ conductance. The only direct effect of α -DaTX was found to be a reduction of a transient, voltage-dependent K^+ current. More recently, α -DaTX has also been shown to inhibit a slowly inactivating K^+ channel present in various neurons (Penner *et al.*, 1986; Stansfeld *et al.*, 1986, 1987). Thus, electrophysiological data obtained from a variety of neuronal sources indicate that α -DaTX recognizes a family of K^+ channels that differ greatly in their inactivation kinetics.

This has been confirmed in experiments in which rat brain cDNA clones encoding different types of voltage-dependent K^+ channels were assayed for their sensitivity to α -DaTX after expression in *Xenopus* oocytes (Stuhmer *et al.*, 1989). RCK1 and RCK5 clones expressing slowly inactivating K^+ channels were much more sensitive to α -DaTX than the more rapidly inactivating channels encoded for by RCK3 and RCK4. In different types of experiments where $^{86}\text{Rb}^+$ efflux from synaptosomes was used to monitor the activity of K^+ channels, α - and δ -DaTX were found to preferentially block a component of flux corresponding to a rapidly inactivating K^+ channel (Blaustein *et al.*, 1988; Sorensen *et al.*, 1989). Moreover, using the same experimental system, β - and γ -DaTX inhibit a noninactivating K^+ channel-mediated component of $^{86}\text{Rb}^+$ flux with greater affinity. Given the high homology in primary amino acid sequences between the different types of voltage-dependent K^+ channels that have been cloned, it is not surprising that the dendrotoxin family can recognize a wide variety of these moieties, just as the scorpion K^+ channel toxins do. It is interesting, however, that $\text{P}_{\text{K,Ca}}$ does not appear to be sensitive to these toxins. To date, the only report concerning an effect of α -DaTX on these channels indicates that toxin may interact with an internal binding site on the channel to produce reversibly a long-lived, inwardly rectifying subconductance state (Lucchesi and Moczydlowski, 1990). In this study, toxin binding was shown to modify ion conduction through the pore without affecting the Ca^{2+} , or voltage dependence of gating.

Using biologically active [^{125}I]DaTX, autoradiography was used to demonstrate that toxin binding sites reside in synapse-rich, white matter regions of the hippocampus, with lower levels in cell body layers (Halliwell *et al.*, 1986). [^{125}I]DaTX has also been used to identify receptor sites in different membrane preparations. [^{125}I]DaTX binds with high affinity to a homogeneous population of sites in rat brain synaptosomes (Mehraban *et al.*, 1984; Black *et al.*, 1986; Halliwell *et al.*, 1986). In preparations of rat synaptic plasma membrane vesicles, both [^{125}I] α -DaTX and [^{125}I] β -DaTX

bind to a single class of receptors (Sorensen and Blaustein, 1989). Although β -DaTX inhibits the binding of [125 I] α -DaTX competitively, inhibition of [125 I] β -DaTX by α -DaTX is more complex, indicating perhaps that β -DaTX binds with similar affinities to two different sites, only one of which may be identical to the α -DaTX receptor. Binding sites for [125 I] δ -DaTX have also been identified in guinea pig synaptosomes (Muniz *et al.*, 1990). α -DaTX inhibits [125 I] δ -DaTX binding in a noncompetitive fashion, suggesting that δ -DaTX interacts at a site distinct from the receptor for the former peptide.

Binding sites for α -DaTX are also modulated by mast cell degranulating peptide (MCD) isolated from venom of the bee *Apis mellifera*, and by β -BTX (Bidard *et al.*, 1987; Black *et al.*, 1988; Rehm *et al.*, 1988; Rehm and Lazdunski, 1988a, b; Breeze and Dolly, 1989). Different binding sites for these agents seem to reside on the same molecular entity since each toxin can allosterically inhibit the binding of the other molecules. Such studies have identified two subtypes of α -DaTX binding proteins. In one subtype (60–70% of the total population), binding of both [125 I] α -DaTX and [125 I]MCD is inhibited by β -BTX with low affinity, while in the second subtype, β -BTX inhibits these interactions with high affinity (Rehm and Lazdunski, 1988a). The α -DaTX binding subtype, with low affinity for β -BTX, usually contains binding sites for [125 I]MCD. The receptor for α -DaTX has been purified from a detergent extract of rat brain membranes (Rehm and Lazdunski, 1988b). Analysis of the purified material reveals the existence of three polypeptide bands of M.W. 76–80 kDa, 38 kDa, and 35 kDa. Interestingly, [125 I]MCD and [125 I] α -DaTX binding activities copurify together, and binding of these two agents in the purified preparation is inhibited by β -BTX. Incubation of this material with the catalytic subunit of c-AMP-dependent protein kinase leads to phosphorylation of the 76–80 kDa toxin-binding subunit (Rehm *et al.*, 1989a). Reconstitution of this receptor complex into artificial bilayers results in the appearance of multiple conductance levels of unitary current. The majority of openings correspond to a channel that displays a conductance of ca. 21 pS in 150 mM symmetrical K^+ medium. However, up to seven different conductance levels have been described, suggesting that reconstitution of the α -DaTX receptor is associated with incorporation of multiple types of functional K^+ channels into the membrane. This observed heterogeneity in conductance levels probably indicates that the purified α -DaTX receptor is a mixture of closely related K^+ channel structures, with similar, but not identical, pharmacological properties (Rehm and Lazdunski, 1988a). Activity of the 21-pS K^+ channel α -DaTX receptor is enhanced by phosphorylation, and the phosphorylated channel is still sensitive to inhibition by toxin. Polyclonal antibodies have been raised against two synthetic peptides derived from different regions of the predicted amino acid sequence of MBK1, the mouse homolog of the *Drosophila* Shaker K^+ channel (Rehm *et al.*, 1989b).

These antibodies cross-react with the toxin-binding subunit of the α -DaTX receptor from rat and bovine brain. This suggests that the α -DaTX binding protein is the product of the mammalian homolog of the Shaker gene.

Conclusions

Over the last few years, combined molecular biology and biochemical investigations have led to the identification and characterization of several different types of K^+ channel proteins. Peptidyl toxins isolated from a variety of organisms are currently being used to define the pharmacology of these channels. For those K^+ channels where molecular biological techniques have not yet identified the corresponding proteins, biochemical approaches will be needed to achieve this end. Therefore, it is significant that toxins which interact specifically with single classes of K^+ channels have been identified. It has only recently become apparent how many different types of K^+ channel toxins can be found in a given venom source. These toxins surely will provide unique tools for future work, and will enable investigators to define the biochemical, pharmacological, and physiological properties of different K^+ channel proteins in target tissues of interest.

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