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# An Emerging Pharmacology of Peptide Toxins Targeted Against Potassium Channels

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## Introduction

Proteins that perform similar functions often exhibit common themes of structural organization. In cases where there is particularly striking homology at the level of primary sequence and tertiary structure, distinct families of related proteins can be recognized and classified. In recent years, substantial progress has been made toward understanding structural relationships within the class of membrane proteins known as ion channels. At the supramolecular level, current thinking holds that water-filled pores are formed across biological membranes in the central cavities of rosette-like arrangements of protein subunits that span the hydrophobic core of the bilayer. Such structures have either been observed or implicated for porin channels (trimeric) (Benz, 1985), gap junction channels (hexameric) (Makowski et al., 1984), acetylcholine-receptor channels (pentameric) (Kistler et al., 1982), voltage-dependent Na channels (tetrameric) (Noda et al., 1984) and voltage-dependent Ca channels (tetrameric) (Tanabe et al., 1987). The first three channels in this list are composed of traditional distinct subunits that are either identical or homologous polypeptides. For the last two channels listed, it is believed that the subunits of the rosette are actually "pseudosubunits": four internally homologous domains of about 300 amino acids within a single 2,000-residue polypeptide. At the level of primary sequence, considerable homology has been found among proteins belonging to the receptor-operated class of channels (acetylcholine-receptor, GABA-receptor, glycine-receptor) (Grenningloh et al., 1987; Schofield et al., 1987) and among the voltage-activated class of channels (Na channel, Ca channel, K channel) (Noda et al., 1984; Tanabe et al., 1987; Tempel et al., 1987). The similarity of sequences revealed in these studies serves as a unifying basis for classifying channels and may ultimately permit the evolution of these proteins to be traced, as envisioned by Hille (1984) in his discussion of the phylogenetic distribution of channel types.

At a deeper level of complexity, a single organism may express variants of a given type of channel in different tissues or even in the same tissue. For example, the postjunctional cleft of mammalian muscle contains nicotinic acetylcholine receptors that are blocked by $\alpha$-bungarotoxin, while similar nicotinic receptors in neurons are not blocked by this classic snake neurotoxin (Carbonetto, Fambrough & Muller, 1978; Ravdin & Berg, 1979). Similarly, tetrodotoxin and $\mu$-conotoxin have been used to discriminate three subtypes of voltage-dependent Na channels that are found in various excitable tissues of mammals (Moczydlowski et al., 1986). Thus, specific neurotoxins serve as a means of pharmacological classification that can be used to identify a unique subtype within a host tissue.

The ability of high-affinity toxins to discriminate among classes and subtypes of channels arises from chemical interactions between functional groups of the toxin and channel protein. A slight modification of one or several of these binding interactions can result in large changes in affinity for the toxin. Toxins can therefore be used to probe structural differences at sites that are often associated with important functions as ion permeation and gating. Toxin pharmacology is also invaluable in dissecting the physiological contribution of one channel type to macroscopic behavior that may be the

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result of many different types of channels operating simultaneously. As molecular studies on channels progress, it may be possible to correlate pharmacological differences among channel types or subtypes with structural differences in toxin binding sites that arise in the synthesis of channel proteins at the pre- or post-translational level.

Of primary concern to this review is the class of channels selective for $K^+$. Although K channels are presently least understood from a biochemical perspective, work in the area of membrane regulation indicates that they may be the most important class of channels from the viewpoint of modulation (Kaczmarek & Levitan, 1987). It is often cited that slow biochemical progress in K-channel research is due to the lack of specific high-affinity toxins or ligands that exist for other types of channels. However, this situation is rapidly changing. It appears that a wide variety of peptide toxins directed against K channels do exist in nature. The lag in development of these toxins has permitted the identification and sequence analysis of K-channel genes by genetic approaches in *Drosophila* (Tempel et al., 1987) to actually proceed in advance of the biochemical purification of K-channel proteins. The breakthroughs in toxin chemistry described in this review, coupled with progress in the genetic analysis of K channels, foreshadows a productive era in K-channel research.

## Remarkable Diversity in Gating Mechanisms and Functions of K channels

One can devise schemes for classifying K channels according to functional properties of ion selectivity and conductance, gating behavior, pharmacology, and regulation. In a previous review in this journal, Latorre and Miller (1983) classified channels according to their permeation properties. They arrived at four classes of channels on this basis: (1) low-conductance, ion-specific channels; (2) valence-selective channels of intermediate conductance; (3) nonselective channels of large conductance; (4) maxi-$K^+$ channels of large conductance. While this system is workable for a limited purpose, we shall see in this review that unitary conductance is not a reliable criterion for assessing the structural relatedness of K channels. For example, one of the peptide toxins that is discussed (charybdotoxin) blocks both maxi Ca-activated K channels with a conductance of 230 pS (Miller et al., 1985) and a smaller conductance Ca-activated K channel in *Aplysia* neurons that is only 35 pS (Hermann & Erxleben, 1987). Since these two channels are associated with a binding site for a rather discriminating toxin, at least part of the protein components of these two channels must be structurally related. However, on the basis of conductance alone, these two channels would be considered distant relatives.

In other previous reviews of K channels (Thompson & Aldrich, 1980; Latorre, Coronado & Vergara, 1984; Hille, 1984; Kaczmarek & Levitan, 1987; Yellen, 1987), $K^+$ currents have been classified according to their voltage dependence and gating kinetics. By this rationale, at least four classes have been recognized: (1) delayed, outward rectifiers; (2) transient, outward rectifiers or A-currents; (3) anomalous or inward rectifiers; (4) Ca-activated K channels.

The delayed rectifier is the classic voltage-activated K channel described by Hodgkin and Huxley (1952) in their analysis of the action potential in squid axon. Because the characteristics of this $K^+$ current are somewhat atypical compared to that of other excitable tissues, Rogawski (1985) has suggested that our conception of this K channel as a prototype would be quite different had Hodgkin and Huxley chosen another tissue for their work. The delayed rectifier assists in the repolarization of fast action potentials in unmyelinated nerves, striated muscle and in myelinated nerves of amphibians. This channel seems to be absent in the nodal regions of most mammalian myelinated nerve fibers (Chiu et al., 1979). In the original description of squid delayed rectifier currents (Hodgkin & Huxley, 1952), the $K^+$ current was considered to be noninactivating as compared to the rapidly inactivating $Na^+$ current. A closer examination of many tissues has revealed that delayed rectifier currents differ greatly in their inactivation characteristics. While $K^+$ current inactivation is slow and incomplete in squid axons, it rapidly inactivates to near-zero levels in frog skeletal muscle (Adrian, Chandler & Hodgkin, 1970). Even within a given tissue, such as frog node of Ranvier or skeletal muscle, multiple components of delayed rectifier $K^+$ currents can be resolved on the basis of voltage dependence of activation and kinetics of deactivation (Dubois, 1981; Lynch, 1985).

Single-channel conductances in the range of 2–200 pS have been reported for delayed rectifier K channels in various preparations (Conti & Neher, 1980; Reuter & Stevens, 1980; Clapham & DeFelice, 1984; Coronado, Latorre & Maunter, 1984; Standen, Stanfield & Ward, 1985). Because the unit conductance of these channels seems to be quite dependent on the ionic conditions of the measurement (Coronado et al., 1984), it is not clear how much of this variation is due to intrinsic differences in channel types. Although tetraethylammonium (TEA) is often cited as a blocker of the delayed rectifier, TEA also blocks practically every type of known K channel and also blocks Na channels from

the inside of the cell. Clearly, more selective pharmacological agents for delayed rectifier K channels are needed to distinguish the wide variety of such channels that seem to belong to this class.

A second class of outward-rectifying K channels activated by depolarization underlies a current called the transient, inward current or A-current. This current was originally described in molluscan neurons (Hagiwara, Kusano & Saito, 1961) and has since been observed in many cell types (Rogawski, 1985). It is often substantially inactivated at normal resting potentials, but can be reprimed by hyperpolarization to remove steady-state inactivation. Other hallmarks of the A-current are an activation range that is usually more negative than delayed rectifier K current in the same cells, and a relative sensitivity to block by 4-aminopyridine (4-AP) and insensitivity to tetraethylammonium (TEA). The A-current has been shown to regulate the firing frequency of neurons (Connor & Stevens, 1971). Its function has been described as an input encoder, because it allows graded depolarization at synapses to be translated into increasing rates of action potential discharge. This K channel is the focus of current notoriety because sequences complementary to the *Shaker* locus in *Drosophila* have recently been determined (Tempel et al., 1987) and found to contain stretches similar to the pseudosubunit domains of Na- and Ca channels (Noda et al., 1984; Tanabe et al., 1987). Mutations at the *Shaker* locus affect A-currents in *Drosophila,* and mRNA transcribed from *Shaker* cDNA clones has been shown to induce functional A-currents when injected into frog oocytes (Timpe et al., 1988). Electrophysiological investigations of cultured embryonic cells of *Drosophila* have identified a 12–16 pS K channel in myotubes and a 5–8 pS K channel in neurons that appear to underlie A-currents in these cells (Solc, Zagotta & Aldrich, 1987). Only the myotube K channel was affected by *Shaker* mutations, suggesting the presence of multiple subtypes of A-current channels. Recent sequence analysis of *Shaker* gene products indicates that alternative splicing of mRNA transcripts may be an operative mechanism for multiple A-channel subtypes (Schwarz et al., 1988). Although A-current channels have been considered as a distinct class of K channels, the similarity of their gating properties to certain types of inactivating delayed rectifiers raises suspicions about the relatedness of these two types of channels. In this review, we shall discuss a group of snake toxins (dendrotoxins) that have been shown to selectively block both a typical A-current in rat hippocampal cells (Halliwell et al., 1986) and a subclass of delayed rectifier current in frog node of Ranvier (Benoit & Dubois, 1986). This is an example of a case where a toxin reveals structural relatedness between K channels grouped in different categories according to their gating behavior.

A third type of $K^+$ current that is recognized by its unique voltage dependence is the anomalous- or inward-rectifying $K^+$ current. This current is activated by hyperpolarizing voltage, in contrast to nearly every other type of voltage-activated channel. Inward rectifiers are found in many cell types including skeletal and heart muscle, egg cells and neurons (Thompson & Aldrich, 1980; Hagiwara, 1983; Kaczmarek & Levitan, 1987). Such channels have been implicated in setting the resting potential of cells, preventing $K^+$ accumulation in the lumen of skeletal muscle T-tubules and allowing for long duration action potentials in heart and egg cells. At the single channel level, unitary conductances in the range of 5–27 pS have been reported for inward rectifiers in various preparations (Ohmori, Yoshida & Hagiwara, 1981; Fukushima, 1982; Sakmann & Trube, 1984; Matsuda, Saigusa & Irisawa, 1987). Discrete blocking events induced by cations such as $Na^+$, $Cs^+$, $Sr^{2+}$ and $Ba^{2+}$ have been used to facilitate observation of single-channel events (Ohmori et al., 1981; Fukushima, 1982). Understanding the mechanism of inward rectification has been a challenging problem in channel biophysics. Because the instantaneous current exhibits strong rectification in some preparations, it was thought that the rectification is due to properties of the open channel. A multi-ion model for permeation of $K^+$, with an intracellular, diffusible blocking ion, has been used to explain this behavior (Hille & Schwarz, 1978). Recently, it has been found that block by intracellular $Mg^{2+}$ can account for inward rectification of an otherwise ohmic K channel in heart ventricular muscle cells (Matsuda et al., 1987). Although no toxins have yet been identified that act directly on inward rectifier K channels, such channels have been found to be regulated by internal adenine nucleotides in pancreatic $\beta$-cells (Misler et al., 1986), by G-proteins in atrial cells (Logotheitis et al., 1987; Yatani et al., 1987), and by cyclic-AMP dependent phosphorylation in sensory neurons of *Aplysia* (Benson & Levitan, 1983). It is hoped that such regulatory mechanisms may lead to biochemical assays for the identification or isolation of these K channels. It has also been found that sufonylureas, a class of anti-diabetic drugs, are potent inhibitors of ATP-modulated K channels in pancreatic $\beta$-cells (Schmid-Antomarchi et al., 1987). A tritiated derivative of glibenclamide has recently been used to identify specific binding sites in insulinoma cells (Schmid-Antomarchi et al., 1988) that may correspond to a protein component associated with these channels.

Ca-activated K channels are a fourth class of K channels that are found in practically every type of animal cell (Schwarz & Passow, 1983; Petersen &

**Table 1.** Amino acid sequences of two classes of bee venom toxins

| | 1---5---10---15---20-- |
|---|---|
| apamin | --CNCK--APETALCARRCQQH-$NH_2$ |
| MCDP | IKCNCKRHVIKPHICRKICGKN-$NH_2$ |
| tertiapin | ALCNCN-RIIIPHMCWKKCGKK |

The sequences are aligned by four cysteine residues (underlined) and are arranged for maximum homology by inserting gaps denoted by a hyphen. Identical residues found in a majority of the peptides at a given position are marked in boldface type. Original references to the sequences are given in Habermann (1972) and Hider and Ragnarsson (1981). Disulfide bonds are formed by cysteine residues 3–15 and 5–19. The single letter amino acid code is: (A) ala, (E) glu, (Q) gln, (D) asp, (N) asn, (L) leu, (G) gly, (K) lys, (S), ser, (V) val, (R) arg, (T) thr, (P) pro, (I) ile, (M) met, (F) phe, (Y) tyr, (C) cys, (W) trp, (H) his.

Maruyama, 1984; Blatz & Magleby, 1987). In neurons, these channels function in the repolarization of action potentials and in the termination of bursts. In nerve and muscle cells they give rise to the after-hyperpolarizing phase of action potentials. In secretory organs, such as salivary glands, sweat glands and tear ducts, these channels are a pathway for release of $K^+$ during fluid secretion and are also responsible for $K^+$ secretion by the cortical collecting tubule of kidney (Hunter et al., 1984). Patch-clamp studies and peptide toxins have been used to distinguish two major types of Ca-activated K channels in mammals. One type is characterized by a large conductance of about 200 pS and is blocked by a class of scorpion peptides called *charybdotoxins* (Miller et al., 1985). The second type has a conductance of 10–14 pS and is specifically blocked by a bee venom peptide called *apamin* (Blatz & Magleby, 1986). The large-conductance Ca-activated K channel is also known as the BK or maxi-$K^+$ channel, while the small-conductance one has been referred to as the SK channel. BK channels are activated by both internal $Ca^{2+}$ and depolarizing voltage. Increasing concentrations of internal $Ca^{2+}$ have been shown to shift the voltage dependence of activation of BK channels along the voltage axis in the negative direction (Barrett, Magleby & Pallotta, 1982). In contrast, SK channels in rat myotubes are activated maximally by 10 $\mu$M internal $Ca^{2+}$ and exhibit little voltage dependence (Blatz & Magleby, 1986). In this article, we shall review the status of apamin and charybdotoxin as specific ligands for these distinct K-channel subtypes.

In addition to the four classes of K channels, distinguished by their gating behavior, other types have been distinguished by their susceptibility to modulation. Examples of these are the M-current of certain vertebrate neurons (Jones & Adams, 1987) and the S-current of *Aplysia* sensory neurons (Siegelbaum, 1987). Specific K-channel toxins will be useful in examining possible structural relationships between modulated K channels and members of the four basic types of gating groups.

## Apamin and MCD, K-Channel Toxins in Bee Venom

The venom of the honeybee, *Apis mellifera,* contains two types of peptide toxins that are directed against K channels. The most widely known of these is apamin, an 18-residue peptide that causes hyperactivity, convulsions and death in mice at doses greater than 1 mg/kg body weight (Habermann, 1972; Romey et al., 1984). As a basic peptide, apamin is unusual in its ability to penetrate the blood-brain barrier. A second group of toxins includes MCD and the homologous peptide, tertiapin (Table 1). In contrast to apamin, the 22-residue MCD peptide is relatively nonlethal when injected intravenously in mice, but it produces similar convulsive symptoms when injected directly into the brain (Banks, Garman & Habermann, 1978). MCD peptide, also known as 401 peptide (Gauldie et al., 1976; Banks et al., 1981), was first noted for its mast cell degranulating activity (Habermann, 1972). It now appears that this activity is unrelated to its neurotoxicity and is a nonspecific property of a variety of polycationic compounds (Taylor, Bidard & Lazdunski, 1984).

The related primary sequences of these basic toxins can be aligned by four cysteine groups that form two intramolecular disulfide bonds in each of the molecules (Table 1). As isolated from the venom, the carboxy termini of apamin and MCD are amidated (Habermann, 1972). A minor form of apamin with a formylated amino terminus has also

been reported (Wemmer & Kallenbach, 1983). Chemical modification studies (Vincent, Schweitz & Lazdunski, 1975) and studies of synthetic peptide analogs (Cosland & Merrifield, 1977; Granier, Pedraso-Muller & van Reitschoten, 1978) have shown that the two adjacent arginine residues near the C-terminal end of apamin are essential for toxin activity. Native apamin has been chemically modified at the N-terminal amino group of $cys_1$, the amino group of $lys_4$, the carboxyl group of $glu_7$ and the imidazole group of $his_{18}$ with only two- to threefold decreases in relative toxicity (Vincent et al., 1975), suggesting that these functional groups are nonessential. A radiolabeled derivative of apamin that is useful for ligand binding studies can be prepared by iodination of the $his_{18}$ residue using chloramine T (Hugues et al., 1982*a*) or iodogen (Seager, Granier & Couraud, 1984). Similar iodination of the two histidine residues of MCD peptide yields a product that binds with high affinity to rat brain membranes (Taylor et al., 1984). Reaction of all six free amino groups in MCD peptide with acetic anhydride or reaction of its two arginine groups with 1,2-cyclohexanedione destroys high affinity binding activity. The effect of selective modification of individual functional groups in MCD has not yet been reported.

Although crystal structures of these peptides are unavailable, spectroscopic studies (Bystrov et al., 1980; Wemmer & Kallenbach, 1983) have shown that the solution structure of apamin contains an $\alpha$-helical core of residues 9–15. Secondary structure predictions for MCD and tertiapin (Hider & Ragnarsson, 1981) also suggest an $\alpha$-helix involving residues in a similar region of the sequence (position 13–19 in Table 1). In addition to the three toxins shown in Table 1, another possibly related peptide of 24 residues called *secapin* has also been isolated from bee venom (Gauldie et al., 1976, 1978), but its biological activity is as yet unknown.

The specific action of apamin against Ca-activated K channels was first suspected when it was found to inhibit $K^+$ efflux in guinea pig hepatocyes (Banks et al., 1979) and hyperpolarization of smooth muscle (Maas & Den Hertog, 1979) that is induced by agonists or the $Ca^{2+}$ ionophore, A23187. At concentrations of 100 nM or less, apamin has been shown to block a slow after-hyperpolarization following action potentials in cultured rat myotubes (Fig. 1*A,B*) (Hugues et al., 1982*d*; Romey & Lazdunski, 1984), mouse neuroblastoma cells (Hugues et al., 1982*c*) and bullfrog sympathetic ganglion cells (Pennefather et al., 1985). Voltage-clamp studies have shown that the slow phase of after-hyperpolarization in these cells is due to a Ca-activated $K^+$ current that is insensitive to TEA. Until recently, the identity of the K channel underlying this current was a mystery because large conductance (200 pS) Ca-activated K channels identified by patch-clamp recording in many cell types were

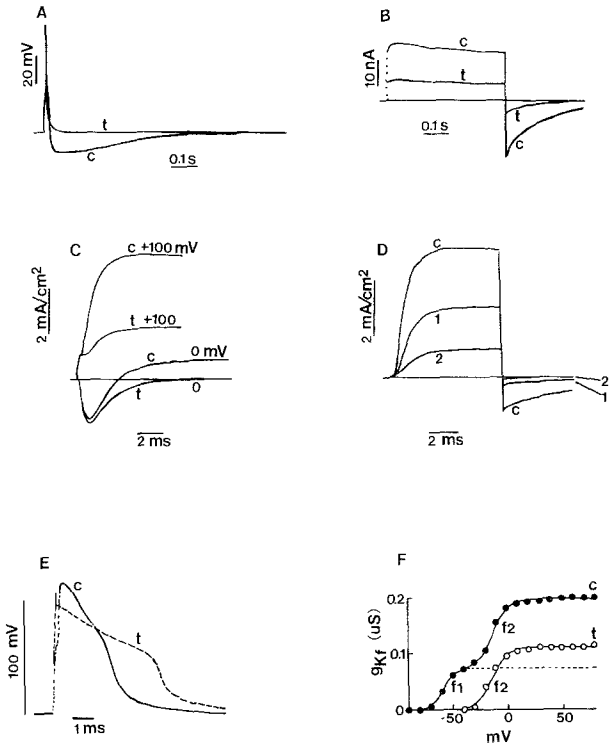


**Fig. 1.** Electrophysiological demonstrations of the specificity of various peptide toxins as K-channel inhibitors. Control records are marked *c* and records taken after the addition of various toxins are marked *t*. (*A* and *B*) Effect of 10 nM apamin on the action potential (*A*) and a slow $K^+$ current (*B*) in cultured rat myocytes. In *A*, apamin specifically inhibited a TEA-insensitive, after-hyperpolarization following the action potential. In *B*, $K^+$ currents were recorded in the presence of 20 mM TEA by depolarization of +40 mV from a holding potential of −50 mV. Tail $K^+$ currents were recorded after repolarization to −50 mV. [Redrawn from Romey and Lazdunski, (1984) with permission of the publisher.] (*C* and *D*) Effect of noxiustoxin (NTX) on membrane currents in a perfused squid giant axon in the absence (*C*) and presence of 0.3 $\mu$M tetrodotoxin (*D*). In *C*, $Na^+$ and $K^+$ currents were evoked by depolarization to the indicated voltage from a holding potential of −80 mV and after preconditioning at −100 mV for 0.1 sec. The records marked *t* were taken 13 min after addition of 5 $\mu$M NTX. In *D*, $K^+$ currents were recorded in the presence of tetrodotoxin by depolarization to +70 mV from a −80-mV holding potential and repolarization to −60 mV after the pulse. Traces *1* and *2* were taken 10 min after the addition of 0.5 and 3 $\mu$M NTX, respectively. [Redrawn from Carbone et al. (1987) with permission of the publisher.] (*E*) Effect of 85 nM dendrotoxin (DTX) on the action potential recorded from a frog motor nerve in normal Ringer solution. [*Redrawn from* Weller et al. (1985) with permission of the publisher.] (*F*) Effect of 53 nM Toxin I on fast $K^+$ conductance in the frog node of Ranvier. [*Redrawn from* Benoit and Dubois (1986) with permission of the publisher]

found to be TEA sensitive and apamin insensitive. To explain this discrepancy, Romey and Lazdunski (1984), hypothesized that there were two classes of Ca-activated K channels in cultured myotubes. The apamin-sensitive after-hyperpolarization was proposed to be mediated by a class of K channels activated at the very low levels of internal $Ca^{2+}$ reached during a single action potential, but inhibited at high internal $Ca^{2+}$. The other class of Ca-activated K channels could be induced to open at higher internal $Ca^{2+}$ achieved with the use of a Ca ionophore. The maintained hyperpolarization observed under these conditions was inhibited by TEA and corresponded to the large conductance Ca-activated K channels. This interpretation was proven correct when Blatz and Magleby (1986) observed a distinct small-conductance Ca-activated K channel of 10–14 pS in cultured rat muscle cells that was blocked by nanomolar apamin and was insensitive to TEA.

$^{125}$I-apamin has been used to identify specific high affinity binding sites for this toxin in the following tissues: brain synaptosomes (Hugues et al., 1982*a*), embryonic neurons (Seagar et al., 1984), neuroblastoma (Hugues et al., 1982*c*), intestinal smooth muscle (Hugues et al., 1982*b*), myotubes (Hugues et al., 1982*d*), and hepatocytes (Cook & Haylett, 1985). In these studies, high affinity $K_D$'s in the range of 10–400 pM were observed, but the maximum site densities ($B_{max}$) are low (1–30 fmol/mg) in comparison to the density of Na channels in rat brain synaptosomes (4000 fmol/mg) (Catterall, Morrow & Hartshorne, 1979). Higher levels of $^{125}$I-apamin binding sites (600 fmol/mg) have recently been reported for undifferentiated pheochromocytoma cells (Schmid-Antomarchi, Hugues & Lazdunski, 1986), making these cells a potential tissue source for eventual purification of the apamin receptor. Along these lines, preparations of detergent-solubilized apamin receptors have been reported (Schmid-Antomarchi et al., 1984; Seagar, Marqueze & Couraud, 1987*b*).

Apparently, the apamin receptor in rat muscle is only expressed in noninnervated myocytes. Specific $^{125}$I-apamin binding is undetectable in normal adult rat muscle but appears in a time-dependent fashion after denervation (Schmid-Antomarchi et al., 1985). Corresponding to the expression of receptor, the action potential in normal rat skeletal muscle fibers is insensitive to apamin, but an apamin-sensitive after-hyperpolarization appears after denervation.

The specific binding of apamin is enhanced by external $K^+$. In rat neurons, $^{125}$I-apamin binding increased threefold as extracellular $K^+$ was increased from 0.1 to 10 mM at constant ionic strength (Seagar et al., 1984). Scatchard analysis of this effect revealed an increase in the maximal binding capacity without an effect on the $K_D$. In experiments with rat brain synaptosomes using varying ionic strength, the dependence on $K^+$ is biphasic with a 1.8-fold stimulation from 10 $\mu$M to 5 mM $K^+$ and a progressive decrease at higher $K^+$ (Hugues et al., 1982*a*). In this preparation, the stimulatory effect was due to a decrease in the $K_D$ for $^{125}$I-apamin rather than an effect on $B_{max}$. $Rb^+$ had essentially the same biphasic effect as $K^+$, but other cations such as $Li^+$, $Na^+$, $Ca^{2+}$ and guanidinium only inhibited apamin binding. These effects of cations have been explained in terms of two sites: an allosteric stimulatory site specific for $K^+$ and $Rb^+$ and a second, nonspecific inhibitory cation site (Hugues et al., 1982*a*). This second site could be an anionic subsite of the apamin binding site that is normally involved in binding the two essential arginine groups of apamin. A variety of other organic cations have been found to compete with $^{125}$I-apamin binding. Among these, the highest affinity has been observed for neurotensin in rat brain synaptosomes ($K_{0.5}$ = 170 nM), which presumably acts by virtue of its two adjacent arginine residues (Hugues et al., 1982*a*). In guinea pig hepatocytes, the ability of a number of organic cations to block Ca-activated K efflux is closely correlated with their ability to inhibit $^{125}$I-apamin binding (Cook & Haylett, 1985).

Since the receptor site for apamin is presumably closely associated with the SK-type Ca-activated K channel, biochemical studies of the apamin receptor are being pursued with the goal of identification and isolation of the channel components. Radiation inactivation studies of specific $^{125}$I-apamin binding to rat brain membranes have yielded a molecular weight of 250 kDa using lyophilized membranes (Schmid-Antomarchi et al., 1984) or 84-115 kDa using frozen membranes (Seagar et al., 1986). Direct crosslinking of $^{125}$I-apamin to rat brain membranes with disuccinimylsuberate labeled only a 33-kDa component in the presence of protease inhibitors (Schmid-Antomarchi et al., 1984), suggesting that the binding site may be associated with a smaller subunit of a multimeric complex. Another laboratory used two photoreactive derivatives of apamin containing aryl azide groups coupled at the $\alpha$-amino of $cys_1$ or the $\varepsilon$-amino of $lys_4$ residues to examine peptide labeling patterns in both cultured neurons and brain membranes (Seagar et al., 1986). These experiments showed that the $lys_4$ derivative labeled bands of 33 and 22 kDa in both tissues, while the $cys_1$ derivative only labeled an 86-kDa peptide in cultured neurons and both 86- and 59-kDa peptides in isolated brain membranes. These results were interpreted to mean that the apamin binding site is situated near the interface of several

subunits such that the observed labeling is dependent on the location of the reactive group in the toxin. Further studies have recently shown that labeling of the 59-kDa component is found in cultured rat astrocytes but not in neurons, suggesting that glial cells may be responsible for the 59-kDa component that was labeled in brain membranes (Seagar et al., 1987*a*). Thus, the apamin receptor protein may be an oligomer containing 86-, 59-, 33- and 22-kDa peptides in various cells. However, the possibility of proteolysis in vivo requires more detailed structural information on these peptides before the oligomeric composition of the receptor complex can be established.

Binding studies using $^{125}$I-MCD peptide have begun to shed light on its possible mode of neurotoxicity. A $K_D$ of 150 pM and a $B_{max}$ of 200 fmol/mg was reported for binding of $^{125}$I-MCD to rat brain membranes (Taylor et al., 1984). This binding could be displaced by tertiapin with a $K_{0.5}$ of 100–150 nM but not by apamin at concentrations as high as 10 $\mu$M. These results show that these two classes of bee peptides recognize distinct receptor sites. The results of autoradiographic localization and the symptoms of MCD toxicity suggest that the hippocampus is a major target of MCD (Bidard et al., 1987*a*). Although voltage-clamp analysis of the effect of MCD on isolated cells has not yet been described, a recent report indicates that a snake neurotoxin of the dendrotoxin class (Toxin I) is able to inhibit binding of $^{125}$I-MCD with a $K_{0.5}$ of 0.16 nM (Bidard, Mourre & Lazdunski, 1987*b*). The mechanism of this inhibition was found to be noncompetitive, with a lowering of the $B_{max}$ but no change in the $K_D$ for $^{125}$I-MCD. Since Toxin I has been found to specifically block an A-current in hippocampal neurons (Halliwell et al., 1986), it appears that these two toxins may act on the same A-type K channel via different but interacting sites.

## Noxiustoxin and Charybdotoxin, Members of a Family of Scorpion Toxins Directed Against K Channels

Scorpion venom has been previously established as a source of peptide toxins specific for voltage-dependent Na channels. The collected sequences of these toxins show that they comprise a family of homologous basic peptides of 60–70 residues (Rochat et al., 1970; Watt & Simard, 1984). Each of these contain eight cysteines that form four intramolecular disulfide bonds. The venom of a given scorpion can contain many distinct Na-channel isotoxins. These toxins act at the extracellular surface of Na channels and fall into two classes based on binding competition and their effects on Na-channel gating (Meves, Simard & Watt, 1986). The $\alpha$-toxins bind with higher affinity at negative membrane potentials and slow the kinetics of Na-channel inactivation. The binding of $\beta$-toxins appears to be independent of membrane potential and shifts the voltage dependence of activation to more negative voltages. Binding competition studies with radiolabeled toxins show that toxins within each class compete for binding to a common site, but $\alpha$-toxins do not compete with $\beta$-toxin binding and vice versa. Thus, these Na-channel toxins recognize two distinct sites that interact with gating processes of the channel. The crystal structure of one of the toxins has been obtained at 1.8 A resolution (Almassy et al., 1983). Recently, Martin et al. (1987) suggested that $\beta$-toxins can be distinguished structurally from the $\alpha$-toxins by characteristic short deletions of 2–5 residues that are evident when sequences of the two types of toxins are aligned by the eight cysteines for maximum homology.

In addition to effects on Na-channel gating, early work with crude scorpion venom showed that $K^+$ currents are also suppressed (Narahashi et al., 1972). It has recently become apparent that inhibition of $K^+$ current by scorpion venom is due to a distinct family of K-channel toxins.

The first of these to be isolated and sequenced was Noxiustoxin (NTX), a 39-residue peptide from the Mexican scorpion, *Centruroides noxius Hoffmann* (Possani, Martin & Svendensen, 1982). This basic peptide has a structure that is quite different from the Na-channel toxins (Fig. 2). NTX contains six cysteine residues instead of eight. It also contains one methionine residue, whereas most of the Na-channel toxins lack this amino acid. NTX is lethal when injected intraperitoneally into mice (20 mg/kg body weight), but the symptoms of toxicity have not yet been described in detail.

A biological site of action of NTX was first located in the classic preparation of voltage-clamped squid giant axon (Carbone et al., 1982). Externally applied NTX was found to depress the delayed rectifier $K^+$ current without affecting $Na^+$ current (Fig. 1*C,D*). This effect on the $K^+$ current was fully reversible and was described by a one-site binding reaction with a $K_D$ of 390 nM.

In addition to NTX, another minor peptide component, called II-10-2, was isolated from *C. noxius Hoffmann* and its N-terminal sequence was found to be highly homologous to NTX (Table 2) (Possani et al., 1982). A similar homologous peptide (II-9) isolated from the Brazilian scorpion, *Tityus serrulatus*, was also found to inhibit squid axon $K^+$ currents without affecting Na currents.

In a more detailed investigation into its mode of

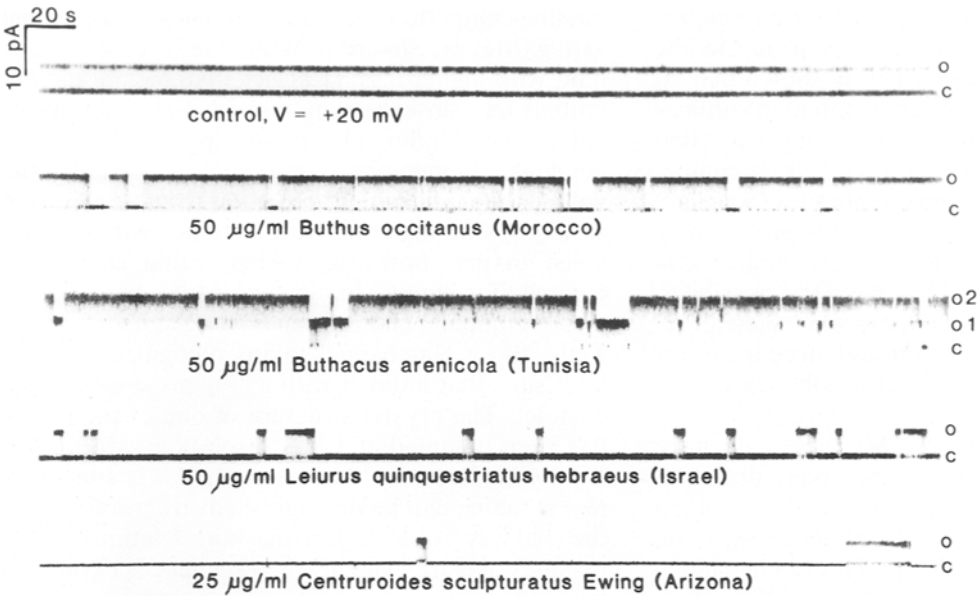


**Fig. 2.** Screening of crude venoms of various scorpions for blocking activity against a large conductance Ca-activated K-channel. Ca-activated K-channels from rat muscle were incorporated into planar lipid bilayers according to Moczydlowski and Latorre (1983). Single channel currents were recorded in the presence of a symmetrical solution of 10 mM MOPS-KOH, pH 7.4, 0.2 M KCl. 100 μM $CaCl_2$ was present on the internal (intracellular) side of the bilayer, and 0.1 mg/ml bovine serum albumin was present on the external side. The control record was taken in the absence of scorpion venom, and the other records were taken in the presence of the indicated external concentrations of various crude venoms. The closed channel level is denoted by *c* and the open channel level by *o*. In each experiment the bilayer contained a single channel except for the middle experiment, which used a bilayer containing two channels (A. Ravindran and E. Moczydlowski, *unpublished results*)

**Table 2.** Amino acid sequences of two classes of scorpion venom toxins

| | 1---5---10---15---20---25---30---35---40 |
|---|---|
| NTX | TIINVKC-TSPKQCSKP-CKELYGSSAGAKCMNGKCKCY?N |
| II-9 | VFINAKCRGSP-ECL-PKCKEA?GKAAG-KCMN |
| II-10-2 | TFIDVKC-GSSKEC?-P |
| ChTX | ZFTNVSCTTS-KECWSV-CQRLHNTSRG-KCMNKKCRCYS |

The sequences are aligned by six cysteine residues as otherwise described in Table 1. At present, only the complete sequences of NTX (Possani et al., 1982) and ChTX from *L. quinquestriatus* (Garcia et al., 1988) are available. The known N-terminal portions of toxins II-9 from *T. serrulatus* and II-10-2 from *C. noxius* are also listed (Possani et al., 1982). Positions at which the identity of the residue is uncertain are noted with a question mark. Z denotes pyroglutamic acid. The disulfide pairing relationships of the six cysteine residues are presently unknown; however, weak homology to α-bungarotoxin of *Bungarus multicinctus* noted by Garcia et al. (1988) suggests that cysteines at residues 14 and 19 are paired.

action, Carbone et al. (1987) found that NTX appears to block squid axon $K^+$ current in a voltage-independent manner at low toxin concentrations, but does exhibit some voltage dependence at concentrations above 1.5 μM. In this high concentration range, block is partially relieved by repetitive depolarization and is enhanced by hyperpolarization. It was also found that not all of the $K^+$ current is blocked at large depolarizations, suggesting incomplete inhibition of the channel or the presence of two classes of K channels.

In addition to its effect on squid axon $K^+$ currents, NTX stimulates release of $^3$H-GABA and inhibits efflux of $^{86}Rb^+$ from mouse brain synaptosomes (Sitges, Possani & Bayon, 1986). These observations are also consistent with a direct action of

NTX on $K^+$ permeability. In these experiments, NTX exhibited a $K_i$ of 3 nM, which is 100-fold higher affinity than that observed in the squid axon. While the specificity of NTX for various types of K channels needs to be examined in greater detail, the available results indicate that this toxin is a potent blocker of certain types of voltage-activated K channels.

The demonstrated activity of scorpion venom against voltage-activated K channels mentioned above prompted Miller et al. (1985) to search for a toxin directed against large conductance Ca-activated K channels. This search led to the discovery of a peptide christened *charybdotoxin* (ChTX). ChTX was first described as a component of an Old World scorpion, *Leiurus quinquestriatus* (LQ), but certain other scorpion venoms possess similar activity. Figure 2 shows results of experiments in which crude venom of various scorpions was assayed on single Ca-activated K channels incorporated into planar lipid bilayers from rat muscle T-tubule membranes. The control record shows an example of a current from a bilayer containing a single channel at +20 mV holding voltage and 100 $\mu$M internal $Ca^{2+}$. Under these conditions the channel rapidly fluctuates between closed and open conductance states and exhibits a time-averaged open-state probability of about 0.5. When 50 $\mu$g/ml LQ venom is added to the external side of the channel, discrete blocking events are observed that are characteristic of reversible binding of a blocker to the channel. Figure 2 also shows that venoms of other Old World scorpions (*Buthus* and *Buthacus*) and a New World scorpion (*Centruroides*) also contain ChTX-like activity and that the potency and kinetics of blocking activity vary markedly among different venoms. Table 3 summarizes the results of screening various venoms for blocking activity against the maxi-$K^+$ channel in our laboratory. These results show that in the four active venoms, the mean blocked time varies from 1.8 sec for the venom of *Buthacus arenicola* to 150 sec for *Centruroides sculpturatus*. Among the scorpion venoms that have been tested thus far, it appears that *Leiurus* and *Centruroides* venom are the best source of ChTX activity. In similar experiments, venoms of other scorpions were found to lack any significant inhibitory activity against the large-conductance Ca-activated K channel. These inactive venoms included those of *Androctonus australis*, *Buthotus judaicus*, *Pandinus imperator* and *Tityus serrulatus*. In addition, bee venom (*Apis mellifera*) was also inactive (A. Ravindran, *unpublished results*).

Purification and sequencing of ChTX from *Leiurus* venom has recently been achieved (Smith, Phillips & Miller, 1986; Garcia et al., 1988). ChTX is a 37-residue basic peptide containing pyroglutamate at its N-terminus. The N-terminal pyroglutamate can be cleaved with pyroglutamate aminopeptidase, and the resulting unblocked peptide can be sequenced by automated methods. The sequence of ChTX (Table 2) indicates that this peptide is highly homologous to NTX and also to other K-channel toxins described by Possani et al. (1982). In view of the close homology of these peptides, the reported action of NTX on delayed rectifier-type K channels and that of ChTX on Ca-activated K channels leads to questions about the specificity of these toxins.

Recently, Valdiva et al. (1988) compared the effect of ChTX and NTX in the planar bilayer assay of single maxi Ca-activated K channels. These workers found that ChTX exhibited a mean blocked time of 10–15 sec and a $K_D$ of 1–3 nM. In similar experiments, NTX induced brief blocking events with a mean blocked time of 0.05 sec and a $K_D$ of 450 nM. On the basis of these results, it appears that NTX also recognizes the maxi Ca-activated K channel with low affinity. Further evidence for cross-specificity of toxins of the NTX/ChTX class for Ca-activated and delayed rectifier K channels was reported by Lewis and Cahalan (1988). In electrophysiological studies of murine thymocytes, it was shown that 5 nM ChTX completely blocked two different types of delayed rectifier K channels with unitary conductances of 17 and 18 pS and had no effect on a third type of delayed rectifier with a conductance of 27 pS. Thus, it appears that the high affinity binding site for ChTX is shared by a selected

**Table 3.** Crude venoms of various scorpions found to have charybdotoxin-like blocking activity

| Active venoms | Relative potency $IC_{50}$ ($\mu$g/ml) | Mean blocked time (sec) |
|---|---|---|
| *Buthacus arenicola* | 910 | 1.8 ± 0.5 |
| *Buthus occitanus* | 490 | 3.0 ± 0.5 |
| *Leiurus quinquestriatus hebraeus* | 3.6, 17 | 18 ± 0.2 |
| *Centruroides sculpturatus Ewing* | 1.9 | 150 ± 15 |

The relative potency and mean blocked time characteristic of each of the venoms in blocking large conductance Ca-activated K channels was determined from experiments described in Fig. 2. The $IC_{50}$ was calculated from the observed steady-state inhibition at one venom concentration using a one-site/one-toxin model of inhibition. The mean blocked time and standard error of the duration of blocking events were calculated from populations of 50–100 events. The relative potency of two different lots of *Leiurus* venom are listed (A. Ravindran and E. Moczydlowski, *unpublished results*).

variety of voltage- and Ca-activated K channels. These pharmacological results predict corresponding structural homology between various ChTX-sensitive subtypes of K channels.

In other planar bilayer studies with purified ChTX, this toxin has no effect on batrachotoxin-activated Na channels and a sarcoplasmic reticulum K channel but blocks large conductance Ca-activated K channels from skeletal muscle and colon smooth muscle with nanomolar affinity (Miller et al., 1985; Latorre, 1986). In a patch-clamp study of cultured kidney epithelial cells, 2 nM ChTX blocked a similar Ca-activated K channel (Guggino et al., 1987).

In rat hippocampal neurons, 25 nM ChTX was found to block a TEA-sensitive, fast, after-hyperpolarizing phase of the action potential, but not a TEA-insensitive, slow, after-hyperpolarizing phase (Lancaster & Nicoll, 1987). Other phases of the action potential appeared to be unaffected by ChTX, suggesting specificity for a distinct class of Ca-activated K channels in these cells.

In the dorsal longitudinal flight muscle of *Drosophila*, ChTX blocks ($K_i$ = 75 nM) a Ca-activated K current that is also abolished by a mutation called slowpoke (slo) (Elkins, Ganetzky & Wu, 1986). In this preparation, ChTX did not appear to affect delayed rectifier or inactivating $K^+$ currents, but a reduction of inward Ca current at high doses of ChTX was noted. This result implies that ChTX can have nonspecific effects on other types of channels at high concentrations.

In single neurons of various ganglia from *Aplysia californica*, Hermann and Erxleben (1987) reported that partially purified ChTX blocked a Ca-activated $K^+$ current but had no effect on $Na^+$ current, $Ca^{2+}$ current, delayed rectifier $K^+$ current and transient $K^+$ current. In this preparation, block by ChTX was voltage dependent, with the $K_D$ for ChTX (30 nM at −30 mV) increasing *e*-fold for 50–70 mV of depolarization. In voltage recordings, ChTX was found to increase the duration of single action potentials up to eightfold in the R-15 neuron, showing that the Ca-activated $K^+$ current contributes to repolarization of the action potential in this cell. Single-channel studies showed that the unit conductance of this ChTX-sensitive K channel in *Aplysia* is 35 pS.

A report concerning Ca-activated K channels from rat brain described three types of channels with conductances of 75, 140 and 240 pS (Reinhart & Levitan, 1987). All of these channels were blocked by nanomolar ChTX, suggesting that there may be a family of related Ca-activated K channels in brain that differ in unitary conductance but share a common binding site for ChTX.

To our knowledge, highly purified ChTX has not yet been tested directly on the apamin-sensitive class of Ca-activated K channels. However, work with crude venom and partially purified fractions suggest that ChTX does not block or has low affinity for apamin-sensitive K channels. Two groups of workers (Abia et al., 1986 Castle & Strong, 1986) reported that crude LQ venom inhibited both apamin-sensitive, Ca-activated $K^+$ efflux from guinea pig hepatocytes and apamin-insensitive, Ca-activated $K^+$ efflux from human erythrocytes. However, upon cation-exchange chromatography of the crude venom, the inhibitory activity separated into two distinct peaks (Castle & Strong, 1986). The most basic fraction, peak X, was a more potent inhibitor of the erythrocyte $K^+$ efflux, while peak VIII was more potent against the hepatocyte $K^+$ efflux. In addition, peak VIII was an inhibitor of $^{125}$I-apamin binding to hepatocytes, while peak X was inactive in this assay. Since ChTX is found in the most basic peak in cation exchange chromatography of LQ venom (Miller et al., 1985), it was suggested that peak X may correspond to ChTX. Recent experiments with highly purified ChTX have confirmed its effectiveness against Ca-activated $K^+$ efflux in the human erythrocyte (K. Lucchesi, *unpublished results*). These results indicate that LQ venom contains at least two toxins active against two different classes of Ca-activated K channels. The toxin specific for the apamin-sensitive class has not yet been purified. However, the structural relationship of this toxin to apamin, NTX and ChTX will be of considerable interest.

Recently, the blocking kinetics of maxi Ca-activated K channels by ChTX was analyzed in detail (Anderson, MacKinnon & Miller, 1988). From measurements of association and dissociation rate constants for ChTX block, these workers found that binding of ChTX is both state dependent and voltage dependent. ChTX was found to bind to both closed and open states of the channel but with higher affinity to the open state. The kinetic basis of this effect was a fivefold faster $k_{on}$ for binding to the open channel *vs.* the closed channel. The observed $k_{off}$ of ChTX bound to closed and open states of the channel appeared to be the same. The effect of voltage on ChTX binding was also examined in experiments designed to keep the probability of channel gating constant. Under these conditions, it was found that depolarization increased the $k_{off}$ of the toxin (*e*-fold per 20 mV), and $k_{on}$ was independent of voltage. It was also found that increasing the ionic strength of the external medium from 20 to 200 mM with various salts reduced the observed $k_{on}$ of ChTX by a factor of 100, while the $k_{off}$ of the toxin was unaffected. This suggests that there is a nega-

**Table 4.** Comparison of amino acid sequences of five snake dendrotoxins, two subunits of β-bungarotoxin and a protease inhibitor, BPTI

```
                1---5---10---15---20---25---30---35---40---45---50---55---60
DTX             ZPRRKLCILHRNPGRCYDKIPAFYYNQKKKQCERFDWSGCGGNSNRFKTIEE-CRRTCIG
C13S1C3         --AAKYCKLPVRYGPCKKKIPSFYYKWKAKQCLPFDYSGCGGNANRFKTIEE-CRRTCVG
Toxin I         ZPLRKLCILHRNPGRCYQKIPAFYYNQKKKQCEGFTWSGCGGNSNRFKTIEE-CRRTCIRK
Toxin K         --AAKYCKLPLRIGPCKRKIPSFYYKWKAKQCLPFDYSGCGGNANRFKTIEE-CRRTCVG
DV14            --AAKYCKLPVRYGPCKKKIPSFYYKWKAKQCLYFDYSGCGGNANRFKTIEE-CRRTCVG

BGT B           RQRHRDCDKPPDKGNC-GPVRAFYYDTRLKTCKAFQYRGCDGDHGNFKTETL-CRCECLVYP
BGT A           GAGGSGRPIDALDRCCYVHDNCYGDAEKKHKCNRKTSQICYGAAGGTCRIVCDCDRTAALCF

BPTI            --RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAED-CMRTCGGA
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The sequences are aligned by six cysteine residues as otherwise described in Table 1. The first group of five toxins are known to have DTX-like neurotoxic activity. Toxins DTX and C13S1C3 are from *D. angusticeps*. Toxin I and Toxin K are from *D. polylepis*. Toxin DV14 is from *D. viridis*. The second group includes the complete sequence of the B-subunit (BGT B) and residues (30–101) of the A-subunit (BGT A) of β-bungarotoxin from *Bungarus multicinctus*. The last sequence is that of bovine pancreatic trypsin inhibitor (BPTI). Z denotes pyroglutamic acid. Homology to the known structure of BPTI suggests that the disulfide pairing relationships in the dendrotoxins are residues 7-58, 16-40 and 32-54. Original references to the sequences are given in Rehm (1984), Dufton (1985) and Harvey and Anderson (1985).

tive electrostatic surface potential at the toxin binding site that controls the observed association rate of this highly basic peptide.

In a companion paper, MacKinnon and Miller (1988) reported the surprising finding that increasing internal $K^+$ relieves ChTX block on the opposite side of the membrane. This effect, due to an increase in the observed dissociation rate of the toxin with increasing internal $K^+$, was termed "*trans*-enhanced dissociation." While the permeant $Rb^+$ ion also caused a similar effect, impermeant $Li^+$, $Na^+$, $Cs^+$ and arginine on the internal side of the channel did not cause this effect. These results led the authors to speculate that ChTX binds to a site near the external mouth of the channel and blocks by direct occlusion. The observed voltage dependence of ChTX binding appears to be due to the voltage-dependent binding of internal $K^+$ rather than a direct effect of voltage on the toxin molecule, as in a conventional Woodhull model (Woodhull, 1973).

In addition to NTX and ChTX, a third type of blocking activity against K channels has recently been described for venom of the African scorpion, *Pandinus imperator* (Pappone & Cahalan, 1987; Pappone & Lucero, 1988). This venom was found to partially block the voltage-activated K current in frog myelinated nerve, and in GH3 pituitary cells but not in frog skeletal muscle. The blocking activity was found to be irreversible, in contrast to that of NTX and ChTX, and was also more effective at negative than at positive potentials. Since only part of the total voltage-activated K current was blocked in these studies, it was suggested that the active toxin component in this venom might be specific for a distinct subclass of K channels or might act by altering gating kinetics of K channels.

## Dendrotoxins, a Family of K-channel Toxins in Snake Venom

Snake venom toxins that facilitate transmitter release from nerve terminals as a result of their actions on K channels have been identified from two species of green mamba, *Dendroaspis angusticeps* and *D. viridis*, and from the black mamba, *D. polylepsis* (Harvey & Anderson, 1985). Dendrotoxin (DTX), which is obtained from the venom of *D. angusticeps*, is a member of a group of homologous basic peptides of 57–61 residues that presently include five examples known to have neurotoxic activity (Table 4).

The facilitory effect of the dendrotoxins on neurotransmission contrasts with the neuromuscular blockade produced by the anti-cholinergic neurotoxins of *Elapidea* snakes, which bind to the α-subunit of acetylcholine receptor-channels (e.g., α-bungarotoxin). The five dendrotoxins are also

distinguished from the β-bungarotoxin class of prejunctional snake neurotoxins because they are without intrinsic phospholipase A2 activity. Dendrotoxins are therefore advantageous in pharmacological studies because the phospholipase activity of β-bungarotoxin can mask its receptor interactions by permeabilizing nerve terminals subsequent to binding (Othman, Spokes & Dolly, 1982; Rugulo, Dolly & Nicholls, 1986). Given their prejunctional facilitory activity, the dendrotoxins are considerably less lethal when administered peripherally than are the curaremimetic toxins from *Elapid* or *Hydrophid* snakes. However, when injected intracerebrally into rats, DTX and the homolog, Toxin I, produce convulsions and death at 1/10,000 of the peripheral lethal dose (Mehraban et al., 1985). This convulsive action has been attributed to facilitation of transmitter release in noncholinergic synapses of the hippocampus (Docherty et al., 1983). Although it is much less lethal when administered peripherally, DTX has been shown to hypersensitize mice to external stimuli and to produce respiratory paralysis. Without causing spontaneous contractures or twitching, DTX, Toxin I and Toxin K augment contractile responses of the chick biventer cervicus nerve-muscle preparation by facilitating the presynaptic release of Ach from the neuromuscular junction (Barrett & Harvey, 1979; Harvey & Karlsson, 1982). In this commonly used bioassay, the K-channel blocker, 3,4-diaminopyridine, produced a similar response to nerve stimulation, but at 1000-fold higher concentrations than the dendrotoxins.

Nanomolar concentrations of DTX induce repetitive firing in rat visceral sensory neurons by inhibiting a slowly inactivating outward $K^+$ current which is similar to the effect of 4-AP (Stansfeld et al., 1986). In fact, all of the above-mentioned excitatory actions of dendrotoxins on central and peripheral nervous tissue appear to be the direct result of inhibition of several related types of outward $K^+$ currents. In this respect, dendrotoxins have been already recognized as valuable pharmacological probes of K channels.

From an evolutionary standpoint, a fascinating aspect of dendrotoxin chemistry is the considerable homology of these neurotoxins to an ubiquitous class of protease inhibitors called Kunitz-type inhibitors. One of the best-known examples of these inhibitors is bovine pancreatic trypsin inhibitor (BPTI). The sequence of BPTI can be exactly aligned with the dendrotoxin sequence by the positions of six cysteine residues common to both of these peptides (Table 4). In addition, there are several other invariant residues. Although the crystal structure of a dendrotoxin peptide has not yet been obtained, the homology to the well-characterized Kunitz-inhibitors has provided a framework on which the structure of the snake toxins has been modeled (Dufton, 1985; Harvey & Anderson, 1985).

Despite the high degree of homology of these two classes of peptides, dendrotoxins do not possess significant inhibitory activity toward proteases. Likewise, the known protease inhibitors do not possess dendrotoxin-like activity. The structural basis for these contrasting activities has been discussed in terms of differences in secondary structure and critical residues that are involved in recognition of the active site of proteases and the inhibitory site of K channels (Dufton, 1985). On the basis of the strong homology of these peptides, Dufton (1985) suggested that the inhibitory target of dendrotoxins was likely to exhibit homology with the serine protease family. This suggested relationship between proteases and ion-channel proteins would indeed be unprecedented. However, examination of the recently deduced sequence of a *Drosophila* A-channel component (Tempel et al., 1987) reveals only minor homology between this sequence and that of trypsinogen. The significance of this neurotoxin/protease inhibitor homology will undoubtedly continue to be explored.

Two of the known dendrotoxin homologs (DTX and Toxin I) are blocked at their N-terminus by pyroglutamic acid, a modification that has also been found for ChTX, the scorpion toxin discussed above. Aside from this, few other structural similarities exist between snake dendrotoxins and the scorpion K channel toxins. However, DTX does show considerable homology with the lighter B-subunit of β-bungarotoxin (Table 4), another type of inhibitory presynaptic neurotoxin from *Bungarus multicinctus*. This latter group of toxins exhibits phospholipase A2 activity that resides in a 13,000 dalton A-subunit linked to the B-subunit by one disulfide bridge. A portion of the A-subunit including residues 45–86 is also faintly homologous to the dendrotoxins (Table 4). A neurotoxic, tritiated derivative of β-bungarotoxin has been prepared by reaction of the native toxin with N-succinimidyl-[2,3-$^3$H]propionate (Othman et al., 1982). This derivative has negligible phospholipase activity but binds with high affinity to rat brain synaptosomes ($K_D$ = 0.6 nM). Toxin I competes with high affinity for [$^3$H] β-bungarotoxin binding, indicating that these two toxins recognize a common receptor site.

The electrophysiological basis for the central convulsive action of DTX has recently been examined by intracellular recordings from single neurons in the $CA_1$ region of hippocampal slices (Halliwell et al., 1986). In the presence of TTX to block $Na^+$

currents, 50 nM DTX induced repetitive firing of $Ca^{2+}$ action potentials in response to depolarizing current. Voltage-clamp experiments revealed that DTX did not affect $Ca^{2+}$ current measured in the presence of internal $Cs^+$ to block outward $K^+$ current. In addition, DTX had no effect on a Ca-activated $K^+$ current, a mixed $Na^+/K^+$ current activated by hyperpolarization and a noninactivating $K^+$ current, activated by depolarization. The only consistent effect of DTX was inhibition of an inactivating outward current that is considered to be an A-type $K^+$ current in these cells. While this A-current was blocked reversibly by 100 $\mu$M 4-AP, 290 nM DTX caused a practically irreversible inhibition of the same current. Thus, the facilitory and convulsive effects of DTX can be explained by removal of a current that normally exerts a hyperpolarizing effect and serves to reduce excitability.

Additional support for an effect on brain K channels can be found in experiments on isolated synaptosomes (Weller et al., 1985). In these experiments, DTX induced release of $^3$H-GABA that occurred due to membrane depolarization as measured by the distribution of tetraphenylphosphonium, a lipophilic cation. Since similar effects are produced by 4-AP, these observations were interpreted as an inhibition of K channels that contribute to synaptosomal membrane potential.

The electrophysiological effects of dendrotoxins have also been examined in peripheral nerve tissues. With the air-gap recording method in frog tibial myelinated nerves, as little as 0.1 nM DTX was found to increase the threshold, reduce the amplitude and prolong the duration of action potentials (Fig. 1*E*) (Weller et al., 1985). These experiments established that DTX was not purely a presynaptic toxin, in spite of its homology and interaction with the binding sites of $\beta$-bungarotoxin. Although this effect was shown to be due to an inhibition of $K^+$ current with no effect on $Na^+$ current, Weller et al. (1985) also found that not all of the total $K^+$ current was sensitive to DTX. In various experiments the fraction of total $K^+$ current resistant to 85 nM DTX ranged from 15–69%.

At the frog node of Ranvier, three distinct $K^+$ currents have been separated by analysis of tail currents: the $f_1$ and $f_2$ components of fast $K^+$ conductance that completely inactivate, and one slowly activating *s* component, that does not inactivate (Dubois, 1981). Toxin I, a dendrotoxin from *D. polylepis*, preferentially blocked the $f_1$ component (Fig. 1*F*) in a voltage-dependent manner; i.e., the block was less complete at depolarizing voltages (Benoit & Dubois, 1986). This $f_1$ component of the fast $K^+$ conductance, that activates between $-80$ and $-40$ mV, was blocked in an essentially irreversible manner by externally applied Toxin I, with an approximate $K_D$ of 0.4 nM. Since the $f_1$ K channels in the node of Ranvier and the channels that give rise to the A-current in neurons have similar characteristics such as sensitivity to 10–100 $\mu$M 4-AP (Dubois, 1981), block by DTX in both tissues is consistent with the notion that these two channels share some common structural features. Unlike the $f_1$ component, the $f_2$ component of the fast $K^+$ conductance that activates between $-40$ and $+30$ mV and the slowly activating *s* component of the $K^+$ current were not affected by Toxin I. In the same preparation, 20 $\mu$M capsaicin was found to reversibly block only the $f_2$ component (Dubois, 1982), providing further support for the pharmacological distinctness of these three types of K channels.

In contrast to the effects on the fast transient currents at the frog node and in the hippocampus, a different type of $K^+$ current in guinea pig dorsal root ganglion neurons was found to be sensitive to DTX (Penner et al., 1986). Here, 0.14 to 1.4 nM externally applied DTX irreversibly blocked a portion of delayed, noninactivating outward $K^+$ current without affecting a fast, transient current that was completely inactivated at a holding potential of $-50$ mV. In these experiments, 3,4-diaminopyridine and TEA blocked both of these currents and thus could not be used to cleanly distinguish either of them. In this case, DTX selectively recognizes a noninactivating subtype of K channel.

In another recent study, Stansfeld et al. (1986) examined the effect of DTX on a subpopulation of rat visceral afferent neurons. Single A-cells of the rat nodose ganglion were found to fire repetitively in response to depolarization in the presence of 1–30 $\mu$M 4-AP or 3–10 nM DTX. The ionic basis for this effect was found to be due to inhibition of a rapidly activating, but slowly inactivating (1–5 sec) $K^+$ current. This current was unusual in that it was not inhibited by other K-channel common blockers such as 10 mM TEA, 3 mM $Ba^{2+}$ or 4 mM $Cs^+$. The kinetics of this unusual current are quite different from those of a conventional transient A-current which inactivates rapidly ($\tau$ = 25 msec) and is present in a different subpopulation of cells in the ganglion. However, this latter current, which behaves kinetically like an A-current, is not inhibited by 100 $\mu$M 4-AP, in contrast to many other examples of A currents. These authors suggested that the slowly inactivating $K^+$ current that is sensitive to DTX and 4-AP might be analogous to the $f_1$ class of $K^+$ currents described in the frog node of Ranvier discussed above. Thus, electrophysiological results in various neuronal cells indicate that DTX recognizes certain types of outward-rectifying K channels that differ greatly in their kinetics.

For use in binding and receptor localization studies, $^{125}$I-DTX can be prepared without significant loss of biological activity using the chloramine-T method of iodination (Dolly et al., 1984; Mehraban, Breeze & Dolly, 1984; Black et al., 1986). The autoradiographic distribution of $^{125}$I-DTX bound to brain slices has been found especially dense in synapse-rich areas of the hippocampus and cerebellum (Halliwell et al., 1986). $^{125}$I-DTX has been shown to bind with high affinity to a single class of sites in rat brain synaptosomes (Mehraban et al., 1984; Black et al., 1986; Halliwell et al., 1986). These studies reported $K_D$'s of 0.3–0.4 nM and a maximal site capacity of 1–1.7 pmol/mg in various brain preparations. Mammalian brain thus appears to be a comparatively rich source of DTX binding sites that could be exploited in attempts to purify possible K-channel components. Binding of $^{125}$I-DTX in rat brain was also found to be inhibited by various non-radioactive dendrotoxin homologs in the potency order that correlates with neurotoxicity, Toxin I > DTX = DV14 > Toxin B (Black et al., 1986), suggesting a functional role of this binding site in the production of convulsive symptoms. While biochemical studies of the DTX binding protein(s) are at an early stage, crosslinking of $^{125}$I-DTX to rat brain membranes using dimethylsuberimidate has identified a specifically labeled band of 65,000 daltons (Mehraban et al., 1984). This size is much smaller than the apparent molecular weight of the DTX binding site as measured by radiation inactivation, which gave a preliminary result of about 250,000 daltons (Dolly et al., 1984). These findings are suggestive of a receptor structure composed of multiple subunits.

The results of $^{125}$I-DTX binding to chick synaptic membranes are somewhat more complicated, showing two classes of binding sites: a high affinity site with a $K_D$ of 0.5 nM and a site density of 90 fmol/mg; and a low affinity site with a $K_D$ of 15 nM and a poorly defined maximal density (Black & Dolly, 1986). While dendrotoxin homologs inhibited both classes of binding sites, β-bungarotoxin inhibited only the high affinity component of $^{125}$I-DTX binding with a $K_i$ of 0.8 nM. In rat brain, β-bungarotoxin does not appear to inhibit $^{125}$I-DTX binding with high affinity (Black et al., 1986), although Toxin I is able to completely inhibit high affinity binding of $^{3}$H-β-bungarotoxin (Othman et al., 1982). It appears that there are two receptor subtypes in both tissues that can be distinguished by their affinity for β-bungarotoxin. In rat, these two sites have very similar affinity for DTX, but in chick they differ by about 30-fold. Similar crosslinking studies with $^{125}$I-DTX in chick membranes identified two labeled bands of 69,000 and 75,000 daltons. However, labeling of both of these bands was partially inhibited by β-bungarotoxin, indicating that both of the bands originated from both classes of the observed binding sites. As of yet, binding studies with $^{125}$I-DTX have not been reported in peripheral tissues but will be of considerable interest in light of the electrophysiological evidence for interactions of dendrotoxins with unique K-channel subtypes.

## Conus Venom as a Potential Source of K-Channel Toxins

Marine snails of the *Conus* genus are venomous predators that have recently attracted attention as a promising source of peptide toxins directed against various types of ion channels (Olivera et al., 1985). Three distinct classes of conotoxins specific for muscle acetylcholine receptors, muscle Na channels and neuronal Ca channels have been purified and sequenced from two snail species, *Conus geographus* and *Conus magus*. In addition to these toxins, other *Conus* peptides with unique biological activity but with an unknown molecular basis of action have also been described (McIntosh et al., 1984).

At present, *Conus* venom remains largely unexplored as a source of K-channel toxins. However, a recent paper by Chesnut, Carpenter and Strichartz (1987) reported effects of whole venom of *Conus striatus* on delayed rectifier K currents in *Aplysia* neurons. These authors observed complex effects on $K^+$ currents that could be explained by multiple components of the venom: a heat labile component of greater than 50,000 daltons appears to be responsible for reduction of the peak $K^+$ current; a heat-stable component of less than 50,000 daltons appeared to increase the peak $K^+$ current; and a heat-stable component of lower molecular weight slowed activation and inactivation kinetics. These results invite attempts to purify the venom constituents responsible for these effects. In collaboration with Dr. Baldomero Olivera's laboratory at the University of Utah, our laboratory recently screened the venom of five *Conus* species (*C. geographus*, *C. striatus*, *C. magus*, *C. textile* and *C. marmoreus*) for ChTX-like blocking activity against the large conductance Ca-activated K channel from rat skeletal muscle. We did not find any evidence of ChTX-like activity in these venoms (A. Ravindran, *unpublished results*).

## Summary

Voltage-dependent ion channels are a difficult class of proteins to approach biochemically. Many such channels are present at low density in relevant tis-

sues and exist as multiple subtypes that can be distinguished electrophysiologically. In particular, K channels appear to be a diverse family of proteins characterized by many different conductance properties, gating behaviors and regulatory phenomena. Fortunately, specific peptide toxins for K channels are present in the venoms of insects, scorpions, snakes and possibly other species. The available sequences of these peptides define several different families of toxins. Electrophysiological and radioligand binding studies suggest that these toxins can be used to distinguish subclasses of K channels that share similar toxin binding sites. The growing databank of sequence homologies for both toxins and channels is, in essence, a codebook for identifying common elements of structure and function. The continuing development of toxins as biochemical probes should help to uncover the molecular basis and physiological significance of K-channel diversity.

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## *Note Added in Proof*

A detailed report on the primary structure of charybdotoxin (Gimenez-Gallego et al., 1988) and two papers on the biochemistry of dendrotoxin-binding proteins (Rehm & Lazdunski, 1988*a*,*b*) have recently appeared.

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