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COMMENTARY

TOXINS THAT AFFECT VOLTAGE-DEPENDENT CALCIUM CHANNELS

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Toxins are poisonous compounds, produced in living organisms, that are used to immobilize or kill and sometimes to digest prey. The death of the prey is often due to either respiratory or circulatory failure, and hence the target sites of the toxic components are often proteins of excitable membranes. Fractionation of several venoms has led to the discovery of molecules that have high affinity and specificity for ion channels in nerve and muscle. These toxins have proven to be invaluable tools in the biochemical characterization of these proteins. Two examples of proteins whose isolation and purification have been made possible by the use of toxins that bind specifically to them are the nicotinic acetylcholine receptor and the voltage gated sodium channel.

A family of small basic polypeptides, known as alpha-neurotoxins, are found in the venoms of a variety of Hydrophiidae and Elapidae snakes and have been used extensively in the purification and characterization of the nicotinic acetylcholine receptor (reviewed in Refs. 1–5). These toxins all appear to share a common binding site on the receptor and inhibit its function by similar mechanisms. A variety of other toxins bind to and consequently alter the function of voltage-dependent sodium channels. The interactions of these toxins with a sodium channel have been reviewed by Lazdunski and Renaud [6] and by Catterall [7]. The sodium channel toxins are a very diverse group of molecules which modulate the activity of the channel in different fashions. Lazdunski and Renaud [6] divide the toxins into five groups: (a) molecules that produce a reversible block of the fast inward sodium current (tetradotoxin and saxitoxin), (b) compounds that prolong the activation of the channels (vertridine, batrachotoxin, aconitine and grananotoxin), (c) polypeptides that slow the inactivation of the channel by a mechanism which is dependent on the membrane potential (the sea anemone and some scorpion toxins), (d) neurotoxins that affect the kinetics of channel opening (pyrethroid toxins), and (e) other scorpion polypeptide toxins that affect the activation of the channel but are not dependent on the membrane potential.

The effectiveness of these toxins in the study of the acetylcholine receptor and the sodium channel has led to the search for toxins specific for other proteins present in excitable membranes. A number of toxins have been identified that alter the activity of potassium channels. Several types of potassium channels exist, however, which differ in their electrophysiological characteristics. Possani *et al.* [8] have purified a polypeptide from the venom of the Mexican scorpion, *Centuroides noxius*. This toxin, noxiustoxin, blocks the voltage-dependent potassium channel in the squid giant axon [9], and in mouse brain synaptosomes [10] and, hence, may prove to be important in the characterization of these potassium channels. Apamin, a polypeptide toxin from bee venom, blocks Ca^{2+}-activated K^+ currents in neuroblastoma cells [11], smooth muscle [12], skeletal muscle [13], and hepatocytes [14]. This toxin has been used to characterize the protein to which it binds in rat synaptic membranes [15]. Another toxin, charybdotoxin, which is a 7000 molecular weight protein from the venom of the scorpion, *Leiurus quinquestriatus*, blocks Ca^{2+}-activated K^+ currents in mammalian skeletal muscle [16, 17]. This K^+ current, however, is distinct from the one blocked by apamin because apamin has no effect on the channel altered by charybdotoxin. Finally, a polypeptide, dendrotoxin, isolated from the venom of the green mamba, *Dendroaspis augusticeps*, facilitates neurotransmitter release from synaptosomes [18, 19] and selectively blocks a non-inactivating potassium current in dorsal root ganglion cells [20]. These toxins will be extremely important in differentiating among the different types of potassium channels and, hopefully, in their purification and biochemical characterization.

The discovery of naturally occurring toxins which affect such diverse proteins as acetylcholine receptors, sodium channels, and potassium channels suggests that it might be possible to find calcium channel specific toxins. Calcium channels play important roles in excitability, excitation–contraction coupling, excitation–secretion coupling and other cellular functions. As there are a number of different types of calcium channels, for the purposes of this review we will confine our discussion to a search for toxins that affect the dihydropyridine-sensitive, high-threshold calcium channels. A toxin which binds specifically and with high affinity to this protein would greatly aid in its purification and characterization. Recently, several toxins that are potential candidates for calcium channel specific toxins have been isolated. In this commentary we would like first to establish criteria by which to identify a toxin as being specific

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for the calcium channel and then discuss and compare the five potential calcium channel toxins.

CRITERIA BY WHICH TO IDENTIFY A TOXIN AS CALCIUM CHANNEL SPECIFIC

Because a number of mechanisms can be involved in altering the function of an ion channel (such as a second messenger or alteration of the membrane potential), it is important to establish that the toxin of interest binds to the calcium channel protein. We suggest that a calcium channel specific toxin should have the following properties:

The toxin should alter calcium channel function and/or the binding of calcium channel specific ligands

A number of different techniques are being used to determine if the toxic action of a venom component is due to its interaction with the voltage-dependent calcium channel. These include tension measurements on aortic and cardiac strips [21], release of neurotransmitter from brain synaptosomal preparations [22], measurement of whole cell calcium currents under voltage clamp conditions [23, 24], measurement of single calcium channel activity using patch clamp techniques [25], and measurement of ion fluxes using radioisotopes [26, 27]. The major difficulty in all of these techniques is establishing unambiguously that the effect is due to a direct interaction with the calcium channel. Effects on calcium channels are generally discriminated from effects on other channels by choosing incubation conditions in which sodium and potassium currents are blocked, either due to the absence of the permeant ion in the incubation buffer or to the blockage of sodium currents with tetrodotoxin and potassium currents with tetraethylammonium chloride, cesium chloride and 4-aminopyridine [28]. It should, however, be established directly that the toxin does not affect sodium or potassium currents over the concentration range at which it alters calcium currents. The interaction of the toxin with calcium channels in the presence of known agonists and antagonists is another approach to determining specificity.

In looking for an effect of a toxin on calcium channels, one must also be aware of the possibility of a voltage dependence of the effect or of the binding of the toxin. For example, the inhibitory dihydropyridines appear to bind preferentially to the inactivated state of the channel [29–31]. Also, as will be discussed below, the action of taicatoxin on the calcium channel is voltage dependent [32]. In addition, voltage-dependent effects have been seen with some of the sodium channel toxins [6]. For this reason, it is important to screen the toxin for effects at several different membrane potentials. As has been observed with the sodium channel toxins, toxins can alter the function of the channel in a variety of ways. They can be activators or inhibitors by modifying either the activation or inactivation process or by stabilizing one of the channel states (resting, open or inactivated).

To assay the ability of the toxins to modulate the binding of calcium channel specific ligands, a number of radiolabeled compounds are available. These include dihydropyridines such as [^{3}H]nitrendipine, [^{3}H]PN200-110, and [^{3}H]202-791, arylalkylamines such as [^{3}H]verapamil, and benzothiazepines such as [^{3}H]diltiazem. In the radioligand binding studies it is necessary to determine whether the toxin sample being studied has any phospholipase or protease activity (see below) as these enzymes will produce inhibition of binding of the dihydropyridines [33].

The number of binding sites for the toxin should be comparable to the number of calcium channels

Electrophysiological estimates of the number of active calcium channels per unit area of the membrane have been made for a variety of cell types [34, 35, *]. If a toxin can be radiolabeled, it is useful to compare the number of binding sites with the number of calcium channels determined electrophysiologically or with the number of binding sites for dihydropyridines. Although discrepancies can be explained by a pool of inactive channels or by the existence of more than one ligand binding site per channel, significant differences should be examined carefully. In addition, the concentration of the toxin which gives rise to a half-maximal effect should correspond to its apparent K_D determined from binding assays (assuming no voltage dependence to the binding and no spare receptors).

The toxin should not act via a second messenger

Because calcium channel activity is altered by β-adrenergic agonists [34, 36, 37], by 8-bromo-cAMP [38], by intracellular injection of cAMP [39, 40], or by injection of the catalytic subunit of protein kinase [41], a cAMP-dependent phosphorylation reaction may modify channel function. It is necessary, therefore, to eliminate the possibility that a toxin with an effect on calcium currents is doing so via a second messenger. One method to rule out this type of indirect interaction is to employ a patch clamp technique [42] in which the toxin may be added either via the pipette or outside of the patch. An application of β-adrenergic agonists to a patch clamped cardiac myocyte has been used to demonstrate that the observed stimulation of calcium channel activity is not due to direct effects of the agonists on the calcium channel [43]. A toxin with a direct effect on calcium channels should only exert this effect when added in the pipette.

The fraction containing the toxic activity should not have any detectable enzyme activities

As most venoms have high levels of enzyme activity (especially proteases and phospholipases), it is important to assay the toxic fraction for the known enzyme activities of the venom. The common enzymes found in snake venoms and techniques to assay their activity have been reviewed by Iwanaga and Suzuki [44]. A procedure used to determine some types of protease activity utilizes Azocoll [20]. Phospholipase activity can be detected by using either the procedure of Wells and Hanahan [45] or that of Habermann and Hardt [46].

Because an effect due to an enzyme activity should

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be essentially irreversible, when possible, it is also helpful to demonstrate that the component altering calcium channel activity or radioligand binding is doing so reversibly. Electrophysiologically, it should be possible to wash out the toxin and have calcium current restored to normal. In practice this is often difficult to do because of the phenomenon of channel rundown [47]. In radioligand binding studies it should be possible to pretreat the membranes with the toxin, wash extensively, and detect no difference in ligand binding as compared to untreated membranes. Washout, however, cannot be accomplished if the toxin has a slow dissociation rate. An example of a toxin with such an extremely slow dissociation rate that it appears irreversible is the alpha-neurotoxin that binds to the nicotinic acetylcholine receptor [48].

TOXINS THAT AFFECT CALCIUM CHANNEL FUNCTION

The toxins in the following sections are those that have been identified to alter the activity of voltage-dependent calcium channels.

Maitotoxin

Maitotoxin is a water-soluble, non-peptide toxin isolated from the dinoflagellate, *Gambierdiscus toxicus* [49]. This toxin at low concentrations causes a dose-dependent contraction of isolated aorta from rabbits [21], rat atria [50], rabbit duodenum [50], guinea pig ileum [51], and taenia caeci [51]. This contractile response is inhibited by verapamil. The toxin also stimulates neurotransmitter release from sympathetic neurons [52] and cultured pheochromocytoma (PC12) cells [27]. The effect on PC12 cells is not altered by tetrodotoxin but is inhibited by verapamil or tetracaine. Maitotoxin also induces the release of prolactin from primary pituitary cultures [53]. In NG108-15 cells [26] and in PC12 cells [27] maitotoxin stimulates uptake of $^{45}Ca^{2+}$, and this uptake in the NG108-15 cells is blocked by nitrendipine, D-600, and diltiazem [26]. In contrast, maitotoxin does not alter the binding of dihydropyridines to these cells [26]. It has been suggested that this toxin is a specific activator of calcium channels. Maitotoxin, however, also stimulates the formation of inositol phosphates (IP) in rat aortic myocytes [54] and, therefore, it cannot be ruled out that its effects on calcium channels are via stimulation of IP formation.

β-Leptinotarsin-h

This protein is isolated from the beetle *Leptinotarsa haldemani* [55] and is the only toxic component of the hemolymph [56]. In rat brain synaptosomes β-leptinotarsin-h produces release of neurotransmitters [22]. The release is blocked by Ba^{2+}, Sr^{2+}, Co^{2+} and Cd^{2+}. The fraction is inactivated by heat, and its action is dependent on the presence of Ca^{2+} in the incubation medium. The main component of the active fraction is an acidic protein with an apparent molecular weight of 57,000.

ω-Conotoxin

ω-Conotoxin is isolated from the venom of the cone snail, *Conus geographus*. In addition to this toxin, the venom of this gastropod also contains a number of neuroactive peptides including toxins that block nicotinic acetylcholine receptors and toxins that specifically inhibit muscle sodium channels [21]. ω-Conotoxin is a basic 27 amino acid peptide [57] with a large number of hydroxylated amino acids. This toxin irreversibly blocks nerve stimulus-evoked release of transmitter at the frog neuromuscular junction and attenuates the Ca^{2+} component of the action potential in dorsal root ganglion neurones from embryonic chick [58] but has no effect on the calcium channels of heart or skeletal muscle [24]. The binding of an iodinated derivative of this toxin to synaptosomal membranes appears to be essentially irreversible and is not inhibited by dihydropyridines or by verapamil [59].

Atrotoxin

The venom of the Western Diamondback rattlesnake, *Crotalus atrox*, upon injection causes a pronounced fall in systemic blood pressure and, at the site of injection, it causes hemorrhage, necrosis and edema [60]. Atrotoxin, a protein isolated from this venom, activates calcium currents in cardiac myocytes and blocks the binding of dihydropyridines to membranes derived from ventricular tissue [20].

The effect of this toxin has also been studied at the single calcium channel level, and it appears to prolong the channel open time [25] in a manner similar to high concentrations of the dihydropyridine agonist Bay K8644 [61]. Atrotoxin has no effects on either sodium or potassium currents in these cells, and its effects on calcium currents are blocked by either organic or inorganic calcium channel blockers but are not altered by β-adrenergic ligands [23]. The effect is reversible, only occurs when the toxin is added externally, and is independent of membrane potential. This toxin, however, has proven difficult to purify to homogeneity. Further steps appear to result in loss of activity. The purification is being pursued currently in our laboratory.

Taicatoxin

Perhaps the most promising calcium channel toxin discovered thus far is a basic polypeptide isolated from the venom of the Australian Taipan snake, *Oxyuranus s. scutellatus*. The venom of this Elapidae snake produces both neurotoxic and cardiovascular effects. The purified toxin, designated taicatoxin (TCX), has a molecular weight of 8000 and blocks high threshold calcium channels in cardiac tissue in a voltage-dependent fashion [32]. The toxic compound is distinct from the toxin taipoxin which has been isolated from this same venom [62]. TCX also has no protease or phospholipase activities associated with it. The blockage occurs at nanomolar concentrations, is only effective when added at the external surface of the membrane, is reversible, and does not work when added outside of a patch pipette in a cell-attached patch clamp experiment. The toxin has been purified to homogeneity and partially sequenced. At the single calcium channel level, TCX appears to suppress calcium currents by increasing the frequency of null records and by reducing the frequency of reopening of channels. TCX, like atro-

toxin, has no effects on either sodium or potassium currents.

SUMMARY

At this time, there are five potential candidates for calcium channel specific toxins. All five of these toxins appear to affect the function of voltage-dependent calcium channels. Atrotoxin, β-leptinotarsin-h and maitotoxin activate channels, whereas both taicatoxin and ω-conotoxin are inhibitors. Neither maitotoxin nor ω-conotoxin alters the binding of dihydropyridines to membranes derived from the cells upon which the toxins exert their effects. In contrast, both atrotoxin and taicatoxin inhibit the binding of dihydropyridines to ventricular membranes. It is not currently known whether β-leptinotarsin-h affects the binding. The effects of maitotoxin and atrotoxin are blocked by dihydropyridines and verapamil. Direct binding studies with radiolabeled toxins have been performed only with ω-conotoxin, and the binding site density for this toxin [58] appears to be at least one order of magnitude greater than the density of dihydropyridine binding sites in synaptosomes [63].

Studies to examine the third and fourth criteria which we have listed (i.e. that the effects are not via a second messenger, or an enzyme activity) have not been reported for either β-leptinotarsin-h or ω-conotoxin. Atrotoxin and taicatoxin, added outside a patch pipette, have no effects on calcium channels within the patch and are, therefore, probably not affecting calcium channels via a second messenger. Maitotoxin, however, affects the formation of inositol phosphates and, hence, could be affecting the channel indirectly. The fractions containing the toxic components atrotoxin and taicatoxin have no phospholipase or protease activity, and this is presumably true also for ω-conotoxin since it has been purified to homogeneity.

Although all of the toxins have the potential to be important tools with which to study calcium channel structure and function, a number of experiments remain to be done in order to establish conclusively that these five toxins bind specifically to the voltage-dependent calcium channel.

In conclusion, we would like to briefly mention why so much effort is being devoted to the search for these calcium channel specific toxins. Such a toxin would provide a very valuable tool in the study of calcium channels for a number of reasons. First, the toxin would be another ligand for the channel and, as such, would provide an alternative to organic ligands such as the dihydropyridines which are lipophilic and, in many tissues, have more than one binding site [64–66, *]. The toxin would greatly aid the purification of the channel protein either by using it attached to a solid matrix as an affinity resin or, when radiolabeled, using it to follow the purification of the protein during conventional chromatography. For the latter purpose, ω-conotoxin should prove to be extremely important since it dissociates from its binding site extremely slowly [59] and would, therefore, remain bound throughout the purification procedure. The use of this toxin is, however, limited to neuronal tissue.

Another potential use would be to identify the polypeptide to which the toxin is bound and which is presumably a subunit of the calcium channel. For this purpose, the radiolabeled toxin could be cross-linked to its binding site such as we have done previously with toxins specific for the nicotinic acetylcholine receptor [67], or it might be possible to use an antibody to the toxin to precipitate the toxin–calcium channel complex from solubilized and radiolabeled membrane proteins. Also, fluorescent derivatives of the toxins could be used to study the distribution and mobility of calcium channels in intact cells. These are only a few of the areas where toxins could be used and, therefore, the five toxins already characterized, and those yet to be discovered, represent a very exciting area of calcium channel research.

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