Effects of δ-Conotoxins PVIA and SVIE on Sodium Channels in the Amphibian Sympathetic Nervous System

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Running title: δ-Conotoxin Effects on Frog Sympathetic Sodium Channels

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ABSTRACT

δ-Conotoxins are a family of small, disulfide-rich peptides found in the venoms of predatory cone snails (Conus). We examined in detail the effects of δ-conotoxin PVIA from the fish hunting cone snail Conus purpurascens on sodium currents in dissociated sympathetic neurons from the leopard frog Rana pipiens. We also compared this toxin’s effects with those of δ-conotoxin SVIE from Conus striatus, another piscivorous cone snail. δ-PVIA slowed the time course of inactivation of TTX-sensitive and TTX-resistant sodium currents and shifted the voltage-dependence of activation and steady-state inactivation to more hyperpolarized potentials. Similar, albeit more pronounced, effects were seen with δ-SVIE. While the effects of δ-PVIA were reversed by washing, those of δ-SVIE were largely irreversible over the time course of these experiments. The effects of δ-PVIA could be suppressed by conditioning depolarizations in a voltage- and time-dependent manner, while the effects of δ-SVIE were largely resistant to conditioning depolarizations. Lastly, in intact sympathetic nervous system preparations, δ-PVIA inhibited evoked trains of compound action potentials. Many of these effects of δ-PVIA and δ-SVIE are remarkably similar to those of toxins that bind to site-3 on voltage-gated sodium channels.
INTRODUCTION

Voltage-gated sodium channels (VGSCs) are transmembrane proteins responsible for the rising phase of action potentials. The importance of VGSCs in the functioning of the nervous system is reflected by the fact that they are often the targets of neurotoxins in the venoms of a variety of organisms such as scorpion, sea anemone, spider, coral, wasp, and cone snail [for review see (Cestele and Catterall 2000)].

Cone snails (Conus) comprise the largest genus of venomous animals known. The majority of their active venom components are small, disulfide-rich peptides called conotoxins (Terlau and Olivera 2004). These snails prey upon a variety of different organisms such as worms, other marine gastropods, and most remarkably fish. In order to capture agile prey such as fish, conotoxins must be fast acting and effective immobilizing agents. To this end, cone snails have evolved a group of conotoxins, the “lightning-strike cabal”, that causes rapid and rigid immobilization of prey (Terlau et al. 1996). When members of this cabal are injected together into fish, they cause a massive depolarization of axonal fibers resulting in immediate tetanic paralysis. One family of toxins that contributes to the lightning-strike cabal is the δ-conotoxin family.

δ-Conotoxins are a large and diverse group of peptides that are found in every fish hunting species of Conus examined so far that employs the hook-and-line strategy for prey capture (Bulaj et al. 2001). The characteristic activity of δ-conotoxins is to slow the time course of VGSC inactivation (Barbier et al. 2004; Bulaj et al. 2001; Fainzilber et
al. 1995; Gonoi et al. 1987; Hasson et al. 1995; Leipold et al. 2005; Terlau et al. 1996). However, other peptide toxins from a variety of different organisms that inhibit inactivation also affect other characteristics of VGSCs such as the voltage-dependence of activation and steady-state inactivation [for examples see (Bergman et al. 1976; Cestele et al. 1999; Gilles et al. 2001; Gonoi et al. 1986; Gonoi et al. 1984; Gordon et al. 1996; Grolleau et al. 2001)]. Comparable detailed data on the activity of δ-conotoxins from fish hunting cone snails remains relatively scarce.

The aim of this investigation was to characterize the activity of δ-conotoxin-PVIA, from the fish hunting cone snail *Conus purpurascens*, in greater detail than has been previously reported. Additionally, we compared this toxin’s effects with that of δ-conotoxin-SVIE from another piscivorous cone snail, *Conus striatus*. We examined the effects of these two conotoxins on sodium currents recorded from voltage-clamped sympathetic neurons of the leopard frog, *Rana pipiens*. Lastly, we used extracellular recording in conjunction with focal toxin application to examine how δ-PVIA affected the conduction of action potentials in the sympathetic nervous system.

A note on the abbreviations PVIA and SVIE: The "P" and "S" refer to *purpurascens* and *striatus*, respectively, the species of *Conus* that produces the peptide; the Roman numeral "VI" designates the cysteine pattern of the conopeptide family (C–C–C–CC–C–C); and the suffix "A" or "E" indicates that the peptide is the first or fifth member of the family identified from the given species of snail. See Terlau and Olivera (2004) for a recent comprehensive review of conopeptides.
A preliminary report of some of this work has appeared (West et al. 2001).
METHODS

*Dissociated neurons*

Lumbar paravertebral ganglia 8 through 10 were dissected from 2.5 to 3 inch adult frogs (*Rana pipiens*) of either sex and processed in a manner similar to that described by others (Jones 1987; Selyanko et al. 1990). Briefly, ganglia were treated with collagenase followed by trypsin. Cells were mechanically dissociated by trituration, washed, suspended in 73% Leibowitz’s L15 solution (supplemented with 14 mM glucose, 1 mM CaCl2, 7% fetal bovine serum, and penicillin/streptomycin), and plated on polylysine-coated coverslips. After incubation at room temperature overnight to allow the cells to adhere to the coverslip, they were stored at 4 °C until used.

*Whole cell patch clamp electrophysiology*

Whole cell patch clamp recordings were made essentially as previously described (West et al. 2002). Briefly, sympathetic neurons were perfused with extracellular solution containing (in mM): NaCl, 117; KCl, 2; MgCl2, 2; MnCl2, 2; HEPES, 5; TEA, 10; pH 7.2. Recording pipettes with resistances of 1-2 MΩ contained (in mM): NaCl, 10; CsCl, 110; MgCl2, 2; CaCl2, 0.4; EGTA, 4.4; HEPES, 5; TEA, 5; MgATP, 4; pH 7.2. These solutions inhibit voltage-gated potassium and calcium currents and thereby permit recording of sodium currents exclusively. Conotoxins were dissolved in extracellular solution and applied to neurons under study by bath exchange. During exposure to toxin, the bath was static to conserve material. Unless
otherwise noted, neurons were held at –80 mV and voltage-gated sodium currents were activated by a 50 ms test pulse to 0 mV applied every 10 s, where each test pulse was preceded by a -120 mV prepulse lasting 50 ms to relieve any steady-state inactivation that may be present at –80 mV. Current signals, acquired at room temperature with an Axopatch 200B amplifier (Axon Instruments, Union City, California), were filtered at 2 kHz and digitized at 10 kHz with a PCI-MIO-16E-4 data acquisition board (National Instruments, Austin, Texas). Series resistance and whole cell capacitance was compensated for by ≥85% to minimize voltage errors. Current signals were leak-subtracted by a P/5 protocol using homemade software written in LabVIEW (National Instruments, Austin, Texas).

**Extracellular electrophysiology**

Extracellular recordings of compound action potentials (CAPs) in sympathetic nerves were made as previously described (Keizer et al. 2003; Tavazoie et al. 1997). Briefly, lumbar paravertebral ganglia 8 through 10 and the adjoining 10th spinal nerve were dissected from 2.5 to 3 inch adult frogs (*Rana pipiens*) of either sex. The recording chamber was fabricated from Sylgard and consisted of seven circular or semicircular compartments with diameters between 4 to 5 mm, each separated from its neighbor by a partition about 1 mm wide. A bead of Vaseline was placed atop each partition between compartments. The chain of sympathetic ganglia and attached 10th nerve were arranged in the compartments, and those portions draped over the Vaseline-topped partitions were covered with additional Vaseline to prevent tissue-drying and to seal-off compartments from each other such that the fluid in each
compartment was isolated and independently maintained; furthermore, electrical stimulation or recording across a Vaseline gap was possible. Each compartment was maintained essentially as a static bath except for the test compartment which was perfused with frog Ringer's when it did not contain toxin. Normally, all compartments contained frog Ringer's solution consisting of (in mM): NaCl, 111; KCl, 2; CaCl₂, 1.8; HEPES, 10; pH 7.2. Conotoxins were dissolved in this solution and applied to the test compartment by stopping its perfusion and replacing the solution with one containing toxin. During toxin-exposure, the test compartment was static to conserve material.

Extracellular platinum wire electrodes were used for stimulation and recording. To stimulate the preganglionic nerve of the 10th ganglion, supramaximal stimuli were applied to the connective between the 8th and 9th ganglia. CAPs in postganglionic fibers of the 10th ganglion were recorded from the 10th spinal nerve. Stimuli (0.1-1 ms rectangular voltage pulses) were provided by an S-88 stimulator (Grass Instruments, West Warwick, Rhode Island) through a stimulus isolation unit. Recordings were made using a P-55 differential A/C preamplifier (Grass Instruments, West Warwick, Rhode Island) with bandpass filter settings of 1 Hz and 1 kHz. Data were acquired at a sampling frequency of 5 kHz with a Macintosh computer fitted with an A/D converter. Stimuli were triggered and responses were stored and processed with homemade software written in LabVIEW (National Instruments, Austin, Texas). All experiments were performed at room temperature. The protocol for use of frogs in these experiments was approved by the Institutional Animal Care and Use Committee at the University of Utah.
Data analysis

Data were analyzed and curve-fit using Graphpad Prism for Macintosh (version 3.0cx, Graphpad Software, San Diego, California) or Kaleidagraph for Macintosh (version 3.6, Synergy Software, Reading, Pennsylvania). Numerical data are presented as mean ± SEM, and error bars on all graphs represent SEM. Statistical significance was assessed at P < 0.05 using a one-sample t test.

Toxins

δ-Conotoxins PVIA and SVIE were chemically synthesized, oxidized and purified as previously described in detail (Bulaj et al. 2001; DeLa Cruz et al. 2003; Shon et al. 1995). These toxins were dissolved in bathing solution and used on the day of the experiment or stored frozen until used.
RESULTS

δ-Conotoxins PVIA and SVIE slow the time course of sodium channel inactivation.

Figure 1 illustrates the effects of δ-PVIA and δ-SVIE on sodium currents activated by a step to 0 mV in two representative neurons. The traces in Fig. 1, A and B, represent averaged responses before, in the presence of 10 µM δ-PVIA or δ-SVIE, and after perfusing the recording chamber for > 10 minutes with toxin-free bathing solution. While neither toxin significantly changed the peak amplitude of the sodium current (103 ± 2%, n = 4, and 111 ± 9%, n = 3, for 1 µM δ-PVIA and 1 µM δ-SVIE, respectively), both toxins clearly slowed the kinetics of fast inactivation. The effect of δ-PVIA fully reversed after washing toxin as indicated by the overlapping control and wash traces. In contrast, the effects of δ-SVIE were largely irreversible over the 45 minute wash period in this experiment as indicated by the superimposition of the wash trace with the trace in the presence of toxin.

The effect of the toxins on inactivation was also examined by plotting the fraction of sodium current remaining 2 ms after the peak, relative to the peak ($I_{Na \, 2ms}/I_{Na \, peak}$), as a function of time (c.f., Rogers et al., 1996). This is illustrated in Fig. 1, C and D. In the presence of either δ-conotoxin, the relative amount of sodium current 2 ms after the peak increased, indicating that these toxins slowed the kinetics of inactivation. Upon washing δ-PVIA out, the toxin's effect reversed and control levels were essentially reestablished in about 15 minutes. In contrast, the effects of δ-SVIE persisted largely
unabated even after perfusing the chamber with toxin-free bathing solution for > 45 minutes.

In addition to slowing the time course of inactivation, δ-conotoxins also prevented a fraction of the sodium current from becoming inactivated. Figure 2A shows traces from a representative neuron in the presence of 0 (control), 0.5, 1, or 2 µM δ-PVIA. The peak sodium currents in the presence of δ-PVIA were no different than that of their controls and were as follows: 0.5 µM = 102 ± 3% (n = 3), 1 µM = 103 ± 2% (n = 4), 2 µM = 103 ± 1% (n = 3), 5 µM = 104 ± 2% (n = 6), and 10 µM = 103 ± 3% (n = 7) of control. In contrast, a non-inactivating or persistent inward current was observed whose amplitude increased in proportion to δ-PVIA concentration. This can be more clearly seen when the y-axis is expanded, as shown in the inset. The dose-response relationship for the induction of the non-inactivating current by δ-PVIA is shown in Fig. 2B. The half-maximum effective concentration (EC$_{50}$) of this dose-response curve was 2.4 µM and the Hill slope was 1.1. A corresponding dose-response relationship was not obtained for δ–SVIE. However, 10 µM δ–SVIE appeared to be a saturating concentration since the magnitude of its effect on inactivation (Fig. 1D: $I_{Na\ 2ms}/I_{Na\ peak} = 0.73$) was no greater than that seen at 1 µM ($I_{Na\ 2ms}/I_{Na\ peak} = 0.81 ± 0.003$, n = 3).

δ-Conotoxins PVIA and SVIE alter the voltage-dependence of sodium channel activation and steady-state inactivation.
The voltage-dependence of sodium channel activation was examined by varying the test pulse potential as illustrated in the voltage-command diagram at the top of Fig. 3. Representative current traces recorded at each test potential under control conditions and in the presence of 10 µM δ-PVIA or 10 µM δ-SVIE are shown in Fig. 3, A and B. Conductances were calculated from the peak inward currents and $E_{Na}$ (empirically determined by interpolation) and are plotted as a function of test potential (Fig. 3C). Both toxins left-shifted (i.e., to more hyperpolarized potentials) the voltage at which half the channels were activated ($V_{50}$), without markedly altering the slope of the conductance-voltage relationship.

To examine the effects of δ-conotoxins on steady-state inactivation, 200 ms prepulses ranging from −140 to 0 mV were used (see voltage-command protocol at the top of Fig. 4). Representative current traces recorded following each prepulse potential under control conditions and in the presence of 10 µM δ-PVIA or 1 µM δ-SVIE are shown in Fig. 4, A and B. In addition to slowing inactivation kinetics, both toxins also induced a non-inactivating current as already seen in Fig. 2. Such currents are evident in Fig. 4, A and B, as currents that persist at the end of the prepulse preceding the start of the test pulse (indicated by the arrow), as well as during the test pulses, in response to prepulse depolarizations normally sufficient to cause complete inactivation. Figure 4C shows the voltage-dependence of steady-state inactivation in control saline, 10 µM δ-PVIA, or 1 µM δ-SVIE. Here, peak current amplitude relative to the maximum amplitude (the latter obtained when prepulse potential was −140 mV) is plotted as a function of prepulse potential. When either toxin was present, the voltage at which half
the channels are inactivated ($V_{50}$) was hyperpolarized, and the slope shallower, relative to control. Furthermore, prepulse potentials $>-20$ mV, which under control conditions fully inactivated all VGSCs in these neurons, failed to produce complete inactivation in the presence of toxin.

Since it was frequently observed that the $V_{50}$ of steady-state inactivation can spontaneously drift to hyperpolarized potentials, we monitored $V_{50}$ over time to track the changes in $V_{50}$ produced by δ-conotoxins. Two examples of such experiments are shown in Fig. 4, D and E. Although the $V_{50}$ gradually drifted to increasingly hyperpolarized potentials over time, the application of either δ-conotoxin produced a larger shift in $V_{50}$ than that which can be accounted for by drift alone ($δ$–PVIA, $n = 3$; $δ$–SVIE, $n = 1$). In the case of $δ$–SVIE, the experiment was performed only once since the magnitude of the toxin’s effect was so large that it was clear to be not due to spontaneous drift. Upon washout, the change in $V_{50}$ induced by $δ$-PVIA reversed to approximately control levels predicted by extrapolation from the rate of drift observed before toxin exposure. In contrast, the change in $V_{50}$ induced by $δ$-SVIE did not reverse following washout. Also plotted in these graphs are the slopes of the Boltzmann fits to the steady-state inactivation data. These slope values did not spontaneously drift over time. While the decrease in the slope induced by $δ$-PVIA was reversible, the decrease in slope induced by $δ$-SVIE was not only more pronounced but also irreversible over the time course of these experiments.
δ-Conotoxin PVIA’s effect on sodium channel inactivation is suppressed by conditioning depolarizations in a voltage- and time-dependent manner.

It has long been appreciated that the binding of site-3 neurotoxins to their receptor site on VGSCs is voltage dependent (Catterall 1977; Catterall et al. 1976; Ray and Catterall 1978). This voltage-dependence in affinity can also be seen as a decrease in the magnitude of the toxin’s effect when sufficiently large conditioning depolarizations are given prior to test pulses (Rogers et al. 1996). Voltage-dependent suppression of toxin-effects was examined here in view of the similarity in the functional effects of δ-conotoxins and site-3 toxins. The voltage-command protocol is illustrated at the top of Fig. 5. Conditioning voltage steps (V<sub>condition</sub>) to depolarized potentials between 20-120 mV and lasting between 20-300 ms were given prior to a –120 mV prepulse and test pulse to 0 mV. Figure 5 shows representative traces before and during exposure to 5 μM δ-PVIA or 1 μM δ-SVIE, following an 80 mV conditioning depolarization with a duration ranging from 0 to 300 ms. The effect of δ-PVIA on inactivation kinetics was fully suppressed when the 80 mV conditioning depolarization lasted 300 ms, and shorter conditioning depolarizations produced correspondingly less suppression (Fig. 5A). Compared to the effects of δ-PVIA, the effects of δ-SVIE are much more resistant to suppression by conditioning depolarizations (Fig. 5B).

Figures 5, C & D, show suppression as a function of the duration of the conditioning depolarizations at several potentials. The ratio of the sodium current 2 ms after the peak, relative to the peak (i.e., I<sub>Na 2ms</sub>/I<sub>Na peak</sub>), was used as an index of the
toxin's effect on inactivation kinetics. In the absence of toxin, 80 mV conditioning depolarizations lasting between 20 - 300 ms did not alter inactivation kinetics (open circles). In contrast, when δ-PVIA was present, toxin-induced slowing of inactivation kinetics was suppressed by conditioning depolarization in a time-dependent manner. The data fit single exponential kinetics (solid lines), and stronger conditioning pulses produced more rapid suppression of the toxin's effects. In experiments where 10 μM δ-PVIA was used, suppression of toxin effect by the 100 mV conditioning step occurred with a time constant of 85 ± 16 ms (n = 3). Figure 5D shows that the effect of δ-SVIE on inactivation kinetics was only mildly suppressed by conditioning depolarizations over the voltage and time ranges tested in these experiments.

δ-Conotoxin PVIA selectively inhibits evoked trains of action potentials in the frog sympathetic nervous system.

In previous studies, various δ-conotoxins were shown to cause action potential broadening and increased excitability in preparations from frog and Aplysia (Barbier et al. 2004; Bulaj et al. 2001; Hasson et al. 1995; Shon et al. 1995). To examine how δ-PVIA affects the function of an intact physiological system, we recorded compound action potentials (CAPs) from postganglionic sympathetic fibers in the 10th spinal nerve in response to electrical stimulation of preganglionic fibers innervating the 10th sympathetic ganglion (see recording arrangement diagram in Fig. 6A).
Although concentrations $\geq 5 \mu M$ were required to inhibit CAPs evoked by a single stimulus applied every 30 seconds ($n = 3$; data not shown), $1 \mu M$ $\delta$-PVIA was sufficient to inhibit trains of B-neuron CAPs evoked at higher stimulation frequencies between 5 and 20 Hz ($n = 2$); in Fig. 6B, the preganglionic nerve was briefly stimulated at a frequency of 20 Hz. Stronger stimuli have to be applied to initiate action potentials in preganglionic axons innervating C-neurons than those innervating B-neurons, and the low stimulus strength used here evoked only B-neuron responses. The first CAP in the train was largely unaffected by $1 \mu M$ $\delta$-PVIA; in contrast, subsequent CAPs were strongly inhibited. This inhibition was reversed upon washout of the toxin.
DISCUSSION

We conclude that δ-conotoxins PVIA and SVIE slow the inactivation kinetics of VGSCs in frog sympathetic neurons. These peptide toxins also shift the voltage-dependence of activation and steady-state inactivation to hyperpolarized potentials. The effects of δ-PVIA are rapidly reversed by washing, while those of δ-SVIE are not. Furthermore, conditioning depolarizations that completely suppress the effects of δ-PVIA minimally suppress the effects of δ-SVIE. The primary manifestation of these effects in the frog sympathetic nervous system is a selective inhibition of trains of action potentials.

These effects of δ-conotoxins on sodium currents are remarkably similar to those of toxins that bind to site-3 on VGSCs, such as those of scorpion and sea anemone. Clearly, both site-3 toxins and δ-conotoxins inhibit the time course of sodium channel inactivation. Additionally, although not universally true (Gordon et al. 1996; Lee et al. 2000; Salceda et al. 2002), site-3 neurotoxins have been shown to shift the voltage-dependence of sodium channel activation to hyperpolarized potentials (Gilles et al. 2000; Goni et al. 1984; Grolleau et al. 2001; Little et al. 1998). Also like the δ-conotoxins reported here, some site-3 toxins have been shown to shift the V_50 of steady-state inactivation to hyperpolarized potentials and decrease the voltage-dependence of this relationship as reflected in a decrease in the slopes of the Boltzmann fits to the steady-state inactivation data (Gilles et al. 2001; Gordon et al. 1996; Salceda et al. 2002). However, other site-3 toxins only affect the slope of the
steady-state inactivation relationship (Bergman et al. 1976; Cestele et al. 1999; Gonoï et al. 1984; Grolleau et al. 2001), and still others can shift the $V_{50}$ to depolarized potentials (Lee et al. 2000). These variations may be due to subtle differences in some toxins’ interactions with sodium channels expressed in various tissues.

Site-3 toxins have also been shown to affect VGSC slow-inactivation. For instance, the classical scorpion $\alpha$-toxin Lqh II has been shown to shift the voltage-dependence of slow-inactivation to hyperpolarized potentials when examined on rat brain II sodium channel expressed in either HEK293 or CHO cells (Gilles et al. 2001). This shift in the voltage dependence of slow-inactivation lead to a 60% decrease in the peak sodium current amplitude when these cells were held at $-70$ mV. Although we did not directly examine the effects of $\delta$-conotoxins on slow-inactivation, we found that the addition of 1 $\mu$M $\delta$-SVIE or 0.5 - 10 $\mu$M $\delta$-PVIA did not decrease the peak sodium current amplitude when sympathetic neurons were held at $-80$ mV. This result suggests that neither $\delta$-conotoxin affected the number of VGSCs that were in a slow-inactivated state at $-80$mV. It remains to be determined if $\delta$-conotoxins affect either the voltage-dependence or kinetics of slow inactivation in these neurons.

As mentioned earlier, it has long been appreciated that the binding of site-3 neurotoxins to their receptor site on VGSCs is voltage dependent (Catterall 1977; Catterall et al. 1976; Ray and Catterall 1978). This voltage-dependent decrease in affinity can be observed as a decrease in the magnitude of the toxin’s effect when sufficiently large conditioning depolarizations are given prior to test pulses (Rogers et al.
1996). A similar voltage-dependent attenuation of δ-conotoxin SVIE’s effect on Na\textsubscript{v1.4} expressed in HEK293 cells has recently been reported (Leipold et al. 2005). In our results from sodium channels natively expressed in frog sympathetic neurons (Fig. 5), δ-PVIA’s activity was attenuated by conditioning depolarizations in a similar manner while δ-SVIE’s activity was minimally affected. Again, these differences may be due to different affinities of these toxins for sodium channels expressed in various tissues.

Earlier studies have demonstrated that a δ-conotoxin from the venom of a mollusk-hunting cone snail, δ-TxVIA, does not compete for binding with site-3 toxins (Fainzilber et al. 1994). Additionally, a much larger peptide from the venom of Conus striatus, CsTX, with an activity similar to that of δ-SVIE, only partially competed with the site-3 toxin alpha-LqTx (Gonoi et al. 1987). For these reasons, the binding site for δ-conotoxins had been defined as site-6 (Cestele and Catterall 2000). In contrast with these earlier studies, δ-SVIE has recently been shown to functionally compete with the site-3 toxin Lqh II and interact with a conserved hydrophobic triad of amino acids in the S3-S4 linker of domain IV (Leipold et al. 2005). Likewise, it has been shown for α-scorpion toxin that critical residues for site-3 binding are found in the S3-S4 linker in domain IV (Rogers et al. 1996). The α-scorpion toxin binding site suggested a mechanism by which these toxins exert their effects; namely, binding of toxin to site-3 inhibits the movement of the domain IV S4 voltage-sensor (Cestele and Catterall 2000; Rogers et al. 1996). “Voltage-sensor trapping” appears to be a mechanism employed by a variety of peptide toxins that interact with VGSCs (Catterall 2002), and these results suggest that δ-conotoxins act in a similar fashion.
The amino acid sequences of δ-conotoxins PVIA and SVIE are shown in Table 1. Of particular interest is the relatively high number of conserved hydrophobic residues present in these peptides, which is a biochemical hallmark of δ-conotoxins. The structures of these peptides are beginning to be solved. Analysis of PVIA and SVIE homology models reveals that both conotoxins share similar three dimensional structures, including distribution of solvent-exposed hydrophobic surface areas (DeLa Cruz et al. 2003). Recently, these hydrophobic patches on the surface of δ-conotoxins were suggested to play a role in the toxins’ interactions with sodium channels (Kohno et al. 2002; Volpon et al. 2004).

X-ray crystallalographic results of MacKinnon and colleagues indicate that the S4 voltage sensors of voltage-dependent K+ channels from a thermophilic archaebacterium reside largely in the lipid membrane (Jiang et al. 2003a; Jiang et al. 2003b; Mackinnon 2004). This is in contrast to the conventional model which depicts the voltage sensors as alpha-helices embedded in the center of each repeat domain. Furthermore, it has been suggested that hydrophobic peptide toxins from tarantula inhibit voltage-dependent K+ channels by partitioning into lipid membranes and subsequently binding to the voltage sensors (Lee and MacKinnon 2004). Assuming that the 3D structure of VGSCs is similar to that of voltage-dependent K+ channels, the unusual hydrophobicity of δ-conotoxins, as well as toxins that target site-3 (Sun et al. 2003), may reflect their interaction with binding sites on the sodium channel that are closely associated with the lipids surrounding the channel.
There is abundant evidence that site-3 toxins from different organisms can discriminate between various VGSC subtypes [for examples see (Gilles et al. 1999; Gilles et al. 2000; Saab et al. 2002; Sahara et al. 2000; Salceda et al. 2002)]. Thus, the different isotypes of the VGSC α-subunit vary sufficiently at site-3 to allow peptide toxins to differentially recognize them. δ-Conotoxins may also have subtype selective pharmacology. In support of this, it has been recently shown that a δ-conotoxin from *Conus ermineus* can discriminate among vertebrate VGSCs (Barbier et al. 2004). Regarding the δ-conotoxins discussed in this report, binding experiments with membrane preparations from rat brain demonstrate that δ-PVIA’s EC$_{50}$ is 50 nM (Shon et al. 1995), while data in Fig. 2B show that the EC$_{50}$ of δ-PVIA for VGSCs expressed in frog sympathetic neurons is 2.4 µM. The latter EC$_{50}$ is relatively high and raises the question as to whether other excitable tissues in the frog (or fish, the natural prey of piscivorous cone snails) may have VGSCs with higher affinity for δ-PVIA. Also, in light of the effect of voltage on the activity of these toxins as seen in Fig. 5, it remains to be seen how holding the membrane potential of these neurons at more hyperpolarized potentials may affect the apparent dose-response relationships of these peptides.

As seen in Fig. 6, inhibition of the propagation of sympathetic CAPs by 1 µM δ-PVIA is limited to those in trains. It is of interest to note that δ-conotoxin intoxication can have different physiological “end-points” depending on the system. Following application of δ-PVIA to a frog nerve-muscle preparation, a single stimulus applied to the motor nerve induces repetitive muscle action potentials (Shon et al. 1995). The
same is observed for a δ-conotoxin from *Conus ermenius*, δ-EVIA (Barbier et al. 2004). This effect is consistent with the tetanic paralysis observed in fish envenomated by *Conus pupurascens* (Shon et al. 1995; Terlau et al. 1996). In contrast, as we report here, δ-PVIA application to the frog sympathetic system does not induce repetitive firing. Instead, at low concentrations δ-PVIA block repetitive B-neuron fiber activity induced by repetitive stimuli, while at high concentrations it blocks B-neuron fiber activity altogether (data not shown).

Our working hypothesis to explain the behaviors of δ-PVIA on the neuromuscular and B-neuron systems is as follows. Inhibition of VGSC inactivation by δ-PVIA produces a long-lasting action potential in the motor nerve ending so that during a single impulse in the presynaptic terminal, release of transmitter is greatly prolonged. This results in a comparably prolonged endplate potential (Bulaj et al., 2001), which in turn induces a train of action potentials in the muscle. This sequence of events was also used to explain the action of δ-EVIA (Barbier et al. 2004). In the case of sympathetic axons, we presume that action potentials are blocked by high concentrations of δ-PVIA because the membrane potential is latched in a depolarized state. On the other hand, low concentrations of δ-PVIA only prolong the action potential duration, which effectively increases the relative refractory period, and this in turn prevents the axon from propagating action potentials at high frequencies.

It is most likely that the primary target of δ-PVIA is the motor system. However, it remains to be seen how important the δ-conotoxin-susceptibility of the sympathetic
system, or sensory system for that matter, is for prey capture by cone snails that possess δ-conotoxins.
ACKNOWLEDGEMENTS

We thank Prof. B.M. Olivera for advice and encouragement. This work was supported by National Institute of General Medical Sciences Grant GM 48677.
REFERENCES


FIGURE LEGENDS

FIG. 1. δ-Conotoxins PVIA and SVIE slow the time course of sodium channel inactivation. Neurons were dissociated from frog sympathetic ganglia and whole-cell voltage clamped as described in Methods. **TOP:** Voltage-command protocol. Neurons were held at –80 mV. A 50 ms step to –120 mV immediately preceded the test pulses to 0 mV. The start of the test pulse is indicated by an arrow. **A** and **B:** Representative sodium currents in control solution, 10 µM δ-PVIA (panel A) or 10 µM δ-SVIE (panel B), and after washing toxin out of the recording chamber (gray traces). All traces are normalized and represent the average of 10 responses. Arrow represents the beginning of the test pulse and corresponds to the time point indicated by the arrow in the voltage-command protocol. **C** and **D:** Ratio of the current 2 ms after the peak, relative to the peak current, plotted as a function of time for cells whose traces are shown in **A** and **B,** respectively. For the most part, test pulses were applied every 10-40 seconds. Black horizontal bar and dashed vertical boundaries indicate duration of toxin exposure.

FIG. 2. A non-inactivating current is induced by δ-PVIA in a dose-dependent manner. **A:** Representative sodium currents in 0 (control), 0.5, 1, or 2 µM δ-PVIA. The voltage-command protocol is diagrammed at the top; neurons were held at –80 mV and stepped to 0 mV for 250 ms. All traces are normalized and represent the average of 3 responses under each condition. Peak sodium current amplitudes in the presence of δ-PVIA were not significantly different than control responses and were as follows: 0.5
µM = 102 ± 3% (n = 3), 1 µM = 103 ± 2% (n = 4), and 2 µM = 103 ± 1% (n = 3) of control. Inset: Same currents, but with the y-axis expanded approximately 10-fold. Note that δ-PVIA induces a non-inactivating current whose amplitude increased in a dose-dependent manner. B: Dose-response relationship for the generation of non-inactivating current in the presence of δ-PVIA. The average of 5-10 traces at each toxin concentration was fit with a double-exponential decay curve. The predicted plateaus of these curves were normalize and are plotted as a function of toxin concentration. Data represent the averages ± SEM of responses at the following concentrations (µM): 0.2 (n = 2), 0.5 (n = 3), 1 (n = 4), 2 (n = 3), 5 (n = 6), 10 (n = 7), 20 (n = 1); where n is the number of cells tested. Solid line represents the best-fit curve with the following parameters: EC_{50} = 2.4 µM and Hill slope = 1.1.

FIG. 3. δ-Conotoxins PVIA and SVIE alter the voltage-dependence of activation. TOP: Diagram of the voltage-command protocol. Neurons were held at –80 mV. A 50 ms prepulse to –120 mV preceded the test pulse, which ranged between –50 and +30 mV. The start of the test pulse is indicated by an arrow. A and B: Representative sodium currents in the presence 10 µM δ-PVIA (panel A) or 10 µM δ-SVIE (panel B) with matching controls from the respective neurons. Currents shown correspond to the first 10 ms of the test pulse starting at the arrow for each group. C: Activation as a function of voltage. Peak sodium conductance, normalized to the maximum conductance (test pulse = +30 mV), is plotted as a function of the test pulse potential. Control (open circles, n = 9), in the presence of 10 µM δ-PVIA (closed circles, n = 8) or 10µM δ-SVIE (closed squares, n = 1), where n is the number of cells tested. Error bars represent
SEM. Solid lines represent Boltzmann fits to the averaged data with the following parameters (± SEM). Control; \( V_{50} = -8.6 \pm 0.4 \text{ mV} \), slope = 5.9 ± 0.4; 10 µM \( \delta \)-PVIA: \( V_{50} = -13.2 \pm 0.3 \text{ mV} \), slope = 5.9 ± 0.3); and 10µM \( \delta \)-SVIE: \( V_{50} = -19.9 \text{ mV} \), slope = 6.7.

FIG. 4. \( \delta \)-Conotoxins PVIA and SVIE alter the voltage-dependence of steady-state inactivation. TOP: Diagram of the voltage-command protocol. Neurons were held at –80 mV. 200 ms prepulses to potentials from –140 mV to 0 mV preceded a 50 ms test pulse to 0 mV. The start of the test pulse is indicated by an arrow. A and B: Representative sodium currents in the presence of 10 µM \( \delta \)-PVIA (panel A) or 1 µM \( \delta \)-SVIE (panel B) with matching controls from the same neurons. Currents shown correspond to the first 10 ms of the test pulse starting at the arrow for each group. C: Steady-state inactivation as a function of voltage. Peak sodium currents relative to the maximum (for prepulses ranging between -100 to -140 mV) are plotted as function of the prepulse potential. Data points represent the averages of responses from several cells: Control (open circles, n = 9), 10 µM \( \delta \)-PVIA (closed circles, n = 6), and 1 µM \( \delta \)-SVIE (closed squares, n = 3). Error bars represent SEM. Solid lines represent Boltzmann fits of the averaged data with the following parameters (± SEM). Control: \( V_{50} = -39.9 \pm 0.3 \text{ mV} \), slope = -6.4 ± 0.3; 10 µM \( \delta \)-PVIA: \( V_{50} = -46.1 \pm 0.6 \text{ mV} \), slope = -9.8 ± 0.5; and 1 µM \( \delta \)-SVIE: \( V_{50} = -66.5 \pm 1.5 \text{ mV} \), slope = -13.7 ± 1.5. D and E: \( V_{50} \) (closed circles) and slope factor (open circles) plotted as a function of time for two representative cells exposed to 10µM \( \delta \)-PVIA (panel D) or 1 µM \( \delta \)-SVIE (panel E). Toxin exposures are indicated by the horizontal black bars flanked by vertical dashed lines.
FIG. 5. The slowing of inactivation by δ-conotoxins PVIA and SVIE is suppressed by conditioning depolarizations in a time- and voltage-dependent manner. TOP: Diagram of voltage-command protocol. Neurons were held at –80 mV, and conditioning depolarizations (V\textsubscript{condition}) ranging from +20 to +120 mV and lasting between 20 to 300 ms were given to test the voltage- and time-dependence of reversal of the toxin’s effect. A –120 mV prepulse lasting 50 ms was given to relieve steady-state inactivation prior to each 50 ms test pulse to 0 mV. A and B: Representative traces showing the first 10 ms of the test pulse in the presence of 5 µM δ-PVIA (panel A) or 1 µM δ-SVIE (panel B) with or without conditioning depolarizations, in comparison to control responses (bold gray traces). Normalized responses, in the presence of toxin, following an 80 mV conditioning depolarization lasting 40, 100, 200, or 300 ms are compared to a response without any conditioning depolarization (the latter is labeled ”0 ms”). C and D: Ratio of the sodium current 2 ms after the peak to the peak current (I\textsubscript{Na 2 ms}/I\textsubscript{Na peak}), plotted as a function of conditioning depolarization duration. The effect of the V\textsubscript{condition} duration was tested at the following voltages (mV): 20 (closed square, panel C only), 40 (closed triangle), 60 (closed inverted triangle), 80 (closed diamond), 100 (closed circle), and 120 (closed square, panel D only). Data were obtained in the presence of 5µM δ-PVIA (panel C), or 1 µM δ-SVIE (panel D). In both panels, data obtained in the absence of toxin at V\textsubscript{condition} = 80 mV are represented by open circles. Solid lines are single-exponential fits to the data. In panel C, the time constants (ms) were 1667, 313, 182, 95, and 63 for V\textsubscript{condition} (mV) = 20, 40, 60, 80, and 100, respectively. In panel D, the
degree of δ-SVIE suppression was insufficient over the time range tested to obtain reliable time constant values.

FIG. 6. δ-PVIA inhibits trains of B-neuron compound action potentials (CAPs). Sympathetic ganglia were dissected and recorded from as described in Methods. A: Sketch of recording chamber and arrangement of electrodes. Stimuli were applied to the connective between the 8th and 9th ganglia while CAPs were recorded from the 10th spinal nerve. Thus, the preganglionic fibers innervating the 10th ganglion were stimulated, while responses from the postganglionic fibers of this ganglion were recorded. δ-PVIA was applied at the indicated concentrations to the test compartment (darkened well) containing the 10th ganglion and its ramus to the 10th spinal nerve. Thus, the cell bodies in the 10th ganglion, as well as portions of both its pre- and postganglionic fibers, were exposed to toxin. B: Recordings of B-neuron CAPs evoked by a train of stimuli before, during, and after exposure to 1 µM δ-PVIA. Each trace represents the average of 10 responses. Only fast and slow B-neuron CAPs are seen because the stimulus strength was adjusted to be below the threshold required to evoke C-neuron CAPs. A train of 8 CAPs were evoked by stimulating at 20 Hz.
Table 1: Sequences of δ-conotoxins PVIA and SVIE

<table>
<thead>
<tr>
<th>Conus species</th>
<th>δ-CTX</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. purpurascens</em></td>
<td>PVIA</td>
<td>EACYAOGTFCGIKOGCLCCSEFCLPGVCFG#</td>
</tr>
<tr>
<td><em>C. striatus</em></td>
<td>SVIE</td>
<td>DGCSGGTFCGIHOGLCCSEFCFLWCIWFID</td>
</tr>
</tbody>
</table>

0 = hydroxyproline; # = C-terminal amidation;
Shaded residues = conserved in fish-hunting species (Bulaj et al. 2001)
FIG. 1.
FIG. 2.

A

B

0 mV
-80 mV

20%
50 ms

Control
0.5 μM
1 μM
2 μM

Predicted Plateau / Peak I_{Na}

[δ-Pvia] (M)
FIG. 3.
FIG. 5.