# δ-Conotoxin Structure/Function through a Cladistic Analysis†

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ABSTRACT: δ-Conotoxins are *Conus* peptides that inhibit inactivation of voltage-gated sodium channels. The suggestion that δ-conotoxins might be an essential component of the venoms of fish-hunting cone snails which rapidly immobilize their prey [Terlau, H., Shon, K., Grilley, M., Stocker, M., Stühmer, W., and Olivera, B. M. (1996) *Nature 381*, 148−151] has not been tested. On the basis of cDNA cloning, all of the fish-hunting *Conus* analyzed yielded at least one δ-conotoxin sequence. In addition, one δ-conotoxin isolated from the venom of *Conus striatus* had an amino acid sequence identical to that predicted from cDNA cloning. This new peptide exhibited properties of δ-conotoxins: it targeted sodium channels and potentiated action potentials by slowing channel inactivation. Homologous sequences of δ-conotoxins from two groups (clades) of related fish-hunting *Conus* species share consensus features but differ significantly from the two known δ-conotoxins from mollusc-hunting *Conus* venoms. Three large hydrophobic amino acids were conserved; analogues of the previously described δ-conotoxin PVIA with alanine substituted for the conserved amino acids F9 and I12 lost substantial biological activity. In contrast, both the T8A and K13A δ-conotoxin PVIA analogues, where substitutions were at nonconserved loci, proved to be biologically active. Taken together, our results indicate that a cladistic approach can identify amino acids critical for the activity of conotoxins and provide extensive information as to which amino acid substitutions can be made without significant functional consequences.

The first peptides belonging to the δ-conotoxin family, characterized from snail-hunting (molluscivorous) *Conus* venoms (*2*−*4*), were shown to inhibit voltage-gated Na channel inactivation in molluscan neurons (*5*, *6*). More recently, δ-conotoxins have been characterized from fish-hunting cone snail venoms as well (*7*, *8*). The role of one δ-conotoxin in prey capture was elucidated for *Conus purpurascens* (*1*): it was shown that the combination of δ-conotoxin PVIA with a potassium channel antagonist (κ-conotoxin PVIIA, in the case of *C. purpurascens*) caused immediate tetanic paralysis of prey. Presumably, firing of axons in the vicinity of the injection site was elicited by the peptides, causing trains of action potentials to emanate from the targeted focal site. In some ways, the strategy might be viewed as introducing an epileptic focal site in the circuitry of the peripheral nervous system that results in tonic/clonic seizure-type symptomatology.

We suggested that groups of toxins present in venom that act together toward the same physiological end be called "cabals" and, specifically, that the set of venom peptides that almost instantaneously immobilize fish by eliciting the seizure-like symptomatology be referred to as the "lightning-strike cabal" (*9*). Thus, for many fish-hunting cone snails, a decisive factor for successful prey capture is to evolve an appropriate lightning-strike cabal of venom peptides. Although aquarium observations are consistent with most fish-hunting cone snails causing immediate tetanic paralysis of prey, it has not yet been established how widely distributed δ-conotoxins are among fish-hunting cone snails and the extent of peptide variation to be found. Since the effects of the lightning-strike cabal are somewhat akin to prey electrocution, it seems reasonable to postulate that δ-conotoxins, which in effect cause a dramatic increase in electrical activity of axons, may well be a key venom component in the lightning-strike cabals of fish-hunting cones.

We have investigated whether δ-conotoxins are generally found in the venoms of fish-hunting *Conus* species by cDNA cloning. Every Indo-Pacific fish-hunting cone snail species examined that is known to use a "harpoon-and-line" strategy for prey capture expressed a δ-conotoxin in its venom duct. A new δ-conotoxin, δ-SVIE from *Conus striatus*, has been purified from venom, cloned, chemically synthesized, and characterized. Thus, we establish that, like other major peptides important for prey capture (e.g., the α-conotoxins and the ω-conotoxins which inhibit synaptic transmission), the δ-conotoxins are widely distributed in the venoms of fish-hunting cones.

The elucidation of a large number of δ-conotoxin sequences identifies which amino acid loci in the δ-conotoxins

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from piscivorous cone snails are highly conserved, as well as which substitutions are allowed at variable loci. We demonstrated by mutation of the conserved residues that these are important for the potent effects of δ-conotoxins on mammalian sodium channels. In contrast, nonconserved residues could be substituted without abolishing activity. Thus, for hypervariable gene products such as conotoxins, this cladistic approach of systematically collecting homologous sequences from a clade of related organisms provides a useful informational resource for deducing structure/function relationships. This cladistic approach should be generally applicable for ~50 000 peptides likely to be found in cone snail venoms.

## EXPERIMENTAL PROCEDURES

*Purification of δ-Conotoxin SVIE from C. striatus Venom*. Lyophilized venom (50 mg) was placed in a 15 mL conical tube; 2 mL of spectra grade TFA[1] was added to the venom, and the tube was placed on ice for 20 min. The venom extract was stirred using a vortex mixer for 5 min and centrifuged at 20 000 rpm for 30 min at 4 °C. The supernatant was saved in a separate tube, and 2 mL of 0.5% TFA−20% ACN in $H_2O$ was added to the pellet. The pellet was incubated at 4 °C for 20 min and sonicated, and the mixture was vortexed for 5 min and centrifuged at 20 000 rpm for 30 min at 4 °C. The second supernatant was pooled with the first, and 2 mL of 0.5% TFA−40% ACN in $H_2O$ was added to the pellet. The steps were repeated to obtain a third supernatant that was pooled with the first two.

Preparative-scale reversed-phase HPLC was used to initially fractionate the venom pool. The venom pool was diluted to 500 mL with 0.1% TFA in $H_2O$ and applied to a $C_{18}$ preparative HPLC column (300 Å, 10 μm, 20 mm i.d. × 250 mm) with a guard column. For both preparative and analytical scale runs, the elution gradient was 0.1% TFA in water to 0.082% TFA in 90% ACN. The relevant fractions were then chromatographed on an analytical HPLC column (300 Å, 5 μm, 4.6 mm i.d. × 250 mm) for further purification. The fraction of interest, which eluted very late, caused spastic paralysis and, at higher doses, lethality.

*Peptide Synthesis*. The linear forms of δ-conotoxins SVIE, PVIA, and PVIA analogues were synthesized using standard Fmoc chemistry. All six cysteine residues were protected with trityl groups. The linear peptide was cleaved from the resin by treatment with cold TFA/water/ethanedithiol/phenol/thioanisole (90/5/2.5/7.5/5 by volume) for 2 h at room temperature. Filtering the reaction mixture into precooled MTBE precipitated the peptide. The reaction tube was rinsed with 1 mL of TFA that was also filtered into MTBE. The precipitate was pelleted by centrifugation. The supernatant was decanted and the pellet washed twice with MTBE. After the last wash and centrifugation, the pellet was dissolved by adding successively 6 mL of ACN, 1 mL of TFA, and 6 mL of water. For δ-PVIA and its analogues, the linear form was purified by reversed-phase HPLC. The peptides were loaded on a semipreparative diphenyl column (Vydac) and eluted with the gradient of acetonitrile in 0.1% TFA.

[1] Abbreviations: ACN, acetonitrile; HPLC, high-performance liquid chromatography; MTBE, methyl *tert*-butyl ether; TFA, trifluoroacetic acid.

The crude linear δ-SVIE was folded in a single-step reaction without prior purification. Cysteine residues in this peptide were oxidized at room temperature for at least 12 h in a folding solution consisting of 5% Tween 40, 1 mM GSH, 1 mM GSSG, 1 mM EDTA, and 25 mM glycine−NaOH, pH 8.0. The pH was adjusted to 8.0 with stock 1 M glycine−NaOH, pH 10.0. The folding reaction was quenched by reducing the pH to 3.0 with formic acid. The oxidative folding of δ-PVIA and the analogues was essentially the same as for δ-SVIE, except the reaction was carried out for 24 h at pH 8.7 and at 0 °C (in ice bath). The peptide concentration was 10 μM.

Preparative-scale reversed-phase HPLC (300 Å, 10 μm, 22 mm i.d. × 250 mm) was used to carry out solid-phase extraction on the folding mixture by running a linear gradient of 0.1% TFA in water to 0.091% TFA in 90% ACN for 5 min. The pooled fractions were then run on an analytical HPLC column (300 Å, 5 μm, 4.6 mm i.d. × 250 mm). The fractions with the correctly folded peptide were pooled and run on a diphenyl semipreparative HPLC column (300 Å, 5 μm, 10 mm i.d. × 250 mm) eluted with a gradient of 22.5% ACN, 0.1% TFA to 67.5% ACN, 0.1% TFA at a rate of 1.8% ACN per minute. The fractions containing the peptide were pooled and subjected to isocratic runs at 45 °C with 43% ACN and 0.082% TFA on a diphenyl analytical column (300 Å, 5 μm, 4.6 mm i.d. × 250 mm) to remove trace detergent.

*cDNA Cloning*. The primers used for cDNA cloning were those for the general O superfamily, and PCR amplification, cloning, and sequencing were carried out as previously described (*8*). In all fish-hunting *Conus* species shown in Table 1, at least one peptide showed close sequence similarity to the δ-PVIA and δ-SVIE precursor sequences. For *Conus magus*, four such sequences were identified. Other hydrophobic O-superfamily peptides were identified which were encoded by the cDNA clones, but these were significantly divergent in sequence from known δ-conotoxins; the functional activities of these latter peptides remain to be elucidated, and these were excluded from further consideration here.

*Biological Assays*. Sixteen- to seventeen-day-old Swiss Webster mice were injected intracranially as previously described (*10*).

*Electrophysiology: Muscle Preparation*. The *cutaneus pectoris* nerve muscle preparations and sciatic nerves (see below) were dissected from *Rana pipiens* frogs (about 2.5 in. body length). Motor nerve stimulation and recording of the resulting compound end plate potential from the muscle were performed essentially as previously described (*11*). To irreversibly and specifically block action potentials in the muscle, it was treated with conotoxin μ-PIIIA and washed (*12*). Thus, following μ-PIIIA treatment, only end plate potentials resulted in response to stimulation from the motor nerve.

*Electrophysiology: Sciatic Nerve Preparation*. Compound action potentials (CAPs) were recorded from the lateral crural branch of the sciatic nerve with extracellular wire electrodes. The stimulating and recording chamber consisted of a Sylgard (Dow Corning) filled dish containing a series of four wells, each separated from the next by a partition which was about 1 mm wide at its thinnest region. The nerve was placed in all four wells, and those portions of the nerve that draped

over the partitions (and were therefore exposed to air) were covered with Vaseline. One of a pair of stimulating electrodes was placed in the well containing the proximal end of the nerve; the other stimulating electrode was placed in the adjacent well, which also contained a ground electrode. The distal end of the nerve was in the fourth well which contained a wire electrode leading to the negative input of an AC (0.1 Hz) preamplifier. The wire electrode leading to the positive input of the preamplifier was located in the well adjacent to that containing the distal end of the nerve. Toxin was applied only to the well containing the distal end of the nerve. Suprathreshold stimuli (about 20 V and 1 ms duration) were used, and the resulting compound action potentials were biphasic (see Figure 3); the second positive phase arises when the action potential has propagated into the well containing the distal end of the nerve; thus, the effect of toxin would be expected to be seen in only this phase of the CAP. All electrodes were stainless steel. Signals were low-pass filtered (3 kHz, 8-pole Bessel) and digitized at a sampling frequency of 10 kHz. Data were acquired with homemade software written in LabVIEW (National Instruments, Austin, TX).

*Electrophysiology: Whole Cell Voltage Clamp of Frog Sympathetic Neurons*. Neurons from *R. pipiens* paravertebral sympathetic ganglia were dissociated essentially as previously described (*13*, *14*). Briefly, the 8th, 9th, and 10th ganglia were treated with 1 mg/mL collagenase at 37 °C for 45 min and then with 0.06 mg/mL trypsin at room temperature for 20 min. Neurons were dissociated by trituration, suspended in modified L-15 media (diluted by 20% with 1 mM $CaCl_2$ and 0.3% glucose and supplemented with 7% fetal bovine serum), plated on poly(L-lysine)-coated coverslips, and stored at 4 °C for no longer than 1 month.

Whole cell recordings were carried out at room temperature with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Extracellular solution contained the following (in mM): NaCl, 117; KCl, 2; $MgCl_2$, 2; $MnCl_2$, 2; HEPES, 5; tetraethylammonium (TEA) chloride, 10; pH 7.2. The recording pipet contained the following (in mM): NaCl, 10; CsCl, 110; $MgCl_2$, 2; $CaCl_2$, 0.4; ethylenediaminetetraacetic acid (EGTA), 4.4; HEPES, 5; TEA, 5; MgATP, 4; pH 7.2. These solutions permit recording of voltage-gated sodium currents exclusively by blocking voltage-gated potassium and calcium channels. Current signals were filtered at 2 kHz and digitized at 30 kHz using homemade software written in LabVIEW (National Instruments, Austin, TX). $Na^+$ currents were leak-subtracted by a P/5 protocol.

*Binding Assay*. The crude membrane fraction was obtained from the whole brain of 3-month-old mice as previously described (*15*). Iodination and storage of the radiolabeled TxVIA and competition binding assay were carried out as previously described (*8*).

*Autoradiography*. Male Sprague−Dawley rats, weighing 175−200 g, were anesthetized with ketamine and xylazine (80 mg/kg and 12 mg/kg ip, respectively) and perfused transcardially with 200 mL of ice-cold phosphate-buffered saline with 5% sucrose (pH 7.4). The brains were removed immediately and quick-frozen with powdered dry ice on top of a microtome tissue holder containing OCT compound. The brains remained at −70 °C until sectioned. Twenty micrometer thick saggital sections were cut with a cryostat microtome at −15 °C, thaw-mounted on chrome-alum-coated microscope slides, and stored at −70 °C until use. For ligand−receptor binding, tissue sections were warmed to room temperature and layered with 200 μL of 5 mM HEPES/Tris buffer (pH 7.4) containing 50 pM carrier-free [$^{125}$I]-δ-TxVIA, 5.5 mM glucose, and 1 mg/mL bovine serum albumin (BSA) for 45 min in a humidified box. Next, the slides were washed three successive times for exactly 2 min in 35 mL of ice-cold wash buffer containing 130 mm NaCl, 5 mM $CaCl_2$, 1.3 mM KCl, 0.8 mM $MgCl_2$, 1 mg/mL BSA, and 5 mM HEPES/Tris (pH 7.4). Nonspecific binding was determined by incubating sections for 45 min with binding buffer containing both [$^{125}$I]-δ-TxVIA (50 pM) and unlabeled δ-TxVIA (10 μM). Competition binding was performed by incubating sections with [$^{125}$I]-δ-TxVIA (50 pM) in the presence of 10 μM δ-SVIE or δ-PVIA for 45 min. Sections were dipped into ice-cold distilled water for 1 s and dried immediately using a stream of warm, dry air. The sections were apposed to Kodak Biomax MR film for 10 days and developed using a Kodak X-OMAT 1000 film processor. The autoradiograms were photographed using a DP-10 Olympus digital camera on a Olympus SZX9 stereo microscope and analyzed using the NIH Image program.

FIGURE 1: Shells of the fish-hunting species analyzed. Top row, left to right: *C. ermineus*, *C. aurisiacus*, *C. bullatus*, and *C. catus*. Bottom row, left to right: *C. consors*, *C. magus*, *C. striatus*, *C. purpurascens*, and *C. stercusmuscarum*.

## RESULTS

*Identification of δ-Conotoxin-Encoding Clones from Fish-Hunting Cone Snails*. Seven different piscivorous *Conus* species not previously analyzed for δ-conotoxins were examined. cDNA clones encoding putative δ-conotoxin precursors were identified and sequenced from them using methods previously described (*8*). The seven species analyzed include the one fish-hunting *Conus* known from the Atlantic, *Conus ermineus*, and six Indo-Pacific species, *Conus aurisiacus*, *Conus catus*, *Conus consors*, *Conus magus*, *Conus stercusmuscarum*, and *Conus striatus*. *C. magus*, *C. stercusmuscarum*, and *C. striatus* are venoms that our laboratories have previously analyzed extensively for other conotoxins. *C. aurisiacus* and *C. consors* are species generally collected in deeper waters, while *C. catus* is a small, shallow water piscivore. The shells of all of the *Conus* species included in this study are shown in Figure 1.

The results of the molecular cloning analysis are shown in Table 1. This table shows the predicted mature toxin regions from each species, including predicted posttransla-

Table 1: Comparison of Mature Toxin Sequences[a]

| *Conus* Species | δ-Conotoxin | Encoding Clone | Actual or Predicted Sequence‡ |
|---|---|---|---|
| Fish-hunting species | | | 5 • 10 • • 15 • • 20 • 25 |
| *C. aurisiacus* | AVIA | A6.5 | DG**C**SNAGAF**C**GIHOGL**CC**SEI**C**IVW**C**T |
| *C. catus* | CVIE | C6.2 | YG**C**SNAGAF**C**GIHOGL**CC**SEL**C**LVW**C**T |
| *C. consors* | CnVIA | Cn6.1 | YE**C**YSTGTF**C**GINGGL**CC**SNL**C**LFFV**C**LITFS |
| *C. ermineus* | EVIA | E6.4 | EA**C**YPOGTF**C**GIKOGL**CC**SEL**C**LPAV**C**VG# |
| *C. magus* | MVIA | M6.3 | DG**C**YNAGTF**C**GIROGL**CC**SEF**C**FLW**C**ITFVDS# |
| *C. magus* | MVIC | M6.6 | DE**C**YPOGTF**C**GIKOGL**CC**SAI**C**LSFV**C**ISFDF |
| *C. magus* | MVIB | M6.7 | EA**C**YNAGSF**C**GIHOGL**CC**SEF**C**ILW**C**ITFVDS# |
| *C. magus* | MVID | M6.8 | EA**C**YNAGTF**C**GIKOGL**CC**SAI**C**LSFV**C**ISFDF |
| *C. nigropunctatus* | NgVIA | ? | SK**C**FSOGTF**C**GIKOGL**CC**SVR**C**FSLF**C**ISFE |
| *C. purpurascens* | PVIA | P6.5 | EA**C**YAOGTF**C**GIKOGL**CC**SEF**C**LPGV**C**FG# |
| *C. stercusmuscarum* | SmVIA | Sm6.4 | DG**C**SSGGTF**C**GIROGL**CC**SEF**C**FLW**C**ITFID |
| *C. striatus* | SVIE | S6.5 | DG**C**SSGGTF**C**GIHOGL**CC**SEF**C**FLW**C**ITFID |
| *C. bullatus** | BVIA | B6.7 | DE**C**SAOGAF**C**LIROGL**CC**SEF**C**FFA**C**F |
| Mollusc-hunting species | | | |
| *C. gloriamaris* | GmVIA | Gm6.1 | VKP**C**RKEGQL**C**DPIFQN**CC**RGWN**C**VLF**C**V |
| *C. textile* | TxVIA | Tx6.16 | W**C**KQSGEM**C**NLLDQN**CC**DGY**C**IVLV**C**T |

[a] Symbols: ‡, proline residues in positions 6 and 14 have been predicted to be hydroxylated on the basis of homology to δ-PVIA, δ-SVIE, and δ-NgVIA; prolines in position 5 are also likely to be hydroxylated, since multiple proline residues in the same inter-Cys "loop" are often (but not always) all hydroxylated. #, C-terminal amidation; •, conserved amino acids. *, possibly fish-hunting—biology not established.

tional modifications (such as C-terminal amidation and proline hydroxylation). For comparison, mature toxin sequences of previously purified δ-conotoxins (PVIA, NgVIA, TxVIA, and GmVIA) are also included. One peptide from a species of unknown biology, *Conus bullatus*, is also shown.

The striking result is that all of the δ-conotoxins from fish-hunting cone snails share a set of conserved amino acids that define a coherent group, clearly divergent from the two δ-conotoxins from snail-hunting *Conus*, δ-conotoxins TxVIA and GmVIA. In particular, there is a region of high sequence similarity between residues 7−19. Apart from the Cys residues, which are conserved over the entire O superfamily, there are seven amino acids that are absolutely conserved in all δ-conotoxins from piscivorous *Conus*, including three large hydrophobic residues, F9, I12, and L16. Six of these amino acids are also conserved in the *C. bullatus* sequence, consistent with the possibility that *C. bullatus* may be a fish-hunting *Conus*. The functional importance of conserved residues was evaluated, as is detailed below.

*Purification and Chemical Synthesis of δ-Conotoxin SVIE from C. striatus.* The sequence of one of the mature peptides shown in Table 1 was determined independently by isolation of the peptide from venom. The δ-conotoxin family member from *C. striatus*, δ-conotoxin SVIE, was purified using two different assays. In the first purification, binding competition with radiolabeled [$^{125}$I]-δ-conotoxin TxVIA was used, and the activity which displaced the radiolabel was purified to homogeneity. In the second purification, the in vivo spastic paralytic activity, also observed with δ-conotoxin PVIA, was used as an assay for purification of δ-SVIE. Both purification schemes have led to the same purified peptide; the second purification is shown in Figure 2.

The purified fraction was analyzed by standard Edman degradation microsequencing procedures, and the sequence obtained was identical to that predicted from the clone, except

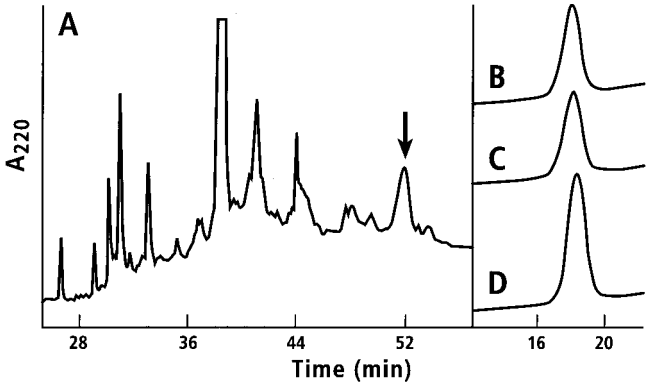


FIGURE 2: Purification of δ-conotoxin SVIE: identity of synthetic and native material. The HPLC chromatogram of crude *C. striatus* venom, eluted as described under Experimental Procedures is shown in panel A (arrow indicates the position of δ-conotoxin SVIE). Panel B shows purified native δ-conotoxin SVIE from *C. striatus* venom. Panel C shows synthetic δ-conotoxin SVIE after folding and purification (see Experimental Procedures). Panel D shows a one-to-one mixture of purified native and chemically synthesized SVIE, demonstrating the coelution of the two peptides.

that the lone proline residue was hydroxylated (the proline residues at the homologous position in δ-conotoxins PVIA and NgVIA are also hydroxylated). In addition, heterogeneity at the N-terminus was also observed (with E replacing D1 in a minor fraction of the peptide). The sequence assignment and the microheterogeneity at the N-terminus were confirmed by mass spectrometric analysis, with masses of 3320 (SVIE) and 3343 (the D1E variant) observed.

The availability of the native peptide purified from venom provided a reference standard for chemical synthesis of δ-conotoxin SVIE. The peptide was chemically synthesized using standard Fmoc chemistry and subsequently folded as described under Experimental Procedures. Synthetic δ-conotoxin SVIE coeluted with the native material (Figure 2).

Table 2: Biological Activity of δ-Conotoxin SVIE[a]

| dose (pmol/g) | SVIE (native) | SVIE (synthetic) |
|---|---|---|
| 12 ± 2 | twitching back limbs | twitching back limbs |
| 70 ± 2 | running in circles; spastic paralysis | running in circles; spastic paralysis |
| 130 ± 2 | not tested | spastic paralysis; lethal in 4 min |

[a] All injections were done ic on 16−17-day-old mice.

*Biological Activity of δ-Conotoxin SVIE*. Availability of synthetic peptide made an evaluation of the biological activity of the peptide feasible. The peptide is a potent excitotoxin when injected into mice (Table 2), producing increasingly severe symptoms including spastic paralysis and lethality as higher concentrations are injected. δ-Conotoxin SVIE elicited clearly assayable symptoms (twitching of hind limbs) even at a dose of 10 pmol/g; this was more potent than observed for δ-conotoxin PVIA (under the same conditions, 20 pmol/g δ-PVIA did not elicit any apparent symptoms). At a higher concentration of δ-SVIE (~70 pmol/g), more severe excitatory symptoms were routinely observed; the mice would run in circles before going into a spastic paralysis. It should be noted that δ-conotoxin TxVIA, from the molluscivorous species *Conus textile*, did not cause any behavioral changes in injected mice, even at much higher doses (>1000 pmol/g).

The activity of δ-conotoxin SVIE on the frog neuromuscular junction is shown in Figure 3A. The activity is consistent with delaying the inactivation of voltage-gated Na channels. In addition to its effect on the neuromuscular junction, δ-conotoxin SVIE also showed a striking effect on a frog sciatic nerve preparation. When the nerve was exposed to δ-conotoxin SVIE (2.5 μM), a greatly prolonged compound action potential was recorded (see Figure 3B). These effects of the peptide are consistent with previous reports for δ-conotoxin PVIA (see also refs *1* and *16*; D. Yoshikami, unpublished results).

The experimental results above strongly suggest (but do not prove) that the molecular target of δ-conotoxin SVIE is a voltage-gated sodium channel. To provide rigorous proof that this peptide acts on voltage-gated sodium channels, intracellular recording using a sympathetic ganglion cell in culture was employed. Conditions were designed so that the only currents elicited would be from voltage-gated Na channels. To eliminate the contribution of voltage-gated $Ca^{2+}$ currents, no $Ca^{2+}$ was present in the extracellular medium, and $Mn^{2+}$, an inhibitor of voltage-gated $Ca^{2+}$ channels, was present. To eliminate the contribution of potassium channels, the $K^+$ channel inhibitor TEA was added to both intracellular and extracellular solutions. The effect of δ-conotoxin SVIE on the $Na^+$ current elicited by membrane depolarization (to 0 mV) is shown in Figure 4. Clearly, a dramatic effect on the rate of inactivation of the voltage-gated Na channel in the cultured cells is seen. This result demonstrates that δ-conotoxin SVIE delays fast inactivation of the voltage-gated sodium channel in voltage-clamped neurons, the characteristic defining feature of δ-conotoxin activity.

Finally, as previously reported for δ-PVIA, δ-conotoxin SVIE completely displaces radiolabeled [$^{125}$I]-δ-conotoxin TxVIA binding (Figure 5) with an $IC_{50} \approx 10$ nM.

*Radiolabeling of Rat Brain Sections with [$^{125}$I]-δ-Conotoxin TxVIA: Competition by Other δ-Conotoxins*. It was previously reported that a major component of *C. striatus* venom, referred to as striatotoxin, displaced [$^{125}$I]-δ-cono-

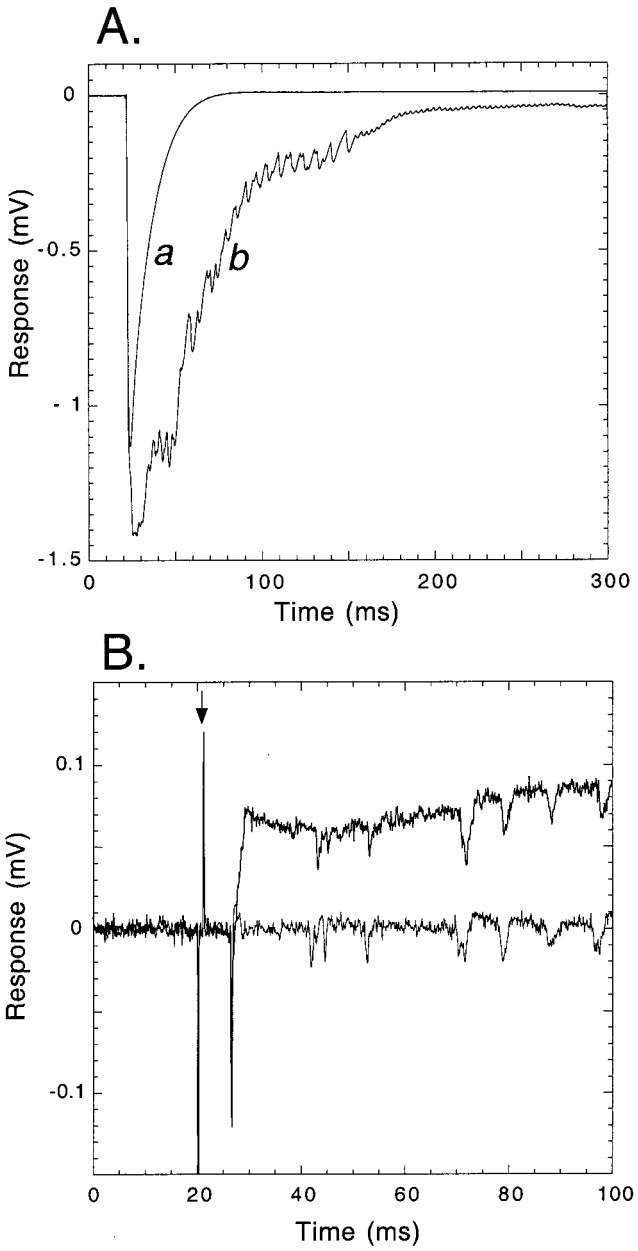


FIGURE 3: (A) Conotoxin δ-SVIE greatly prolongs evoked-transmitter release at the frog neuromuscular junction. Compound endplate potentials were recorded extracellularly in response to electrical stimulation of the motor nerve. Traces: a, control response; b, response acquired 4 min following exposure to 4 μM δ-SVIE. The preparation was previously treated with conotoxin μ-PIIIA to irreversibly block voltage-gated Na channels in the muscle (*12*). The extended time course of transmitter release is consistent with a drastic prolongation and repetition of the presynaptic action potential. (B) δ-Conotoxin SVIE greatly prolongs the compound action potential (CAP) recorded from the frog sciatic nerve. Traces: lower, control response; upper, response acquired 4 min after exposure to 2.5 μM SVIE. The first transient, at arrow, is the stimulus artifact. The second transient is the fast A-CAP, and subsequent transients are CAPs with slower conduction velocities. The negative phase of A-CAP was largely unaffected by the peptide, as would be expected since it arises from the action potential in the well which was not exposed to toxin (see Experimental Procedures). The positive phase of the A-CAP, produced by the action potential in the distal well, is small in the control but dramatically potentiated and prolonged when the peptide was present in the distal well.

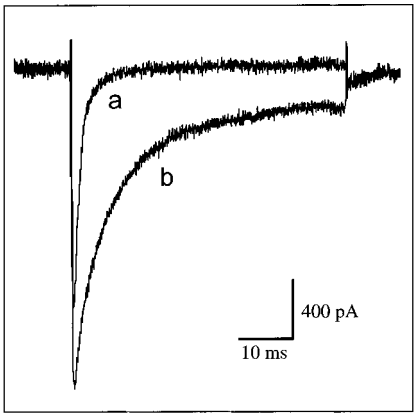


FIGURE 4: δ-Conotoxin-SVIE inhibits fast inactivation of voltage-gated sodium channels in dissociated frog sympathetic neurons. The cell was voltage clamped as described in Experimental Procedures. The membrane potential was held at −80 mV, and sodium channels were activated with a 50 ms step to 0 mV following a 50 ms hyperpolarizing prepulse to −120 mV. Traces: a, control response; b, response acquired at 3.5 min following exposure to 10 μM δ-SVIE. The toxin greatly prolonged the falling phase of the inward $Na^+$ current.

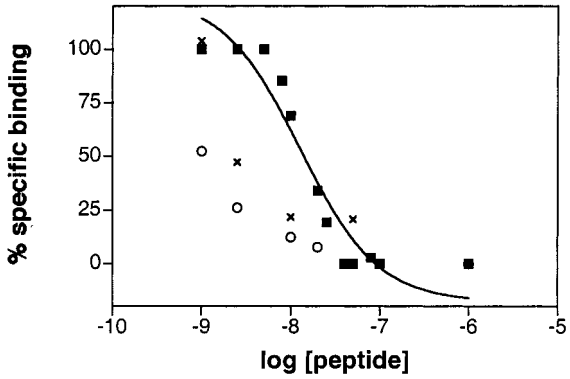


FIGURE 5: Binding competition between δ-conotoxins and [$^{125}$I]-δ-conotoxin TxVIA, a probe for high-affinity sites on rat brain sodium channels (*19*). The displacement of radiolabeled δ-conotoxin TxVIA by δ-conotoxin SVIE (■) was fit using nonlinear regression to a single site competition model (GraphPad Prism Software), yielding an apparent $IC_{50}$ of 12 nM. Note the unusually sharp displacement curve; the solid line represents the expected curve for a 1:1 displacement. The displacement of [$^{125}$I]-δ-conotoxin TxVIA by two δ-PVIA analogues, F9A (○) and I12A (×), is also shown.

toxin TxVIA binding to rat brain membranes. δ-Conotoxin SVIE appeared to be the major activity we detected in *C. striatus* venom that competed for δ-TxVIA binding to rat brain membranes. To examine binding specificity more thoroughly, we have carried out autoradiographic experiments to define δ-TxVIA binding sites in the mammalian CNS and evaluated with greater anatomic resolution which of these binding sites other δ-conotoxins compete for. $^{125}$I-Labeled δ-conotoxin TxVIA was prepared as described under Experimental Procedures. The radiolabeled peptide was applied to sagittal sections of rat brain (see Figure 6A), and specific binding was determined by displacement of the radiolabel using a large excess of unlabeled δ-conotoxin TxVIA (10 μM) (Figure 6B). Similar competition experiments were carried out with two δ-conotoxins from fish-hunting cone snails, δ-conotoxin SVIE (Figure 6C) and δ-conotoxin PVIA (Figure 6D).

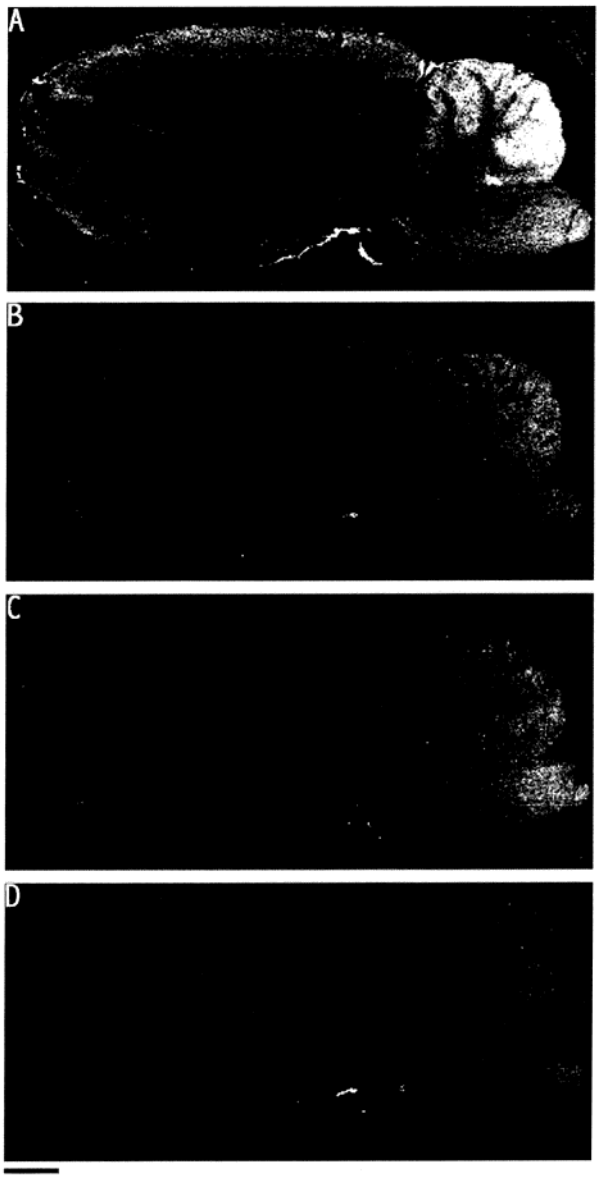


FIGURE 6: Distribution of [$^{125}$I]-δ-TxVIA binding sites in sagittal sections of rat brain: displacement by other δ-conotoxins. (A) Total binding with [$^{125}$I]-δ-TxVIA. (B) Nonspecific binding in the presence of 10 μM δ-TxVIA. (C−D) Competition with unlabeled δ-SVIE (10 μM) and δ-PVIA (10 μM). Autoradiography was performed as described in Experimental Procedures. The highest density of binding sites is noted in the cerebellar gray matter; the low-density binding sites are observed in the upper layers of the cortex and brain stem. Bar = 2 mm.

The highest density of specific [$^{125}$I]-δ-TxVIA binding sites is found in the gray matter of the cerebellum. Binding sites were also observed in the upper layer of cortex and in the brain stem. Competition with unlabeled δ-TxVIA, SVIE, and PVIA gave similar results: all peptides competed out most sites, including most of the binding in the cerebellum and in the cortex. There may be small quantitative differences between panels B, C, and D, but the most striking result is that all three of the unlabeled δ-conotoxins displaced most [$^{125}$I]-δ-conotoxin TxVIA binding.

*Demonstration of Amino Acid Residues Critical for Activity*. The δ-conotoxin sequences in Table 1 reveal conserved amino acid residues in the peptides from fish-hunting cone snails (though not from the snail-hunting *Conus*), a result

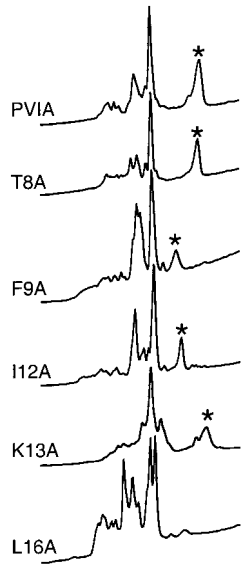


FIGURE 7: Folding mixtures of PVIA and its analogues. For each analogue, the folding reaction was quenched by acidification and fractionated by reversed-phase HPLC. The asterisk indicates the correctly folded δ-conotoxin. The biological activity of the native analogues is summarized in Table 3 and described in more detail in Results. Note the lack of accumulation of the native peptide for the L16A analogue.

that implies that these conserved amino acid loci may be important for activity on vertebrate Na channels. Of the seven conserved non-cysteine δ-conotoxin residues, the most frequently observed amino acid was Gly, probably structurally important for β-turns. However, three large hydrophobic residues are conserved in all sequences. We therefore synthesized and folded δ-conotoxin PVIA analogues in which alanine replaced the conserved large hydrophobic amino acids: F9, I12, and L16.

All alanine analogues of PVIA were folded in essentially the same way as wild-type PVIA (see Experimental Procedures). After the folding reaction was quenched, the mixture was separated by reversed-phase HPLC using an analytical diphenyl column. The correctly folded form of synthetic δ-conotoxins has been previously shown by Shon and coworkers (*4*, *8*) to possess the following disulfide connectivity: Cys(I)−Cys(IV), Cys(II)−Cys(V), and Cys(III)−Cys(VI). There are three characteristic properties of the correctly folded form of δ-conotoxins: (i) the longest HPLC retention time, as compared to the other folded species, suggesting that in the native form the hydrophobic residues are forced to face outward (*4*), (ii) a well-shaped peak in reverse-phase HPLC (*8*), and (iii) sensitivity of the accumulation of the correctly folded form to the presence of nonionic detergents such as Tween (Bulaj and DelaCruz, manuscript in preparation).

As shown in Figure 7, folding of four analogues (out of five) yielded a well-shaped peak (marked with an asterisk), also characterized by the longest retention time. Moreover, the accumulation of this peak was observed only when folding was carried out in the presence of Tween detergent (data not shown). Therefore, on the basis of these diagnostic features, we believe that the indicated characteristic peaks correspond to the correctly folded forms of PVIA analogues. No such peak was observed for [L16A]-δ-PVIA, suggesting that the yield of the correctly folded isomer was very low.

Table 3: Biological Activity of δ-PVIA Analogues

| Part A | |
|---|---|
| analogue | activity (dose) |
| PVIA | spastic paralysis (50 pmol/g) |
| T8A | spastic paralysis (250 pmol/g) |
| F9A | no visible effects (up to 500 pmol/g) |
| I12A | no visible effects (up to 500 pmol/g) |
| K13A | spastic paralysis (up to 200 pmol/g) |
| PVIA (linear/alkylated) | no visible effects (up to 3000 pmol/g) |

| Part B | |
|---|---|
| injection | observed effects of injection |
| control | mice normal |
| 0.5 nmol of δPVIA | immediate spastic paralysis, death |
| 15 nmol of [F9A]-PVIA | mice normal |
| 0.5 nmol of δPVIA + 15 nmol of [F9A]-PVIA | normal behavior (3 mice); spastic paralysis, recovered after 10 min, then normal behavior (1 mouse) |

However, we cannot eliminate the possibility that the L16A analogue with the correct disulfide connectivity elutes earlier than expected.

The four correctly folded analogues were evaluated for biological activity using an in vivo mouse injection assay (Table 3A). Injection of each of two analogues, T8A and K13A, resulted in symptoms similar to those elicited by wild-type toxin. However, both F9A and I12A analogues were inactive even at the highest concentrations tested. However, when assayed by competition binding with [$^{125}$I]-δ-conotoxin TxVIA, even the inactive F9A and I12A analogues were able to displace radioligand (see Figure 5).

The results suggested that inactive analogues could still bind to the target site of δ-conotoxins even though they could not functionally alter the activity of voltage-gated sodium channels. We therefore tested the F9A analogue for its ability to protect mice from the toxic effects of δ-conotoxin PVIA. The results of this experiment are shown in Table 3B. Coinjection of the F9A analogue did indeed protect against δ-conotoxin PVIA, suggesting that the analogue binds competitively to the δ-conotoxin ligand site even though [F9A]-δ-PVIA binding does not affect voltage-gated sodium channel function. However, such a protective effect does not occur for all physiological targets. When tested on the sciatic nerve preparation (described in Figure 3), the F9A analogue had no detectable effects on the compound action potential and did not prevent the native δ-PVIA from eliciting a prolonged depolarization.

We also synthesized and characterized two analogues of PVIA in which alanine was substituted for T8, a locus at which alanine is in fact found in some natural peptides [see *C. aurisiacus* (A6.5), *C. consors* (C6.2), and *C. bullatus* (B6.7) in Table 1], and for K13, a locus that has positively charged amino acid residues in almost all δ-conotoxins. As shown in Table 3, both of these analogues were biologically active.

## DISCUSSION

In this work, δ-conotoxin family members expressed in the venom ducts of fish-hunting *Conus* were identified. Like other major conotoxin families (such as the ω-conotoxins which inhibit presynaptic Ca channels and the α-conotoxins which are competitive nicotinic antagonists), we have shown that δ-conotoxins are widely distributed in piscivorous cone

snail venoms. δ-Conotoxins presumably play a major role in successful prey capture by fish-hunting cone snails that use a "harpoon-and-line" strategy (these species extend their proboscis and harpoon a fish at a distance)—all such fish-hunting cone snails analyzed express a δ-conotoxin in their venom ducts. It is noteworthy that in one species, *C. magus*, four distinct δ-conotoxins were found. The results suggest that more than one voltage-gated Na channel subtype may be targeted, a situation analogous to what has been established for ω-conotoxins, which target multiple subtypes of presynaptic Ca channels (*17*).

We have analyzed the relationship between the δ-conotoxin sequences shown in Table 1 in several ways. In a phylogenetically based analysis to be presented elsewhere (*18*), we show that the δ-conotoxins from non-Indo-Pacific species, *C. ermineus* and *C. purpurascens*, evolved in a separate lineage from all of the Indo-Pacific δ-conotoxins. However, when the mature toxin sequences are analyzed, the δ-conotoxin sequences from fish-hunting *Conus* venoms have shared sequence features that are absent from δ-conotoxins from mollusc-hunting species.

The elucidation of multiple δ-conotoxin sequences from piscivorous cone snail venoms provides an informational resource for structure/function analyses. We refer to a collection of homologous sequences from a species clade as a "clade scan"; because of the hypervariability of *Conus* peptides, the clade scan reveals considerable latitude for substitutions at most loci. However, a few amino acids are absolutely conserved; thus, such a cladistic analysis identifies residues that might be functionally important. We attempted to make alanine substitutions in δ-conotoxin PVIA for all of the conserved hydrophobic residues in δ-conotoxin sequences from fish-hunting cone snails. In addition, as a control, two nonconserved residues were also substituted with an alanine residue. We had expected that the replacements of the conserved residues would result in a substantial decrease of the biological activity, whereas those of the nonconserved residues would not significantly change the activity. These predictions have been fulfilled.

Finally, we demonstrate that [F9A]-δ-conotoxin PVIA, despite being functionally inactive, binds to voltage-gated sodium channels (see Figure 5). It has previously been shown that the radioligand used for these experiments binds to a novel ligand site on voltage-gated Na channels [called site 5 by Gordon and co-workers (*7*)]. Occupancy of the ligand site would prevent the natural δ-conotoxin PVIA from binding, and therefore the homologue would antagonize the activity of δ-PVIA.

Toxin analogues that have no functional effects on their own, but which block the activity of a toxin by binding to the same ligand site, may have potential applications. It should be possible to generate such analogues targeted to homologous sites, each specific for a different subtype of voltage-gated sodium channel (to take one example of a specific target family). Inactive analogues derived from subtype-specific *Conus* peptides have potential for defining specificity of various toxins and pharmacological agents acting at homologous ligand sites and, ultimately, may have important pharmacological, diagnostic, and therapeutic applications.

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