Structural Basis for Tetrodotoxin-resistant Sodium Channel Binding by \(\mu\)-Conotoxin SmIIIA

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SmIIIA is a new \(\mu\)-conotoxin isolated recently from Conus sterculiuscarum. Although it shares several biochemical characteristics with other \(\mu\)-conotoxins (the arrangement of cysteine residues and a conserved arginine believed to interact with residues near the channel pore), it has several distinctive features, including the absence of hydroxyproline, and is the first specific antagonist of tetrodotoxin-resistant voltage-gated sodium channels to be characterized. It therefore represents a potentially useful tool to investigate the functional roles of these channels. We have determined the three-dimensional structure of SmIIIA in aqueous solution. Consistent with the absence of hydroxyprolines, SmIIIA adopts a single conformation with all peptide bonds in the trans configuration. The spatial orientations of several conserved Arg and Lys side chains, including Arg14 (using a consensus numbering system), which plays a key role in sodium channel binding, are similar to those in other \(\mu\)-conotoxins but the N-terminal regions differ, reflecting the trans conformation for the peptide bond preceding residue 8 in SmIIIA, as opposed to the cis conformation in \(\mu\)-conotoxins GIIMA and GIIIB. Comparison of the surfaces of SmIIIA with other \(\mu\)-conotoxins suggests that the affinity of SmIIIA for TTX-resistant channels is influenced by the Trp9 side chain, which is unique to SmIIIA. Arg17, which replaces Lys in the other \(\mu\)-conotoxins, may also be important. Consistent with these inferences from the structure, assays of two chimeras of SmIIIA and PIIIA in which their N- and C-terminal halves were recombined, indicated that residues in the C-terminal half of SmIIIA confer affinity for tetrodotoxin-resistant sodium channels in the cell bodies of frog sympathetic neurons. SmIIIA and the chimera possessing the C-terminal half of SmIIIA also inhibit tetrodotoxin-resistant sodium channels in the postganglionic axons of sympathetic neurons, as indicated by their inhibition of C-neuron compound action potentials that persist in the presence of tetrodotoxin.

Polypeptide toxins typically interact with their target receptors with high potency and exquisite selectivity and as such are valuable tools in elucidating the physiological functions of their targets and in probing the size and shape of their cognate binding sites. Toxins from the genus Conus have been especially valuable in this respect, and, of the various classes of conotoxins that have been characterized to date (1, 2), the \(\mu\)-conotoxins represent a particularly good example. Their targets are the voltage-gated sodium channels (VGSCs),1 which are responsible for the influx of sodium ions during action potentials in excitable tissues.

Three families of conotoxins target VGSCs, causing either inhibition (\(\mu\)- and \(\omega\)-conotoxins) or delayed inactivation (\(\delta\)-conotoxins), but to date a detailed understanding of their interactions with the channel has been achieved only in the case of the \(\mu\)-conotoxins. These toxins bind to Site 1 on VGSCs, one of several toxin binding sites identified on these channels (3). The pore-forming \(\alpha\)-subunit of each VGSC consists of four homologous domains, each containing six putative transmembrane helices S1–S6, with the Na+ channel thought to be formed by the S5–S6 loops from all four domains (these S5–S6 linkers are further subdivided into the S5-P, P, and S6-P loops, with the P loop containing the SS1 and SS2 segments). Site 1, located on the extracellular surface of this pore, binds the guanidinium alkalioid tetrodotoxin (TTX) and saxitoxin as well as the \(\mu\)-conotoxins. Not all VGSCs, however, bind TTX. Of the nine well characterized \(\alpha\)-subunits cloned to date from mammals (4, 5), at least three, Na1.5, Na1.8, and Na1.9, can be classified as TTX-resistant. To probe the physiological roles of these VGSCs, and indeed of the different subtypes of TTX-sensitive VGSCs, additional blockers are needed. The \(\mu\)-conotoxins offer considerable promise in this respect.

The first \(\mu\)-conotoxin characterized, \(\mu\)-conotoxin GIIMA from the fish-hunting snail Conus geographus, competes with TTX and saxitoxin for binding to Site 1 on the skeletal muscle Na+ channel (6, 7) but is much more selective, targeting largely the skeletal muscle subtype Na1.4 (8). Of the neuronal subtypes studied thus far, Na1.1.1 is the most readily blocked by GIIMA, but the EC50 is still an order of magnitude higher than that for Na1.4 (9). GIIB, from the same species, has similar selectivity, binding preferentially to skeletal muscle over neuronal or cardiac subtypes (6, 7, 9–11). Another \(\mu\)-conotoxin, PIIMA from the fish-hunting snail Conus purpurascens, also has a strong preference for the skeletal muscle subtype but can block other

1 The abbreviations used are: VGSC, voltage-gated sodium channel; GIIMA and GIIIB, \(\mu\)-conotoxins GIIMA and GIIIB, respectively, from C. geographus; PIIMA, \(\mu\)-conotoxin PIIMA from C. purpurascens; SmIIIA, \(\mu\)-conotoxin SmIIIA from C. sterculiuscarum; Sm-P and P-Sm, chimeric \(\mu\)-conotoxins; TTX, tetrodotoxin; CAP, compound action potential; TOCSY, total correlation spectroscopy; COSY, correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser and exchange spectroscopy; r.m.s.d., root mean square deviation.

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TTX-sensitive subtypes as well, albeit with lower affinity (8, 12, 13). Very recently, West et al. (14) described a novel \( \mu \)-conotoxin, SmIII A, from the fly speck cone snail \( C. \) stercusmuscarum, which blocked TTX-resistant VGSCs in frog sympathetic and dorsal root ganglia but had little effect on TTX-sensitive currents. SmIII A thus represents the first specific antagonist for TTX-resistant Na\(^+\) channels.

The amino acid sequences of SmIII A is compared with those of GIHA, GIIHB, GIIHC, and PIHA in Fig. 1. The locations of the six half-cystines, and thus of three disulfide bridges, are conserved, as is the key residue Arg\(^{14}\) (using the consensus numbering of Fig. 1). The important basic residues at positions 2, 9, 17, and 21 are also conserved or conservatively substituted. On the other hand, SmIII A lacks the hydroxyproline residues found in all other \( \mu \)-conotoxins described to date, contains a Trp residue at position 15 in contrast to Gln or Arg, and has an atypical sequence between the fourth and fifth half-cystines. As these changes might be expected to affect the three-dimensional structure of SmIII A we have determined its structure in solution using NMR spectroscopic data. Comparison of the structure with those of GIHA (15–17), GIIHB (18), and PIHA (13) confirms that its overall fold and the spatial disposition of conserved Arg and Lys residues on the surface are similar but also allows some inferences to be drawn about the basis for the selectivity of SmIII A for TTX-resistant channels. These inferences have been confirmed by the construction and assay of chimeras of SmIII A and PIHA in which the N- and C-terminal halves were recombined.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Sample Preparation—Conotoxins SmIII A and PIHA and chimeras were synthesized, folded, and purified using the protocols described previously (12, 14). The peptides were purified using C18 reversed-phase HPLC and their identities confirmed by mass spectrometry.**

**NMR Spectroscopy—**Two-dimensional homonuclear TOCSY spectra with a spin-lock time of 50 ms and E-COSY spectra were collected on a Bruker AMX-500 spectrometer, and NOEY spectra with mixing times of 300, 200, and 76 ms were collected on a Bruker DIX-600 spectrometer, essentially as described previously (19). A 300-ms NOESY spectra at pH 3.1 was also acquired. A series of one-dimensional spectra at 5°C temperature and no allowances were made for isotope effects. 

In the SmIII A and PIHA sequences, Z represents pyroglutamate. All peptides are C-terminally amidated. The locations of disulfide bonds previously determined in other \( \mu \)-conotoxins are indicated, as well as the biologically important Arg\(^{14}\). Cys and basic residues common to all sequences are in **bold**.

**Fig. 1. Amino acid sequences of SmIII A, GIHA, GIIHB, GIIHC, and PIHA.** SmIII A is from \( C. \) stercusmuscarum, GIHA, GIIHB, and GIIHC are from \( C. \) geographus (Swiss-Prot accession numbers P01523, P01524, and P05462, respectively) and PIHA is from \( C. \) purpureascens (Swiss-Prot accession number P58925). Note that a consensus numbering system has been adopted to encompass all \( \mu \)-conotoxin sequences described to date. A series of one-dimensional spectra at 5°C temperature and no allowances were made for isotope effects. A 300-ms NOESY at pH 3.1, 300-ms NOESY at pH 4.7, and 300-ms NOESY at pH 75 ms were collected on a Bruker DRX-600 spectrometer. A pH titration was performed over the range 1.5–9.5. All spectra were collected at 5°C unless otherwise stated and were referenced to an impurity peak at 0.15 ppm or the water resonance. Spectra were processed using XWINNMR (Bruker Biospin) and analyzed using XEASY (21). Chemical shift assignments for SmIII A have been deposited in the BioMagResBank data base (22).

**Structural Constraints—**SmIII A contains three disulfide bonds linking the following pairs of half-cystines: Cys\(^{4}\)–Cys\(^{16}\), Cys\(^{5}\)–Cys\(^{19}\), and Cys\(^{9}\)–Cys\(^{22}\). Appropriate distance restraints for each of these disulfides were included at each stage of the structure calculations. Distance restraints were obtained from the following spectra: 300- and 75-ms NOESY spectra at pH 4.7, 300-ms NOESY at pH 3.1, and 300-ms NOEY at pH 4.4 in \( \text{H}_2\text{O} \) all acquired at 600 MHz. \( J_{\text{HNH}} \) coupling constants were measured from a double quantum filtered COSY acquired at 500 MHz and then converted to dihedral restraints (\( J_{\text{HNH}}>8\) Hz, \( \phi =-120±30^\circ \), \( J_{\text{HNNH}}<6\) Hz, \( \phi =-60±30^\circ \)). If a positive \( \phi \) angle could be excluded on the basis of NOE data (23), \( \phi \) angles were restricted to the range –180 to 0°. \( \chi \) angles were determined based on analysis of a 75-ms NOESY spectrum.

**Structure Calculations—**Two sets of structural calculations were performed in CYANA (24). Both used a set of four peak lists derived from the NOEY spectra described above. The first set of calculations used manual assignments for each peak. Intensities for each peak were calibrated using the conventional CALIBA script supplied with CYANA. These were then scaled by a factor of 1.2 before use in structural calculations to allow for possible effects of spin diffusion. A family of 200 structures was calculated using torsion angle dynamics, and 20 structures with the lowest target function were then chosen for further analysis. No dihedral violations greater than 5° or NOE violations greater than 0.2 Å were observed in this family.

In addition to the traditional manual assignment of NOE cross peaks, we used the CANDID module of CYANA to assign NOE cross-peaks and calculate a structure for SmIII A. The peak lists used for the manual assignments were supplied to CANDID and NOE assignments and structures were determined using the default scripts. The peak intensities from CANDID were equivalent to those obtained after scaling the peak intensities in the manually assigned peak lists by a factor of 1.2. Of the 1193 peaks supplied to CANDID, 1174 were assigned automatically, with the remaining being unassigned predominantly because of ambiguity among several possible assignments. Only eight of the assignments made by CANDID differed from those made manually. The backbone r.m.s.d. between structures calculated in CYANA using a manually assigned peak list and those calculated with the peak list produced by CANDID was 0.66, indicating that the methods were in excellent agreement.

The final NOE and dihedral restraint lists from CYANA were then used to calculate a family of 200 structures in Xplor-NIH (25) using the standard distance geometry and simulated annealing scripts. The library files supplied with Xplor-NIH were modified to include a pyroGlu residue. The lowest 30 structures in energy were chosen for further refinement. A water box was built around the molecule and then energy minimized on the basis of NOE and dihedral restraints and the geometry of bonds, angles, and improper. A final family of 20 structures was then chosen for further analysis based on stereochemistry and energy considerations. This family has been deposited in the RCSB protein structural data base (Protein Data Base accession number 1Q2J). Structures were analyzed using the programs PROCHECK (26) and MOLMOL (27). Structural figures were prepared using MOLMOL (27),
GRASP (28), and InsightII (Accelrys, San Diego, CA).

**Dissociated Neuron Preparation**—Lumbar paravertebral ganglia 8–10 were dissected from 6–7.5 cm adult frogs (Rana pipiens) of either sex and processed as described previously (29, 30). Briefly, ganglia were treated with collagenase followed by trypsin. Cells were mechanically dissociated by trituration, washed, and suspended in 73% Leibowitz’s L15 solution (supplemented with 14 mM glucose, 1 mM CaCl2, 7 mM fetal bovine serum, and penicillin/streptomycin), plated on polylysine-coated coverslips, and stored at 4 °C.

**Whole Cell Patch Clamp Electrophysiology**—Whole cell patch clamp recordings were made essentially as described previously (14). Briefly, sympathetic neurons were perfused with extracellular solution containing (in mM): NaCl, 117; KCl, 2; MgCl2, 2; MnCl2, 2; HEPES, 5; TEA, 10; MgATP, 1; MgEGTA, 1; pH 7.2. Recording pipettes contained (in mM): NaCl, 10; CaCl2, 110; MgCl2, 2; CaCl2, 0.4; EGTA, 4.4; HEPES, 5; TEA, 5; MgATP, 4; pH 7.2. These solutions inhibit voltage-gated K+ and Na+ currents, thereby permitting recording of TTX-resistant Na+ currents exclusively. Conotoxins were dissolved in extracellular solution and applied to neurons by bath exchange. Toxin exposures were conducted in static baths. To evoke voltage-gated TTX-resistant Na+ currents, the neuron was held at −80 mV, while 50-ms test pulses to 0 mV were applied every 10 s. Each test pulse was preceded by a −120 mV prepulse lasting 50 ms to relieve steady-state inactivation. Current signals, acquired at room temperature with an Axopatch 200B amplifier (Axon Instruments), were filtered at 2 kHz, digitized at 10 kHz, and leak-subtracted by a P/5 protocol using in-house software written in LabVIEW (National Instruments, Austin, TX).

**Extracellular Electrophysiology**—Extracellular recordings of compound action potentials (CAPs) in sympathetic nerves were made essentially as described previously (31). Briefly, lumbar paravertebral ganglia 8–10 and the adjoining 10th spinal nerve were dissected from 6–7.5 cm adult frogs (R. pipiens) of either sex. The recording chamber was fabricated from Sylgard and consisted of seven circular or semicircular compartments with diameters of 4–5 cm (see Fig. 4A), each separated from its neighbor by a partition about 1-mm wide. A bead of Vaseline was placed atop each partition between compartments. The compartments as illustrated in Fig. 4A. Portions of the fluid in each compartment was isolated and independently maintained and electrical stimulation or recording across compartment partitions was possible. Each compartment was maintained essentially as a static bath except for the test compartment, which was perfused when it did not contain toxin. All compartments contained normal 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 toxin.

Platinum wire electrodes were used to convey supramaximal stimuli to the preganglionic sympathetic chain between the 8th and 9th ganglia and to record postganglionic CAPs in the 10th spinal nerve. Stimuli (0.1–1 ms rectangular voltage pulses) were provided by a stimulator (S-88, Grass Instruments) through a stimulus isolation unit. Recordings were made using a differential AC preamplifier (P-55, Grass Instruments, with bandpass filter settings of 1 Hz and 1 kHz). All experiments were performed at room temperature.

**RESULTS**

**NMR Spectroscopy**—Broad NH chemical shift dispersion in one-dimensional spectra indicated that SmIIIA was well structured, and two-dimensional homonuclear spectra recorded at 5, 15, and 25 °C showed that only one conformation was present in aqueous solution at these temperatures. Sequence-specific resonance assignments for backbone and side-chain protons in SmIIIA were made at 500 and 600 MHz using standard two-dimensional homonuclear NMR experiments. Assignments at pH 4.7 and 3.1 are tabulated in Supplementary Material and have been deposited with BioMagResBank (22) with accession number 5881. Distance constraints were taken from the volumes of NOE cross-peaks in NOESY spectra acquired at 5 °C at 600 MHz.

**NMR Assessment of Hydrogen Bonds**—The temperature dependence of each amide proton chemical shift was determined to probe for hydrogen bonding. The amide protons of Cys5, Gly5, Cys1, Cys3, Arg, His19, Ser20, and Cys23 exhibited temperature coefficients of magnitude ≤4 ppb/°C (see Supplementary Material), consistent with their possible involvement in hydrogen bonds (32). However, amide exchange experiments conducted at 5 °C and pH 4.4 indicated that all amide protons, except for those of Cys16 and Ser20, exchanged with solvent deuterium within minutes of dissolution of the polypeptide in D2O. Amide protons from these residues were apparently in NMR spectra for several hours following dissolution. Because even the most slowly exchanging amides in SmIIIA exchanged relatively quickly, no hydrogen bonds were used as distance constraints in the structure calculations.

**Solution Structure**—Parameters characterizing the final 20 structures of SmIIIA and structural statistics are summarized in Table I, and stereo views of the structures superimposed over the backbone are shown in Fig. 2. Inspection of Table I indicates that the final 20 structures fit well with experimentally derived distance and angle constraints and are well defined over the entire length of the polypeptide. The structures of SmIIIA have been deposited with the RSCB Protein Data Bank (accession number 1IQ2J) (33).

The angular order parameters, S, for the backbone φ and ψ angles and the side-chain χ1 angles of SmIIIA are presented in Fig. S1 in the Supplementary Material. The φ and ψ angles are well ordered (Sφ,ψ > 0.9) for all residues except Arg12, for which Sφ,ψ is 0.82, indicating that the structure is well defined. Of the three disulfide bridges in SmIIIA, two, Cys4–Cys16 and Cys5–Cys22 have a left-handed (ψ80–90°) configuration and Cys11–Cys23 is right-handed (ψ80 +90°).

In the N-terminal region of SmIIIA the pattern of NOEs and hydrogen bonds is consistent with a series of turns, including the inverse γ-turn at Cys13 and γ-turn at Gly13. There is no evidence of the β-hairpin seen in the N-terminal region of GIIB (18). The backbone between Ser13 and Ser17 adopts a distorted helical structure, with an i + 2 to i hydrogen bond from Arg17 to Trp15 implying a β-strand rather than an α-helical character, but this does not continue through to the C terminus. In its lack of a β-hairpin in the N-terminal region and limited helical character in the C-terminal half, SmIIIA appears to be more similar to PIIBA (13) than GIIB. However,
when we subjected the structures of GIIIB to analysis in PRO-CHECK, little secondary structure was predicted, with only a turn of helix over residues Arg14 to Cys16 and Lys21 to Cys23 being present in 4 out of 20 structures in Protein Data Bank entry 1GIB. In fact, the backbone of SmIIIA over residues 11–17 overlays with the corresponding region of GIIIB with an r.m.s.d. of 1.00 Å (1.07 Å in the case of GIIIA), indicating that the structures are quite similar despite the different descriptions of their ordered secondary structure. By contrast, when the N-terminal region is included, the r.m.s.d. values are 2.46 and 3.00 Å to GIIIB and GIIIA, respectively, confirming that there are significant differences between SmIIIA and GIIIA/B in this region, as was the case for PIIIA, r.m.s.d. values to PIIIA cannot be calculated because the PIIIA structure has not been deposited with the Protein Data Bank.

SmIIIA is a highly basic polypeptide as a consequence of its 6 Arg residues. The only other charged side chains are those of Asp18 and His19, and both the N and C termini are blocked. As illustrated in Fig. 3, the surface of SmIIIA is highly positively charged, relieved only by the negatively charged Asp18 on one face. The indole ring of Trp15, which is unique to SmIIIA, is flanked by the imidazolium ring of His19 on one side and the guanidinium moiety of Arg2 on the other (Fig. 2). These side chain interactions would be favored not only by interactions between the π orbitals of the indole and imidazolium rings but also by aromatic-cation interactions (34). Interestingly, the relative orientation of Trp and Arg side chains in SmIIIA shows some similarity to those of the Trp and Arg side chains in the “WSWX” structures of hemopoietic cytokine receptors, such as that for erythropoietin (35). Analysis of ring current shifts in the final structures was performed in MOLMOL using both the Johnson-Bovey and Haigh-Mallion methods (27, 36). As a consequence of the close proximity of His19 and Arg2 to the indole ring, resonances of both are perturbed by ring current effects, with deviations upfield of more than 0.5 ppm predicted for Cα-H, Cδ-H, and Cγ-H of Arg2 and Cα-H, Cδ-H, and ring protons of His19.

The CαH resonance of His19 is shifted upfield significantly, but in Arg2 only the CαH2 resonance is shifted, and by only 0.2 ppm compared with other Arg side chains, perhaps reflecting some flexibility in the Arg2 position.

**pH Dependence**—A limited pH titration was undertaken to determine the pKα values for the Asp18 carboxyl and His19 imidazolium groups. The Asp18 pKα of 3.8 is very similar to that of the carboxyl group of Asp in small peptides (37, 38). In small histidine-containing peptides the imidazolium pKα was 7.0 at 35 °C (38), while in the uncharged model compound Ac-His-NHMe it was 6.38 at 37 °C (39), 6.43 in H2O, and 6.54 in 2H2O, each containing 0.1 M NaCl at 30 °C (40). The intrinsic pKα cited by Shire et al. (41) is 6.3. The His19 pKα of 6.8 is slightly higher than the model values. Given the highly basic nature of SmIIIA, a reduced pKα might have been expected for His19, but these influences must be offset by proximity of the imidazolium ring to the negatively charged side chain of Asp18 and by cation-aromatic interactions with the indole ring of Trp15, both of which would tend to stabilize the imidazolium form.

**Minor Conformers and Conformational Averaging**—Previous NMR studies of μ-conotoxins have found evidence of conformational flexibility, manifested in line broadening of one or more resonances (15, 17, 18). By contrast, no significant line broadening was observed for SmIIIA at pH 4.7 or 3.1 over the temperature range 5–25 °C, from which we infer that SmIIIA does not undergo significant conformational averaging in aqueous solution.

Minor peaks with intensities ≤10% of the main peaks were present in spectra of SmIIIA but may have arisen from impurities in the synthetic sample used for structure determination (although its purity was >95% according to reversed-phase HPLC). Moreover, analysis of backbone NOEs confirms that all peptide bonds in SmIIIA adopt the trans conformation. This contrasts with the behavior of other μ-conotoxins investigated by NMR, where multiple conformations associated with cis-
trans isomerism have been observed. In PIIIA the ratio of major to minor conformer was about 3:1 (13). Minor conformers associated with cis-trans isomerism were not noted for GIIIB by Hill et al. (18), although there is a statement by Nielsen et al. (13) that minor (trans) conformers of GIIBA and GIIBB were “masked by broadening associated with intermediate exchange occurring on the NMR time scale.”

Electrophysiological Analysis of μ-Conotoxins and Chimeras—Unlike SmIII A, μ-conotoxin PIIIA does not inhibit TTX-resistant VGSCs in rat DRG neurons (13) or in frog sympathetic neurons (Fig. 4A). To test the hypothesis that residues in the C-terminal half of SmIII A determine affinity for TTX-resistant VGSCs, two complementary chimeras of SmIII A and PIIIA were generated (Table II). The C-terminal half of SmIII A, beginning at Arg134, was replaced by the corresponding PIIIA residues in μ-chimera Sm-P, while in the second, P-Sm, the C-terminal half of PIIIA was replaced by the corresponding SmIII A residues.

Although SmIII A inhibits TTX-resistant VGSCs with minimal effects on TTX-sensitive VGSCs in frog sympathetic neurons (14), it does inhibit CAPs in frog skeletal muscle (summarized in Table II; data not shown). Inhibition by SmIII A of muscle CAPs is reversed by washing, unlike the irreversible inhibition by PIIIA (12). In contrast, SmIII A inhibition of TTX-resistant VGSCs in frog sympathetic and DRG neurons is essentially irreversible (14), indicating that its affinity for these VGSCs is greater than its affinity for those in frog skeletal muscle. Since both PIIIA and SmIII A inhibit CAPs in frog skeletal muscle, this tissue was used to assess the baseline activities of the Sm-P and P-Sm chimeras. As summarized in Table II, both chimeras reversibly inhibited muscle CAPs, indicating that the structures of these peptides resembled those of their parent compounds to the extent that they retained their biological activities.

The effects of the chimeric μ-conotoxins on TTX-resistant VGSCs were compared on voltage-clamped frog sympathetic neurons (Fig. 5). Neurons were held at −80 mV, and 50-ms test pulses to 0 mV were applied at 0.1 Hz. Each test pulse was immediately preceded by a 50 ms prepulse to −120 mV (see voltage-command protocol diagrammed at the top of Fig. 5) to relieve any steady-state inactivation that may have occurred at the −80 mV holding potential. The beginning of the test pulse is indicated by an arrow both in the voltage-command diagram and in the current response traces. All illustrated responses in the presence of toxin represent those obtained only after the preparation had equilibrated with peptide for >10 min.

Although 1 μM PIIIA does not inhibit TTX-resistant currents in these neurons (Fig. 5A), 1 μM SmIII A irreversibly inhibited nearly all of the TTX-resistant current (Fig. 5B), verifying our previous findings (14). Like PIIIA, 1 μM Sm-P did not inhibit TTX-resistant currents (Fig. 5C). In contrast, 1 μM P-Sm (the “inverse” chimera) did inhibit the TTX-resistant current in a manner similar to that of SmIII A (Fig. 5D). As seen in the bottom half of Fig. 5D, the time course of this inhibition was slower than that of native SmIII A, and complete inhibition was not achieved during the 20-min toxin exposure in this experiment. Nevertheless, the inhibition was not reversed when the preparation was perfused with toxin-free bathing solution for 30 min, indicating that the P-Sm chimera, like SmIII A, has a high affinity for TTX-resistant VGSCs in these neurons.

To further characterize the activity of these peptides, CAPs were recorded from sympathetic postganglionic axons in the 10th spinal nerve essentially as described previously (31). These CAPs were evoked by stimulating the preganglionic axons (for review, see Ref. 42). These differences in action potentials on TTX-resistant VGSCs in rat DRG neurons (13) or in frog sympathetic neurons (Fig. 4A). To test the hypothesis that residues in the C-terminal half of SmIII A determine affinity for TTX-resistant VGSCs, two complementary chimeras of SmIII A and

![Surface representation of SmIIIA](Image)
TABLE II

| Sequences and activities of SmIIIA and PIIIA and chimeric μ-conotoxins Sm-P and P-Sm | Inhibition of frog muscle CAP (1 μM) |
| --- | --- |
| Native μ-conotoxins |  |
| SmIIIA | ZR-CCNGRRGCSRWSHDHSC |
| PIIIA | ZRLCGFOKSRSQCKH-

| μ-Conotoxin chimeras |  |
| Sm-P | ZR-CCNGRRGCSRWSHDHSC |
| P-Sm | ZRLCGFOKSRSQCKH-

When 10 μM TTX was applied to postganglionic axons in this preparation, fast B-neuron and slow B-neuron CAPs were inhibited, while C-neuron CAPs were spared (Fig. 4B, 10 μM TTX). This indicates that sympathetic C-neurons express sufficient levels of TTX-resistant VGSCs in their axons to allow action potential propagation, albeit at a slower conduction velocity (note latency difference of C-CAPs in control versus TTX-treated preparations in Fig. 4). Since SmIIIA inhibits TTX-resistant VGSCs, it was predicted that SmIIIA would also inhibit C-neuron CAPs that persist in 10 μM TTX. Indeed, as seen in Fig. 4B (10 μM TTX + 1 μM SmIIIA), application of 1 μM SmIIIA inhibited the C-neuron CAP that persisted in 10 μM TTX. This inhibition did not reverse when the preparation was perfused with bathing solution containing only TTX (not shown), consistent with the irreversible activity of SmIIIA on TTX-resistant currents.

In Fig. 4C, the Sm-P and P-Sm μ-chimeras were tested for their ability to inhibit C-neuron CAPs that persist in TTX. As in Fig. 4B, only the C-neuron CAP remained after exposing the preparation to 10 μM TTX. When 10 μM Sm-P was added, the C-neuron CAP remained largely unaltered (Fig. 4C, 10 μM TTX + 10 μM Sm-P). In contrast, when 10 μM P-Sm was added to the same preparation, the C-neuron CAP was inhibited (Fig. 4C, 10 μM TTX + 10 μM P-Sm). This indicates that the P-Sm chimera, but not the Sm-P chimera, inhibits TTX-resistant VGSCs expressed in the axons of sympathetic C-neurons, in agreement with the voltage-clamp results presented in Fig. 5.

DISCUSSION

SmIIIA blocks TTX-resistant channels whereas GIIIA, GIIIB, GIIIC, and PIIIA block various categories of TTX-sensitive channels. It is instructive to compare their solution structures to ascertain the extent to which these different subtype specificities are reflected in structural differences as opposed simply to different side chains displayed on similar scaffolds. In making this comparison we have used the consensus numbering system presented in Fig. 1. The major structural features of GIIIB (18) were described as a distorted helix from residues 18–24 (using the consensus numbering of Fig. 1) and a small β-hairpin involving residues 4–10, with a turn centered around residues 7–8. A type I β-turn is centered on Cys4-Cys8, although this coincides with the first segment of the β-hairpin and is somewhat distorted. A second turn involving residues 6–9 is located in the β-hairpin, but it does not fit any of the standard turn types, no doubt partly because of the presence of trans-Hyp7 and cis-Hyp8. A third turn involving residues 10–13 joins the β-hairpin to the helix but low backbone angular order parameters for this region imply that it accesses more than one conformation.

Hill et al. (18) also made a detailed comparison of the structure of GIIIB with previously reported structures of GIIIA.
GIIIA (15–17) was described as consisting of β-turns (Asp<sup>3</sup>–Thr<sup>6</sup> and Thr<sup>6</sup>–Lys<sup>9</sup>), a linear extension (Lys<sup>9</sup>–Asp<sup>13</sup>), a non-hydrogen-bonded loop (Asp<sup>13</sup>–Cys<sup>16</sup>), and a single right-handed helical turn (Cys<sup>16</sup>–Gln<sup>19</sup>), with a final loop directing the C terminus away from the core in the opposite direction to the N terminus. By contrast, GIIIB was described as containing a small β-hairpin involving residues 4–10 and 3<sub>10</sub>-helix from residues 14–24. In fact, the backbones of the two structures superimpose quite well (r.m.s.d. 1.36 Å) and the differences between them lie more in the descriptions of the structures than the structures themselves.

Very recently, Nielsen et al. (13) determined the solution structure of PIIIA. As this μ-conotoxin is able to block Na<sub>a</sub>1.4, many of the structural features in GIABA and GIIIB are likely to be conserved in PIIIA, although some structural differences might be expected to account for the high affinity of PIIIA for both neuronal and muscle forms of TTX-sensitive VGSCs. Although the C-terminal regions of PIIIA and GIABA overlap and the functionally important Arg<sup>14</sup> is exposed in a similar manner, marked differences are apparent in the orientation of the N-terminal region to the end of loop 1 at Cys<sup>11</sup>. The distorted β-hairpin in GIIB and possibly GIABA was replaced in the major conformation of PIIIA by a series of loops, reflecting the presence of a trans conformation at Hyp<sup>8</sup> in the major conformer of PIIIA in contrast to the cis conformation in GIABA and GIIB, as discussed above. This affects the locations of residues such as Arg<sup>2</sup> and Lys<sup>9</sup>, which are moderately important for GIABA binding to the Na<sup>+</sup> channel. Effects of cis-trans isomerization on the C-terminal region of PIIIA are minimal, however, with the orientations of functionally important residues such as Arg<sup>14</sup>, Lys<sup>17</sup>, and Arg<sup>20</sup> being similar to those of their GIIBA counterparts.

In fact, we believe that the C-terminal halves of all of these μ-conotoxins have similar structures despite the different descriptions in the literature. By contrast, the N-terminal regions do differ, reflecting the trans conformation for the peptide bond preceding residue 8 in GIABA and the major form of PIIIA, as opposed to the cis conformation in GIABA, GIIB, and the minor form of PIIIA. Taking into account these structural differences and differences in amino acid sequence, how do the surfaces of these toxins present to the Na<sup>+</sup> channel differ? Fig. 6 compares two views of the surface of GIABA with the corresponding surfaces of GIABA and GIIB. The disulfide bridges (shown in yellow) are conserved across the three structures but their surface exposure varies slightly according to the position of surface loops and side chains. Of the basic residues, 5 (Arg<sup>2</sup>, Arg/Lys<sup>9</sup>, Arg<sup>14</sup>, Arg/Lys<sup>17</sup>, and Arg/Lys<sup>21</sup>) are conserved. GIABA has an additional Arg at position 8, GIABA and GIIB have an additional Lys at positions 10 and 12, and GIIB has an Arg at position 15 (Fig. 1). The upper part of Fig. 6 shows that the orientations of Arg<sup>14</sup> and Arg/Lys<sup>21</sup> are conserved across all three structures, and the lower half shows that this is also true for Arg/Lys<sup>17</sup> (where this side chain is adjacent to Arg<sup>14</sup> and pointing toward the viewer). By contrast, Arg/Lys<sup>9</sup> is oriented quite differently in GIABA compared with GIABA and GIIB.

![Comparison of native and chimeric μ-conotoxin effects on TTX-resistant sodium currents](image-url)
GIIB, as evident in the lower view in Fig. 6, and Arg is located just above Trp in SmIIIA but near Lys in GIIB, which is in turn near Lys. In these respects, SmIIIA appears to be more similar to the major form of PIIIA than to GIIB/GIIB, although a detailed comparison of these structures is not possible because the PIIIA structure is not available. These similarities reflect the different orientation of the N terminus in SmIIIA arising from the trans conformation for the peptide bond preceding residue 8. Arg and Lys residues are Asp10 and Asp18 in GIIB and GIIBB occupy different positions in the sequence and on the surface compared with Lys14 in SmIIIA. Given their proximity to Arg and their different locations relative to this key side chain, they could be expected to influence sodium channel binding.

In addition to these differences in the locations of charged side chains, there are also differences in shape between SmIIIA and GIIB/GIIBB, particularly at the top of the structure as viewed in Fig. 6. Again, these reflect the different orientation of the N terminus in SmIIIA. Ser is buried in GIIB, consistent with it playing a structural role that ensures the exposure of Arg14 (13). In SmIIIA, Ser12 and Ser13 are located on the surface of the protein, and the side chain of Ser13 is involved in a hydrogen bond. The O'H resonances of both of these residues are exchanging slowly enough with solvent to be resolved in the spectrum, indicative of some protection from solvent. Analysis of solvent accessibility shows that most backbone amides are shielded from solvent but when the accessibility of entire residues is considered, Cys, Gly10, Cys16, and Ser stand out as being the least exposed.

Mutarial analysis of GIIB shows that Arg is a particularly important residue for binding as well as for blockade of channel function (43–45). When the toxin is bound, Arg appears to occupy the mouth of the channel and inhibits Na+ flux by acting as both a steric and electrostatic barrier (46–48). The GIIB mutant R14Q binds with poor affinity and when bound reduces channel conductance by only 70% compared with the native toxin which blocks conductance 100% (44); this has been taken to indicate that the toxin binds to the pore with Arg very close to the selectivity filter. Replacement of numerous other residues of the toxin also affects its function, indicating that many parts of the toxin surface interact with the Na+ channel (49). Charges grouped on one side of the toxin at positions 2, 13, and 15 had a weaker influence, whereas residue 17, on the opposite face, was more important. It appears that one side of the toxin is masked by binding to the pore, but Lys is exposed to an aqueous cavity accessible to entering ions (50).

Figure was prepared using InsightII (Accelrys).

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