Neuronally Selective $\mu$-Conotoxins from Conus striatus Utilize an $\alpha$-Helical Motif to Target Mammalian Sodium Channels

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$\mu$-Conotoxins are small peptide inhibitors of muscle and neuronal tetrodotoxin (TTX)-sensitive voltage-gated sodium channels (VGSCs). Here we report the isolation of $\mu$-conotoxins SIIB and SIIB by $^{125}$I-TIIIA-guided fractionation of milked Conus striatus venom. SIIB and SIIBB potently displaced $^{125}$I-TIIIA from native rat brain Na$_{1.2}$ (IC$_{50}$ values 10 and 5 nM, respectively) and muscle Na$_{1.4}$ (IC$_{50}$ values 60 and 3 nM, respectively) VGSCs, and both inhibited current through Xenopus oocyte-expressed Na$_{1.2}$ and Na$_{1.4}$. An alanine scan of SIIBB-(2–20), a pyroglutamate-truncated analogue with enhanced neuronal activity, revealed residues important for affinity and selectivity. Alanine replacement of the solvent-exposed Trp-12, Arg-14, His-16, Arg-18 resulted in large reductions in SIIBB-(2–20) affinity, with His-16 replacement affecting structure. In contrast, [D15A]SIIBB-(2–20) had significantly enhanced neuronal affinity (IC$_{50}$ 0.65 nM), while the double mutant [D15A/H16R]SIIBB-(2–20) showed greatest Na$_{1.2}$ versus 1.4 selectivity (136-fold). $^1$H NMR studies revealed that SIIBB adopted a single conformation in solution comprising a series of turns and an $\alpha$-helical motif across residues 11–16 that is not found in larger $\mu$-conotoxins. The structure of SIIBB provides a new structural template for the development of neuronally selective inhibitors of TTX-sensitive VGSCs based on the smaller $\mu$-conotoxin pharmacophore.

Voltage-gated sodium channels (VGSC) are crucial for the excitability of nerve and muscle cells (1). Nine different VGSC isoforms (Na$_{1.1}$–1.9) have been identified and characterized as either tetrodotoxin-sensitive (TTX-S) or tetrodotoxin-resistant (TTX-R) (2, 3). Of these isoforms, the TTX-R Na$_{1.8}$ and Na$_{1.9}$ are implicated in neuropathic pain states (4, 5), the TTX-S Na$_{1.7}$ is implicated in allodynia and neuropathic pain (6–10), and the TTX-S Na$_{1.3}$ is up-regulated in inflammatory pain (5). To learn more about the different roles played by each of the VGSC subtypes in normal and disease states requires new inhibitors with improved VGSC subtype selectivity.

Inhibitors of TTX-S VGSCs include tetrodotoxin (TTX) from puffer fish, the saxitoxins (STXs) from marine dinoflagellates, and the $\mu$-conotoxins from the venom of cone snails. These toxins act competitively at Site 1 in the P-loop region of the ion-conducting pore of the $\alpha$-subunit of the VGSC (3, 11). While TTX and STX act with nanomolar potency across the six TTX-S VGSCs (Na$_{1.1}$–1.4, 1.6, and 1.7), $\mu$-conotoxins are more discriminating. Currently 12 $\mu$-conotoxins have been identified from nine species of fish hunting cone snails (Table 1), $\mu$-Conotoxins GIILA, GIIB, and GIILC from Conus geographus selectively target the skeletal muscle Na$_{1.4}$, PIILA from Conus purpurascens (12–14) and TIIIA from Conus tulipa (15) target Na$_{1.2}$ and 1.4, and $\mu$-conotoxins SmIILA from Conus stercusmuscarum (16, 17), SIILA from Conus striatus (18, 19), and KIIILA from Conus knoshtai (18) target amphibian TTX-R sodium channels. More recently, KIIILA has also been shown to inhibit TTX-S sodium channels in mouse DRG and rat TTX-S sodium channels expressed in Xenopus oocytes (20). Lastly, $\mu$-conotoxins CnIIILA, CnIIIB from Conus consors, CIIILA from Conus catus, and MIIILA from Conus magus were found to inhibit TTX-R and TTX-S sodium channels in frog DRG but not rat or mouse DRG neurons (21). Most reported $\mu$-conotoxins have a conserved arginine in loop 2, which is essential for high affinity interactions at TTX-S sodium channels (15, 22–25). In contrast, SIILA and KIIILA have a much shorter loop 2 and a lysine instead of an arginine in the corresponding position. Recent structure-activity studies on TIIIA (15) and KIIILA (20) have confirmed that the arginine in loop 2 is crucial for TIIIA affinity at both neuronal and skeletal muscle sodium channels, whereas the structurally equivalent lysine was only important for affinity at the skeletal muscle VGSC. Three-dimensional structures of $\mu$-conotoxins GIILA, GIIB, PIILA, TIILA, and SmIILA have been determined by NMR (14, 15, 17, 26, 27). These structures reveal a similar fold for the $\mu$-conotoxins despite high sequence divergence. At present, structures of $\mu$-conotoxins SIILA and KIIILA are modeled from SmIILA (18, 20, 21).

This study reports the isolation and structure-activity of $\mu$-conotoxins SIILA and SIIB from the milked venom of C. striatus. To define aspects of their structure that contribute...
to binding to mammalian VGSCs, we determined the NMR structure of SIIIA. Distinct from other \( \mu \)-conotoxins, SIIIA adopts a single conformation in solution that includes an \( \alpha \)-helical motif not previously seen in other members of this class. This structural change was associated with a significant pharmacophore shift from the dominant arginine residue in loop 2 of the larger \( \mu \)-conotoxins to a series of five similarly important residues, including four associated with the \( \alpha \)-helical region, in the smaller SIIIA. Interestingly, a number of SIIIA analogues had significantly enhanced neuronal selectivity, opening the way to the development of subtype-selective neuronal VGSC inhibitors.

**EXPERIMENTAL PROCEDURES**

*Isolation of \( \mu \)-Conotoxins SIIIA and SIIIB—Crude C. striatus milked venom was separated into 80 1-min fractions by semi-preparative RP-HPLC using a Vydac C18 column (250 × 10 mm, 5 \( \mu \)) eluted at 3 ml/min with a 1% gradient from 100% A to 80% B over 80 min (solvent A 0.05% trifluoroacetic acid, solvent B 90% ACN + 0.045% trifluoroacetic acid). The eluant was monitored at 230 nm and \( ^{125} \)-TIIIA-guided fractionation (15) of the crude milked venom RP-HPLC fractions of C. striatus used to isolate SIIIA and SIIIB to homogeneity. The sequences of SIIIA and SIIIB (Table 1) were determined by Edman sequencing only after pyroglutamate aminopeptidase digestion, indicating that the N-terminal residue of both these conotoxins was a pyroglutamate. The observed masses for native SIIIA and SIIIB (2206.8 and 2120.7 Da, respectively) were consistent with the Edman sequencing results for peptides with an amidated C terminus. Co-injected synthetic and native SIIIA and SIIIB (2:1 ratio) co-eluted on a Vydac C18 reversed-phase HPLC column (250 × 4.6 mm, 5 \( \mu \)) eluted with a 1% gradient from 100% A to 50% B over 50 min, confirming the identity of the native peptides.

*Peptide Synthesis—\( \mu \)-Conotoxins SIIIA and SIIIB and analogues were prepared by Boc chemistry (28) using methods described previously (14). Side-chain protecting groups chosen were Arg(Tos), Asp(OcHex), Lys(CIZ), Ser(Bzl), Asn(Xan), His(DNP), and Cys(p-MeBzl). The crude reduced peptides were purified by preparative RP-HPLC using a 1% gradient (100% A to 80% B over 80 min) and UV monitoring (230 nm), and oxidized at 0.02 mM in either aqueous 0.33M NH₄OAc/0.5 mM NaCl (90%), were used to study toxin effects on TTX-S sodium ion current after complete washout of TTX block. Toxins were diluted in extracellular solution and added via a gravity-fed perfusion system.

*Xenopus Oocyte Experiments—Depolarization-activated sodium ion currents were recorded from small DRG neurons (<25 \( \mu \)m diameter) in the presence of 300 nM TTX. Large neurons (>40 \( \mu \)m diameter), in which the sodium ion current exhibited fast activation and inactivation kinetics and was mostly TTX-sensitive (>90%), were used to study toxin effects on TTX-S sodium ion current after complete washout of TTX block. Toxins were diluted in extracellular solution and added via a gravity-fed perfusion system.
direct bath application (static bath) followed by extensive mixing to reach the final concentrations stated. The steady-state block was reached after ~8 min of toxin exposure, and washout of SIIIA was followed for 20–30 min.

$^3$H Nuclear Magnetic Resonance (NMR) Spectroscopy—All NMR experiments were recorded on a Bruker ARX 500 spectrometer equipped with a z-gradient unit, a Bruker DMX 750, or a Bruker 600 Avance spectrometer equipped with a $x,y,z$-gradient unit. Peptide concentrations were ~2 mM. SIIIA and SIIIB were examined in 95% H$_2$O/5% D$_2$O (pH 3.0) and in 100% D$_2$O (260–293 K). SIIIA analogues were examined in 90% H$_2$O/10% D$_2$O, pH 3.0. For SIIIA, $^1$H NMR experiments were recorded on a Bruker ARX 500 spectrometer equipped with a Bruker DMX 750 spectrometer equipped with a $z$-gradient unit, a Bruker 600 Avance spectrometer equipped with a $x,y,z$-gradient unit. Peptide concentrations were ~2 mM. SIIIA and SIIIB were examined in 95% H$_2$O/5% D$_2$O (pH 3.0) and in 100% D$_2$O (260–293 K). SIIIA analogues were examined in 90% H$_2$O/10% D$_2$O, pH 3.0. For SIIIA, $^1$H NMR experiments recorded included NOE, (mixing times 120, 300, and 400 ms), TOCSY (mixing time 80 ms), double-quantum filtered (DQF)-COSY, and E-COSY in 100% D$_2$O. Slowly exchanging amide protons were detected by acquiring one-dimensional NMR spectra immediately after dissolving the peptide in 100% D$_2$O at pH 3.5. For all other analogues, TOCSY experiments were only run if ambiguities arose during assignment. The solvent block was reached after 120 s. Chemical shift values of SIIIA and analogues were obtained in 90% H$_2$O, 10% D$_2$O and referenced internally to DSS at 0.00 ppm. Secondary Ha shifts were measured and compared with values for random coil shifts (33).

SIIIA was assigned in sparky (34), and distance information obtained by integration of a 300 ms mixing time NOESY spectrum. Backbone dihedral restraints were derived from $^3$J$_{HN-Ha}$ coupling constants from a DQF-COSY spectrum or a one-dimensional $^1$H NMR spectrum. The $\phi$ dihedral angle restraints included were restrained to 120$\pm$30$^\circ$ for $^3$J$_{HN-Ha}$ > 8 Hz and $-60 \pm 30^\circ$ for $^3$J$_{HN-Ha}$ < 5.8 Hz. $\chi_1$ dihedral angles were determined from intraresidue nuclear Overhauser effect (NOE) and $^3$J$_{Ha-Hb}$ coupling patterns for an E-COSY spectrum. Initial structures were generated using dyana (35), and final structures were calculated in explicit water with CNS (36) as previously described (37). An initial 50 structures were calculated, and the 17 lowest overall energy structures selected to represent the solution structure of SIIIA.

RESULTS

Isolation of $\mu$-Conotoxins SIIIA and SIIIB—To identify $\mu$-conotoxins selective for neuronal VGSCs, fractionated milked C. striatus venom was assayed for the ability to displace $^{125}$I-TIIIA from rat brain sodium channels (15). Three active fractions with retention times of 20, 21, and 24 min (Fig. 1) were purified to homogeneity, digested using pyroglutaminase, and Edman-sequenced to reveal three peptides with a CC-C-C-CC motif characteristic of $\mu$-conotoxins (38). Fraction 24 contained the sequence of TIIIA, previously identified in C. tulipa by PCR (15), fraction 20 the sequence for SIIIA, while fraction 21 contained a sequence for a new $\mu$-conotoxin we named SIIIB (Table 1). SIIIA and SIIIB each comprised 20 residues with a net charge of +2 and +3, respectively. Mass spectrometry and co-elution studies of the native and synthetic peptides confirmed the sequences and C-terminal amidation of both peptides (data not shown).

Radioligand Binding Studies—Synthetic $\mu$-conotoxin SIIIA and SIIIB each potently displaced $^{125}$I-TIIIA from VGSCs in rat brain and rat skeletal muscle (Fig. 2). The pIC$_{50}$ values for SIIIA and SIIIB and selected additional $\mu$-conotoxins are summarized in Table 2. Similar to TTX and STX, SIIIA had a small but

![Image](https://example.com/image.png)

**FIGURE 1. Identification of native SIIIA and SIIIB in milked crude C. striatus venom.** Active fractions eluting from RP-HPLC using a 1% gradient (0–80% B solvent B: 90% ACN, 0.045% trifluoroacetic acid) displaced $^{125}$I-TIIIA (21623) binding to rat brain. Dashed line indicates non-specific binding, and inset shows Conus striarius venom being collected by milking.

**TABLE 1**

$\mu$-Conotoxin sequences and VGSC selectivity

| Peptide\* | Sequence | Selectivity | Ref. |
|-----------|----------|-------------|------|
| SIIIB     | 2N-CCECN1--OCSSRCCGKHHARCCb | 1.4 ≈ 1.2 | This work |
| SIIIA     | 2N-CCECN1--OCSSRCCGKHHARCCb | 1.2 ≈ 1.4 | This work (18, 19) |
| KIIIA     | --CCECN1--OCSSRCCGKHHARCCb | 1.2 ≈ 1.4 | Amphibian (16) |
| SmIIIA    | 2-CCECN1--OCSSRCCGKHHARCCb | 1.2 ≈ 1.4 | Amiphibian (21) |
| MIIIA     | 2-CCECN1--OCSSRCCGKHHARCCb | 1.2 ≈ 1.4 | Amiphibian (21) |
| CIIIA     | --CCECN1--OCSSRCCGKHHARCCb | 1.2 ≈ 1.4 | Amphibian (21) |
| CalIIIA   | --CCECN1--OCSSRCCGKHHARCCb | 1.2 ≈ 1.4 | Amphibian (21) |
| TIIIA     | --CCECN1--OCSSRCCGKHHARCCb | 1.2 ≈ 1.4 | Amphibian (21) |
| PIIIA     | 2RCCFROCRSROCRK-CHHHARCCb | 1.4 ≈ 1.2 | (15) |
| GIIIA     | RD--CTOOCRKCDROCKRROCCb | 1.4 ≈ 1.2 | (24) |
| GIIIB     | RD--CTOOCRKCDROCKRROCCb | 1.4 ≈ 1.2 | (38) |
| GIIIC     | RD--CTOOCRKCDROCKRROCCb | 1.4 ≈ 1.2 | (38) |

\* SIIIA, SIIIB, and KIIIA are smaller $\mu$-conotoxins, whereas the others are defined as larger $\mu$-conotoxins.

\* C-terminal amidation.

\* Free acid C-terminal. Cysteines 1–4, 2–5, 3–6 are paired.
significant preference for neuronal Na\textsubscript{1.2} over the skeletal muscle Na\textsubscript{1.4}, whereas SIIIB had a preference for Na\textsubscript{1.4}. Both SIIIA and SIIIB were full inhibitors of \textsuperscript{125}I-TIIIA binding to rat brain sodium channels. All displacement curves were best fitted with a Hill slope of 1.

**TABLE 2**

| \(\mu\)-Conotoxin | Brain (Na\textsubscript{1.2}) | Muscle (Na\textsubscript{1.4}) | Selectivity\(^a\) |
|--------------------|----------------|----------------|----------------|
| SIIIA              | 8.02 ± 0.12    | 7.67 ± 0.13    | 0.35           |
| SIIIB              | 8.29 ± 0.15    | 8.49 ± 0.11    | -0.20          |
| TIIIA              | 9.27 ± 0.06    | 9.72 ± 0.08    | -0.45          |
| PIIIA              | 8.72 ± 0.07    | 9.05 ± 0.05    | -0.33          |
| GIIB               | 6.47 ± 0.27    | 10.0 ± 0.05    | -3.53          |
| GIIC               | 7.85 ± 0.15    | 10.0 ± 0.05    | -2.15          |
| STX                | 9.35 ± 0.05    | 9.80 ± 0.06    | 0.45           |
| TTX                | 8.16 ± 0.21    | 7.32 ± 0.18    | 0.84           |

\(^a\) Neuronal selectivity calculated as brain pIC\(_{50}\) - muscle pIC\(_{50}\).

**FIGURE 2**. Displacement of \textsuperscript{125}I-TIIIA from (A) rat brain Na\textsubscript{1.2} and (B) rat skeletal muscle Na\textsubscript{1.4} VGSCs by SIIIA (●), SIIIB (■), and selected SIIIA analogues, SIIIA-(2–20) (▲), SIIIB-(2–20) (▼), and [R11,A15]SIIIA-(2–20) (◆), as well as TIIIA for comparison (◇).

**FIGURE 3**. Inhibition of recombinant VGSCs expressed in Xenopus oocytes by \(\mu\)-conotoxin SIIIA. A, superimposed depolarization-activated sodium ion currents mediated by Na\textsubscript{1.2}, Na\textsubscript{1.3}, and Na\textsubscript{1.4} in the absence and presence of 1 \(\mu\)M SIIIA. Oocytes were held at -80 mV and depolarized to 0 mV every 5 s. B, concentration-response curves obtained for SIIIA inhibition of Na\textsubscript{1.2}, Na\textsubscript{1.3}, and Na\textsubscript{1.4} (n = 3–5). C, kinetics of SIIIA inhibition of Na\textsubscript{1.2}. The on-rate curve was fitted with a single exponential function, yielding a \(t_{1/2}\) of 4.0 min for 3 \(\mu\)M SIIIA. Inhibition was not reversed upon washout for up to 30 min (n = 3 ± S.E.).

**Effects of SIIIA on Whole Cell Sodium Ion Currents**—The effects of \(\mu\)-conotoxin SIIIA was investigated on both TTX-S and TTX-R sodium ion current in neonatal rat DRG neurons (31). SIIIA at 3 \(\mu\)M produced no detectable inhibition of either TTX-S or TTX-R sodium ion currents in rat DRG neurons (data not shown).

**Effects of \(\mu\)-Conotoxins on Oocyte-expressed VGSC Subtypes**—SIIIA was examined for inhibition of sodium ion current through VGSC subtypes expressed in Xenopus oocytes. SIIIA at 3 \(\mu\)M caused 95% inhibition of Na\textsubscript{1.2} current, 45% inhibition of Na\textsubscript{1.3} current (Fig. 3A), but no detectable inhibition of sodium ion current through Na\textsubscript{1.5}, Na\textsubscript{1.7}, or Na\textsubscript{1.8} (data not shown). The IC\(_{50}\) values for SIIIA was 790 nM (95% CI 630–990 nM) at Na\textsubscript{1.2}, 330 nM (95% CI 300–360 nM) at Na\textsubscript{1.4}, and 5.3 \(\mu\)M (95% CI 3.6–7.6 \(\mu\)M) at Na\textsubscript{1.3} (Fig. 3B). SIIIB at 3 \(\mu\)M caused 93% inhibition of Na\textsubscript{1.2} current and 10% inhibition of Na\textsubscript{1.7} current (data not shown) but was not tested on other VGSC subtypes. The inhibitory effect of SIIIA was not reversed upon washout (Fig. 3C), in contrast to TIIIA which was fully reversible upon washout at Na\textsubscript{1.2} (15).

**Affinity of \(\mu\)-Conotoxin Analogues**—The affinity of \(\mu\)-conotoxin analogues of SIIIA and SIIIB are shown in Fig. 4. SIIIA-(2–20) had decreased affinity at Na\textsubscript{1.4}, but increased binding affinity at Na\textsubscript{1.2} sodium channel, whereas SIIIB-(2–20) had increased affinity at both subtypes. In an attempt to identify residues contributing to the neuronal selectivity, we produced an alanine scan of SIIIA-(2–20), except for the small Asn, Gly, and Ser residues in loops 1 and 2. Surprisingly, [K11A]SIIIA-
SIIIA-(2–20) had only a small 6-fold reduction in affinity at Na\textsubscript{a,1.2} and Na\textsubscript{a,1.4}, compared with the ~100–300-fold reduction in affinity for the equivalent substitution in TIIIA ([R14A]TIIIA at rat Na\textsubscript{a,1.2}) (15) and GIIIA ([R13A]GIIIA at rat Na\textsubscript{a,1.4}) (39). In contrast, the adjacent mutant [W12A]SIIIA-(2–20) had 22- and 87-fold reduced affinity compared with SIIIA-(2–20) at Na\textsubscript{a,1.2} and Na\textsubscript{a,1.4}, respectively, similar to that reported for KIIIA (20). Removing the negative charge at position 15 in [D15A]SIIIA-(2–20) produced a 10-fold increased affinity at Na\textsubscript{a,1.2} without affecting Na\textsubscript{a,1.4} affinity, resulting in 70-fold neuronal selectivity. This enhanced affinity was lost if Asp-15 was replaced with a positive charge (e.g. lysine) or an aromatic residue (e.g. tyrosine). The double mutant [K11R/D15A]SIIIA-(2–20) had a similar potency and slightly enhanced Na\textsubscript{a,1.2} selectivity compared with [D15A]SIIIA-(2–20), indicating that the lysine in loop 2 of SIIIA can be interchanged with arginine without loss of affinity.

Of the mutants tested, [H16A]SIIIA-(2–20) showed the largest drop in affinity at both Na\textsubscript{a,1.2} and Na\textsubscript{a,1.4}, confirming the results reported for KIIIA (20). Interestingly, the double mutant [D15A/H16R]SIIIA-(2–20) where the negative charge from Asp was removed and an adjacent positive charge was introduced, had similar affinity at Na\textsubscript{a,1.2} but a 30-fold loss of affinity at Na\textsubscript{a,1.4} compared with SIIIA-(2–20), making it the most neuronally selective \(\mu\)-conotoxin identified in this study (137-fold selective). In contrast, the corresponding Asp-16 analogue [D15A/H16D]SIIIA-(2–20) had dramatically (100,000-fold) reduced affinity at Na\textsubscript{a,1.2} and 400-fold reduced affinity at Na\textsubscript{a,1.4}, compared with native SIIIA-(2–20), producing a Na\textsubscript{a,1.4}-selective peptide. The C-terminal arginine analogue [R18A]SIIIA-(2–20) had ~30-fold reduced affinity for Na\textsubscript{a,1.2} compared with SIIIA-(2–20) and ~500-fold reduced affinity for Na\textsubscript{a,1.4} to yield a second neuronally (116-fold) selective peptide. As anticipated, [A11,A14,A15,D16,A18]SIIIA-(2–20), [A11,A14,A15,Y16,A18]SIIIA-(2–20), and [A11,A14,A15,A18]SIIIA-(2–20) had significantly reduced affinity compared with SIIIA-(2–20), particularly at Na\textsubscript{a,1.2}.

To identify the structural determinants underlying these shifts in affinity and selectivity, the three-dimensional solution structure of SIIIA was calculated using \(^1\)H NMR spectroscopy. In addition, \(\mathrm{H}_\alpha\) chemical shifts were determined to establish that each analogue adopted the native fold found in SIIIA.

\(^1\)H NMR Spectroscopy—Apart from TIIIA, spectral assignment and structure calculations have proved difficult for \(\mu\)-conotoxins due to Pro/Hyp cis/trans isomerization that produces line-broadening and multiple conformations (14, 26, 27). Consistent with the absence of Pro/Hyp residues in SIIIA, NMR data indicated that a single conformation for SIIIA and analogues dominated in solution. Only [H16A]SIIIA-(2–20) showed a significant change in backbone \(\mathrm{H}_\alpha\)-chemical shift, indicating that His-16 provides structural stabilization, especially to the C-terminal-half of the peptide (Fig. 5). Interestingly, [A15,R16]SIIIA-(2–20) and [A11,A14,A15,Y16,A18]SIIIA-(2–20) did not show a change in secondary \(\mathrm{H}_\alpha\)-chemical shifts, apart from minor local shifts at residue 15 or 11, respectively, suggesting that arginine and tyrosine (but not Ala) can replace His-16 as a structural stabilizer. The three-dimensional SIIIA structure is described below.

SIIIA was examined by NMR spectroscopy under aqueous conditions over a range of different temperatures to confirm proton assignments (data not shown). NMR solution structures were then calculated based on a total of 288 distance restraints derived from 102 intra-residue, 93 sequential, 89 medium- and long-range NOEs, 4 hydrogen bond restraints defining 2 hydrogen bonds, and 12 \(\phi\) and one \(\chi_1\) dihedral angle restraints. A total of 46 of 50 structures converged to a consensus structure. These structures con-
Structure-Activity of μ-Conotoxin SIIIA

The 17 lowest energy structures of SIIIA are superimposed globally in Fig. 6A, and a representative ribbon structure highlighting secondary structural motifs is shown in Fig. 6B. Overall, the structures are well-defined with a backbone root mean-square deviation (RMSD) of 0.21 across residues 3–20 and 0.35 across residue 1–20. The angular order parameters for φ and ψ angles are also well ordered and >0.92 across residues 6–20. The slightly disordered N-terminal residues do not appear to arise from conformational exchange or structural flexibility, because no line-broadening was observed for these residues, and are more likely due to a lack of NOEs to loop 1, which contains two Gly residues. Unlike the longer loop 1 in other μ-conotoxins, which protrudes outward, loop 1 of SIIIA comprises a tightly folded β-turn-like motif that is positioned to interact with loop 3 (see Fig. 7). Analysis of the surface exposure of each residue illustrates this structural shift, with loop 1 and the first two residues in loop 2 of SIIIA being relatively buried compared with the equivalent region of TIIIA (15), whereas Lys-11, Trp-12, Arg-14 within the α-helical region, together with Arg-18, are relatively exposed to solvent (supplemental Fig. S1B). As expected, all Cys residues are buried in the core of the molecule. Comparing the NMR structures of SIIIA and TIIIA reveals that the key binding determinants of SIIIA are distributed across the α-helical motif (Lys-11, Trp-12, Arg-14), and Arg-18 with each contributing similarly to affinity, whereas a single arginine (Arg-14) is the key determinant of TIIIA affinity (Fig. 7).

DISCUSSION

The venoms of cone snails provide a rich source of μ-conotoxin inhibitors of VGSCs, including several recently isolated μ-conotoxins with a smaller loop 2 (18, 19). In this study we used assay-directed fractionation of milked C. striatus venom to identify a new μ-conotoxin SIIIB, together with the recently reported SIIIA (18) and TIIIA (15). Interestingly, the smaller μ-conotoxins from C. striatus exploit a new pharmacophore...
that allows the development of peptides with selectivity for Na$_{1.2}$ and potentially other TTX-S subtypes.

SIIIA and SIIIB both displaced $^{125}$I-TIIIA and $[^3]$HSTX binding to rat brain (Na$_{1.2}$) and rat skeletal muscle membrane (Na$_{1.4}$), indicating they act at site 1 of the VGSC like the larger $\mu$-conotoxins PIIIA (24), TIIIA (15), GIIIA, and GIIB (38). SIIIA also inhibited current through oocyte-expressed Na$_{1.2}$ and Na$_{1.4}$ at nanomolar potencies, confirming it acts as a pore blocker of mammalian VGSCs. Like KIIIA (20), inhibition of Na$_{1.2}$ was not reversed by toxin washout. SIIIA also inhibited current through Na$_{1.3}$ at higher concentrations, showing that these smaller $\mu$-conotoxins have the potential to target additional neuronal subtypes. $\mu$-Conotoxins SIIIA, KIIIA, SmIIIA, CnIIIA, and CIIIA all potently inhibit frog TTX-R sodium ion current (18, 21). However, we were unable to detect any inhibition of TTX-R or TTX-S sodium ion current in rat DRG neurons by SIIIA, in contrast to an earlier study showing inhibition of the TTX-R current in adult rat DRG by SIIIA (19). The inability of SIIIA to inhibit TTX-S sodium ion current in neonatal rat DRG indicates that Na$_{1.2}$ and 1.3 are not expressed at significant levels in these neurons.

A number of analogues of SIIIA and SIIIB, and the neuronally selective truncated SIIIA-(2–20), were synthesized to identify residues contributing to VGSC affinity and selectivity. A loop 2 lysine is present in SIIIA and SIIIB instead of the corresponding arginine previously found to be critical for high affinity interactions of the larger $\mu$-conotoxins GIIIA (39–41), PI11A (24), SmI11A (17), and TIIIA (15). However, the [K11A]SIIIA-(2–20) analogue revealed that this loop 2 lysine contributes only modestly to VGSC affinity, producing a 6-fold reduction in affinity for Na$_{1.2}$ or Na$_{1.4}$ compared with SIIIA-(2–20). Additional SIIIA analogues [W12A]SIIIA-(2–20), [K14A]SIIIA-(2–20), and [R18A]SIIIA-(2–20) revealed that Trp-12, Lys-14, and Arg-16 might play a similar structural role to His-16.

Comparing the NMR solution structure of SIIIA with TIIIA it is apparent they adopt distinct conformations in solution (Fig. 7). Superimposition of loop 2 residues 8–13 of SIIIA with the corresponding residues 11–16 of TIIIA and PI11A and gave high RMSDs of 1.08 1.53, respectively. This affect is even more dramatic when SIIIA and SmI11A are compared across loops 2 and 3 (RMSD of 2.58), despite having almost identical sequences. In contrast, comparing the same residues between PI11A and TIIIA gave a lower RMSD of 0.64. Examining the secondary structures of the different $\mu$-conotoxins provides an explanation. GIIIA, GIIB, PI11A, SmI11A, and TIIIA each comprise a series of turns and loops (14, 15, 17, 25, 27, 42), with a $\beta$-hairpin seen in the N-terminal part of GIIIA and GIIB and the minor conformation of PI11A (14). These toxins in GIIB, SmI11A, PI11A, and TIIIA produce a $\alpha^3$-helix across residues 13–22, 13–20, 13–17, and 12–15, respectively (numbering specific to each conotoxin) (14, 15, 17, 25, 27, 42) compared with the $\alpha$-helix found in SIIIA. Previously described structures of SIIIA and KIIIA (18, 20, 21), as well as CnI11A, CnI11B, CIIIA, and MIIIA (21), were modeled from the NMR structure of SmI11A (17). However, the NMR solution structures of SIIIA reveal that loop 1 of SIIIA is not extended as seen in TIIIA (Fig. 7) and other larger $\mu$-conotoxins (supplemental Fig. S2) but instead folds in toward the $\alpha$-helical region. This altered conformation, supported by a series of long-range NOEs between loops 1 and 3, within loops 2 and 3, and between loops 2 and 3, permits a more extended C-terminal region not seen in the larger $\mu$-conotoxins. Interestingly, these structural shifts allow Lys-11, Arg-14, and Arg-18 of SIIIA and Arg-14, Arg-17, and Lys-6 of TIIIA to adopt similar relative positions (Fig. 7) that might allow binding to a negatively charged crevice in or near the mouth of the VGSC. A comparison of the sequences of KIIIA, SIIIA, and SIIIB reveals they are similar across loops 2 and 3, and include all important binding determinants, suggesting they likely have similar binding modes and similar conformations in solution.

The present study of SIIIA and SIIIB extends our knowledge of the structure-activity relationship of smaller $\mu$-conotoxins and defines key residues contributing to Na$_{1.2}$ selectivity. SIIIA contains an $\alpha$-helix across residues 11–16 unlike previously determined $\mu$-conotoxin NMR structures. The reduction in size of loop 1 from five residues in the larger $\mu$-conotoxins to three in smaller $\mu$-conotoxins like SIIIA appears to underlie a shift from a $\alpha^3$-helical motif to the $\alpha$-helix. SIIIA targets Na$_{1.2}$, 1.3, and 1.4, whereas the even smaller KIIIA additionally targets Na$_{1.1}$, 1.6, and 1.7 (20), indicating that the size of loop 1 can influence not only the structure but also the selectivity of $\mu$-conotoxins. The range of VGSC subtypes inhibited by these smaller $\mu$-conotoxins opens the door to the development of peptides with novel subtype specificity and analgesic potential.

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Structure-Activity of \(\mu\)-Conotoxin SIIA

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