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Structure, dynamics and selectivity of the sodium channel blocker μ -conotoxin SIIIA^{†,‡}

Shenggen Yao[§], Minmin Zhang^{||}, Doju Yoshikami^{||}, Layla Azam^{||}, Baldomero M. Olivera^{||}, Grzegorz Bulaj[⊥], and Raymond S. Norton^{§,¶}

[§]The Walter & Eliza Hall Institute of Medical Research

^{||} Department of Biology, University of Utah

[⊥]Department of Medicinal Chemistry, University of Utah

Abstract

μ -SIIIA, a novel μ -conotoxin from *Conus striatus*, appeared to be a selective blocker of tetrodotoxin-sensitive sodium channels in frog preparations. It also exhibited potent analgesic activity in mice, although its selectivity profile against mammalian sodium channels remained unknown. We have determined the structure of μ -SIIIA in aqueous solution and characterized its backbone dynamics by NMR and its functional properties electrophysiologically. Consistent with the absence of hydroxyprolines, μ -SIIIA adopts a single conformation with all peptide bonds in the trans conformation. The C-terminal region contains a well-defined helix encompassing residues 11–16, while residues 3–5 in the N-terminal region form a helix-like turn resembling 3_{10} helix. The Trp12 and His16 side chains are in close proximity, as in the related conotoxin μ -SmIIIA, but Asn2 is further away. Dynamics measurements show that the N-terminus and Ser9 have larger magnitude motions on the sub-ns timescale, while the C-terminus is more rigid. Cys4, Trp12 and Cys13 undergo significant conformational exchange on μ s - ms timescales. μ -SIIIA is a potent, nearly irreversible blocker of $\text{Na}_V1.2$, but also blocks $\text{Na}_V1.4$ and $\text{Na}_V1.6$ with submicromolar potency. The selectivity profile of μ -SIIIA, including poor activity against the cardiac sodium channel, $\text{Na}_V1.5$, is similar to that of the closely related μ -KIIIA, suggesting that the C-terminal regions of both are critical for blocking neuronal $\text{Na}_V1.2$. The structural and functional characterization described in this paper of an analgesic μ -conotoxin that targets neuronal subtypes of mammalian sodium channels provides a basis for the design of novel analogues with an improved selectivity profile.

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[‡]Chemical shift assignments and the family of structures for α -SIIIA have been deposited in the BioMagResBank with accession number 20023.

[¶]Please address editorial correspondence to: Raymond S. Norton, The Walter & Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria, 3050, Australia. Phone: +61 3 9345 2306. Fax: +61 3 9345 2686. ray.norton@wehi.edu.au.

SUPPORTING INFORMATION AVAILABLE

NMR data and structure comparisons referred to in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Toxins from the genus *Conus* are valuable tools in elucidating the physiological functions of their targets and in probing the size and shape of their cognate binding sites {Jones, 2000 #3; Norton, 2006 #2}. Conotoxins that target voltage-gated sodium channels (VGSCs)¹, which are responsible for the influx of sodium ions during action potentials in excitable tissues, represent a particularly good example. Four families of conotoxins target these channels, causing activation (ι -conotoxins), inhibition (μ - and μ O-conotoxins) or inhibition of fast-inactivation (δ -conotoxins). Among the most extensively studied of these in terms of their interactions with the channel are the μ -conotoxins. These toxins bind to Site 1 on VGSCs {Cestele, 2000 #4}, which is located on the extracellular surface of the pore-forming α -subunit and also binds the guanidinium alkaloids tetrodotoxin (TTX) and saxitoxin. Of the nine α -subunits cloned from mammals {Catterall, 2000 #71; Goldin, 2001 #5}, six bind TTX with high affinity (IC_{50} in the nM range), and three, $Na_v1.5$, $Na_v1.8$, and $Na_v1.9$, are classified as TTX-resistant ($IC_{50} > 1 \mu M$).

The μ -conotoxins are a validated source of selective sodium channel blockers. The first μ -conotoxin characterized, μ -GIIIA, targets mainly the skeletal muscle subtype $Na_v1.4$ {Cruz, 1985 #6; Moczydlowski, 1986 #7; Safo, 2000 #8; Li, 2003 #9}. μ -PIIIA also has a strong preference for $Na_v1.4$, but can block other TTX-sensitive subtypes as well, albeit with lower affinities {Safo, 2000 #8; Shon, 1998 #12; Nielsen, 2002 #13}. Recently, a new group of μ -conotoxins was discovered that targets neuronal sodium channels with greater selectivity and potency than μ -GIIIA or μ -PIIIA {West, 2002 #14; Keizer, 2003 #66; Bulaj, 2005 #87; Zhang, 2007 #88; Lewis, 2007 #100}. This group includes μ -SmIIIA, which irreversibly inhibited TTX-resistant sodium currents in amphibian sympathetic and dorsal root ganglion (DRG) neurons {West, 2002 #14}, as well as μ -KIIIA and μ -SIIIA {Bulaj, 2005 #87}. The selectivity of these new μ -conotoxins towards mammalian sodium channel subtypes was defined only recently, when it was shown that μ -KIIIA was a potent blocker of the neuronal subtype $Na_v1.2$, and that several amino acid residues, including Lys7 and His12, were critical for activity {Zhang, 2007 #88}. Thus, the new group of μ -conotoxins consisting of μ -KIIIA, μ -SIIIA and μ -SmIIIA is a promising source of subtype selective blockers of neuronal sodium channels.

Relatively little is known about biological activity or structural properties of μ -SIIIA. μ -SIIIA was shown to potently block TTX-insensitive sodium currents in amphibian DRG and sympathetic neurons, but it was a relatively poor inhibitor of TTX-sensitive currents recorded from frog skeletal muscle preparations {West, 2002 #14}. Micromolar concentrations of μ -SIIIA (5–25 μM) partially blocked TTX-sensitive currents in mice DRG neurons, whereas 1 μM of the peptide blocked almost completely A-compound action potentials in mouse sciatic nerve preparations {Green, 2007 #86}. μ -SIIIA appeared to significantly reduce the acute and the inflammatory pain responses in the formalin assay in mice at doses < 1 mg/kg following intraperitoneal administration {Green, 2007 #86}. These

¹Abbreviations: μ -GIIIA, μ -GIIIB, μ -conotoxins GIIIA and GIIIB, respectively, from *Conus geographus*; μ -KIIIA, μ -conotoxin KIIIA from *C. kinoshitai*; μ -PIIIA, μ -conotoxin PIIIA from *C. purpurascens*; μ -SIIIA, μ -conotoxin SIIIA from *C. striatus*; μ -SmIIIA, μ -conotoxin SmIIIA from *C. stercusmuscarum*; MD, molecular dynamics; RMS, root mean square; TTX, tetrodotoxin; VGSC, voltage-gated sodium channel.

results strongly suggested that μ -SIIIA has therapeutic potential as a potent blocker of mammalian sodium channels, but the subtype selectivity or structural properties of this μ -conotoxin remained unknown.

Previous NMR studies of μ -conotoxins have found evidence of conformational flexibility, manifested in line broadening of one or more resonances {Lancelin, 1991 #15; Wakamatsu, 1992 #17; Hill, 1996 #18}, although this was not the case for μ -SmIIIA {Keizer, 2003 #66}. However, when μ -SmIIIA was subjected to molecular dynamics simulations, the resulting structure showed several differences from the one calculated on the basis of NMR restraints {Bulaj, 2005 #87}. In particular, the side chain of Arg2 moved away from that of Trp14, in conflict with the observed NOEs between these side chains {Keizer, 2003 #66}, and the first loop (between Asn5 and Gly9) sampled a larger region of conformational space than in the NMR structure. Apart from these differences, the rest of the backbone was largely constant throughout the simulation, and consistent with the NMR structure. Nonetheless, these observations raise questions about the flexibility of μ -conotoxin structures, which are germane to their use as a basis for modelling new structures in this superfamily and for the design of non-peptidic mimetics {Green, 2007 #86}.

NMR relaxation measurements provide a means of characterising protein solution dynamics over a broad range of timescales. Experimental procedures for measuring ^{15}N relaxation parameters (R_1 , R_2 and steady-state ^{15}N - $\{^1\text{H}\}$ NOE) as well as protocols for data analysis using *Modelfree* formalism are well established for the study of protein backbone dynamics at ps to ns timescales {Palmer, 1997 #56; Fischer, 1998 #61}. There is also compelling evidence that protein dynamics in solution, particularly on the ms timescale, correlate closely with protein function {Feher, 1999 #58; Volkman, 2001 #59; Eisenmesser, 2002 #89}. This is significant because one of the mechanisms underlying protein function and protein-protein interaction is conformational exchange, which typically occurs at the sub-ms to ms timescales {Frauenfelder, 1988 #60}.

Most of these studies have been conducted using isotopically enriched proteins produced in bacteria grown on minimal media. Toxins with multiple disulfide linkages such as the μ -conotoxins can be expressed and isotopically labelled in heterologous expression systems, eg. {Goldenberg, 2001 #83}, but most toxins that are the subject of structural studies are produced by solid-phase synthesis without labelling. With the widespread availability of NMR cryoprobes, it has become straightforward to obtain ^{13}C and ^{15}N chemical shifts for unlabelled samples {Buczek, 2007 #72; Ellison, 2008 #73}, and these parameters are useful in defining backbone and potentially also side chain dihedral angles {Cornilescu, 1999 #65}. Given that ^{13}C has a higher natural abundance and sensitivity than ^{15}N (1.11 and 0.37 %, and sensitivities for the same number of nuclei relative to ^1H of 1.59×10^{-2} and 1.04×10^{-3} , respectively), it has also become feasible to monitor ^{13}C relaxation on unlabelled samples. In the past this has required large sample tubes and/or concentrated samples (e.g. {Oldfield, 1975 #82; Norton, 1977 #77}), but such studies can now be undertaken in a reasonable time with *ca* 1 mM samples. In this paper we describe the results of a ^{13}C NMR relaxation study of the conotoxin μ -SIIIA, which was undertaken to determine whether such measurements could detect internal motions in this class of toxin on timescales relevant to conformational averaging and biological activity. In order to interpret the relaxation data we have also

determined a high-resolution structure for μ -SIIIA and compared it with the structures of other μ -conotoxins.

EXPERIMENTAL

Peptide Synthesis & Sample Preparation

μ -SIIIA was synthesized, folded and purified using the protocols described previously {Bulaj, 2005 #87}. The peptide was purified using C18 reversed-phase HPLC and its identity confirmed by mass spectrometry. Samples used for structure determination and ^{13}C relaxation measurements were prepared by dissolving μ -SIIIA in 350 μl of 95% H_2O /5% $^2\text{H}_2\text{O}$ or 100% $^2\text{H}_2\text{O}$, respectively, to a final concentration of approximately 0.9 mM. The pH was adjusted to 4.2 without allowances for isotope effects.

NMR Spectroscopy

For structure determination, 2D TOCSY (spin-lock time 60 ms), NOESY (mixing time 250 ms) and DQF-COSY spectra were recorded on a Bruker DRX600 spectrometer at 25 °C. Complex data matrixes of 2048 $\times$ 225 for TOCSY, 2048 $\times$ 300 for NOESY and 2048 $\times$ 300 for DQF-COSY and 40,112 and 40 scans per t_1 increment in the indirect detected dimension, respectively, were used. Assignments of aliphatic carbon resonances were established from ^{13}C HSQC, ^{13}C HMQC and ^{13}C HMQC-TOCSY {Palmer, 1991 #90} spectra whereas backbone ^{15}N resonances of μ -SIIIA were assigned from a ^{15}N HSQC spectrum based on the corresponding amide proton chemical shifts, which were well resolved in the F2 (^1H) dimension. All of these heteronuclear correlation spectra were recorded at natural abundance on a Bruker Avance500 using a TXI cryoprobe. ^{13}C R_1 , R_2 and ^{13}C - $\{^1\text{H}\}$ NOE were also measured at natural abundance on the Bruker Avance500 at 283, 298 and 310 K using standard pulse sequences in the Bruker pulse sequence library. Relaxation-weighted ^{13}C -HSQC spectra were acquired in an interleaved manner, i.e. a 3D data set was acquired with the pulse sequence stepping through the relaxation durations prior to signal averaging and the time increments of the indirectly detected dimension of the spectra. Twelve relaxation delays, including two duplicated delays, ranging from 10 to 800 ms for R_1 and 22.4 to 268.8 ms for R_2 , respectively, were measured. A complex data matrix of 2048 $\times$ 32 together with 48 scans per t_1 increment for R_1 and R_2 and 192 for the NOE were used. The recycle times were 2.8 s for R_1 and R_2 and 4.0 s for the steady-state ^{15}N - $\{^1\text{H}\}$ NOE. All NMR spectra were processed using TOPSPIN (Version 1.3, Bruker BIOSPIN) and analyzed using XEASY (Version 1.3; {Bartels, 1995 #55}).

Structure Calculations

Volumes of cross peaks in a 250 ms mixing time NOESY spectrum recorded at 25 °C were converted to distance restraints using the script CALIBA supplied with CYANA {Herrmann, 2002 #23}. The resultant values were then scaled by a factor of 1.2 to allow for potential effects of spin diffusion. Restraints were added for all three disulfide bonds (Cys3-Cys13, Cys4-Cys19 and Cys8-Cys20). Backbone ϕ angle restraints were derived from $^3J_{\text{HNNA}}$ coupling constraints measured from the DQF-COSY spectrum ($^3J_{\text{HNH}\alpha} > 8$ Hz, $\phi = -120 \pm 30^\circ$ $^3J_{\text{HNH}\alpha} < 6$ Hz, $\phi = -60 \pm 30^\circ$) and combined with those derived from the program TALOS {Cornilescu, 1999 #65} based on chemical shifts of (N, H α , C α and C β) of

μ -SIIIA. All other residues were restricted to a negative ϕ value except for Gly6 and Gly7, which were not restrained. Initial structures were generated using the torsion angle dynamics program CYANA {Herrmann, 2002 #23} for refinement of structural restraints. The final distant angular restraints were then used to calculate a family of 400 structures in XPLOR-NIH {Schwieters, 2003 #24} using standard distance geometry and simulation annealing scripts. Residue pyroGlu was added into the XPLOR library as previously {Keizer, 2003 #66}. A group of 50 structures out of those 400 calculated structures with lowest NOE energies were then further refined using simulated annealing. The resultant structures were further refined in a water box built around the peptide and then energy minimized on the basis of experimental distance and angular restraints as well as the geometry of bonds, angles and impropers. A family of 20 structures, based on their stereochemical energies and NOE energies, was then chosen for structural analysis using programs PROCHECK {Laskowski, 1996 #85} and MOLMOL {Koradi, 1996 #84}. This family of final structures and associated structural restraints have been deposited in the BioMagResBank database {Ulrich, 2008 #103} with accession no. 20023.

Analysis of ^{13}C relaxation data

^{13}C relaxation rates R_1 and R_2 were obtained by fitting peak intensities at a series of relaxation durations to a two-parameter single exponential decay curve using the program SigmaPlot. Errors of the relaxation rates were standard deviations from fittings in SigmaPlot. The steady-state ^{13}C - $\{^1\text{H}\}$ NOE values were calculated from peak intensity ratios obtained from spectra acquired in the presence and absence of proton saturation with uncertainties of 5 % for peak intensities determined from background noise of the spectra {Farrow, 1994 #67}.

^{13}C relaxation parameters were first analyzed using *Modelfree* formalism. Given that the rotational correlation time of μ -SIIIA is in the low ns range, fittings to the extended spectral density function which includes a slower timescale of internal motion were not attempted. ^{13}C R_1 , R_2 and NOE were fitted to one of three models of (1) S^2 , (2) S^2 and τ_e and (3) S^2 and R_{ex} using criteria similar to model selection approach commonly used in ^{15}N relaxation analysis {Mandel, 1995 #68}.

In contrast to *Modelfree* analysis, reduced spectral density mapping extracts information on molecular dynamics without approximations made regarding molecular shape and global rotational behavior. While reduced spectral density mapping has been commonly used in the analysis of backbone ^{15}N relaxation data, it is more limited for ^{13}C relaxation data than for ^{15}N . It has been noted previously that, while reduced spectral density mapping introduced few errors for ^{15}N relaxation analysis, it was less valid for ^{13}C as the derivation of reduced spectral density functions (Eqs 1b–1c) assuming spectral density function $J(\Omega)$ is flat around Ω_{H} , i.e. $J(\Omega_{\text{H}} - \Omega_{\text{N}}) = J(\Omega_{\text{H}}) = J(\Omega_{\text{H}} + \Omega_{\text{N}})$ for ^{15}N and $J(\Omega_{\text{H}} - \Omega_{\text{C}}) = J(\Omega_{\text{H}}) = J(\Omega_{\text{H}} + \Omega_{\text{C}})$ for ^{13}C , respectively {Atkinson, 1999 #91}. Nevertheless, it provides a complementary picture of the local dynamics of the molecule to that obtained from *Modelfree* analysis, provided the value for $J(1.563\Omega_{\text{H}})$ is not interpreted {Slupsky, 2007 #64}.

The values of spectral density functions $J(0)$, $J(\Omega_C)$ and $J(1.56\Omega_H)$ were calculated from measured ^{13}C R_1 , R_2 and steady-state NOE values similar to those described previously for reduced spectral density mapping of ^{15}N relaxation parameters.

$$\sigma_{\text{C}\alpha\text{H}} = R_1(\text{NOE} - 1)\gamma_C/\gamma_H \quad (1a)$$

$$J(0) = (6R_2 - 3R_1 - 2.72\sigma_{\text{C}\alpha\text{H}})/(3d^2 + 4c^2) \quad (1b)$$

$$J(\omega_C) = (4R_1 - 5\sigma_{\text{C}\alpha\text{H}})/(3d^2 + 4c^2) \quad (1c)$$

$$J(1.563\omega_H) = 4\sigma_{\text{C}\alpha\text{H}}/(5d^2) \quad (1d)$$

where $d = (\mu_0 h \gamma_C \gamma_H / (8\pi^2)) / \langle r_{\text{C}\alpha\text{H}}^3 \rangle$, $c = \Omega_C(\sigma_{\parallel} - \sigma_{\perp})/3^{1/2}$, μ_0 is the permeability of free space, h is Planck's constant, γ_H and γ_C are the gyromagnetic ratios of ^1H and ^{13}C , respectively, $r_{\text{C}\alpha\text{H}} = 1.09 \text{ \AA}$ is the average amide bond length, and $(\sigma_{\parallel} - \sigma_{\perp}) = 25 \text{ ppm}$ is the chemical shift anisotropy for $^{13}\text{C}^\alpha$ nuclei {Wei, 2001 #104}.

Electrophysiology of mammalian Na_V clones expressed in *Xenopus* oocytes

Oocytes expressing VGSCs were prepared and two-electrode voltage clamped essentially as described previously {Zhang, 2007 #88; Fiedler, 2008 #102}. Briefly, oocytes were placed in a 30 μL chamber containing ND96 and two-electrode voltage clamped with a holding potential of -80 mV . To activate Na channels, the membrane potential was stepped to a value between -20 and 0 mV (depending on Na_V subtype) for a 50 ms period every 20 sec. To apply toxin, the perfusion was halted, 3 μL of toxin solution (at ten times of the final concentration) was applied to the 30 μL bath, and the bath manually stirred for about 5 s by gently aspirating and expelling a few μL of the bath fluid several times with a micropipette. Toxin exposures were in static baths to conserve material. On-rate constants were obtained from the slopes of k_{obs} versus [peptide], where k_{obs} was determined from the single-exponential fit of the time course of block by a given toxin concentration. Off-rate constants were determined from single-exponential fits of the time course of recovery from block following toxin washout. All recordings were made at room temperature ($\sim 21^\circ\text{C}$).

RESULTS

Chemical Shift Assignments

A one-dimensional ^1H NMR spectrum of $\mu\text{-SIIIA}$ in aqueous solution (Figure S1) showed a single major species with good spectral dispersion. Chemical shifts assignments were obtained for resonances from all amide and aliphatic ^1H , aliphatic ^{13}C and backbone ^{15}N of $\mu\text{-SIIIA}$. These assignments have been deposited in the BioMagResBank database (accession number 20023) and are tabulated in Supplementary Material (Table S1). Plots of secondary chemical shifts of backbone H^N , N , $\text{C}\alpha$ and $\text{H}\alpha$ versus the sequence of $\mu\text{-SIIIA}$ are shown in Figure S2; the $\text{H}\alpha$ and $\text{C}\alpha$ shifts for residues 11–16 are consistent with the presence of α -helical structure in this region of the molecule.

Solution Structure

Parameters characterizing the final 20 structures of μ -SIIIA and structural statistics are summarized in Table 1. The final 20 structures fit well with experimentally derived distance and angle constraints and are well defined over the entire length of the polypeptide, with the N-terminal pGlu1 and Asn2 being slightly less ordered than the rest of the polypeptide chain. A summary of backbone RMS deviations and backbone angular order parameters of μ -SIIIA is shown in Figure S4 and stereo views of the structures are shown in Figure 1.

The main secondary structure element is a well-defined C-terminal helix (residues 11–16), but the N-terminal residues 3–5 also appear to form a helix-like turn resembling 3_{10} helix. The side chain of Lys11, which may be important for binding to Site 1 on VGSC in μ -SIIIA, is only partially exposed owing to its proximity to the side chains of Trp12 and Asp15.

^{13}C Relaxation Parameters and Rotational Correlation Times

After excluding Gly6 and Gly7, as well as residues whose measurements were compromised by the residual water resonance in the directly detected dimension (F2), ^{13}C R_1 , R_2 and ^{13}C - $\{^1\text{H}\}$ NOE values were measured for a total of 15, 16, and 16 $\text{C}\alpha$ of μ -SIIIA at 283, 298 and 310 K, respectively, as shown in Figure 2. A ^{13}C - ^1H HSQC spectrum of μ -SIIIA at 298 K, representative decay curves of R_1 and R_2 , and all measured $^{13}\text{C}\alpha$ relaxation parameters are given in Supplementary Material (Figure S5, Figure S6 and Table S2). The average values of R_1 , R_2 , and NOE as well as effective rotational correlation times of μ -SIIIA estimated from the average ^{13}C R_2/R_1 ratio (residues with angular parameters S_ϕ and $S_\psi > 0.9$ and their R_2/R_1 values within one standard deviation of the averaged R_2/R_1 ratio) are listed in Table 2. The rotational correlation time of μ -SIIIA from hydrodynamics calculations based on its closest-to-mean structure is also shown.

Dynamics Parameters

Dynamics parameters of μ -SIIIA resulting from *Modelfree* analysis and reduced spectral density mapping from its $^{13}\text{C}\alpha$ relaxation parameters measured at 283, 298, and 310K are summarized in Figures 3 and 4, respectively, and tabulated in Supplementary Material (Tables S3 and S4). Average S^2 values over the entire molecule (from residues with S^2 values available at all three temperatures) and over the C-terminal helical region (residues 11–16) are also summarized in Table 2.

Temperature Dependence of Dynamics Parameters

The observed decrease in order parameters at higher temperature reflects increased amplitudes of motion of the $\text{C}\alpha$ -H bond vectors, with several residues (Cys8 and Ser9, and to a lesser extent Lys11, Arg14 and Asp15) exhibiting more pronounced temperature dependence than the average over the entire polypeptide chain or the C-terminal helical region (residues 11–16) (Figure 3). Figure 5 summarizes the temperature dependence of the order parameters (shown as $1 - S$) of μ -SIIIA for the average over the entire molecule, the C-terminal helical region, Ser9 and Lys11 (Figure 5A), as well as the temperature dependence of apparent chemical exchange, R_{ex} , for Cys4, Trp12 and Cys13 (Figure 5B). Ribbon

presentations of μ -SIIIA with its structural characteristics and dynamics parameters highlighted are shown in Figure 6.

Functional Studies

The activity of μ -SIIIA on cloned α -subunits of rat (r) or mouse (m) sodium channels expressed in oocytes was assessed by voltage-clamp protocols. Table 3 summarizes dose-dependent inhibition by μ -SIIIA of seven sodium channels subtypes, including skeletal muscle rNa_v1.4 and heart muscle rNa_v1.5. Four neuronal subtypes, rNa_v1.1, rNa_v1.2, rNa_v1.3, mNa_v1.6, were blocked by μ -SIIIA with IC₅₀ values ranging from < 0.76 to 11 μ M. The block of rNa_v1.2 was only very slowly reversible (Figure 7); in contrast, the block of rNa_v1.1, rNa_v1.3 and mNa_v1.6 was readily reversible. Of the channels tested, rNa_v1.4 was blocked with the fastest on-rate and second slowest off-rate. Inhibition of rNa_v1.5 and rNa_v1.7 was characterized by very low affinity, with IC₅₀ values of 251 and 65 μ M, respectively. Of the five IC₅₀ values determined in Table 3, the corresponding K_d values (calculated from k_{off}/k_{on}) for four were within a factor of 2. For Na_v1.7, however, the K_d (15 μ M) was four-fold lower than the IC₅₀; we attribute this discrepancy to the fact that the block of Na_v1.7 had the slowest on-rate and a relatively slow off-rate, which created a long period to reach steady-state and therefore a less reliable determination of IC₅₀.

DISCUSSION

Conotoxins μ -SmIIIA, μ -KIIIA and μ -SIIIA are potent sodium channel blockers, indeed the latter two were shown recently to possess potent analgesic activity in the inflammatory pain assay in mice {Zhang, 2007 #88; Green, 2007 #86}. μ -KIIIA and μ -SIIIA share high sequence homology in the C-terminal part of their structure. Consistent with this, the selectivity profile of μ -SIIIA for the various Na_v subtypes was similar to that described previously for μ -KIIIA {Zhang, 2007 #88}. Both were nearly irreversible blockers of Na_v1.2, and reversible blockers of skeletal muscle subtype Na_v1.4. However, the neuronal subtype Na_v1.7 was more readily blocked by μ -KIIIA (K_d 0.29 μ M {Zhang, 2007 #88}), than by μ -SIIIA (IC₅₀ 65 μ M or K_d 15.0 μ M; see Table 3 and last paragraph of Results), suggesting that either Ser13 in μ -KIIIA is an important determinant of the interactions with Na_v1.7 (the homologous residue in μ -SIIIA is Ala17), or that some minor conformational differences between the two peptides may be responsible for their different potencies. In any event, our current functional data suggest that the N-terminal region of μ -conotoxins SIIIA, KIIIA and SmIIIA is important for determining subtype selectivity in these peptides, whereas the C-terminal region contains a common pharmacophore for the interactions with the neuronal subtypes of sodium channels.

Comparison with Structures of μ -SmIIIA and Other μ -Conotoxins

The structures of several μ -conotoxins have been solved previously, including μ -SmIIIA (PDB access code: 1Q2J){Keizer, 2003 #66}, μ -PIIIA (1R9I){Nielsen, 2002 #13}, μ -GIIIA (1TCJ){Wakamatsu, 1992 #17} and μ -GIIIB (1GIB){Hill, 1996 #18}. A sequence alignment of these μ -conotoxins is shown in Figure 8 along with a comparison of their structures. Superposition of these five structures over their C-terminal halves is significantly better than that over their N-terminal halves (Figure 8B). As noted by Keizer et al. {Keizer,

2003 #66}, this is at least partly a consequence of the trans conformation for the peptide bond preceding residue 8 in μ -SmIIIA and the major form of μ -PIIIA, as opposed to the cis conformation in μ -GIIIA, μ -GIIIB and the minor form of μ -PIIIA. All peptide bonds in μ -SIIIA are also in the trans conformation. The C-terminal region of μ -SIIIA contains a well-defined helix (residues 11–16), and the N-terminal region contains a helix-like turn resembling 3_{10} helix at residues 3–5.

In μ -SmIIIA the indole ring of Trp14 is flanked by the imidazolium ring of His18 on one side and the guanidinium moiety of Arg2 on the other {Keizer, 2003 #66}, these side chain interactions presumably being favoured not only by interactions between the π orbitals of the indole and imidazolium rings, but also by aromatic-cation interactions. The equivalent Trp in μ -SIIIA, Trp12, is also close to His16 (Figure 1B) but these residues are some distance away from residue 2, which is Asn in μ -SIIIA. The $H^{\delta 2}$ resonance of His16 of μ -SIIIA has a very similar chemical shift to that of His18 in μ -SmIIIA (Table S1), implying that it is influenced by ring-current shifts from the Trp indole ring as a consequence of their close proximity in both structures. Analysis of ring current shifts on the final family structures of μ -SIIIA performed using the program MOLMOL {Koradi, 1996 #26} resulted in very similar upfield shifts for resonances from $C\alpha H$, $C^{\beta} H$ and the ring protons of His16, as previously reported for His18 in μ -SmIIIA {Keizer, 2003 #66}.

Comparison with Model Structure

Models of both μ -SIIIA and μ -KIIIA were generated in the course of MD simulations performed to gain insight into the potential structural consequences of deleting a number of residues in the first loop (the region flanked by the second and third Cys residues) {Bulaj, 2005 #87}. Figure S7 compares stereo views of the μ -SIIIA model with our solution structure. The C-terminal halves of the two are very similar (backbone RMSD 1.07 Å for residues 11–20, compared with 2.58 Å for residues 1–10), and the helical region (residues 11–16) is conserved. The difference seen in the N-terminal region may be attributed to apparent structural differences between μ -SIIIA and μ -SmIIIA, as the model structure of μ -SIIIA was generated based on the reported structure of μ -SmIIIA. As described in the Introduction, the simulated structure of μ -SmIIIA showed several differences from the structure determined from NMR data {Keizer, 2003 #66}.

Backbone Dynamics of μ -SIIIA

Reduced flexibility is observed for μ -SIIIA at lower temperatures, as reflected in its overall S^2 (Figure 3A) and $J(0)$ values (Figure 4A). Relatively lower S^2 values compared with the average over the entire backbone were observed for pyro-Glu1, Asn2 and Ser9. The angular order parameters S_{ϕ} and S_{ψ} (Figure S3) also show relatively lower values for pyro-Glu1 and Asn2 but not for Ser9. It appears that the N-terminus shows larger magnitude motions on the sub-ns timescale, while the C-terminus is more rigid, although the difference is small if the first two residues are ignored. As disulfide bridges connect Cys3 and Cys4 to the C-terminal half of the molecule, there is no simple correlation between the locations of the disulfides and relative backbone flexibility. Greater flexibility is anticipated near the chain termini, which is in accord with our observations for residues 1 and 2, but the C-terminal residue, Cys20, participates in a disulfide bond and has S^2 values essentially identical with other

residues in the C-terminal half. The only other residue to show greater motion on the sub-ns timescale is Ser9 (Figure 3A), which is located in the middle of the polypeptide chain and adjacent to Cys8.

Conformational Exchange of μ -SIIIA- Motion on the Microsecond to Millisecond Timescales

Larger $J(0)$ values were observed for the backbone at 283 K compared with 298 and 310 K, indicating reduced sub-ns motions of the C^α -H bond vectors at lower temperature (Figure 4A). Slower μ s to ms motions due to conformational exchange experienced by backbone $C\alpha$ may also be reflected in the spectral density function at angular frequency, $\Omega = 0$, which give rise to elevated values for $J(0)$. For μ -SIIIA, significantly higher $J(0)$ values were observed for Cys4, Trp12 and Cys13 at 283 K, which agrees very well with the result from *Modelfree* analysis that significant apparent R_{ex} terms are needed in order to fit the relaxation data of these residues (Figure 3C).

The interpretation of R_{ex} in terms of chemical/conformational exchange contributions to the transverse relaxation can be significantly affected by the anisotropic global reorientation of the molecule. However, in μ -SmIIIA, both *Modelfree* analysis and reduced spectral density mapping (which does not assume a particular model from rotational reorientation of the molecule) indicate that Cys4, Trp12 and Cys13 can undergo significant exchange on μ s to ms timescales. It has been shown previously that the sign of dR_{ex}/dT indicates whether microscopic exchange is faster or slower than $3.2/\tau_{cp}$, where τ_{cp} is the interval between two consecutive 180° pulses in the CPMG segment of the pulse sequence used for $^{13}C\alpha$ R_2 measurements {Mandel, 1996 #92}. As can be seen in Figure 5B, the magnitudes of R_{ex} for Cys4, Trp12 and Cys13 decrease with increasing temperature. Thus, for a τ_{cp} of 900 μ s for μ -SIIIA, an estimate of $k_{ex} > 3.2/\tau_{cp} = 3.6 \times 10^3 \text{ s}^{-1}$ can be obtained {Mandel, 1996 #92}.

Given that Trp12 and Cys13 are adjacent to the biologically important Lys11, their observed propensity for conformational exchange implies that structural changes in this region may be possible upon binding to the sodium channel. As Lys11, Trp12 and Cys13 are part of the C-terminal helix, the conformation of this helix may also be subject to change upon sodium channel binding. Two of the residues showing significant exchange, Cys4 and Cys13, are involved in disulfide bridges (although not to each other), raising the possibility that conformational transitions involving disulfide bonds may contribute to the observed exchange processes {Otting, 1993 #94}. The presence of conformational exchange and reduced angular order parameters in the loop between Cys8 and Cys13 is also not a simple function of loop size, as the least flexible region in the molecule is actually the longest loop, encompassed by Cys13 and Cys19.

The backbone dynamics of an ^{15}N -labelled Ω -conotoxin MVIIA precursor have been studied previously using ^{15}N relaxation measurements {Goldenberg, 2001 #83}. The backbone proved to be well ordered on the nanosecond time scale but residues 9–15 experienced conformational exchange processes with a time constant of about 35 μ s. These observations were consistent with the results of ^{13}C relaxation measurements on unlabelled Ω -MVIIA by Atkinson et al. {Atkinson, 2000 #93}, who detected conformational exchange in the loop between Cys8 and Cys15 and suggested that this was associated with

conformational exchange of the Cys8-Cys20 disulfide bridge. As this region of the Ω -conotoxins contains residues essential for Ca^{2+} channel binding {Norton, 1999 #95; Nielsen, 2000 #96}, it is possible that these exchange processes may influence activity.

Conclusions

The structure determined in this study for μ -SIIIA extends our understanding of the conformational space available to this class of conotoxins. Its well-defined C-terminal region closely matches the corresponding regions of all μ -conotoxins investigated to date, but its N-terminal half is distinct, sharing some features with the related μ -conotoxin μ -SmIIIA but showing a more extended structure for the N-terminal residues. Internal motion of the backbone has been detected over various timescales, with pyroGlu1, Asn2 and Ser9 showing larger magnitude motions on the ps-ns timescale and Cys4, Trp12 and Cys13 undergoing conformational exchange on the μ s to ms timescale. Even the presence of three disulfide bonds in a short polypeptide of just 20 residues is not sufficient to confer rigidity on the polypeptide backbone. Indeed, it may even be that conformational averaging associated with the disulfide bridges is the cause of the observed conformational exchange, as also suggested for Ω -MVIIA {Atkinson, 2000 #93}. An awareness of the presence of conformational exchange is valuable in several ways. Interpreting measured NOEs as structural restraints requires some caution if either or both of the interacting nuclei are from regions undergoing such exchange. Knowing which regions of a structure are potentially flexible is also important if modelling studies are to be undertaken based on that structure. And, most important of all, it must be taken into account when using structures determined in solution as a basis for understanding receptor binding or constructing mimetics {Baell, 2001 #98}. Whether conformational exchange serves any important biological function, for example in allowing the toxin to undergo required conformational rearrangements as it engages its cognate binding site, or whether it is simply an adventitious consequence of the presence of multiple disulfide bridges in certain environments, remains to be established. One way of addressing this question is to replace some of the disulfide bridges with less flexible homologues, and means to achieve this are now available {Robinson, 2007 #99}. In parallel with such efforts, greater access to backbone relaxation data afforded by high-field spectrometers equipped with more sensitive probes should contribute to a better understanding of the relationships among structure, flexibility and activity. Our results on the structure and dynamics of μ -SIIIA also provide a foundation for further structure-function studies on this therapeutically important group of conotoxins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
 μ -SIIIA structure. (A) Stereo view of backbone μ -SIIIA (a family of 20 final solution structures of μ -SIIIA superimposed over backbone heavy atoms (N, C α and C') of all 20 residues). The letters N and C refer to the N and C termini, respectively. (B) Stereo view of closest-to-mean structure in the family of 20 structures shown in (A) with backbone of μ -SIIIA in light grey and side chains of several residues highlighted in colour. Those residues with side chains highlighted are also labeled. The three disulfide bonds (residues 3–13, 4–19 and 8–20) are shown in yellow.

Figure 2.

C^α ^{13}C relaxation parameters, R_1 , R_2 and NOE, of μ -SIIIA measured at 283 K (●), 298 K (▲) and 310 K (◆). Data are not shown for Gly6 and Gly7, and residues whose resonances lay too close to the residual water resonance in the F2 dimension (Figure S4 and Table S2).

Figure 3. Dynamics parameters of μ -SIIIA. (A) S^2 , (B) τ_e , and (C) R_{ex} at 283 K (●), 298 K (▲) and 310 K (◆) derived from ^{13}C C^α relaxation parameters shown in Figure 2 using the *Modelfree* formalism.

Figure 4.

Reduced spectral density functions of μ -SIIIA. (A) $J(0)$, (B) $J(\Omega_C)$, and (C) $J(1.563\Omega_H)$ at 283 K (●), 298 K (▲) and 310 K (◆) calculated from ^{13}C relaxation parameters depicted in Figure 2 using Eqs 1a–1d. Dashed lines in (A) corresponding to $(\frac{2}{5})\tau_m$, indicating $J(0)$ values for completely rigid molecule at the given rotational correlation times (283 K in red, 298 K in blue, and 310 K in green, respectively, for μ -SIIIA).

Figure 5.

Temperature dependence of chemical exchange and order parameters for μ -SIII A. (A) Temperature dependence of chemical exchange are shown as Arrhenius plot, ($\ln R_{\text{ex}}$ versus $1/T$), for residues Cys4 (), Trp12 (○), and Cys13 (). (B) Order parameters are shown as ($1-S$) versus T for (●) the average value of overall sequence (12 residues with S^2 values at all three temperatures were determined), (■) the C-terminal helix (residues 11–16), () Ser-9, and () Lys11.

Figure 6.

(A) Ribbon diagram of μ -SIIIA (closest-to-mean) with three disulfide bridges (Cys3-Cys13, Cys4-Cys19 and Cys8-Cys20) highlighted in yellow. (B) Mean structure of the final family of 20 structures of μ -SIIIA (BioMagResBank access code 20023) with radii of backbone atoms proportional to their averaged displacement to the mean structure. Note that this structure is not the same as that shown in parts A, C and D of this figure. (C) Ribbon diagram of SIIIA (closest-to-mean) with radii of backbone atoms proportional to $1-S^2$, where S^2 is the order parameter resulted from *Modelfree* analysis of ^{13}C relaxation parameters

measured at 298 K (Figure 3). Residues 2, 6, 7 and 17, whose S^2 values were not determined, are shown in grey with their bond radii shown as the averaged values from their neighboring residues. (D) Backbone C α atoms of μ -SIIIA, shown in spheres, are color coded based on their R_{ex} values at 283 K. Color scheme: $R_{ex} > 10 \text{ s}^{-1}$ in red, $0 \text{ s}^{-1} < R_{ex} < 10 \text{ s}^{-1}$ in pink, and residues whose dynamics data were not determined in grey. This figure was prepared using MOLMOL {Koradi, 1996 #26}.

Figure 7. $\mu\text{-SIIIA}$ blocks $\text{rNa}_V1.2$ expressed in *Xenopus* oocytes. Oocytes were two-electrode voltage clamped as described under Experimental. (A) Representative recordings, each trace represents the average of 5 responses before (control, gray trace) and during (black trace) exposure to $1 \mu\text{M}$ $\mu\text{-SIIIA}$. (B) Time course of block and recovery; black bar above plot indicates when $\mu\text{-SIIIA}$ was present.

Figure 8.

Sequence alignment and structure comparison of μ -conotoxins. (A) Amino acid sequences of μ -SIIIA, μ -SmIIIA, μ -PIIA, μ -GIIIA, and μ -GIIIB. Numbers shown at the top are those for μ -SIIIA. Z represents pyro-glutamate and O hydroxyproline. Conserved cysteines are highlighted in yellow and partially conserved residues in light grey. The Arg or Lys residues in the C-terminal half of the polypeptide (boxed in dark blue) have been suggested to play a key role for the potency of these peptides. (B) Structures of μ -SIIIA ((BioMagResBank access code 20023), μ -SmIIIA (PDB access code: 1Q2J), μ -PIIA (PDB access code: 1R9I),

μ -GIIIA (PDB access code: 1TCJ), and μ -GIIIB (PDB access code: 1GIB) with side chains of Arg or Lys shown in dark blue and disulfide bonds in yellow. All these structures are shown in a similar orientation after superimposition over backbone heavy atoms (C, C $^{\alpha}$, N) of aligned residues (Figure 8A); superposition over the C $_{\alpha}$ and C $^{\beta}$ atoms of the six Cys residues gave a similar result. Side chains of Asn2, Trp12 and His 16 of μ -SIIIA and Arg2, Trp14 and His18 of μ -SmIIIA are shown in magenta. Figure 8B was generated using the program MOLMOL {Koradi, 1996 #26}.

Table 1Structural statistics for μ -SIHA in aqueous solution at 298 K.

Distance restraints	164
Intra ($i = j$)	53
Sequential ($ i - j = 1$)	68
Short ($1 < i - j < 6$)	31
Long	12
Dihedral restraints	22
Energies (kcal mol ⁻¹) ^a	
E _{NOE}	7.5 $\pm$ 0.8
Deviations from Ideal Geometry ^b	
Bonds (Å)	0.0065 $\pm$ 0.0002
Angles (°)	0.743 $\pm$ 0.014
Impropers (°)	0.551 $\pm$ 0.021
RMS Deviations (Å) ^c	
All heavy atoms	1.22
Backbone heavy atoms (N, C ^{α} , C ^{γ})	0.74
Average pairwise RMS deviations	
All heavy atoms	1.83 $\pm$ 0.43
Backbone heavy atoms (N, C ^{α} , C ^{γ})	1.06 $\pm$ 0.36
Ramachandran plot ^d	
Most favored (%)	91.6
Allowed (%)	8.4
Additionally allowed (%)	0
Disallowed (%)	0

^aThe values for E_{NOE} are calculated from a square well potential with force constants of 50 kcal mol⁻¹ Å².

^bThe values for the bonds, angles, and impropers show the deviations from ideal values based on perfect stereochemistry.

^cRMSD over the backbone heavy atoms (N, C ^{α} , C) of all residues.

^dAs determined by the program PROCHECK-NMR {Laskowski, 1996 #25} for all residues except Gly and Pro.

Table 2

Summary of average ^{13}C relaxation parameters, rotational correlation times and order parameters of μ -SIIIA

T (K)	$\langle R_1 \rangle$ (s ⁻¹)	$\langle R_2 \rangle$ (s ⁻¹)	$\langle \text{NOE} \rangle$	$\langle R_2/R_1 \rangle^a$	τ_c (ns)	τ_c^b (ns)	$\langle S^2 \rangle^c$ (overall)	$\langle S^2 \rangle^d$ (helix 11–16)
283	3.91 ± 0.43	14.38 ± 6.59	1.30 ± 0.13	3.04 ± 0.41 (11/15)	2.29 ± 0.25	2.72	0.91 ± 0.17	0.97 ± 0.01
298	4.28 ± 0.54	8.07 ± 2.2	1.38 ± 0.11	1.87 ± 0.43 (16/16)	1.45 ± 0.19	1.80	0.80 ± 0.21	0.85 ± 0.02
310	4.12 ± 0.63	6.06 ± 1.59	1.54 ± 0.12	1.45 ± 0.22 (16/16)	0.98 ± 0.15	1.32	0.76 ± 0.20	0.84 ± 0.07

^a Average value over residues whose R_2/R_1 within 1.5 SD of averaged value of R_2/R_1 of all measured residues, number of residues satisfy the criteria versus total number of residues whose ^{13}C relaxation parameters are measured is given in the parentheses.

^b Effective rotational correlation times of μ -SIIIA from hydrodynamic calculation, using program HYDRONMR with a radius of atomic elements 3.1 Å and viscosities of 100% $^2\text{H}_2\text{O}$ $R_h = 9.0$ Å. Previously published values of viscosities for 100% $^2\text{H}_2\text{O}$ of 1.679×10^{-3} , 1.110×10^{-3} , and $0.850 \times 10^{-3} \text{ N s m}^{-2}$ at 283, 298, and 310 K, respectively, were used {Cho, 1999 #69}.

^c Averaged over a group of 12 residues with order parameters determined at all three temperatures.

^d Average value over the helical region (residues 11–16)

Table 3

Inhibition by μ -SIIIA of cloned sodium channels expressed in *Xenopus* oocytes. Rate constants were determined as described under Experimental. Standard deviation and 95% confidence intervals (95% C.I) were calculated from at least three independent experiments using Prism software.

Sodium channel ^a	k_{on} ($\mu M^{-1} \cdot min^{-1}$)	k_{off} (min^{-1})	IC ₅₀ (95% C.I.) (μM)
rNa _V 1.1	0.011 $\pm$ 0.004	0.2 $\pm$ 0.06	11 (9.3–12.6)
rNa _V 1.2	0.10 $\pm$ 0.015	Irreversible ^b	N.A. ^c
rNa _V 1.3	0.017 $\pm$ 0.002	0.12 $\pm$ 0.018	11 (9.0–12.9)
rNa _V 1.4	0.29 $\pm$ 0.036	0.04 $\pm$ 0.008	0.13 (0.11–0.16)
rNa _V 1.5	-- ^d	-- ^d	251 (204–308) ^e
mNa _V 1.6	0.06 $\pm$ 0.012	0.082 $\pm$ 0.006	0.76 (0.69–0.85)
rNa _V 1.7	0.005 $\pm$ 0.0005	0.075 $\pm$ 0.012	65 (57.8–73.1)

^a α -Subunit cloned from rat (r) or mouse (m).

^b Although the block was essentially irreversible within the experimental time frame, the k_{off} was estimated as $0.0047 \pm 0.0015 \text{ min}^{-1}$ based on residual recovery after 20 min of wash and assuming exponential decay.

^c Not available because slow kinetics precluded steady state from being achieved within the experimental time frame.

^d Unable to be determined because block was too small and apparent kinetics too fast to be measured accurately.

^e Extrapolated value assuming 100% block at saturating peptide concentration.