Solution Structure of μ-Conotoxin PIIIA, a Selective Inhibitor of Persistent TTX-Sensitive Sodium Channels

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Abstract

μ-Conotoxins are peptide inhibitors of voltage-sensitive sodium channels (VSSCs). Synthetic forms of PIIIA and PIIIA(2-22) were found to inhibit TTX-sensitive VSSC current but had little effect on TTX-resistant VSSC current in peripheral ganglia. In rat brain neurons, these peptides preferentially inhibited the persistent over the transient VSSC current. Radioligand binding assays revealed that PIIIA, PIIIA(2-22) and μ-conotoxin GIIIB discriminated among TTX-sensitive VSSCs in rat brain, that these and GIIIC discriminated among the corresponding VSSCs in human brain, while GIIIA had low affinity for neuronal VSSCs. ^{1}H NMR studies revealed that PIIIA adopts two conformations in solution due to *cis/trans* isomerisation at hydroxyproline8. The major *trans* conformation results in a 3D structure that is significantly different from the major conformation of other μ-conotoxins that selectively target TTX-sensitive muscle VSSCs. Comparison of the structures and activity of PIIIA to muscle-selective μ-conotoxins provides an insight into the structural requirements for inhibition of different TTX-sensitive sodium channels by μ-conotoxins.

Introduction

Voltage-sensitive sodium channels (VSSCs) underlie the influx of sodium ions responsible for action potentials in excitable cells (1). Based on their susceptibility to block by tetrodotoxin (TTX), VSSCs can be divided into TTX sensitive (TTX-S) and TTX-resistant (TTX-R) classes. Members of both classes share considerable sequence homology and are closely related structurally (2). These include the neuronal TTX-S type I/Na_v1.1, type II/Na_v1.2, type III/Na_v1.3, PN1/Na_v1.7 and PN4/Na_v1.6, and the skeletal muscle TTX-S μ1/Na_v1.4. The TTX-R sodium channels include the cardiac H1/Na_v1.5 which is partially TTX-resistant, and the neuronal TTX-R channels SNS/PN3/Na_v1.8 and NaN/PN5/Na_v1.9 (2). A number of these VSSC subtypes are implicated in clinical states such as pain (3–6), stroke (7, 8) and epilepsy (9, 10). Persistent (non-inactivating) forms of the TTX-S sodium channel current which underlie repetitive firing (11, 12) have less well-defined origins, but may involve Na_v1.3 (13) or Na_v1.6 (11) and are enhanced by hypoxia (14–16) and nitric oxide (17). Most TTX-S sodium channels types have a heterogeneous distribution in human brain (18).

VSSCs are inhibited by local anaesthetics and modulated by toxins that act at one inhibitory site (Site 1) and at least four other sites that result in excitatory actions. μ-Conotoxins from the venom of marine cone snails act selectively to occlude the pore of the VSSC by competing with TTX and saxitoxin (STX) for binding to Site 1 in the P-loop region of the α subunit. To date, sequences for four members of the three-loop μ-conotoxin class have been published (Table 1). GIIIA–GIIIC from *Conus geographus* venom are potent blockers of skeletal muscle, but not neuronal VSSCs. The three-dimensional (3D) structures of selected μ-conotoxins (19, 20) have been used to describe the architecture of the outer vestibule of the VSSC (21–25). The most recently described member of this class, μ-conotoxin PIIIA (26) from *C. purpurescens* (Fig. 1). PIIIA is notable for its ability to inhibit neuronal as well as muscle TTX-S sodium channels (26), and to discriminate among VSSCs in rat brain (27). Thus PIIIA is the first peptide toxin for investigating the architecture of Site 1 of neuronal VSSCs.

Previous studies on GIIIA (21, 22) have revealed that the cationic residues, particularly Arg 13, are important for the high potency of this peptide at Na_v1.4 (see Fig. 1). The high sequence identity and similarities in 3D structure of GIIIA and GIIIB (19, 20) provide a rational basis for comparison with

PIIIA, which also contains a number of conserved residues and the same disulfide connectivities as GIIIA and GIIIB (and GIIIC). However, a number of primary structural differences are apparent between PIIIA and other μ-conotoxins, which may affect the relative position and orientation of backbone loops and their projecting sidechains, and thus allow PIIIA to interact with both neuronal and muscle forms of TTX-sensitive VSSCs.

To further investigate the potential of PIIIA as a probe of VSSCs, we determined its structure by ^{1}H NMR spectroscopy and investigated its mode of action on native tissues using electrophysiological and ligand binding approaches. These studies revealed that PIIIA and PIIIA(2-22) preferentially inhibited the persistent TTX-S currents in rat hippocampus, while in rat DRG the TTX-R current was spared. Comparisons of the 3D structures of PIIIA, GIIIA and GIIIB revealed important structural differences, including an alternative major conformation accessed by PIIIA, which had not been identified previously in μ-conotoxins.

Experimental Procedures

Peptide Synthesis—Peptides were prepared by Boc chemistry (28) using methods described for ω-conotoxins (29). The sidechain protection chosen was Arg(tos), Asp(OcHex), Lys(ClZ), Ser(Bzl) and Cys(p-MeBzl). The crude reduced peptides were purified by preparative chromatography, using a 1% gradient (100% A to 80% B, 80 min) and UV detection at 230 nm. The reduced peptides were oxidised at a concentration of 0.02 mM in either aqueous 0.33 M $NH_4$0Ac/0.5 M GnHCl, or aqueous 2 M NH_4OH. The solution was stirred for 3 to 5 days at (pH 8.1). Purification of oxidised peptide was completed using preparative RP-HPLC.

Radioligand Binding—Whole rat brain (29), the human frontal cortex (30), and rat skeletal muscle (31) were homogenised in 50 mM HEPES (pH 7.4), filtered though 100 micron nylon mesh (muscle only), and centrifuged at 28,000 × g (10 min). The pellet was suspended in 50 mM HEPES, 10 mM EDTA (pH 7.4) for 30 min, centrifuged, and resuspended in 50 mM HEPES (pH 7.4). Radioligand binding studies were conducted in assay buffer (mM) 130 choline chloride, 5.4 KCl, 5.5 glucose, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 50 HEPES (pH 7.4 with Tris base). Assays were conducted on rat brain (18 μg protein, 150 μl total volume), human brain (12 μg protein, 150 μl) and rat muscle (50 μg protein, 300 μl) that contained 5.6 nM [^{3}H]-STX (14.9 Ci/mmol, Amersham) and varying concentrations of μ-conotoxins in assay buffer. Assays were incubated for 1 h at 4ºC and filtered through GFB filters on a Tomtec harvester (brain) or a Millipore manifold (muscle) using wash buffer (mM) 163 choline chloride, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 5.0 mM HEPES (pH 7.4 with Tris base). Filters were dried, scintillant added, and retained radioactivity measured on a Microbeta counter (Wallac).

Electrophysiological Experiments—Electrophysiological experiments were conducted to further investigate the effect of PIIIA on TTX-S and TTX-R sodium channels in native tissue.

(i) Dissociation of Nodose and DRG Neurons—Sensory neutrons from rat nodose ganglia and dorsal root ganglia (DRG) were isolated as previously described (32, 33). Briefly, young rats (10–21 days) were killed by cervical dislocation and the nodose and DRG were carefully removed. The ganglia were placed in physiological saline solution containing collagenase (~ 1.0 mg/ml type 2 Worthington Biochemical Corp., NJ) and incubated for 1 h at 37°C in 95% air and 5% CO_2 for 24–48 hr. Neurons from the nodose ganglia that were clear and round were selected for experiments. Small diameter cells

(~20 μm) from the DRG were used as these have been previously been reported to predominantly express TTX resistant Na^+ currents (34).

(ii) Dissociation of Hippocampal CAI Neurons—Young rats (14–21 days) were anaesthetised under CO_2 and decapitated with an animal guillotine. The brain was removed and transferred to ice cold artificial cerebrospinal fluid (ACSF containing 124 mM NaC1, 26 mM NaH_2CO_3, 3 mM KC1, 1.3 mM $MgSO_4$, 2.5 mM NaH_2PO_4 and 20 mM glucose). The brain was mounted in a vibratome and bathed in ice cold ACSF equilibrated with 95 % 0_2 and 5% $C0_2$ while the 500 μm thick slices were prepared. Brain slices incubated for 30 min with 200 U/ml papain (Worthington Biochem.), 1.1 mM cysteine (Sigma), 0.2 mM EDTA and 13.4 mM mercaptoethanol, at 35°C. Following incubation the CA1 region was located, removed and gently triturated using a fire polished Pasteur pipette. Neurons of 10–15 μm were used, with cells that were flat, swollen or grainy in appearance avoided.

(iii) Electrophysiological Recordings—Whole cell Na^+ currents were recorded using the patch clamp technique. Patch pipettes (GC150F, Clarke Electromedical Instruments) were prepared that had resistances of between 1–2 MΩ (nodose and DRG neurons) and 6–10 MΩ (CA1 neurons) when filled with pipette solution. Whole cell Na^+ currents from nodose and DRG neurons were made using a List EPC 7 amplifier (List Medical), voltage steps were generated by a PC (Dell Optiplex GXM) running clamp (Axon Instruments Inc, Foster City, CA). Whole cell Na^+ currents from CA1 neurons were made using a Axopatch 1D amplifier (Axon Instruments Inc, SF), with voltage steps generated using a PC (Osborne 486-SX) running custom software (14, 15, 35).

(iv) Solution and Toxins—To record Na^+ currents from DRG and nodose neurons, patch pipettes were filled with the following solution (mM); CsF 135, NaC1 10, N-hydroxyethylpiperazine-N-ethanesulphonic acid (HEPES) 5, with pH adjusted 7.2 with CsOH. The bath solution contained (mM); NaC1 50, KCl 3, tetraethylammonium chloride (TEA) 90, $CdC1_2$ 0.1, glucose 7.7, HEPES 10, with pH adjusted to 7.4 with TEA-OH. To record Na^+ currents from CA1 neurons, the patch pipette solution contained the following solution (mM); CsF 125, NaF 5, KC1 10, TES 10, with pH adjusted to 7.4 with KOH. The bath solution contained (mM); NaC1 135, KC1 5, $MgC1_2$ 3, $CaC1_2$ 1, $CoC1_2$ 5, CsC1 5, TES 10, with pH adjusted to 7.4 with NaOH

(v) Data Analysis—Three distinct Na^+ currents were measured; a transient TTX sensitive Na^+ current (TTX-S I_{NaT}), a transient TTX resistant Na^+ current (TTX-R I_{NaT}), and a persistent TTX sensitive Na^+ current (TTX-S I_{NaP}). The amplitude of evoked TTX-S I_{NaT} was measured at its peak after subtraction of the current evoked in the presence of TTX (0.5–1 μM). The amplitude the TTX-R I_{NaT} was measured at least 2 min following the addition of 0.5–1 μM TTX. The amplitude of TTX-S I_{NaP} was measured at the end of a 400 ms voltage step after subtraction of the current evoked in the presence of TTX (0.5–1 μM). All values are expressed as means (±) SEM with n indicating the number of cells in a given series of experiments. Comparisons of two means were made using Student's two-tailed unpaired *t* test.

^{1}H NMR Spectroscopy—All NMR experiments were recorded on a Bruker ARX 500 spectrometer equipped with a *z*-gradient unit or on a Bruker DMX 750 spectrometer equipped with a *x,y,z*-gradient unit. Peptide concentrations were ~2 mM. PIIIA was examined in 95% H_20/5% D_20 (pH 3.0 and pH 5.5; 275-298 K) and in 50% aqueous CD_3CN (260-293 K). ^{1}H NMR experiments recorded were NOESY (36, 37) with mixing times of 150, 200 and 400 ms and TOCSY (38) with a mixing time of 80

ms, DQF-COSY (39), and E-COSY in 100% D_20 (40). All spectra were run over 6024 Hz (500 MHz) or 8192 Hz (750 MHz) with 4K data points, 400-512 FIDs, 16-64 scans, and a recycle delay of 1 s. The solvent was suppressed using the WATERGATE sequence (41). Spectra were processed using UXNMR as described previously (29) and using Aurelia, subtraction of background was used to minimise T_1-noise. Chemical shift values were referenced internally to DSS at 0.00 ppm. Secondary Hα shifts were measured using random coil shift values of Wishart et al. (42). $^3J_{NH\text{-}H\alpha}$ coupling constants were measured as previously described (29).

Distance Restraints and Structure Calculations—Peak volumes in NOESY spectra were classified as strong, medium, weak and very weak corresponding to upper bounds on interproton distance of 2.7, 3.5, 5.0 and 6.0 Å, respectively. Lower distance bounds were set to 1.8 Å. Appropriate pseudoatom corrections were made (43) and distances of 0.5 Å and 2.0 Å were added to the upper limits of restraints involving methyl and phenyl protons, respectively. $^3J_{NH\text{-}H\alpha}$ coupling constants were used to determine ϕ dihedral angle restraints (44), and in cases where $^3J_{NH\text{-}H\alpha}$ was 6–8 Hz and it was clear that a positive dihedral angle was not present, ϕ was restrained to – 100 ± 70°. $^3J_{H\alpha\text{-}H\beta}$ coupling constants, together with relevant NOESY peak strengths, were used to determine *x*1 dihedral angle restraints (45). Where there was no diastereospecific assignment for a prochiral pair of protons, the largest upper bound for the two restraints was used. Where stereospecific assignments were established, these distances were specified explicitly.

Structures were calculated using the torsion angle dynamics/simulated annealing protocol in X-PLOR (46) version 3.8 using a modified geometric forcefield based on parhdg.pro. Structure refinements were performed using energy minimisation (200 steps) under the influence of a full forcefield derived from Charmm (47) parameters. Structure modelling, visualisation and superimpositions were done using InsightII (MSI). Surface calculations, RMSDs and H-bond analysis was done using MOLMOL (48). The quality of the structures was analysed using procheck-NMR (49).

Results

Effects of PIIIA and PIIIA(2-22) on Whole Cell Na^+ Currents—The effects of μ-conotoxin PIIIA, and a truncated analogue PIIIA(2-22), were investigated on three distinct voltage dependent VSSCs found in tissues of the peripheral and central nervous system. Rat nodose ganglia were used to investigate the transient TTX-sensitive voltage dependent Na^+ current (TTX-S I_{NaT}), the rat dorsal root ganglia were used to investigate the transient TTX-resistant sodium current (TTX-R I_{NaT}) (34), while hippocampal neurons in the CAI region were used to investigate the persistent TTX-sensitive sodium current (TTX-S I_{NaP}) (15, 50, 51).

PIIIA(2-22) caused a concentration dependent reduction in the peak amplitude of the TTX-S I_{NaT} in rat nodose ganglia neurons (Fig. 2). In contrast, in neurons from rat dorsal root ganglia, PIIIA(2-22) produced only a small reduction in the peak amplitude of the TTX- R I_{NaT} (Fig. 2). High frequency stimulation has been shown to modify the degree of block by some neurotoxins which act in a use-dependent manner. Compared to control. PIIIA(2-22) at 1 μM failed to produce any use dependent inhibition of peak TTX-S I_{NaT} during 20 depolarising pulses from a holding potential of –70 mV to a test potential of –30 mV for 25 ms delivered at a frequency of 20 Hz (n=6).

In rat CAI neurons, addition of PIIIA(2-22) to the bathing solution caused a concentration dependent reduction in the peak amplitude of the TTX-S I_{NaT} and the TTX-S I_{NaP} (Fig. 3). Interestingly, PIIIA(2-22) had a greater effect on the TTX-S I_{NaP} than on the TTX-S I_{NaT} (Fig. 3 inset). At 1 μM, the peak amplitude of the TTX-S I_{NaT} was unaffected, whereas the amplitude of the TTX-S I_{NaP} was reduced by ~75%. PIIIA also reduced the TTX-S I_{NaT} in nodose and CAI neurons (data not shown) with a similar potency to PIIIA(2-22). PIIIA also had a preferential effect on the transient compared with the persistent sodium current, being slightly more potent at reducing the amplitude of the TTX-S I_{NaT} current in CAI neurons than PIIIA(2-22).

Radioligand Binding Studies—The ability of μ-conotoxins to displace [^{3}H]-STX from VSSCs in human and rat brain and rat skeletal muscle is shown in Fig. 4. All peptides were more potent at the rat skeletal muscle than rat brain VSSCs, with GIIIA and GIIIC showing most selectivity and PIIIA least selectivity. The pIC_{50}values and % inhibition for these peptides are given in Table 1. The data show that PIIIA and PIIIA(2-22) have greatest potency at rat and human brain VSSCs, GIIIB has intermediate potency, and GIIIA and GIIIC are least potent. These peptides were less potent than TTX, with none able to fully displace [^{3}H]-STX from rat or human brain (relative to TTX displacement). PIIIA and PIIIA(2-22) produced the largest displacement of [^{3}H]-STX, and GIIIA and GIIIC the least displacement. GIIIB was more effective at displacing [^{3}H]-STX from rat compared with human brain (Fig. 4A and B). All displacement curves were best fitted with a Hill slope of –1.

^{1}H NMR spectroscopy—PIIIA was examined by ^{1}H NMR spectroscopy in a range of different solvent conditions. In aqueous solution at pH 2.5–5.5 over 275–298 K, it was apparent that two conformations of PIIIA were present in a ~3:1 ratio. In aqueous solution at low pH over 283–298 K, the NH resonances of several residues, including 4–7, 10–12, 20 and 22, were broad, and that of Cys21 was not observable. At higher pH values and lower temperatures (275 K), these peaks sharpened (residues 4 and 5) or separated into two distinct sets of peaks (residues 6–7, 10–12, 20 and 22) so that complete assignment of the major and a partial assignment of the minor conformations was possible. The assignment of PIIIA was improved by the addition of up to 50% CD_3CN, where the set of peaks arising from the minor conformation was less evident, and all resonances from the major conformation were present. Chemical shift assignments for PIIIA are given in Table 2.

The two hydroxyproline (Hyp) residues in PIIIA are assigned as *trans* from the observation of strong Hδ-Hα_{i-1} NOEs in the case of Hyp8 and weak-medium Hδ-Hα_{i-1}, together with the stronger Hδ-Hα_{i-1} NOEs in the case of Hyp 18 (52). The minor conformation of PIIIA results from a *cis* conformation of Hyp8, indicated by a Hδ-Hα_{i-1} NOE to the preceding residue. PIIIA(2-22) was also examined under similar conditions and found to have almost identical chemical shifts and to adopt two conformations in proportions similar to those observed for PIIIA. The remainder of this paper describes the major conformation observed for both PIIIA and PIIIA(2-22), unless otherwise specified.

Secondary Hα shifts were used to examine the effects of solvent conditions on the backbone structure of PIIIA (Fig. 5). The shifts of PIIIA(2-22) are also shown. These results clearly indicate that the backbone conformation is the same over a range of pH and solvent conditions for the native and truncated sequences. Similarly, the differences in Hβ shifts for AMX-bearing sidechains, the Hδ shifts of the two Hyp residues and the Hα shifts of Gly6 remain largely unchanged over these conditions for PIIIA and PIIIA(2-22) (data not shown), indicating that the conformations of the sidechains are not significantly affected by changes in the solution environment. One exception is the Hβ protons of

Cys4, where the chemical shift differences between the Hβ2/Hβ3 protons increase with pH from 0.22 ppm at pH 3 to 0.66 ppm at pH 5. This is likely to arise from a ring current effect from an aromatic ring in proximity to the side chain of Cys4 at higher pH.

Comparison of the Hα shifts of the minor conformation of PIIIA show significant differences from residues 7–11, indicating differences in backbone conformation in these regions. This is supported by differences in Hβ shifts that are evident from residues 6–11, and also at Cys16. Due to low signal intensities, it was not possible to observe peaks for both Hβ and protons of Cys4, Cys5 and Cys21. The ring current effects observed for Hβ protons of the major conformations of PIIIA were not present in the minor conformations, indicating a difference in the positions of either Phe7 or His19 relative to Cys4.

Figure 5 also compares the secondary Hα shifts of PIIIA with those of GIIIB, which adopts the same structure in solution as GIIIA (20). Overall, the trends are similar, indicating that the global fold of PIIIA and GIIIB are similar, as may be expected based on their identical disulfide pairings and loop sizes (Fig. 1). However, differences observed at residues 5–11 and 19–20 indicate that in some regions significant structural divergence exists. To directly address potential structural differences, we determined the 3D structure of the major conformation PIIIA (see below). Interestingly, the secondary shifts of the minor conformation of PIIIA at residue 10 are more like those of GIIIB than the major form of PIIIA (Fig. 5), suggesting that the structure of the minor conformation of PIIIA is similar to the major conformation of GIIIB and GIIIA.

The local medium-range NMR data that provide information on the secondary structure of PIIIA are given in Fig. 6. The presence of several Hα-NH_{i+2}, NH-NH_{i+2} and Hα-NH_{i+3} NOEs are indicative of the presence of several turns over the entire peptide, and perhaps helix over residues 13–17. Although several long-range NOEs are present, these did not correspond to the long-range NOEs prescribing the β-hairpin of GIIIB (20). In fact, a number of long-range NOEs were present which preclude a corresponding β-hairpin in the major conformation of PIIIA.

At higher pH values (>4.0 at 293 K) in aqueous solution or in 50% aqueous CD_3CN, the hydroxyl proton of Ser13 sidechain was observed. This resonance sharpened considerably with the lowering of temperature (275 K in H_20; 260 K in CD_3CN) to reveal several medium-range NOEs to residues 15 and 16, indicative of a H-bond involving the sidechain of Ser13. These flanking residues apparently stabilize the position of Arg14, which has been shown to be crucial to the potency of PIIIA (26). No equivalent interaction has been observed previously for either GIIIA or GIIIB, although an Asp in the equivalent position could conceivably stabilize the crucial Arg 13 through the formation of a H-bond with Gln14 (GIIIA), or through a salt-bridge with Arg14 (GIIIB).

3D Structure of PIIIA—A total of 372 NOE-derived distance restrained (149 intraresidual, 98 sequential, 125 long/medium range) and 27 dihedral (16 ϕ and 11 χ1) were used to generate a set of 50 structures of PIIIA. Of these, 46 converged to a similar fold with no NOE violations greater than 0.2 Å and no dihedral violations greater than 3^o. The structural analysis and data indicating the quality of the structures are summarised in Table 3. From this it is apparent that the backbone structure is highly defined over residues 3–22, a conclusion that is supported by high average angular order parameters (S = 0.96) over this region for the ϕ and ψ backbone dihedral angles and low backbone RMSDs (Fig. 7A). Figure 8 shows an overlay of the 20 lowest energy structures, which indicate that PIIIA is dominated

by a series of turns over the N-terminal part of the molecule. From Ser13 to the C-terminus the structure adopts a distorted helix, with deviations from ideality at residues 18 and 19. Figure 8B shows the positions of the sidechains of residues 13–15, where it is clear that the exposure of Arg14 is facilitated by the proximity of Ser13 and Gln15. Analysis of the structures indicate the presence of hydrogen bonds between the sidechain oxygen of Ser13 and the backbone NH proton of Cys 15 (16/20), Gln15 (11/20), or the side chain amide protons of Gln15 (1/20 strcutures), which would assist in stablising this configuration.

Examination of the structures of PIIIA reveals that the positions of some sidechains are less precisely defined than others. Although there has been no quantitative investigation of the correlation between surface exposure and geometric precision within families of NMR-derived structures, it might be expected that more surface-exposed residues are less conformationally constrained than buried residues. To investigate this correlation for PIIIA, the surface area of each residue are compared with the heavy atom RMSDs for each residue in Fig. 7B. From this plot it is evident that surface exposure correlates with RMSD values ($r^2 = 0.83$ for all residues, $r^2 = 0.90$ for residues 3–22). This comparison provides an additional means of checking and comparing NMR-derived structures, beyond a comparison of RMSD values alone.

Discussion

The present study confirms that μ-conotoxins PIIIA and PIIIA(2-22) are potent blockers of neuronal VSSCs. It has been previously shown that PIIIA and GIIIA discriminate amongst subtypes of the TTX-sensitive VSSC found in rat brain (26). This discrimination now extends to PIIIA(2-22) and GIIIB in rat brain, and to all μ-conotoxins except GIIIA in human brain. These differences in potency and extent of inhibition of rat and human brain VSSCs arise from relatively small sequence differences, with positions 14 and 18 influencing to neuronal activity among the muscle-selective μ-conotoxins. Despite differential effects on neuronal TTX-S sodium channels in brain, GIIIA, GIIIB and GIIIC have similar potency at skeletal muscle VSSCs. In the peripheral nervous system, PIIIA and PIIIA(2-22) inhibit peripheral TTX-S VSSCs without significantly affecting the TTX-R sodium current in this tissue. Since μ-conotoxins have been shown to bind higher in the pore of Nav1.4 than TTX (53), it would appear that in addition to residue differences deep within the pore of the VSSC which render the channel TTX-R (54), additional changes occur further out in the pore to render TTX-R VSSCs insensitive to block by μ-conotoxins.

PIIIA and its analogue PIIIA(2-22) are the first μ-conotoxins shown to distinguish between transient and persistent TTX-sensitive subtypes. Selective inhibition of persistent over transient VSSCs may control seizures, where the accompanying slow persistent sodium currents might be blocked without affecting the transient action potentials (7). It has been postulated that the persistent sodium channels are the same as those that generate transient sodium currents, and that a small fraction of these channels enter a non-inactivating mode to generate the persistent sodium current (55, 56). This type of persistent current has been observed in cells lines transfected with cDNA for $Na_v1.6$ (11), Nav1.3 (13) or Nav1.2 (57). The persistent Na^+ current is also thought to play an important role in pacemaking currents and setting rythmicity in central neurons (58). During hypoxia or in the presence of free radicals (oxidative stress) these channels become more active (15, 17, 35), and could thus serve as a prominent pathway for Na^+ influx, triggering a cascade of damaging events which eventually cause cell damage and cell death (59). Hence, specific inhibitors of persistent Na^+ current may have neuroprotective effects. TTX,

lidocaine and quinidine can also inhibit persistent Na^+ channels without blocking transient Na^+ channels (14, 15). The μ-conotoxins extend the list of blockers able to discriminate between persistent and transient sodium currents.

Insights into the structure of the outer vestibule of the Nav1.4 channel have been obtained using the 3D structure GIIIA and GIIIB as molecular calipers (21–23). The fact that PIIIA is also able to block Nav1.4, indicates that many of the structural features found in GIIIA and GIIIB might also be conserved in PIIIA. However, additional structural differences must also exist to account for the affinity of PIIIA at both neuronal and muscle forms of TTX-sensitive VSSCs. The 3D structures of PIIIA are compared to those of GIIIA is shown in Fig. 9. Although the positions of the C-terminal regions overlap, and the positions of the functionally important Arg14 (Arg13 in GIIIA and GIIIB) are exposed in a similar manner, further comparison indicates a marked differences in the orientation of the N-terminal region to the end of loop 1 at Cys 11. In GIIIB, this loop was described as forming a distorted β-hairpin that was suggested to exist also in GIIIA (20). This structural feature is not present in the major conformation of PIIIA, where instead a series of loops exist. The structural difference between the major and minor forms arises from a differences at Hyp8 (Hyp7 in GIIIA), which adopts a predominately *trans* conformation in PIIIA, but a *cis* conformation in GIIIA and GIIIB (19, 20). Importantly, residues including Lys8/9 and Arg1/22, which have been shown in GIIIA to be of moderate importance to binding, are placed in an entirely different position in the major conformation of PIIIA (Fig. 9). However, the effects of the *cis/trans* isomerisation on the C-terminal region of PIIIA are minimal, with the conformation of the putatively important binding residues Arg14, Arg20 and Lys 17 not being significantly different from their GIIIA counterparts. The structural difference between the major forms of PIIIA, GIIIA and GIIIB are unexpected, given that these peptides share the same disulfide connectivity and loop sizes, and have considerable sequence homology. In contrast, the minor conformation of PIIIA, like GIIIA and GIIIB, arises from the *cis* form of Hyp8/7, indicating that it adopts a 3D structure more closely resembling GIIIA and GIIIB.

Comparison of the major conformation of PIIIA to a model of the minor conformation of PIIIA derived from the 3D structure of GIIIA (Fig. 10) reveals that the positions of several sidechains differ markedly between the two forms. Apart from the aforementioned structural differences at Arg2 and Lys9, the hydrophobic residues Leu3 and Phe7 are exposed to the solvent in the *cis* form, yet hug the surface in the *trans* form, providing a different surface profile. In addition, the sidechain of Cys4 lies above the plane of the His19 ring in the *trans* conformation (accounting for the ring current effects mentioned previously) but lies away from His19 in the *cis* form, despite the fact that the position of His19 is unchanged in either conformation. Thus, a simple *cis/trans* isomerisation not only effects the surface of this peptide, but somewhat surprisingly also alters the shape of part of the cysteine framework.

Conformational flexibility was proposed as a possible reason for the broadness of resonances associated with residues in the loop 2 of GIIIB (20). The present study shows that there are differing relative proportions and different rates of interconversion between the *cis/trans* forms. In GIIIA and GIIIB, it is apparent that the *cis* form predominates, with the *trans* form being masked by broadening associated with intermediate exchange occurring on the NMR time-scale. In PIIIA the *trans* for predominates, but the minor form is detectable because the two forms are in slow exchange. It is possible that the bulky Phe residue adjacent to Hyp8 in PIIIA acts to slow the rate of Hyp isomerisation. Two questions arise from the conformational heterogeneity found in PIIIA. Firstly, which of the possible μ-conotoxin conformations binds to the VSSC? Secondly, what role is played by these conformational differences in determining VSSC selectivity among μ-conotoxins? Given that the

broadened lines observed in GIIIA and GIIIB are indicative of alternative conformations, it is possible that a minor conformation of these muscle-selective μ-conotoxins binds to the VSSCs. If this is indeed correct, it could impact on studies investigating the structure of the outer vestibule of the VSSC using the currently available structures of μ-conotoxins.

Apart from Arg14, which has been shown to be important for the activity of PIIIA, it is not known which other residues in this peptide are involved in VSSC binding. An examination of the 3D structures and the surface profile (Figs 7B) PIIIA reveals residues that are on the surface (Fig. 9A), and are hence potentially available for interactions with the sodium channel. Along with Arg14, these include Lys17, Hyp18 and Arg20, which parallel residues Lys16, Hyp17 and Arg19 in GIIIA (Fig. 9B), thus defining a common pharmacophore, as previously suggested (26). Note that Ser13 is buried, consistent with it playing a structural role that ensures the exposure of Arg14. In GIIIA, the residue Asp12, which corresponds to Ser13 in PIIIA, may also play a structural role. The other exposed residues Lys9 and Arg2 have structural counterparts in GIIIA (Lys8 and Arg1) but adopt quite different positions in the predominant conformations of these two peptides.

It is interesting that the residues which differ between PIIIA and GIIIA cluster on one face of the peptide, perhaps forming a functionally significant pocket or cavity (Fig. 9 C–D). It is possible that one or more of these mostly hydrophobic and polar residues contribute to binding to the neuronal VSSCs, and thus confer broader specificity to PIIIA (and PIIIA(2–22)). Thus core residues, and the positioning of exposed residues that differ between the μ-conotoxins, may contribute to selectivity differences of μ-conotoxins at VSSCs. The results from this study show that the μ-conotoxin framework is less conformationally conserved than previously suspected, and illustrates the need for careful analysis of the range of structures this class of conotoxins can access. The structure of PIIIA described here provides a new molecular caliper for neuronal and muscle VSSCs.

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Table 1. Potency (pIC_{50}) and inhibition (%) of [^{3}H]-STX binding to VSSCs* by μ-conotoxins.

μ-Conotoxin	**Human brain**	**Rat brain**	**Rat muscle**
PIIIA	7.1 (7.6-6.6)	6.5 (6.8-6.3)	6.8 (7.1-6.5)
	47 (34-61)%	79 (68-91)%	81 (67-96)%
PIIIA(2-22)	6.6 (7.0-6.2)	6.4 (6.6-6.3)	7.2 (7.4-7.0)
	70 (58-82)%	90 (86-94)%	81 (73-93)%
GIIIA	inactive	5.1 (5.5-4.7)	7.1 (7.3-6.9)
		40 (28-52)%	82 (76-89)%
GIIIB	5.5 (6.4-4.5)	5.9 (6.2-5.7)	7.2 (7.5-7.0)
	36 (17-54)%	87 (72-101)%	85 (78-92)%
GIIIC	6.0 (6.6-5.4)	inactive	7.3 (7.5-7.1)
	30 (20-41)%		93 (86-101)%
TTX	7.6 (7.8-7.4)	7.8 (7.9-7.7)	7.8 (7.9-7.7)
	98 (91-105)%	100 (97-103)%	100 (96-104)%

*95% CI range for pIC_{50} and % inhibition given in parenthesis

Table 2. ^{1}H chemical shifts of PIIIA.*

Residue	NH	Hα	Hβ	Other
pGlu1	(7.98)	(4.29)[4.33]		
Arg2	(8.53)[8.59]	3.93 (4.25)[4.19]	1.79	Hγ 1.52; Hδ 2.92, 2.97; δNH 7.07
Leu3	8.47 (8.45)[8.48]	4.40 (4.41)[4.32]	1.57	Hδ 0.88
Cys4	8.41 (8.43)[8.43]	4.47 (4.58)	2.34, 2.56	
Cys5	7.97 (8.03)	4.14 (4.28)	3.0 , 3.47	
Gly6	8.38 (8.65)[7.84]	3.83 (3.81, 3.82) [3.63, 4.11]		
Phe7	7.38 (7.43)[8.35]	4.98 (5.05)[4.72]	2.98, 3.13	Hδ, Hε 7.19; Hζ 7.23
Hyp8	-	4.41 (4.49)[4.28]	2.05, 2.34	Hγ 4.67; Hδ 3.75, 3.90
Lys9	8.55 (9.02)[8.87]	3.98 (4.07)[4.11]	1.93	Hγ 1.34; Hδ 1.6; Hε 2.90; εNH_3 7.49
Ser10	7.78 (7.92)[8.63]	3.98 (4.05)[4.79]	3.82, 3.37	
Cys11	8.08 (8.25)[8.34]	4.39 (4.51)[4.62]	3.12, 2.91	
Arg12	7.50 (7.52)[8.43]	4.19 (4.28)[4.31]	1.84, 1.98	Hγ 1.66, 1.72; δNH 7.26
Ser13	7.73 (7.86)[7.90]	4.48 (4.53)[4.43]	3.99, 4.18	(OH 5.72)
Arg14	9.04 (9.19)[9.16]	3.95 (4.01)[3.99]	1.88	Hγ 1.67; Hδ 3.18; δNH 7.27
Gln15	8.51 (8.77)[8.72]	4.1 (4.15)[4.16]	2.01	Hγ 2.39, 6.84; δNH_2 7.46
Cys16	7.46 (7.62)[7.62]	4.81 (4.81)[4.88]	3.00, 3.18	
Lys17	8.06 (7.99)[8.06]	4.21 (4.20)[4.16]		Hγ 1.65, 1.70; Hδ 1.84
Hyp18	-	4.58 (4.70)	2.32, 1.79	Hγ 4.46; Hδ 3.74, 3.24
His19	8.18 (8.17)	4.37 (4.25)[4.24]	3.31, 3.34	Hε 7.35
Arg20	8.93 (9.22)[8.96]	4.03 (4.12)[4.11]	1.89	Hγ 1.70; Hδ 3.17; δNH 7.24
Cys21	8.49	4.47 (4.48)	3.11, 3.63	
Cys22	7.87 (8.13)	4.86 (4.91)[4.89]	3.11, 3.29	NH_2 7.30

*Values not in parenthesis are for the major conformation in acetonitrile:H_2O (1:1) at pH 3.0 and 280 K; values in round brackets (major conformation) and square brackets [minor conformation] in H_2O at pH 5.0 and 285 K.

Table 3. Geometric and energetic statistics for the 20 structures of μ-conotoxin PIIIA

Mean RMSDs from experimental restraints	
NOE (Å)	0.009 ± 0.002
dihedral (º)	0.21 ± 0.10
Mean RMSD from idealised covalent geometry	
bonds (Å)	0.0079 ± 0.0004
angles (º)	2.14 ± 0.06
impropers (º)	0.19 ± 0.02
Energies (kcal mol^{-1})	
E_{NOE}	0.92 ± 0.37
E_{cdih}	0.05 ± 0.04
E_{L-J}	–92.9 ± 3.4
$E_{bond} + E_{angle} + E_{improper}$	47.3 ± 3.1
Restraint violations	
mean NOE violation (Å)	0.029
maximum NOE violation (Å)	0.17
mean dihedral angle violation (º)	0.94
maximum angle violation (º)	2.45

Figure legends

Figure 1. Primary sequence of μ-conotoxins GIIIA, GIIIB, GIIIC (60) and PIIIA (26) with disulfide connectivity indicated. Standard one letter code used for amino acids, except pE is pyroglutamic acid, O is hydroxyproline. Common residues found important for binding in GIIIA (21) are boxed. PIIIA(2-22) is a truncated derivative of PIIIA in which the terminal pyroglutamate is absent.

Figure 2. Effects of PIIIA(2-22) and PIIIA on transient TTX-sensitive and TTX-resistant Na^+ currents recorded in nodose and DRG neurons. Na^+ currents recorded from (A) a nodose ganglia neuron and (B) a DRG neuron in control solution and in the presence of 0.1–10 μM PIIIA(2-22). The records shown in (B) were obtained in the continued presence of 1 μM TTX to measure effects on the TTX-R current. (C) Dose response relationship showing the effects of PIIIA(2-22) on the transient TTX-sensitive Na^+ current recorded from DRG neurons (○), and the effects of PIIIA on the transient TTX-sensitive Na^+ current recorded from nodose neurons (●). Each point represents the mean current amplitude from at least three cells obtained following a voltage step to –30 mV from a holding potential of –80 mV.

Figure 3. Effects of PIIIA(2-22) on the transient and persistent TTX-sensitive Na^+ currents recorded from hippocampal CA1 neurons. Na^+ currents recorded from a hippocampal CA1 neuron showing the effects of PIIIA(2-22) on the transient TTX-sensitive Na^+ current and the persistent Na^+ current. Inset shows the differential effects of PIIIA(2-22) on the amplitude of the transient and persistent Na^+ current. Peak amplitudes of the transient and persistent current was normalised to the transient and persistent currents recorded in control solution. Each point represents the mean current amplitude from at least three cells obtained following a voltage step to –30 mV from a holding potential of –80 mV (prepulsed to –130 mV for 300 ms).

Figure 4. Displacement of [^{3}H]-STX from brain and skeletal muscle VSSC by TTX and μ-conotoxins PIIIA, PIIIA(2-22), GIIIA, GIIIB and GIIIC. (A) Human brain, (B) rat brain, (C) rat skeletal muscle, and (D) neuronal versus skeletal muscle selectivity.

Figure 5. Secondary Hα shifts of PIIIA in (A) aqueous solution at pH 3; (B) PIIIA(2-22) in aqueous solution at pH 3; (C) PIIIA in 50% CD_3CN; (D) PIIIA in aqueous solution at pH 5.2 (E) minor conformation of PIIIA in aqueous solution at pH 3; and (F) GIIIB (20). The numbering is based on PIIIA residues and alignment on cysteine residues (see Fig. 1).

Figure 6. Local and medium-range NOE, $^3J_{NH\text{-}H\alpha}$ coupling constant, and slow exchange data for PIIIA (pH 3, 280 K, 100% D_2O). Open circles represent NH protons that are present 2 hr after addition of D_2O. Open squares, $^3J_{NH\text{-}H\alpha} \leq 6$ Hz; filled squares, $^3J_{NH\text{-}H\alpha} \geq 8.5$ Hz; partially filled squares $6 < {}^3J_{NH\text{-}H\alpha} < 8$ Hz. For NOE data, the height of bars indicates the strength of NOE. Open bars indicate peak overlap. Sequential NOEs involving $H\delta_i$-$H\alpha_{i-1}$ and $H\delta_i$-HN_{i+1} distances, where i = Hyp are represented in the dαN(i, i+1) and dNN(i, i+1) sections, respectively.

Figure 7. (A) Backbone angular order parameters for the ϕ and ψ didedral angles and average backbone RMSDs vs PIIIA residue number. (B) Heavy atom RMSDs and the percent surface exposure ($Å^2$) vs PIIIA residue number.

Figure 8. Structure of PIIIA. (A) Superimposition of the 20 lowest energy structures over the backbone region (residues 2-22) shown in stereo. (B) $180_y°$ rotation of the structures shown in (A) with the sidechains of Ser13 (pink), Arg14 (blue), Gln15 (purple), and Cys residues (orange) indicated.

Figure 9. Comparison of the positions of surface residues in (A) PIIIA and their counterparts in (B) GIIIA. Note that surfaced exposed residues are identical in PIIIA, and GIIIA and are all considered important for the potency of GIIIA to rSkM1 VSSCs.
(C) and (D) show the comparison of core residues in PIIIA and GIIIA, respectively. Core residues differ between these two peptides, and thus may contribute to selectivity differences at muscle and neuronal sodium channels. Sidechains shown are Leu/Thr (yellow), Hyp/Ser (pink), Arg/Lys (dark blue), His (light blue); Gln (purple), Phe (brown), and Asp (red).

Figure 10. Comparison of the major conformation of PIIIA to a modeled structure of its minor conformation. The minor conformation was modeled from the structure of the major conformation of GIIIB (20). Structures are superimposed for Arg12 to Cys22. Side chains are labeled as in Fig. 9.