Articles

αC-Conotoxin PrXA: A New Family of Nicotinic Acetylcholine Receptor Antagonists†

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ABSTRACT: We have purified a novel paralytic peptide with 32 AA and a single disulfide bond from the venom of *Conus parius*, a fish-hunting species. The peptide has the following sequence: TYGIYDAK-POFSCAGLRGGCVLPONLROKFKE-NH_2, where O is 4-*trans*-hydroxyproline. The peptide, designated αC-conotoxin PrXA (αC-PrXA), is the defining member of a new, structurally distinct family of *Conus* peptides. The peptide is a competitive nAChR antagonist; all previously characterized conotoxins that competitively antagonize nAChRs are structurally and genetically unrelated. (Most belong to the α- and αA-conotoxin families.) When administered to mice and fish *in vivo*, αC-PrXA caused paralysis and death. In electrophysiological assays, αC-PrXA potently antagonized mouse muscle nicotinic acetylcholine receptors (nAChRs), with IC_{50} values of 1.8 and 3.0 nM for the adult ($\alpha1\beta1\epsilon\delta$ subunits) and fetal ($\alpha1\beta1\gamma\delta$ subunits) muscle nAChR subtypes, respectively. When tested on a variety of ligand-gated and voltage-gated ion channels, αC-PrXA proved to be a highly specific inhibitor of the neuromuscular nAChR. The peptide competes with α-bungarotoxin for binding at the α/δ and α/γ subunit interfaces of the nAChR, with higher affinity for the α/δ subunit interface. αC-PrXA is strikingly different from the many conopeptides shown to be nicotinic antagonists; it is most similar in its general biochemical features to the snake toxins known as Waglerins.

Nearly 35 years ago, the first venom peptides (conotoxins) purified from the fish-hunting cone snail species, *Conus geographus and Conus magus*, proved to be competitive antagonists of the nicotinic acetylcholine receptor (nAChR[1]) at the neuromuscular junction (*2*, *3*). Those small, multiply disulfide-bonded peptides were called α-conotoxins, on the basis of their pharmacological similarity to the previously characterized α-neurotoxins from snake venoms, such as α-bungarotoxin, isolated from the venom of *Bungarus multicinctus* (the Taiwanese banded krait) (*4*). Since those early discoveries, additional families of multiply disulfide-bonded conotoxins have proven to be competitive antagonists of the neuromuscular nAChR: the αA-conotoxins (*5*−*8*) and one novel peptide with 5 disulfide bonds, αS-conotoxin RVIIIA (*9*).

In this work, we report the purification and characterization of a singly disulfide-bonded peptide from the venom of *Conus parius* that defines a new family of *Conus* peptides (αC-conotoxins) targeted to the vertebrate muscle nAChR. Characterization of the peptide, named αC-conotoxin PrXA (αC-PrXA), revealed that it is a highly potent and selective competitive inhibitor of the neuromuscular nAChR.[2]

The purification of a new class of nAChR inhibitors from the venom of *Conus parius* was unexpected. We have noted previously that species closely related to *Conus parius* (e.g., *Conus radiatus*) do not appear to have the typical conotoxin antagonists of neuromuscular nAChRs (α-conotoxins and αA-conotoxins) (*9*) in their venoms; the identification of an unusual neuromuscular nAChR antagonist, αS-conotoxin

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[1] Abbreviations: ACh, acetylcholine; ACN, acetonitrile; C.I., confidence interval; DTT, dithiothreitol; i.c., intracranial; i.m., intramuscular; i.p., intraperitoneal; MALDI, matrix-assisted laser desorption ionization; MTBE, methyl-*tert*-butyl ether; nAChR, nicotinic acetylcholine receptor; N-Fmoc, *N*-(9-flourenyl)methoxycarbonyl; SE, standard error; TFA, triflouroacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

[2] The peptide characterized in this article has been designated as αC-PrXA; subsequently, all characterized peptides that belong to the rapidly diversifying conoidean gene superfamily with a single disulfide bond will be designated with the number 10 (if the target is not yet known) or X (if the target has been defined). This nomenclature will not be used for conopeptide families that are not rapidly diversifying, such as conopressins and contryphans. The conopeptide mr10a, which was described earlier (*1*), has been renamed mr1a; all conoidean peptides with the Cys pattern −CC−C−C− will be referred to with the number 1 or 2 (or I/II), regardless of the gene superfamily that encodes them.

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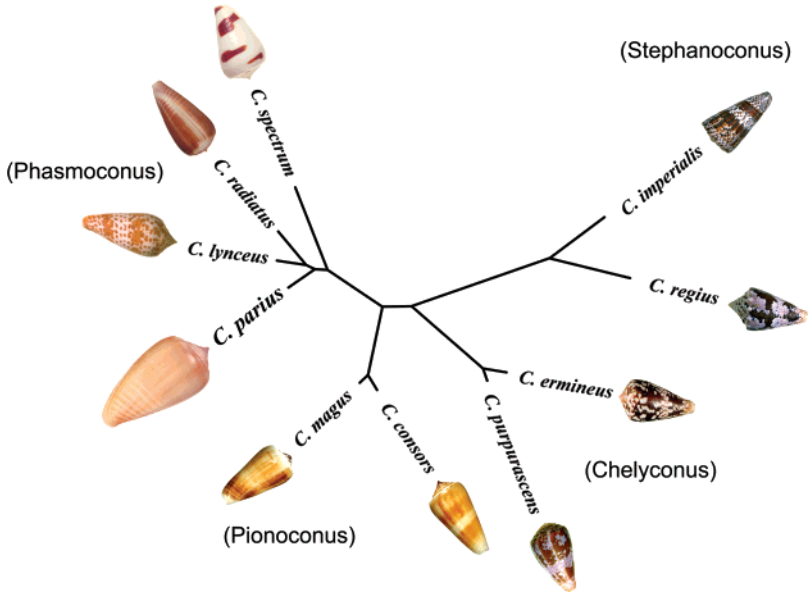

FIGURE 1: Molecular phylogeny of *Conus parius*. Shown are four different clades of *Conus*. *Conus parius*, the species used in this analysis, belongs to one of the four clades shown, *Phasmoconus*, which appear to be primarily fish-hunting snails. Two other fish-hunting clades, *Pionoconus* (e.g., *Conus magus*) and *Chelyconus* (e.g., *Conus purpurascens*) are shown as well as one worm-hunting clade, *Stephanoconus* (e.g., *Conus imperialis*). In *Pionoconus*, *Chelyconus*, and *Stephanoconus*, all of the known paralytic nicotinic antagonists are members of the A-superfamily. This article demonstrates that in *Conus parius*, a novel superfamily is involved in nicotinic antagonism.

RVIIIA (*9*) led us to expect that we would find an αS-conotoxin in *Conus parius*. Despite the expectation, it appears that this clade of fish-hunting cone snails (*Phasmoconus;* see Figure 1) has evolved at least two classes of structurally unrelated nAChR antagonists to facilitate prey capture and/or defense: the αS- and αC-conotoxins.

MATERIALS AND METHODS

Purification of αC*-PrXA.* Venom ducts of *Conus parius* were dissected from the cone snails as described previously (*10*). The collected venom ducts were lyophilized and stored at −80 °C. Fifteen lyophilized venom ducts were ground over liquid nitrogen using a mortar and pestle. Venom was extracted sequentially with 5 mL of $H_2O$and 3 mL each of 20% acetonitrile (ACN), 40% ACN, and 60% ACN. The venom suspension was sonicated in each extracting solvent for five 30-s periods at 0 °C and centrifuged at 5000*g* for 5 min at 4 °C. The combined supernatant (crude venom extract) was lyophilized and stored at −20 °C until further purification.

The crude venom extract was resuspended in a mixture of 0.1% triflouroacetic acid (TFA) (solvent A) and 90% ACN in 0.085% TFA (solvent B) and applied into a Vydac C18 analytical column (4.6 × 250 mm, 5 μm particle size). The peptide was eluted with a gradient of ACN using solvents A and B. The effluents were monitored at 220 nm. A major peak containing the peptide was purified by two more analytical HPLC runs. The purified peptide solution was lyophilized and stored at −20 °C.

Peptide Sequencing. The purified peptide (2 nmol) was dissolved in 500 μL of a mixture of 80% solvent A and 20% solvent B. The pH was adjusted to 7.5 with 0.5 M tris(hydroxymethyl)-aminomethane (Tris) base before the addition of dithiothreitol (DTT) to a final concentration of 10 mM. The solution was flushed with argon, incubated at 65 °C for 20 min, and cooled to room temperature. Then, 4-vinylpyridine (2 μL) was added to the solution. The tube was wrapped in aluminum foil and incubated at room temperature for 25 min. The peptide solution was diluted with 500 μL of solvent A and applied into a Vydac C18 analytical column. The purified alkylated peptide was sequenced using standard Edman chemistry (*11*) on an Applied Biosystem Model 492 Sequenator, courtesy of Dr. Robert Schackmann of the DNA/Peptide Facility of the University of Utah. The 3-phenyl-2-hydantoin (PTH) derivatives were identified by HPLC.

Peptide Synthesis. Linear αC-PrXA was synthesized on solid support in an ABI Model 430A Peptide Synthesizer using standard N-Fmoc (*N*-(9-flourenyl)methoxycarbonyl) chemistry, courtesy of Dr. Robert Schackmann of the DNA/Peptide Facility of the University of Utah. Peptide cleavage was done by treating 25 mg of peptide resin with 500 μL of reagent K (82.5% TFA, 5% H_2O, 5% thioanisol, 2.5% 1,2-ethanedithiol, and 5% phenol) with continuous agitation for 1 h at room temperature. The mixture was filtered under vacuum into cold methyl-*tert*-butyl ether (MTBE). The linear peptide was collected by centrifugation at 5,000*g* for 5 min. The pellet was washed with MTBE, centrifuged again, and dissolved in 10% ACN in 0.1% TFA. The linear peptide was purified on a Vydac C_{18} semipreparative HPLC column (10 × 250 mm, 5 μm particle size) with a gradient of ACN using solvents A and B at a flow rate of 5 mL/min.

Oxidative folding of the linear peptide was done by gradually adding the purified linear peptide solution (∼15 mL) to 25 mL of 1 mM I_2 in 90% solvent A and 10% solvent B mixture with continuous stirring for about 1 min at room temperature. The reaction was quenched by adding 1 mL of 0.1 M ascorbic acid. The oxidized peptide solution was diluted up to 50 mL with solvent A and purified on a C18 semipreparative column with a gradient of 15−40% solvent B for 25 min at a flow rate of 5 mL/min.

Mass Spectrometry. Matrix-assisted laser desorption ionization (MALDI) mass spectra were obtained at the Mass Spectrometry and Proteomic Core Facility of the University of Utah using a Voyager DE STR mass spectrometer.

Biological Assays. The peptide was dissolved in normal saline solution (NSS) and administered to mice and fish using 29-gauge insulin syringes as described earlier (*12*). Swiss Webster mice (18 days old, 11.8−12.4 g) were injected intracranially (i.c.) and intraperitoneally (i.p.), whereas gold fish (∼1.0 g) were injected i.p. and intramuscularly (i.m.). Control mice and fish were similarly injected with NSS. Proper animal care and use protocols were followed in accordance with the guidelines set by the University of Utah Institutional Animal Care and Use Committee.

Heterologous Expression of Receptors in Xenopus Oocytes. Steven M. Sine, Mayo Clinic College of Medicine, USA, provided mouse muscle nAChR clones in the CMV-based pRBG4 vector ((*13*, *14*) and references therein). Stephen F. Heinemann, The Salk Institute, USA, provided the majority of rat neuronal nAChR clones ((*15*) and references therein). The α9 and α10 neuronal nAChR subunit clones were provided by A. Belén Elgoyhen, Universidad de Buenos Aires, Argentina (*16*, *17*). Human neuronal nAChR clones were provided by Jim Garrett, Cognetix, Inc., USA. NMDA receptor clones were provided by Michael Hollmann, Ruhr-Universität Bochum, Germany. Voltage-gated sodium channel clones were provided by Alan Goldin, University of California Irvine, USA.

With the exception of the mouse muscle nAChR clones, all of the expression clones were used to make capped RNA (cRNA) for injection into the oocytes of *Xenopus laevis* frogs. The harvesting of *Xenopus* oocytes was described previously in detail (*15*). cRNA was prepared using *in vitro* RNA transcription kits from Ambion, Inc, according to the manufacturer's protocols. For expression of neuronal nAChRs and NMDA receptors, typically 5 ng of each subunit cRNA was injected per oocyte. For expression of skeletal muscle nAChRs, 1 ng of each subunit cDNA was injected into the nucleus of each oocyte. The plasmid constructs encoding mouse muscle nAChR subunits contain genes expressed from a CMV promoter. Oocyte recordings were obtained 1−6 days postinjection.

Electrophysiology. Voltage-clamp recording of *Xenopus* oocytes was conducted as described in detail previously (*15*). Briefly, oocytes were voltage clamped at −70 mV while gravity perfused with ND96 buffer (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES at pH 7.2−7.5). One micromolar atropine was added to ND96 buffer to block endogenous muscarinic acetylcholine receptors for all nAChR recordings, with the exception of α7 nAChRs, which atropine inhibits (*18*). Bovine serum albumin (BSA) was added to the ND96 buffer at a final concentration of 0.1 mg/mL to reduce the nonspecific adsorption of peptide. $MgCl_2$ was not included in the ND96 buffer used for testing NMDA receptors because Mg^{2+} blocks NMDA receptors. For ligand-gated ion channels (nAChRs and NMDA receptors), currents were elicited by 1-s pulses of gravity-perfused agonist solution: 100 μM ACh for nAChRs and 200 μM glutamate and 20 μM glycine for NMDA receptors. Currents were elicited from voltage-gated sodium channels by a 50 ms step to −10 mV. All data recordings were conducted at room temperature. Data acquisition was automated by a virtual instrument made by Doju Yoshikami of the University of Utah.

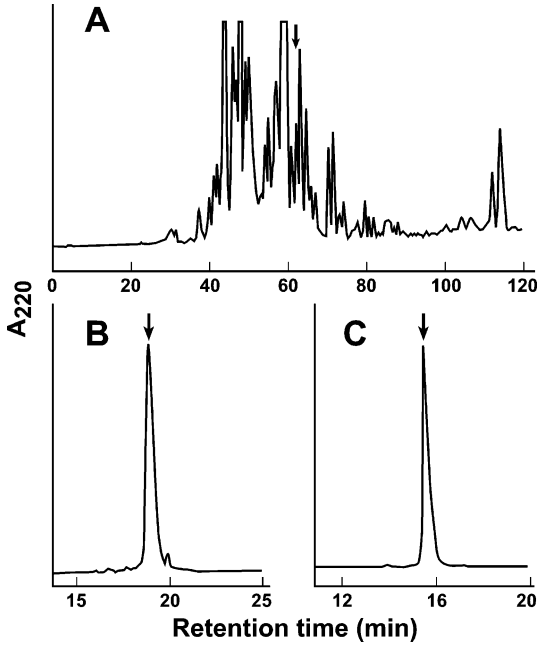

FIGURE 2: Purification of αC-PrXA by reversed-phase HPLC. (A) Fractionation of crude venom extract in a Vydac C_{18} analytical column with a gradient of 0−60% solvent B (90% ACN in 0.085% TFA) over 120 min at a flow rate of 1 mL/min. The solid arrow indicates the fraction containing αC-PrXA. (B) Elution of the fraction indicated by an arrow in A using the same column at 20−45% solvent B over 25 min with a flow rate of 1 mL/min. (C) Elution of the fraction indicated by an arrow in B using the same column with a gradient of 20−60% B over 20 min at a flow rate of 1 mL/min.

T*o* identify the effect of the peptide on a particular receptor subtype, a predetermined concentration of αC-PrXA was applied to an oocyte in a static bath for 5−10 min to allow the peptide to reach equilibrium with receptors. The amplitude of the elicited current, following the peptide application and equilibration period, was calculated as a percentage of the amplitude of the elicited current prior to toxin application. A 10-min equilibration period was used for all peptide concentrations in generating dose−response curves. Dose−response curves were generated using Prism software (GraphPad Software, Inc.), with the following equation, where *nH* is the Hill coefficient, and IC_{50} is the concentration of peptide causing half-maximal block: $\%\ \text{response} = 100/\{1 + ([\text{peptide}]/IC_{50})^{nH}\}$.

RESULTS

Purification and Biochemical Characterization of αC-PrXA. A venom fraction from *Conus parius* caused uncoordinated movement and subsequent paralysis when injected i.c. in mice. The major peptide component of this fraction was purified as described in Materials and Methods. The purification of this peptide, which involved three sequential steps by reversed-phase HPLC, is shown in Figure 2. The purified peptide (designated αC-conotoxin PrXA after its physiological characterization; see following sections) was sequenced by Edman chemistry, which revealed the following primary sequence of 32 amino acid residues: TYGIY-DAKPOFSCAGLRGGCVLPONLROKFKE, where O = 4-*trans*-hydroxyproline. Mass spectrometry indicated an

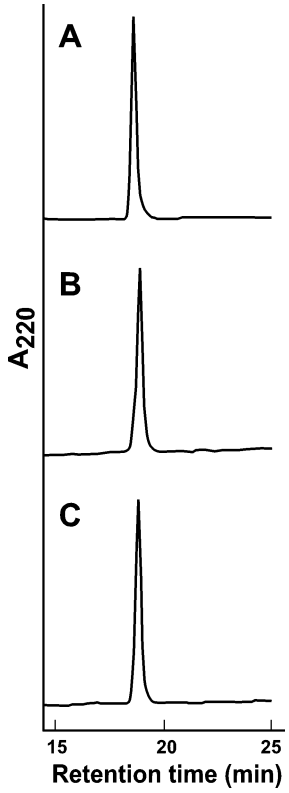

FIGURE 3: HPLC coelution of synthetic and natural αC-PrXA. Elution of the peptide: (A) synthetic, (B) natural, and (C) mixture of synthetic and natural, using a C_{18} analytical column with a gradient of 20−45% B for 25 min at a flow rate of 1 mL/min.

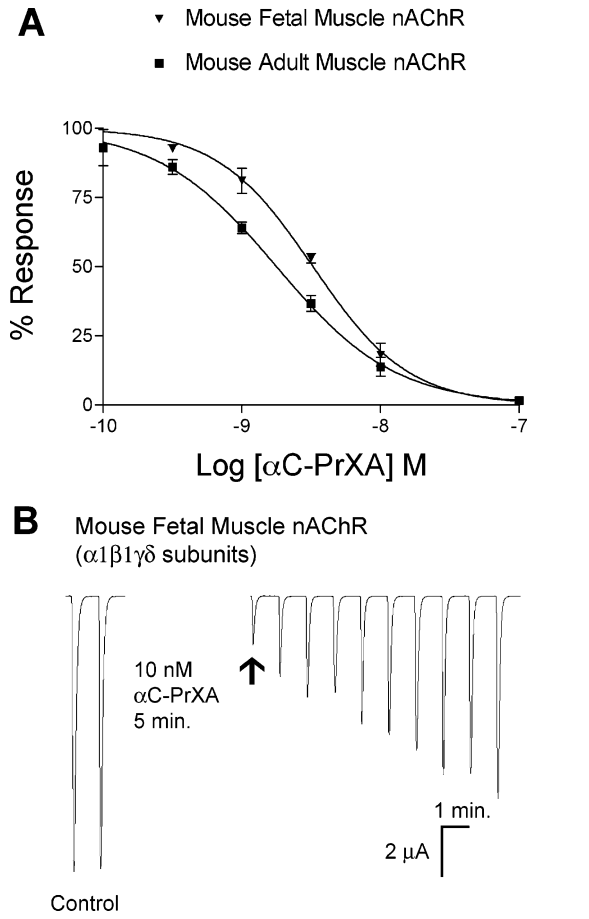

FIGURE 4: Inhibition of mouse fetal and adult muscle nAChRs by αC-PrXA. (A) Dose−response curves were generated from two-electrode voltage clamping *Xenopus* oocytes as described in Materials and Methods. The peptide was applied to oocytes expressing either the mouse fetal muscle nAChR ($\alpha 1\beta 1\gamma\delta$ subunits) or mouse adult muscle nAChR ($\alpha 1\beta 1\epsilon\delta$ subunits) at various concentrations. Each peptide concentration was tested on three different oocytes to generate the average percent response shown (±SE). Percent response for each test was determined by comparing ACh-elicited current amplitude before and after peptide application. αC-PrXA blocked the mouse fetal muscle nAChR with an IC_{50} of 3.3 nM (2.8−3.7 nM, 95% C.I.) and the mouse adult muscle nAChR with an IC_{50} of 1.8 nM (1.5−2.2 nM, 95% C.I.). (B) Representative current traces for 10 nM αC-PrXA on the $\alpha 1\beta 1\gamma\delta$ nAChR. Control traces are shown prior to the application of peptide. The arrow marks the first current trace elicited after a 5-minute application of peptide. Subsequent current traces show peptide dissociation and washout.

average mass of 3540.7, implying that the C-terminus was amidated (calculated average mass = 3541.1). The sequence was confirmed by identification of a cDNA encoding this peptide (Garrett and Olivera, unpublished data).

Synthesis of αC-PrXA. The peptide was synthesized, and the single disulfide bridge was formed by oxidative folding as described in Materials and Methods. The oxidized peptide was compared to the natural peptide in terms of mass and HPLC co-elution. As shown by MALDI mass spectrometry, the synthetic peptide has an average mass of 3540.9 consistent with that of the natural peptide (average mass = 3540.7). When co-injected into a C18 analytical HPLC column, the natural and synthetic peptides co-eluted as shown in Figure 3. The mass spectrometry and co-elution data confirmed the native peptide sequence, including post-translational modifications (4-*trans*-hydroxyproline and amidated C-terminus), and demonstrated that the native and synthetic peptides were chemically identical.

Biological Activity. When αC-PrXA was administered i.c. or i.p. to 18-day-old Swiss Webster mice, ~90 pmol/g body weight caused uncoordinated movement and paralysis followed by death ~30 min after injection. When injected i.p. in goldfish, 1 nmol/g body weight caused paralysis that led to lethality ~10 min after injection; when administered i.m. in fish, 400 pmol/g body weight caused paralysis that resulted in lethality ~35 min after injection. Similar symptoms were observed for both the synthetic and natural peptides.

Electrophysiological Characterization. Because of the paralytic effects observed upon injection into fish and mice, we tested αC-PrXA for the block of mouse adult muscle nAChRs ($\alpha 1\beta 1\epsilon\delta$ subunits) and mouse fetal muscle nAChRs ($\alpha 1\beta 1\gamma\delta$ subunits) expressed in *Xenopus* oocytes. Consistent with its paralytic effects *in vivo*, αC-PrXA potently inhibited the mouse adult and fetal muscle nAChRs *in vitro*, with IC_{50} values of 1.8 and 3.3 nM, respectively (Figure 4). In contrast to the nanomolar affinity for vertebrate muscle nAChRs, 10 μM αC-PrXA had little effect on a variety of neuronal nAChRs, NMDA receptors, and voltage-gated sodium channels tested (IC_{50} values ≫10 μM in all cases), suggesting that αC-PrXA is highly specific for muscle nAChRs (Figure 5).

Because αC-PrXA belongs to a new family of neuromuscular nAChR antagonists, we conducted experiments to determine whether the peptide is a competitive inhibitor at the ACh binding interfaces between the α/δ and α/γ subunits of the nAChR. Many antagonists of neuromuscular nAChRs such as α-bungarotoxin are known to compete with ACh

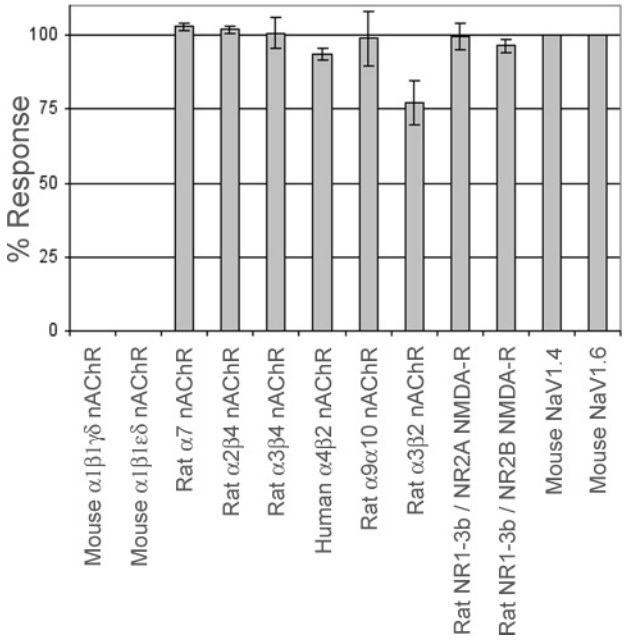

FIGURE 5: αC-PrXA is highly selective for muscle nAChRs. Each bar indicates the average percent response (±SE) after the application of 10 μM αC-PrXA to *Xenopus* oocytes expressing a variety of nAChRs, NMDA receptors, or voltage-gated sodium channels (NaV). To determine the average percent response, the peptide was tested ≥3 times against each receptor subtype. In contrast to the low nanomolar affinity of αC-PrXA for muscle nAChRs (see Figure 4), it demonstrated ≫10 μM IC_{50} values for all other receptors/ion channels tested.

for binding (*4*, *19*). In Figure 6, we demonstrate that αC-PrXA competes with α-bungarotoxin for binding. In this assay, we exploited differences in dissociation rates from the mouse α1β1$\gamma\delta$ nAChR in order to show that pre-block of the nAChR with excess αC-PrXA prevented the very slow dissociation rate characteristic of α-bungarotoxin binding. The sigmoidal shape of the dissociation curves generated in Figure 6 (A and B) also indicated that the peptide binds two interfaces, at least at the high peptide concentration tested. Thus, we conclude that αC-PrXA is a competitive inhibitor at both the α/δ and α/γ interfaces.

We also conducted experiments to identify any significant differences in affinity between binding interfaces. To do so, we expressed muscle nAChRs lacking individual subunits in *Xenopus* oocytes: α1β1δ, α1β1γ, or α1β1ϵ. As shown in Figure 7, αC-PrXA demonstrated the highest affinity for the α/δ interface. In fact, 100 nM αC-PrXA blocked nearly all of the current from the α1β1δ receptor, similar to its block of wild-type α1β1$\gamma\delta$ and α1β1$\epsilon\delta$ nAChRs at the same concentration (see Figures 4 and 7). αC-PrXA at 1 μM concentration also blocked almost all of the current from the α1β1γ receptor, which supported the result indicating competitive binding at both α/δ and α/γ interfaces. However, 100 nM αC-PrXA blocked the α1β1γ nAChR less potently than α1β1δ nAChR (Figure 7). At least part of the difference in potency was due to a difference in peptide dissociation rates (Figure 7, B and C). Because the dose−response curves in Figure 4 were generated primarily with αC-PrXA concentrations ≤10 nM, binding at the α/γ interface presumably had little effect on the toxin potency observed for the α1β1$\gamma\delta$ nAChR dose−response curve. Thus, we conclude that the nearly identical dose−response curves for both fetal (α1β1$\gamma\delta$ subunits) and adult (α1β1$\epsilon\delta$ subunits) nAChRs were generated primarily through the high-affinity inhibition of the α/δ interface that is shared by both adult and fetal receptor subtypes. Interestingly, αC-PrXA demonstrated less potency

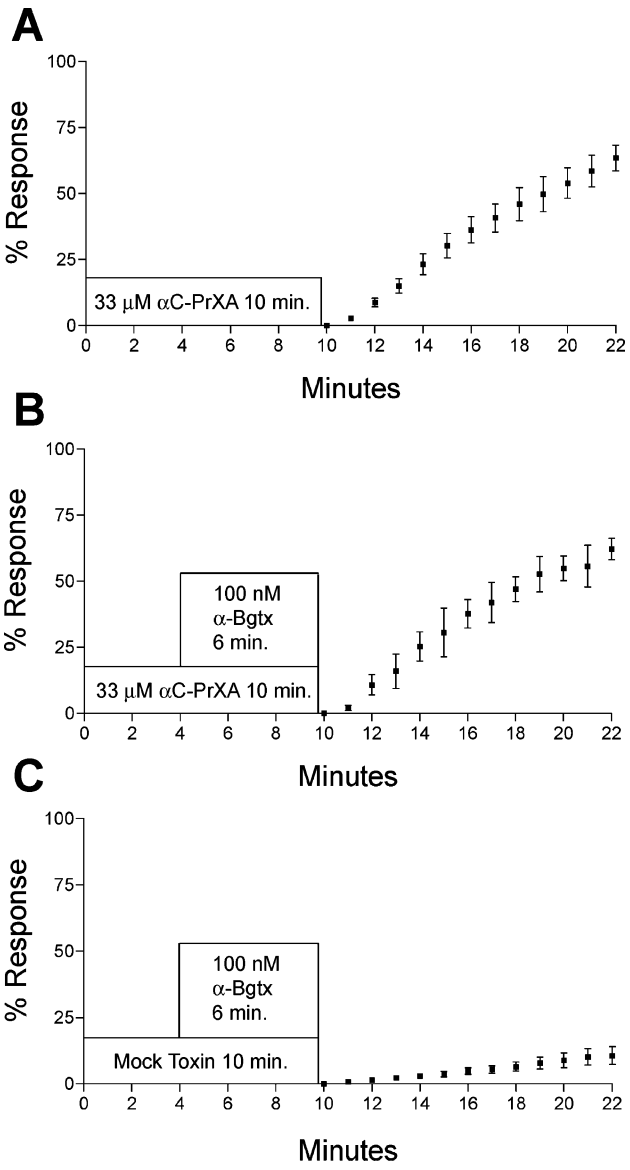

FIGURE 6: αC-PrXA competes with α-bungarotoxin for binding the α1β1$\gamma\delta$ muscle nAChR. (A) αC-PrXA was applied to oocytes expressing the α1β1$\gamma\delta$ muscle nAChR in a static bath for 10 min at a final concentration of 33 μM. The peptide completely blocked ACh-elicited currents. Following the block, ND96 buffer was perfused continuously over the oocyte for 12 min, with the exception of 1 s pulses of ACh once per minute to elicit currents. The data points shown are the average responses of ACh-elicited currents as percent of controls (±SE, $n = 4$). These data points are indicative of the dissociation rate for αC-PrXA. (B) αC-PrXA was applied to oocytes expressing the α1β1$\gamma\delta$ muscle nAChR in a static bath for 10 min at a final concentration of 33 μM. During the final 6 min of this equilibration period, α-bungarotoxin was applied to the same oocytes at a final concentration of 100 nM. Following the block, the data points shown are the average responses of ACh-elicited currents as percent of controls (±SE, $n = 6$). The dissociation rate appeared to be the same as that in A. (C) ND96 buffer was applied to oocytes expressing the α1β1$\gamma\delta$ muscle nAChR for 10 min in a static bath as a mock toxin. During the final 6 min of this equilibration period, α-bungarotoxin was applied to the same oocytes at a final concentration of 100 nM. Following the block, the data points shown are the average responses of ACh-elicited currents as percent of controls (±SE, $n = 6$). In this case, the dissociation rate was indicative of the much more slowly dissociating α-bungarotoxin.

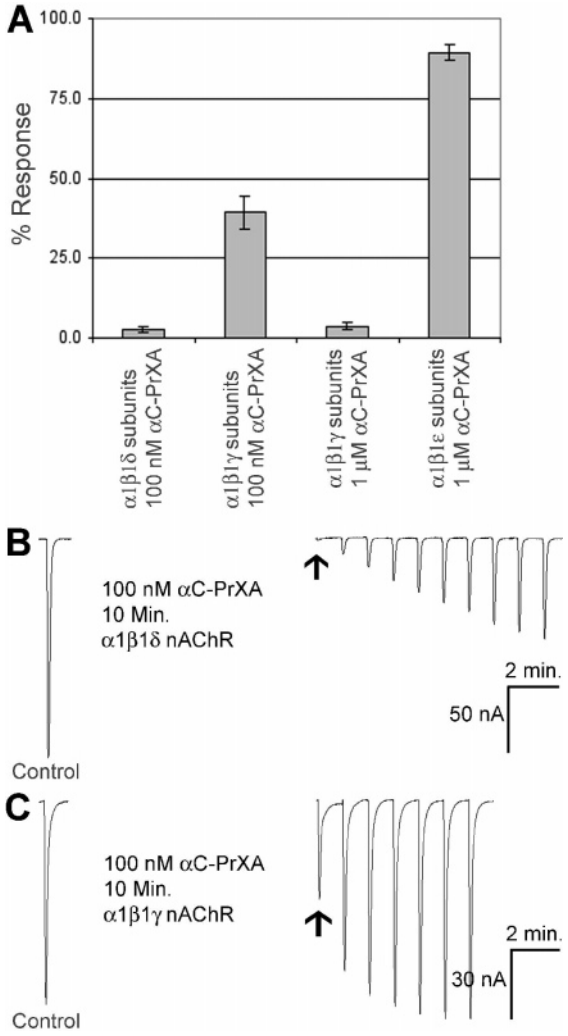

FIGURE 7: αC-PrXA demonstrates the highest affinity for the α/δ interface of the muscle nAChR. (A) Muscle nAChRs lacking a particular subunit (e.g., α1β1δ, α1β1γ, or α1β1ε) were expressed in *Xenopus* oocytes. Each bar shows the average response (±SE) for the respective peptide concentrations and receptor subunits indicated in the Figure ($n \geq 4$ tests for each bar). (B and C) Representative current traces. The arrow marks the first current trace elicited after a 10-min application of peptide. Subsequent current traces show peptide dissociation and washout. (B) 100 nM αC-PrXA blocked nearly all current from the α1β1δ nAChR and dissociated slowly; (C) 100 nM αC-PrXA partially blocked current from the α1β1γ nAChR and dissociated rapidly.

on the α1β1ε nAChR than the α1β1γ nAChR, as shown in Figure 7.

DISCUSSION

The discovery and characterization of αC-conotoxin PrXA has revealed an unexpected functional convergence in the evolution of structurally distinct conotoxin families. Conotoxin families are defined by two criteria: (1) They share a common arrangement of cysteine residues in their primary amino acid sequences called the Cys pattern (e.g., α-conotoxin family Cys pattern: −CC−C−C−) forming a conserved disulfide-bonding framework (e.g., α-conotoxin disulfide-bonding framework: Cys1-Cys3, Cys2-Cys4). (2) They target a common family of receptors or ion channels. Thus, members of a conotoxin family are both structurally and functionally similar. For example, members of the α-conotoxin family inhibit skeletal-muscle and/or neuronal nAChR isoforms (*20*). In fact, the vast majority of nAChR antagonists found in *Conus* venoms belong to the α-conotoxin family (see Table 1), which is broadly distributed throughout the entire *Conus* genus.

Conotoxin families belong to larger superfamilies of genetically related peptides that typically share a single Cys pattern (or two), although the gene products (peptides) may have diverged functionally into different conotoxin families with dissimilar targets. For example, the A-superfamily comprises an extensive group of genetically related conotoxins with demonstrable homology in their precursor sequences. In addition to the α-conotoxins, the A-superfamily includes peptides that have added an additional disulfide bond to the α-conotoxin framework. These peptides, αA- and κA-conotoxins, share a common Cys pattern (−CC−C−C−C−C−) but target nAChRs and K^+ channels, respectively (examples of αA-conotoxins are shown in Table 1). For an overview of conotoxin families and superfamilies see ref *21*.

The characterized αA-conotoxins were all discovered in fish-hunting cone snail venoms. We had therefore expected that venom peptides targeted to paralyze prey by blocking nicotinic receptors at neuromuscular junctions would generally be A-superfamily peptides (α- or αA-conotoxins) in all *Conus*. Even distantly related *Conus* species such as *Conus imperialis* (that belongs to the *Stephanoconus* clade, and hunts amphinomid worms, fireworms), which probably diverged from fish-hunting *Conus* early in the evolutionary history of the genus, apparently use peptides of the α-conotoxin family to block nicotinic receptors at the neuromuscular junctions of their annelid prey. Thus, we had not expected to discover a nicotinic antagonist encoded by an entirely different gene superfamily with 10 Cys residues and 5 disulfide bonds, αS-conotoxin RVIIIA from the fish-hunting species *Conus radiatus* (in the *Phasmaconus* clade) (*9*).

αC-PrXA shares the targeting selectivity of the α-, αA-, and αS-conotoxins for nAChRs, and therefore has the designation α in its conotoxin family name, like snake α-neurotoxins. However, it has a different Cys pattern and disulfide framework than any of the previously characterized A-superfamily (e.g., α- and αA-conotoxins) or S-superfamily (e.g., αS-conotoxins) peptides, indicating that it belongs to an entirely different conotoxin family and superfamily. Thus, it is the defining member of the αC-conotoxin family (C-superfamily).

The evidence we provide above that *Conus parius* a species closely related to *Conus radiatus* in the *Phasmoconus* clade uses an unrelated peptide that belongs to a third gene superfamily as a major nicotinic antagonist in its venom was particularly unexpected. The αS- and αC-peptides from closely related species are not at all structurally similar: S-superfamily peptides have five disulfide bonds; in contrast, the C-superfamily peptide has a single disulfide linkage. Thus, although they are both paralytic to fish and mice, they differ dramatically in their biochemistry and yet come from very closely related species (see Figure 1). Given the fact that A-superfamily peptides are the major paralytic nicotinic antagonists in all other clades of *Conus* examined, two unrelated non-A superfamily nicotinic antagonists in the *Phasmconus* species suggest unusual evolutionary pressures on this clade of *Conus*. There are A-superfamily peptides that have the disulfide patterns of α-conotoxins in these *Conus* species (Teichert, R., unpublished work). However,

Table 1: Some Competitive Inhibitors of Vertebrate Muscle nAChR[a]

A. Comparison of αC-PrXA to other Conopeptides

Conus species (Clade)	peptide	amino acid sequence	super-family	ref
C. magus (*Pionoconus*)	α-MI	GR**C**C**C**HPA**C**GKNYS**C**[b]	A	(*3*)
C. geographus (*Gastridium*)	α-GI	E**C**C**C**NPA**C**GRHYS**C**[b]	A	(*2*)
C.purpurascens (*Chelyconus*)	α-PIB	ZSOG**C**C**C**WNPA**C**VKNR**C**[b]	A	(*26*)
C. obscurus (*Gastridium*)	αA-OIVA	**C**C**C**GVONAA**C**HO**C**V**C**KNT**C**[b]	A	(*7*)
C. ermineus (*Chelyconus*)	αA-EIVA	G**C**C**C**GPYONAA**C**HO**C**G**C**KVGROOY**C**DROSGG[b]	A	(*6*)
C. radiatus (*Phasmoconus*)	αS-RVIIIA	K**C**NFDK**C**KGTGVYN**C**GγS**C**S**C**γGLHS**C**R**C**TYNIGSMKSG**C**A**C**I**C**TYY[c]	S	(*9*)
C. parius (*Phasmoconus*)	αC-PrXA	TYGIYDAKPOFS**C**AGLRGG**C**VLPONLROKFKE[b]	C	this work

B. Comparison of αC-PrXA to Waglerin-1[d]

peptide	amino acid sequence	ref
Waglerin-1	GGKPDLRP**C**HPP**C**HYIPRPKPR[c]	(*24*, *25*)
αC-PrXA	TYGIYDAKPOFS**C**AGLRGG**C**VLPONLROKFKE[b]	this work

[a] O, 4-*trans*-hydroxyproline; Z, pyroglutamate; γ, γ -carboxyglutamate. [b] C-terminal amidation. [c] C-terminal free acid. [d] In B, proline and 4-*trans*-hydroxyproline residues are underlined for Waglerin-1 and αC-PrXA sequences.

they are not paralytic and do not inhibit the muscle nicotinic receptor.

A biochemical feature of αC-PrXA, the unusual pattern of post-translational modification of Pro residues, merits comment. The hydroxylation of Pro residues to 4-*trans*-hydroxyproline (Hyp) is a frequently encountered post-translational modification in *Conus* peptides. In αC-PrXA, there are 5 Pro residues, and three are modified. The two that are not modified are both found in pairs of Pro residues, in each case, the more N-terminal Pro residue is unmodified (thus, a Pro-Hyp sequence). This result might be interpreted to mean that the modification enzyme processes adjacent Pro residues by always modifying the more C-terminal one and not the more N-terminal Pro residue. However, it should be noted that in unrelated conotoxins such as αA-EIVB and μ-conotoxin GIIIA, adjacent Pro residues are both found to be modified to Hyp (*6*, *22*). Thus, the pattern of post-translational modification of Pro residues in PrXA suggests a level of sophistication in the determinants for post-translational modification that would not have been predicted from previously sequenced conopeptides (*23*).

How widely distributed αC-conotoxins are in the ~700 species of *Conus* remains to be established. The gene superfamily (the C-conotoxin superfamily) encoding PrXA has been characterized; surprisingly, as will be detailed elsewhere, the only other conopeptide of the gene superfamily that has been functionally defined to date has proven to target a G-protein-coupled receptor (GPCR), not a ligand-gated ion channel such as the target of αC-PrXA.

αC-PrXA is a highly potent and highly specific competitive antagonist of neuromuscular nAChRs as demonstrated by both *in vivo* and *in vitro* experiments. It demonstrates the highest affinity for the α/δ interface of the nAChR and the lowest affinity for the α/ϵ interface. αC-PrXA defines a new family of singly disulfide-bonded *Conus* peptides that target the vertebrate muscle nAChR.

αC-PrXA caused paralysis in mice when injected both i.p. and i.c. The i.c. injection result requires some explanation because the peptide would not necessarily be expected to escape through the blood−brain barrier to the periphery. When i.c. injections are performed by hand, some of the solution injected tends to leak back out of the injection site where blood vessels have been exposed by the injection, allowing the peptide to be distributed to the periphery. We have observed paralysis consistently with i.c. injections of many other inhibitors of neuromuscular nAChRs (unpublished observations).

As shown in Table 1, αC-PrXA is strikingly divergent in both its amino acid sequence and disulfide framework from other conotoxin inhibitors of neuromuscular nAChRs. However, it shares some notable biochemical features with a family of snake-venom toxins from *Trimeresurus wagleri* (Wagler's pit viper), known as Waglerins (*24*). For example, αC-PrXA and Waglerin-1 both have a single disulfide bond, and both are rich in proline residues (and hydroxyproline residues in the case of αC-PrXA). In contrast to these biochemical similarities, αC-PrXA and Waglerin-1 diverge functionally in their affinity for particular subunit interfaces of the neuromuscular nAChR. Waglerin-1 is a highly selective competitive antagonist at the α/ϵ interface of the nAChR (*25*), whereas αC-PrXA demonstrated the lowest affinity for the α/ϵ interface and the highest affinity for the α/δ interface. It should be instructive to explore the structure−function relationships that determine selectivity for αC-PrXA and Waglerin-1 at the α/δ and α/ϵ interfaces of the nAChR.

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