A Noncompetitive Peptide Inhibitor of the Nicotinic Acetylcholine Receptor from *Conus purpurascens* Venom†

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ABSTRACT: A paralytic peptide, ψ-conotoxin PIIIE has been purified and characterized from *Conus purpurascens* venom. Electrophysiological studies indicate that the peptide inhibits the nicotinic acetylcholine receptor (nAChR). However, the peptide does not block the binding of α-bungarotoxin, a competitive nAChR antagonist. Thus, ψ-conotoxin PIIIE appears to inhibit the receptor at a site other than the acetylcholine-binding site. As ascertained by sequence analysis, mass spectrometry, and chemical synthesis, the peptide has the following covalent structure: HOO**CC**LYGK**C**RRYOG**C**SSAS**CC**QR* (O = 4-*trans* hydroxyproline; * indicates an amidated C-terminus). The disulfide connectivity of the toxin is unrelated to the α- or the αA-conotoxins, the *Conus* peptide families that are competitive inhibitors of the nAChR, but shows homology to the μ-conotoxins (which are Na^+ channel blockers).

Marine snails of the genus *Conus* paralyze their prey by injecting venom through a hollow harpoon-like tooth. The active paralytic agents in these venoms, the conotoxins, are small, multiply disulfide-bonded peptides. Most of the 500 cone snails prey on various invertebrates; however, there are ca. 50−70 *Conus* species which prey exclusively on fish.

In the highly venomous fish-hunting *Conus geographus* (the geography cone), the major paralytic conotoxins fall into three distinct classes (for reviews, see refs 1 and 2). The μ-conotoxins, 22 amino acids in length, have three disulfide bonds and inhibit skeletal muscle voltage-gated sodium channels at site I, the tetrodotoxin-saxitoxin binding site. Another class of *C. geographus* paralytic conotoxins are the ω-conotoxins, 25−29 amino acids in length, with three disulfide bonds, which specifically inhibit presynaptic voltage-sensitive calcium channels. Finally, several α-conotoxins have been characterized; these peptides are 13−15 amino acids in length and block the acetylcholine binding sites of nicotinic acetylcholine receptors. These are the same sites to which α-neurotoxins from snakes, and curare, the South American Indian poison arrow toxin, bind to manifest their neurotoxicity.

We have recently been investigating the venom of another *Conus* species, the purple cone *Conus purpurascens* (3−5). It is found in the eastern Pacific, from the Gulf of California to Peru, including outlying islands such as the Galapagos and Clipperton Atoll, and is the only fish-hunting *Conus* species known from the eastern Pacific marine province. *C. purpurascens* is believed to have long been geographically isolated from the major line of fish-hunting *Conus* species found in the Indo-Pacific, such as *C. geographus*.

Previous analysis of the venom of *C. purpurascens* revealed that this species simultaneously uses two broad physiological strategies for prey immobilization: excitotoxic shock and neuromuscular block (*5*). Among the toxins in *C. purpurascens* venom which effect neuromuscular block is a peptide that inhibits the acetylcholine receptor at the ligand binding site, αA-conotoxin PIVA (*3*). Although this peptide is structurally distinct from the α-conotoxins from *C. geographus* and other piscivorous Indo-Pacific *Conus* species, it nevertheless appears to target the same ligand-binding site on the acetylcholine receptor complex. Recently, a μ-conotoxin in *C. purpurascens* venom with significant homology to the *C. geographus* μ-conotoxin has been characterized (K.-J. Shon et al., manuscript in preparation). We have not yet identified an ω-conotoxin from *C. purpurascens* venom.

In this report, we describe the isolation and characterization of a third type of paralytic peptide in *C. purpurascens* venom. It is distinct in its physiological activity, pharmacology, and specificity from previous classes of paralytic conotoxins characterized from fish-hunting cone snails. The peptide therefore defines a novel family of conotoxins, which we designate ψ-conotoxins.

METHODS

C. purpurascens Specimen Collection and Venom Extraction. Specimens of the purple cone *C. purpurascens* were collected from the Gulf of California. The venom was acquired by milking the snails as previously described (*3*). The snails were milked twice a week, and an average-sized *C. purpurascens* (∼4 cm) yielded approximately 10−15 μL of venom from each milking. Milked venom stored at −70 °C was pooled. Most large-scale purification runs were carried out with ca. 0.5 mL of milked venom per run.

Peptide Purification by HPLC. Preparative scale reversed-phase HPLC was used as the first step in the purification of the milked venom. The venom was diluted with 0.1% trifluoroacetic acid (TFA), spun for a few minutes with a

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benchtop microfuge, and the supernatant applied to a preparative HPLC column (Vydac C_{18}, 2.5 × 25 cm; flow rate, 20 mL/min) with a guard column (22 × 50.8 mm). The crude fractions from the first HPLC column were further purified on an analytical column (Vydac C_{18}, 218TP54, 4.6 × 250 mm; flow rate, 1 mL/min). A gradient of 0.1% TFA in water and 0.085% TFA in 90% CH_3CN (preparative runs) or 0.092% TFA acid in 60% CH_3CN (analytical runs) was used. A linear gradient of 1% increase of the CH_3CN-containing buffer per min was used for elution. The analytical column was also employed to purify alkylated peptides for amino acid sequence analysis.

Sequence Analysis and Disulfide Linkage. The reduced peptide (reduced with TCEP) was HPLC-purified then alkylated with 4-vinyl pyridine as described by Gray (*6*). The pyridylethylated peptide was repurified by HPLC, and a sample analyzed in an ABI model 477A sequencer.

ψ-Conotoxin PIIIE was analyzed by the partial reduction method of Gray (*6*). Suitable reduction conditions were established by small-scale trials. The disulfide connectivity was determined as previously described (*3*).

Mass Spectrometry. Positive ion LSIMS spectra were obtained with a JEOL JMS HX110 double-focusing spectrometer, fitted with a cesium ion gun operated at +30 KV.

Peptide Synthesis. Linear ψ-PIIIE was built on Rink amide resin using fmoc chemistry with equimolar amounts of dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) as coupling reagents on an ABI model 431A peptide synthesizer. All amino acids were purchased from Bachem (Torrance, CA) with side chain protection as follows: Hyp, Ser, Tyr protected with *tert*-butyl; Lys (boc); Arg (pmc); His, Gln, Cys (trityl). At the completion of synthesis, the terminal fmoc group was removed *in situ* by treatment with 20% piperidine in N-methylpyrrolidone.

The linear peptide amide was cleaved from 200 mg of resin by treatment with 4 mL of TFA/H_2O/ethanedithiol/phenol/thioanisole (90/5/2.5/7.5/5 by volume) for 3.5 h at 20 °C. Released peptide was precipitated by filtering the reaction mixture into 200 mL of methyl-*tert*-butyl ether (MTBE) cooled to −10 °C. The reaction vessel was rinsed with 1 mL of TFA, and the rinse was also filtered into MTBE. The precipitate was pelleted by centrifugation and washed once with MTBE. The supernatant was discarded and the pellet dissolved in 10 mL of 0.092% TFA and 60% CH_3CN in H_2O. Peptide was then purified on a preparative column (Vydac C_{18}, 2.2 × 25 cm), eluted with a 6 to 30% CH_3CN gradient and TFA buffer system at a flow rate of 20 mL/min.

Cysteine residues were oxidized with glutathione as previously described (*3*). The major isomeric product (comprising 65% of the isomer mixture) was purified by the HPLC procedures described above. This oxidized peptide was tested for both biological activity (a paralytic effect when injected intraperitoneal (ip) into goldfish) and HPLC comigration with native material. It behaved identically to native peptide in both tests.

Biological Assays. Ip injections of toxin into goldfish and Swiss Webster mice were performed as previously described (*7*, *8*).

Binding Assays. Binding assays were performed using a membrane fraction enriched in nAChR from *Torpedo californica* electroplax as described previously (*3*). The membrane preparation (0.38 pmol of α-bungarotoxin binding sites/μg of protein; 2 μg of protein/0.1 mL of assay) was diluted with 0.02 M HEPES, pH 7.4, 5 mM EDTA, 0.5 mg/mL bovine serum albumin, and 0.5 mg/mL lysozyme and preincubated for 30 min at room temperature with or without unlabeled synthetic ψ-conotoxin PIIIE, followed by a 30 min incubation with [^{125}I]ψ-PIIIE (13 000 cpm/assay). Unbound radioligand was separated from receptor by centrifugation in a microcentrifuge for 3 min at 15000*g*, and radioactivity in pellet and supernatant was separately determined with a γ counter.

Extracellular Recording from Electrocytes in the Teleost Fish Eigenmannia. Recording of synaptically mediated action potentials (i.e., the electric organ discharge) in this modified muscle preparation was performed as previously described (*7*). Briefly, the skinned tail of the fish was placed in a small chamber (volume, <100 μL). The motor nerves innervating the electrocytes were stimulated (once every 15−60 s) by electrical pulses applied to the spinal cord while extracellular platinum wire electrodes were used to monitor action potentials generated by the electrocytes. The preparation was perfused with Hickman's solution (110 mM NaCl, 2 mM KCl, 3 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mM $NaHCO_3$, 1 mM $NaHPO_4$, pH 7.2) at a rate of about 1 mL/min. When toxin was applied, the perfusion was stopped, and the solution bathing the preparation was removed and replaced with one containing toxin. Thus, during exposure to toxin, the preparation was in a static bath. Toxin was washed out by restarting the perfusion.

Recording of ACh-Gated Currents from Voltage-Clamped Xenopus Oocytes Previously Injected with Cloned mRNA for ACh Receptors. Preparation of mRNA, its injection into oocytes, and subsequent analyses of ACh-gated currents by voltage clamping of the oocytes that were perfused with ND-96 buffer was performed as previously described (*9*). The cDNA clones for *Torpedo* AChR subunits were obtained from W. Green (University of Chicago), and those for mouse nAChR subunits were obtained from S. Heinemann and D. Johnson (Salk Institute, San Diego, CA).

Iontophoreses of ACh to Voltage-Clamped Oocytes. To provide a rapidly exchanging oocyte bath of minimal volume, the following "perfused gap" system was used. Two horizontal polyethylene capillaries (i.d. ≈ 1.6 mm) were aligned so that their open ends opposed each other, separated by a gap ∼2 mm wide. The gap was perfused (5−10 μL/s) from both directions with ND-96 supplied through both capillaries. A syringe needle (24 gauge) connected to a vacuum line was used to remove perfusate from the gap; the position of its tip was adjusted so that a horizontal fluid column, suspended in air, was produced. An oocyte was placed in the fluid column, impaled with a pair of intracellular electrodes and voltage clamped. ACh was applied iontophoretically to the oocyte through a microelectrode (tip diameter < 1 μm) filled with 1 M ACh (acetylcholine chloride dissolved in distilled water). The pipette was connected to a 20 Megohm current-limiting resistor and a battery powered "brake box" in series with a stimulus isolation unit (cf. ref 10). A braking current of ∼25 nA was applied to the ACh pipette to retard diffusion of ACh out its tip. To apply ACh, the tip of the iontophoretic pipette was placed against the oocyte surface, and rectangular current pulses (duration, 200 ms) were applied at a frequency of 0.1 Hz. The amplitude of the current pulse was adjusted to provide suitable responses (∼100 nA peak amplitude, <1 s

duration). To apply toxin, the perfusion with ND-96 was halted, and toxin was delivered directly to the fluid column from a microburette connected to a motorized syringe.

Identification and Sequencing of Conus purpurascens cDNA Clones. Venom duct RNA (5 μg) from *C. purpurascens* was annealed to 3 pmol of a polyT oligonucleotide, and cDNA was synthesized by AMV reverse transcriptase (5 units; Promega, according to the manufacturer's suggested protocol). The resulting cDNA was used as a template for PCR reaction in ten 10 μL sealed capillary tubes using an Idaho Technology air thermocycler. Each reaction contained 50 ng of template cDNA, 5 pmol each of oligonucleotides corresponding to 5′ and 3′ UTR sequence of μ-conotoxin prepropeptides, 6 nmol of each of the four dNPTs and 0.5 unit of Taq polymerase (Boehringer Mannheim, Inc.) in a buffer consisting of 50 mM Tris, pH 8.3, 250 μg/mL BSA, and 2 mM $MgCl_2$. The PCR consisted of 40 cycles (94 °C pulse; 54 °C pulse; and 72 °C, 15 s).

The PCR product was gel-purified and recovered from agarose using the Bio-Rad Prep-A-Gene kit according to the manufacturer's protocol. The eluted DNA fragment was ligated to *Sma*1 (BRL)-digested pUC plasmid pTZ18U by first generating blunt ends. The purified PCR product (44 ng) was mixed with *Sma*1-digested pTZ18U DNA (40 ng) and 0.2 mM of each of the four dNTPs in a buffer containing 25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.25 mM spermidine, 1 mM ATP, 1.25 mM hexamine cobalt chloride, and 10 μg/mL BSA in a final volume of 16 μl. Three units of T_4 polymerase (New England Biolabs) were added, and the reaction incubated for 5 min at 37 °C. The enzyme was inactivated by incubation for 15 min at 75 °C and the reaction cooled to 25 °C. Ligation was carried out in the above reaction mixture to which 3 units of T_4 Ligase (Promega) and 2 μL of 1 mM ATP were added at room temperature overnight.

One-tenth of a milliliter of competent DH5αF′IQ cells (BRL) was transformed with the entire ligation mix and spread on B agar plates (X-gal and IPTG) containing 50 μg/mL each of ampicillin and kanamycin (all from Sigma Chemical). After incubation overnight at 37 °C, colorless colonies produced by recombinant plasmids were screened for the presence of the correct sized insert via PCR with vector-targeting primers in an Idaho Technology air thermocycler. Colonies that contained the correct sized inserts were prepared for DNA sequencing.

Sequencing. Single-stranded DNA was prepared from putative PIIIE clones for sequencing by PCR amplification of 50 ng of plasmid with a pair of vector primers, one of which is biotinylated, and binding the resulting PCR product to Streptavidin-bound magnetic polystyrene beads (DYNAL, Dynabeads M-280 Streptavidin). Material for solid-phase sequencing was prepared according to the manufacturer's suggested protocol, generating single-stranded nucleic acid which was sequenced using the Sequenase version 2.0 DNA sequencing kit, the nonbiotinylated vector primer and ^{35}S dATP, according to standard Sequenase protocol.

RESULTS

Purification, Characterization and Chemical Synthesis of ψ-Conotoxin. C. purpurascens specimens from the Sea of Cortez were maintained in our laboratories; because this species has a relatively small venom duct, many hundreds of specimens would have to be collected if venom from the dissected ducts were to be used for isolating toxins. In order to collect sufficient quantities of venom for successful biochemical purification from the relatively few *C. purpurascens* available to us, we resorted to milking of the snails. Preliminary biological assays of milked venom demonstrated that it caused paralysis of both fish and mice. The venom was fractionated, and the paralytic components were purified.

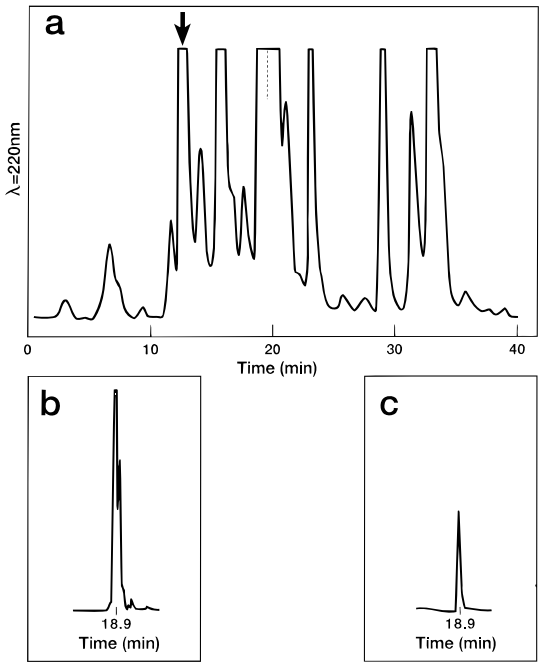

FIGURE 1: Panel a, chromatography of milked *Conus purpurascens* venom. Crude *C. purpurascens* venom (0.5 mL), milked from snails maintained in an aquarium, was injected onto an HPLC preparative column (Vydac C_{18}). Peptides were eluted with a linear gradient of acetonitrile (0 to 36% in 40 min, flow = 20 mL/min). The arrow indicates the peak that eventually yielded ψ-conotoxin PIIIE. Panels b and c, further purification of ψ-conotoxin PIIIE. The peak in Figure 1a was applied to an analytical column (Vydac C_{18}) and eluted with an acetonitrile gradient of 3 to 21% in 30 min, flow = 1 mL/min (panel b). The major peak was collected, reloaded onto the column and eluted under the same conditions to yield the single homogeneous peak shown in panel c. All HPLC conditions are detailed in the Methods.

A major paralytic component in *C. purpurascens* venom was the first major peak in the HPLC elution profile of the venom (see Figure 1). This activity was purified to homogeneity by following fractions which caused paralysis of fish, as shown in Figure 1, panels B and C. The purified peptide was then analyzed by standard microsequencing methods as described in the Methods.

Initial attempts at microsequencing were complicated by inefficient Edman cleavage after the first residue. As a result, although the peptide was homogeneous in most sequencing runs, the incomplete cleavage at the first step resulted in each subsequent step containing a mixture of amino acids (e.g., the fourth Edman step had a mixture of PTH amino acids derived from positions 1−4). In order to facilitate the interpretation of the sequencing runs, the peptide was also cleaved by Lys-C into two fragments, one containing amino acids 1−9, and residues 10−23 in the other. The N-terminal fragment showed the same incomplete Edmund cleavage as the intact peptide, but the C-terminal fragment sequenced cleanly. The amino acid composition of each fragment was verified by amino acid analysis and mass spectrometry. Other

Table 1: Sequence Analysis of ψ-Conotoxin PIIIE[a]

step	amino acid	yield (pmol)
1	H	30.1
2	O	75.4
3	O	85.0
4	C	71.4
5	C	73.2
6	L	42.2
7	Y	37.2
8	G	37.9
9	K	41.9
10	C	40.0
11	R	29.7
12	R	50.2
13	Y	16.8
14	O	44.0
15	G	35.7
16	C	36.4
17	S	7.6
18	S	6.9
19	A	21.1
20	S	4.9
21	C	17.9
22	C	21.3
23	Q	15.7
24	R	9.9

[a] O = hydroxyproline, C = pyridylethylcysteine. Reduced and alkylated ψ-PIIIE was prepared for sequencing as described in the Methods. The tabulation above shows the yield of PTH−amino acid derivative during each step of an Edman degradation sequencing run.

Table 2: cDNA Clone Encoding a ψ-Conotoxin[a]

arg arg his pro pro cys cys met tyr gly arg cys arg arg
AGG AGA CAC CCG CCC TGT TGC ATG TAC GGC AGA TGC CGT CGA

tyr pro gly cys ser ser ala ser cys cys gln gly gly OCH
TAT CCC GGA TGC TCT AGT GCC TCT TGT TGC CAG GGA GGA TAA

predicted toxin sequence: HOOCCMYGRCRRYOGCSSASCCQG*
ψ-PIIIE: HOOCCLYGKCRRYOGCSSASCCQR*

[a] O = hydroxyproline; * = amidated C-terminus. The arrow indicates the predicted point of proteolytic cleavage to generate the mature toxin. The predicted toxin sequence is generated assuming all Pro residues are converted to hydroxyproline, and that a C-terminal Gly is processed to an amide group.

cleavage fragments were also analyzed (including Arg-C products which included two N-terminal fragments, 1−11 and 1−12, and the two corresponding C-terminal fragments). Natural peptide was later isolated from a separate pool of *C. purpurascens* milked venom and produced a clean sequencing run as shown in Table 1. All of the sequencing data are consistent with a 24 amino acid peptide containing six Cys residues.

In order to determine the status of the C-terminus, mass spectrometry was carried out as described in the Methods. The value obtained (MH^+ = 2716.0) is consistent with amidation at the C-terminus. In addition, a cDNA clone encoding the toxin was sequenced (see Table 2). The cDNA encoded a polymorphic variant, differing by three amino acids from the peptide purified from pooled venom. In congruence with the mass spectrometry data, the cloning data predict amidation at the C-terminus.

A peptide containing the amino acid sequence predicted by sequence analysis was synthesized as described in the Methods. The linear peptide was released from the resin and oxidized in a mixture of 1 mM/0.5 mM reduced/oxidized glutathione, pH 7.5, at room temperature. The equilibrium shift between two glutathione forms fully oxidized the linear peptide into a mixture of isomers. The progress of the oxidation was monitored for 5−6 h by analytical HPLC, and the major product after this duration was purified as described in the Methods. An amino acid analysis of the purified synthetic material gave a composition consistent with the sequence assignment.

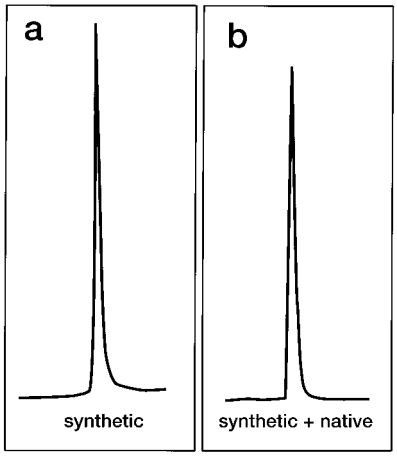

FIGURE 2: HPLC chromatograms of ψ-conotoxin PIIIE. Panel a, The synthetic peptide, synthesized and folded as described in the Methods, was chromatographed using a Vydac C_{18} analytical column (3 to 21% gradient of acetonitrile over 30 min, flow = 1 mL/min). Panel b, coelution experiment. Equal amounts of synthetic and native peptide were mixed and coeluted under conditions identical to those described above. Peptides eluted at t = 18.9 min. Absorbance was measured at 220 nm.

As shown in Figure 2, the native and synthetic peptides were compared by coelution on an analytical HPLC column. The paralytic activity of native and synthetic peptides when injected ip into goldfish was also comparable (data not shown). These criteria indicate that the native and synthetic peptides are identical to each other. Thus, the chemical synthesis confirms the sequence assignment.

Determination of Disulfide Connectivity. The disulfide bonding of the peptide was determined by the method of Gray (*6*). The peptide was reduced with TCEP as described in Figure 3; small-scale reactions were monitored to obtain incubation conditions under which partially reduced species could be detected in significant amounts. An HPLC elution profile of a reaction mixture is shown in Figure 3A. Three species were separated and purified to homogeneity, and each was then alkylated with iodoacetamide, fully reduced, and further alkylated with 4-vinylpyridine. Sequencing of the doubly alkylated species provided a self-consistent determination of disulfide connectivity based on the pairwise deduction strategy developed by Gray (*6*). A typical sequence analysis of a doubly alkylated peptide is shown in Figure 3B. The results indicate that the disulfide connectivity of ψ-conotoxin PIIIE is the same as that found for μ-conotoxin GIIIA.

Effect of ψ-Conotoxin on a Teleost Neuromuscular Preparation. The paralytic effects of the peptide suggest that the toxin probably interferes with some step in neuromuscular transmission. The effect of ψ-conotoxin on a freshly dissected electrocyte preparation from the weakly electric fish *Eigenmannia* was assessed (Figure 4). The

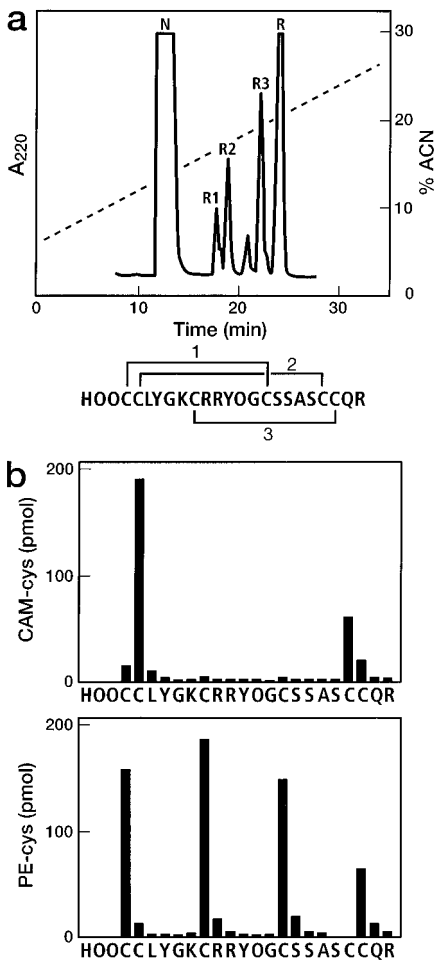

FIGURE 3: Panel a, reverse-phase HPLC chromatogram of a partial reduction of ψ-PIIIE with 10 mM TCEP, pH 3 (5 min incubation, 65 °C). Peaks representing native (N), fully reduced (R), and major partially reduced (R1-R3) species are labeled. The established disulfide bonding arrangement is represented below with bonds labeled 1−3. Partially reduced intermediates were identified as follows: R1, bond 3 reduced; R2, bond 2 reduced; R3, bonds 2 and 3 reduced. Panel b, sequence analysis of partially reduced species R2. This peptide was first alkylated with iodoacetamide to produce carboxyamidomethyl(CAM)-Cys at all reduced Cys residues, then fully reduced and alkylated with 4-vinylpyridine to produce pyridylethyl(PE)-Cys at the remaining cys residues. The analyses of CAM-Cys and PE-Cys are plotted separately. These results indicate that Cys-5 and Cys-21 form a disulfide bond.

electric organ of this teleost, as in electric rays such as *Torpedo*, is derived from muscle.

Synaptically mediated action potentials in *Eigenmannia* electrocytes evoked by electrical stimulation of the motor nerve in the spinal cord was monitored extracellularly as described in the Methods. When the electrocyte preparation was exposed to ψ-conotoxin PIIIE, the amplitude of the action potential was attenuated 100% by 10 μM and ca. 80% by 1 μM of the toxin. These results demonstrate that ψ-conotoxin is a potent inhibitor of neuromuscular transmission in this teleost preparation. Such inhibition would explain the paralytic activity observed *in vivo* when the peptide is injected into teleost fish.

Effect of ψ-Conotoxin on the Nicotinic Acetylcholine Receptor. Although the results above suggest that the toxin is blocking neuromuscular transmission, they do not identify the molecular target of the toxin. The following experiments show that ψ-conotoxin directly inhibits the function of the nicotinic acetylcholine receptor (nAChR).

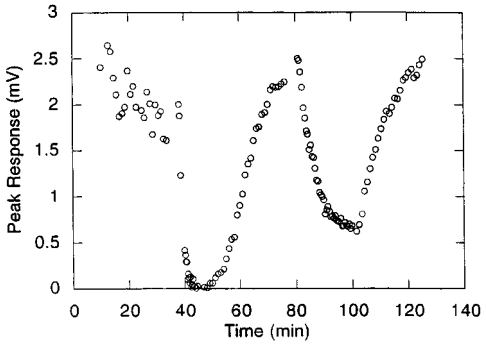

FIGURE 4: Block of synaptically mediated action potentials in *Eigenmannia* electrocytes by ψ-conotoxin PIIIE. Action potentials from electrocytes, elicited by electrical stimulation of motor nerves, were monitored extracellularly from a freshly dissected preparation as described in the Methods. After obtaining a series of control responses, the solution bathing the electrocytes was replaced with one containing 10 μM ψ-conotoxin (at t = 38 min), and after 10 min the toxin solution was washed out (at t = 47 min). After the response had essentially fully recovered, the bathing solution was replaced with one containing 1 μM ψ-conotoxin (at *t* = 81 min), and after 20 min the toxin was washed out (at *t* = 102 min). It is evident that the synaptically evoked action potential in this preparation is reversibly blocked by ψ-conotoxin PIIIE in a dose-dependent fashion.

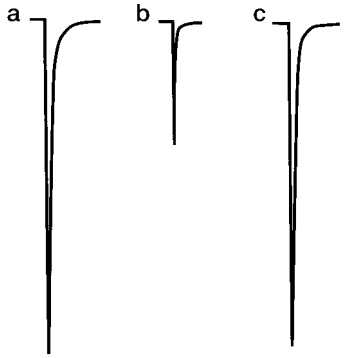

FIGURE 5: Effects of ψ-conotoxin PIIIE on cloned mouse nAChR. ACh-gated currents were monitored in a two-electrode voltage-clamped *Xenopus* oocyte expressing mouse skeletal muscle nAChRs. The oocyte was perfused at an approximate flow rate of 1 mL/min, and 1 s pulses of ACh (1 μM) were applied every 5 min. After stable response to repeated applications of ACh was achieved, the oocyte was incubated in a static bath with either control buffer or ψ-conotoxin PIIIE for 5 min. After 5 min exposure to toxin, the oocyte was subjected to test pulses of ACh with the first pulse applied immediately upon washout of toxin. Typically, response to successive ACh applications varies ≤10%. (a) Control response to the ACh pulse; (b) ACh response after 5 min incubation with ∼14 μM ψ-conotoxin; (c) ACh response after 5 min washout of ψ-conotoxin.

The peptide was tested on nAChRs expressed in *Xenopus* oocytes which had been injected with nAChR mRNA cloned from mouse skeletal muscle or *Torpedo* electric organ. ψ-Conotoxin PIIIE inhibited the current normally elicited by acetylcholine, indicating that the toxin inhibits the nicotinic acetylcholine receptor. Results for the mouse receptor are shown in Figure 5 and for the *Torpedo* receptor in Figures 6 and 7. For the *Torpedo* receptor, the data in Figure 7 indicate that the peptide has an IC_{50} of ∼127 nM .

The peptide had no significant effect on a major neuronal nicotinic acetylcholine receptor subtype (α4β2) expressed in the oocyte system (results not shown). Thus, the peptide inhibits the skeletal muscle subtype of nicotinic acetylcholine receptors, and the results suggest that it may be relatively

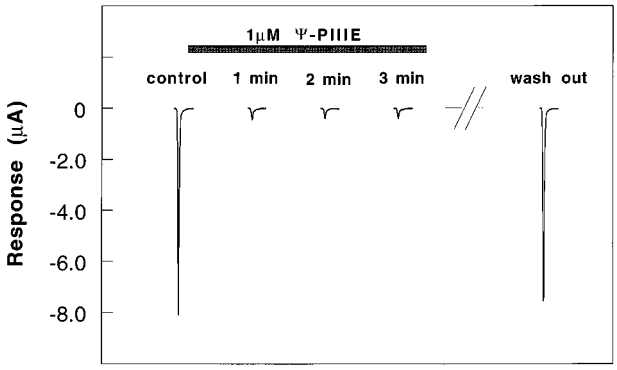

FIGURE 6: ψ-PIIIE blocks ACh-gated currents in *Xenopus* oocytes expressing *Torpedo* nAChRs. The oocyte was continuously perfused at a flow rate of ~1 mL/min with ND-96 buffer either with or without toxin. Responses to 1 s pulses of 10 μM ACh applied at 1 min intervals were measured. One micromolar ψ-PIIIE blocked ~90% of ACh currents within 1 min (before first ACh pulse) of application. Complete toxin washout was typically achieved in 5−7 min. These results were obtained reproducibly in experiments on three separate oocytes.

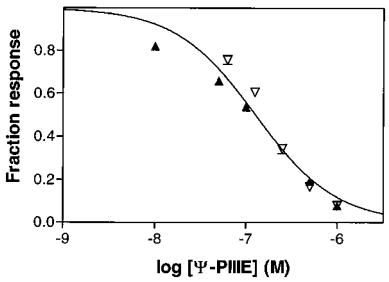

FIGURE 7: A dose−response curve of ψ-conotoxin PIIIE on *Xenopus* oocytes expressing *Torpedo* nAChRs. ACh was applied iontophoretically (open triangles) or using a gravity perfusion system (closed triangles) as detailed in the Methods. The IC_{50} for the combined data is 127 nM, each data point representing the mean of at least three experiments on different oocytes.

specific for this subtype; however, a comprehensive survey of neuronal nAChR subtypes remains to be carried out.

Binding Experiments. Given the results indicating that the peptide functionally inhibits skeletal muscle nicotinic acetylcholine receptors, a binding competition experiment was carried out using a membrane preparation from *Torpedo* electric organ and [^{125}I]α-bungarotoxin, the standard competitive ligand for the acetylcholine binding site. The results, shown in Table 3, demonstrate that the peptide does not compete for α-bungarotoxin binding. In contrast, when another *C. purpurascens* venom peptide targeted to the acetylcholine binding site (αA-conotoxin PIVA) was used, the binding of α-bungarotoxin was abolished. With the converse experiment using [^{125}I]ψ-conotoxin, the radiolabel is displaced by unlabeled ψ-conotoxin (with an apparent IC_{50} of ~25 nM), but is not displaced by α-bungarotoxin or the competitive nAChR ligand from *C. purpurascens* (Table 3). Thus, we conclude that although ψ-conotoxin functionally inhibits the acetylcholine receptor, it does so by a mechanism other than competitive binding to the acetylcholine ligand site.

DISCUSSION

In this report, we describe the identification, purification, characterization and chemical synthesis of a novel paralytic peptide from *C. purpurascens*, which we have named

Table 3: Binding Competition Experiments[a]

	[^{125}I]α-bungarotoxin (cpm bound)	[^{125}I]ψ-PIIIE (cpm bound)
no addition	4652 ± 86	2104 ± 124
10 μM α-bungarotoxin	714 ± 37	2058 ± 202
10 μM ψ-PIIIE	4981 ± 104	556 ± 55
10 μM αA-P_{IVA}	845 ± 129	2014 ± 173

[a] Binding assays were performed using a membrane fraction enriched in nAChR prepared from *Torpedo californica* electroplax as described previously (*3*). For ψ-PIIIE, the membrane preparation (0.38 pmol of α-bungarotoxin binding sites/μg of protein; 2 μg of protein/0.1 mL assay) was diluted into 0.02 M HEPES, pH 7.4, 5 mM EDTA, 0.5 mg/mL bovine serum albumin, and 0.5 mg/mL lysozyme and preincubated for 30 min at room temperature with or without nonradiolabeled PIIIE, followed by a 30 min incubation with [^{125}I]PIIIE (13 000 cpm/reaction). Unbound radioligand was separated from receptor by centrifugation in a microcentrifuge for 3 min at 15 000*g*, and radioactivity in pellet and supernatent was determined using a Packard Multi-Prias γ counter. [^{125}I]α-Bungarotoxin binding was as in Hopkins et al. (*3*).

ψ-conotoxin PIIIE. Structurally, ψ-conotoxin PIIIE is most similar to the μ-conotoxins which affect voltage-sensitive sodium channels of skeletal muscles; ψ-conotoxin has an arrangement of cysteine residues like the μ-conotoxins (CC−C−C−CC), in contrast to the patterns for the ω-conotoxins (C−C−CC−C−C), the α-conotoxins (CC−C−C), or the αA-conotoxins (CC−C−C−C). In parallel with the similarity in the Cys pattern, the disulfide connectivities of ψ- and μ-conotoxins are the same.

However, in contrast to all of the families of paralytic conotoxins previously characterized, ψ-conotoxin PIIIE appears to have a unique physiological mechanism of action. Experiments with cloned nAChRs expressed in oocytes demonstrate that ψ-conotoxin functionally inhibits the nAChR (see Figures 5−7). In addition, results of a photoactivated cross-linking experiment using homogenized *Torpedo* membrane suggest that ψ-conotoxin PIIIE derivatized with [^{125}I]ASA (azidosalicylic acid) at Lys9 cross-links to a protein with a molecular weight corresponding to the β-subunit of the *Torpedo* nAChR (M. Grilley, unpublished results). However, the peptide differs from the α- and αA-conotoxins in that it does not inhibit the binding of [^{125}I]α-bungarotoxin to *Torpedo* membranes, nor does unlabeled α-bungarotoxin inhibit specific [^{125}I]ψ-conotoxin PIIIE binding to these membranes.

The results are consistent with a model in which ψ-conotoxin does not competitively inhibit the acetylcholine binding site of the nicotinic receptor, but acts by inhibiting the nAChR by binding to a different site on the receptor complex. Given the homology of ψ-conotoxin with μ-conotoxin (which blocks voltage-gated Na channels), one attractive hypothesis is that the site of ψ-conotoxin binding is the extracellular end of the channel pore. This mechanistic possibility needs to be explored by a more detailed electrophysiological analysis, which is in progress.

This peptide, ψ-conotoxin PIIIE, represents the third family of paralytic peptides characterized in *C. purpurascens* venom. The two other paralytic conotoxins identified are μ-conotoxin PIIIA (K.-J. Shon et al., manuscript in preparation), which inhibits voltage-gated Na channels, and αA-conotoxin PIVA (*3*). Both ψ-conotoxin PIIIE and αA-conotoxin PIVA target the nicotinic acetylcholine receptor; however, unlike the αA-conotoxin, the ψ-conotoxin does not inhibit the ligand-

binding site. Thus, a combination of αA-conotoxins and ψ-conotoxins should very efficiently inhibit acetylcholine receptors at the neuromuscular synapse. It is remarkable that *C. purpurascens* has evolved two toxins targeting the same receptor complex at two different binding sites, wherein occupation of either site functionally blocks the complex.

The αA-conotoxins from *C. purpurascens* venom have a novel structure, unrelated to the α-conotoxins characterized from venoms of Indo-Pacific *Conus* species. Thus, two of the three major *C. purpurascens* peptides (ψ- and αA-conotoxins) appear to have evolved independently from the paralytic toxins of previously characterized piscivorous *Conus* species.

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