THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 38, Issue of September 22, pp. 22361–22367, 1995
Printed in U.S.A.


# A New Family of *Conus* Peptides Targeted to the Nicotinic Acetylcholine Receptor*

(Received for publication, December 27, 1994, and in revised form, July 7, 1995)

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**In this work, a new family of *Conus* peptides, the αA-conotoxins, which target the nicotinic acetylcholine receptor, is defined. The first members of this family have been characterized from the eastern Pacific species, *Conus purpurascens* (the purple cone); three peptides that cause paralysis in fish were purified and characterized from milked venom. The sequence and disulfide bonding pattern of one of these, αA-conotoxin PIVA, is as follows:**


**where O represents *trans*-4-hydroxyproline. The two other peptides purified from *C. purpurascens* venom are the under-hydroxylated derivatives, [Pro$^{13}$]αA-conotoxin PIVA and [Pro$^{7,13}$]αA-conotoxin PIVA.**

**The peptides have been chemically synthesized in a biologically active form. Both electrophysiological experiments and competition binding with α-bungarotoxin demonstrate that αA-PIVA acts as an antagonist of the nicotinic acetylcholine receptor at the postsynaptic membrane.**

Many venomous animals paralyze their prey by inhibiting communication between motor neurons and the skeletal muscles they innervate. In many instances, a key molecular target of the neurotoxins in the venom is the nicotinic acetylcholine receptor (nAChR)[1] in the postsynaptic membrane at the neuromuscular junction. The first nAChR-targeted venom components to be extensively characterized were the α-neurotoxins from snakes, typified by α-bungarotoxin from the Formosan krait, *Bungarus multicinctus*. Another large class of toxin molecules that inhibit the nAChR at the vertebrate neuromuscular junction are the α-conotoxins, which have been characterized from three different fish-hunting Indo-Pacific *Conus* species: *Conus geographus* (1), *Conus magus* (2), and *Conus striatus* (3, 4).

All of the α-conotoxins from fish-hunting Indo-Pacific cone snails show high structural homology and a conserved sequence motif. The seven peptides that have been purified share the consensus sequence *X*CC(H/N)PACG*XX*(Y/F)*X*C. All of these peptides appear to be potent blockers of neuromuscular transmission in teleosts but differ significantly in their potency when tested in other vertebrates; thus, α-conotoxin GI appears to be very potent in blocking the nicotinic acetylcholine receptor at the neuromuscular junction of all vertebrates tested, while α-conotoxins SI and SII are highly potent in teleosts, but not mammals (3, 4). In addition to the α-conotoxins from Indo-Pacific fish-hunting *Conus*, two α-conotoxins have been purified from non-fish-hunting Indo-Pacific species (5, 6). Although the peptides purified differ significantly from the consensus sequence given above, they do retain the same pattern of Cys residues (the "Cys framework"), *i.e.* $X_n$CC$X_n$C$X_n$C$X_n$.

Because this basic motif was found in both fish-hunting and non-fish-hunting *Conus* species, it seemed reasonable to expect that all nAChR-targeted peptides in *Conus* venoms would share the same conserved α-conotoxin Cys framework. In this report, we describe a novel nAChR-targeted conotoxin from *C. purpurascens*, the purple cone (Fig. 1). *C. purpurascens* is definitely a piscivorous snail, and it was therefore a surprise to find that in this species, the group of peptides that blocks nicotinic acetylcholine receptors does not have a Cys framework typical of α-conotoxins.

A unique feature of the study described below is that in contrast to all previous biochemical studies on *Conus* venoms, which were carried out with dissected venom ducts, the venom used here was milked from living animals. The milking procedure we have developed allows us to harvest much larger amounts of venom from relatively few cone snail specimens.

## MATERIALS AND METHODS

*Venom Collection and Preparation—C. purpurascens* specimens (3–5 cm in length) were collected from the Gulf of California. Because a sufficient number of *C. purpurascens* specimens to prepare venom from dissected ducts for biochemical purification was not available, *C. purpurascens* were milked approximately twice a week. A goldfish was placed in front of the snail to be milked and in most instances, this elicited full extension of the proboscis (see Fig. 1*B*). A surrogate fish consisting of a microcentrifuge tube covered with a latex membrane, cut out from a condom or a glove and overlaid with a fresh goldfish fin, was then substituted for the goldfish at the tip of the proboscis (see Fig. 2). The snail would harpoon the surrogate and typically eject about 5 μl of venom; the harpoon was cut with scissors after ejection of the venom. The goldfish was fed to the snail, and the snail was returned to its aquarium. The venom was centrifuged to the bottom of the tube and pooled together with previous milkings from the same snail. For the general purification described below, crude milked venom from many snails was combined.

There seemed to be considerable variation in how often it was possible to milk individual snails. Some specimens seemed unable or unwilling to harpoon the surrogate at all under these conditions, but the

* This work was supported by National Institute of General Medical Sciences Grant PO1 GM 28677. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: nAChR, nicotinic acetylcholine receptor; Cys(cam), *S*-carboxyamidomethylcysteine; Cys(pe), *S*-pyridylethylcysteine; HPLC, high performance liquid chromatography; LSIMS, liquid secondary ionization mass spectrometry; TCEP, tris-(2-carboxyethyl)phosphine; TEAP, triethylammonium phosphate; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl).

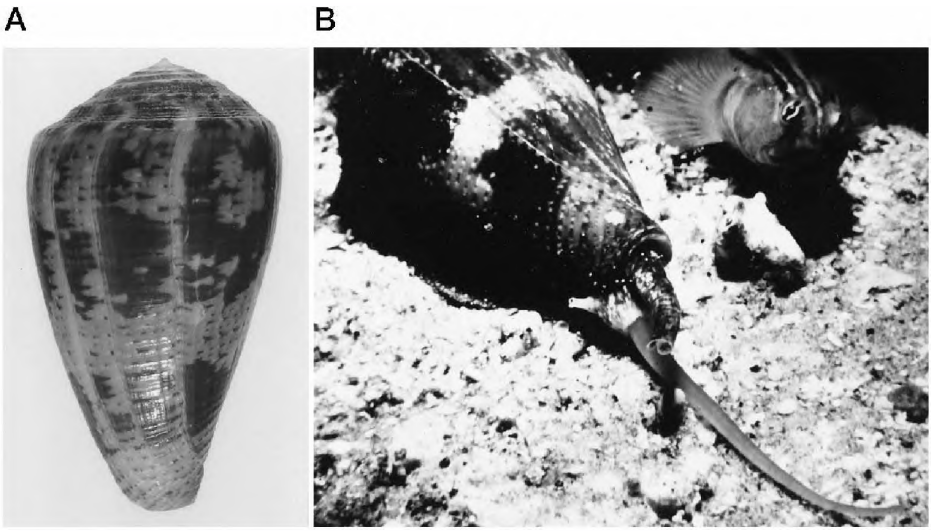


FIG. 1. *A*, a specimen of the purple cone, *C. purpurascens*, from the Cocos Islands, in the Eastern Pacific marine province. *B*, *C. purpurascens* with its proboscis extended, in response to the presence of a fish. A dose of venom (about 5 $\mu$l) has been transferred from the venom duct to the proboscis. If the snail is able to strike the fish, it will inject the venom through a disposable harpoon-like tooth, which it has already transferred from a quiver-like organ, the radula sac, to the tip of the proboscis. Photograph by Alex Kerstitch.

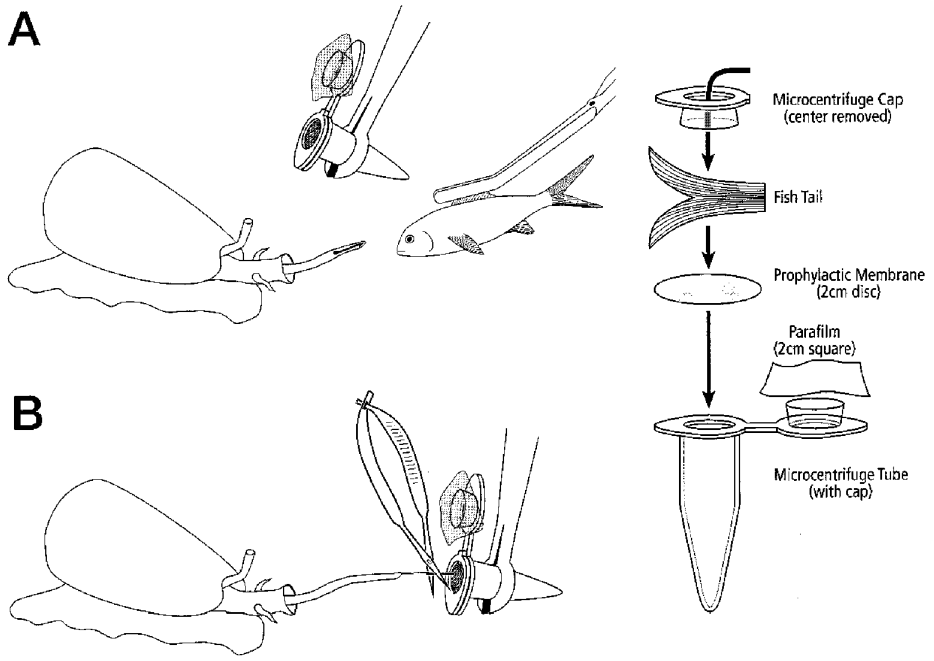


FIG. 2. **Milking procedure.** *A*, a fish is placed in front of the snail until it extends its proboscis. The collection tube (surrogate fish) is quickly substituted for the fish as the snail is about to strike. *B*, once the snail has harpooned the collection tube, the harpoon is cut off. *Right panel* shows the collection tube assembly. The cap of a microcentrifuge tube is hollowed out and placed over a membrane with a cut up fish tail to seal the top of the tube. *C. purpurascens* will generally be reluctant to strike unless the tip of its proboscis actually contacts fish tissue; the fish tail suffices for this purpose. Once the venom has been collected, the microcentrifuge tube is resealed with its own cap and some parafilm, and the venom is collected by centrifugation.

majority of the specimens of *C. purpurascens* could be milked regularly twice a week. Most of the specimens of *C. purpurascens* could be milked on this schedule for 6 months to a year. Among the other *Conus* species milked by this procedure are: *C. striatus*, *Conus obscurus*, *Conus stercusmuscarum*, *Conus ermineus*, *Conus monachus* and *Conus catus*.

*Peptide Purification by HPLC*—A preparative scale reversed-phase HPLC was used for the first line purification of the milked venom. One-half ml of milked venom was diluted with 10 ml of 0.1% trifluoroacetic acid solution and spun for a few minutes in a bench top microfuge. The supernatant was applied to a $C_{18}$ Vydac preparative column (22 × 250 mm; 20 ml/min) with a guard column (22 × 50.8 mm). As a secondary purification, a $C_{18}$ Vydac analytical column (218TP54, 4.6 × 250 mm;1 ml/min) was used. HPLC buffers were 0.1% trifluoroacetic acid in water (buffer A) and 0.085% trifluoroacetic acid in 90% $CH_3CN$ (buffer B) for preparative runs and 0.092% trifluoroacetic acid in 60% acetonitrile for analytical runs. For both preparative and analytical runs, the peptides were eluted with a linear gradient of 1% buffer B increase/min. The $C_{18}$ analytical column was also used for purifying alkylated peptides for amino acid sequence analysis.

*Analysis of Covalent Structure*—Three closely related peptides were isolated, which differ solely in hydroxylation of their proline residues; fully hydroxylated peptide is designated $\alpha$A-conotoxin PIVA, with the others being assigned derivative status.

*Primary Structure*—Disulfides were reduced by TCEP, and the linear peptide was purified and alkylated with 4-vinylpyridine as described by Gray (7). The pyridylethylated peptide was repurified by HPLC, and a sample was analyzed in an ABI model 477A sequencer. A second sample was digested with endoproteinase Lys-C (18 h, 37 °C); the fragments were isolated by HPLC and subjected to sequencing and/or mass analysis.

*Disulfide Connectivity*—Natural $\alpha$A-conotoxin PIVA was analyzed by the partial reduction method of Gray (7, 8), suitable reduction conditions being established by small scale trials. To obtain partially reduced intermediates, 10 nmol of peptide in HPLC effluent and 20 mM TCEP in 0.17 M sodium citrate (pH 3) were preequilibrated to 61 °C for 5 min. Equal volumes of the reactants were mixed and incubated a further 2 min before injection onto the HPLC column. Eluted peptides were immediately frozen (pH 2.0) at −20 °C to minimize disulfide exchange. They were individually thawed, repurified, and immediately alkylated with 2.2 M iodoacetamide using the rapid procedure of Gray (7). The carboxyamidomethylated peptides were desalted, and their residual disulfides were reduced and alkylated with 4-vinylpyridine. Sequencer analysis was then used to determine the positions of Cys(cam) and Cys(pe) residues, thus establishing which residues had been free or bridged. Analysis of two intermediates was sufficient to deduce the disulfide connectivity.

*Mass Spectrometry*—Positive ion LSIMS spectra were obtained using a Jeol JMS HX110 double-focusing spectrometer, fitted with a $Cs^+$ ion gun operated at +30 kV.

*Solid Phase Peptide Synthesis*—$\alpha$A-Conotoxin PIVA was built using standard Fmoc chemistry on an ABI model 430A peptide synthesizer, couplings were carried out with equimolar amounts of amino acid

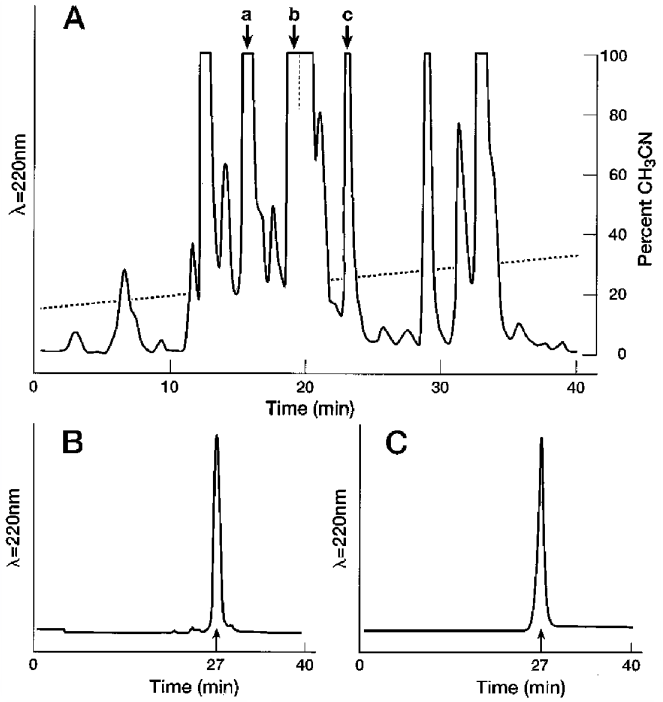


FIG. 3. *Panel A*, reversed-phase HPLC chromatogram of *C. purpurascens* milked venom. 0.5 ml of milked venom was injected into a $C_{18}$ Vydac preparative column, and peptides were eluted with a gradient of $CH_3CN$ (0–36%) in trifluoroacetic acid as described under "Materials and Methods." *Peaks A*, *B*, and *C* with *arrows* correspond to three forms of αA-conotoxin PIVA. *Panel B*, further purification of *peak A* with an analytical $C_{18}$ Vydac column. A small portion of *peak A* was purified using a gradient of $CH_3CN$ (6~24%) in trifluoroacetic acid as described under "Materials and Methods." *Panel C*, the peptide purified from *panel B* was reloaded on the column and eluted under the same conditions as *panel B*. A single homogeneous peak was obtained.

derivative, dicyclohexylcarbodiimide, and hydroxybenzotriazole. All amino acids were from Bachem (Torrance, CA) with side-chain protection: pentamethylchromansulfonyl (Arg); trityl (Asn, Cys, Gln, His); *t*-butyl (Asp, Hyp, Ser, Tyr); *t*-butyloxycarbonyl (Lys). At the completion of synthesis, the terminal Fmoc group was removed by a standard machine cycle.

Peptide was removed from the resin and deprotected by treatment (2 h, 20 °C) with trifluoroacetic acid/$H_2O$/ethanedithiol/phenol/thioanisole (36/2/1/3/2 by volume), and the whole mixture was filtered rapidly into *t*-butyl methyl ether at −10 °C. The precipitate was collected by centrifugation, dissolved in 60% $CH_3CN$ containing 0.092% trifluoroacetic acid, and diluted 10-fold with 0.1% trifluoroacetic acid in water. Linear peptide was purified by reversed-phase HPLC on a $C_{18}$ preparative column (Vydac 218TP1022, 20 ml/min), with a gradient of $CH_3CN$ (6–30%) in 0.1% trifluoroacetic acid. The peptide solution was diluted to approximately 20 μM, glutathione was added to give final concentrations of 1.0 mM reduced and 0.5 mM oxidized, and the pH was adjusted to 7.5 with NaOH.

[$Pro^{7,13}$] Conotoxin PIVA was built using standard *t*-butyloxycarbonyl chemistry on a Beckman 990B synthesizer; couplings were carried out with dicyclohexylcarbodiimide in dimethylformamide or dichloromethane/dimethylformamide. All amino acids were from Bachem (Torrance, CA). Side-chain protection, synthetic methodology, cleavage, air oxidation and desalting on Bio-Rex 70 were essentially as described for ω-conotoxin GVIA (9). Histidine was protected as the *p*-toluenesulfonyl derivative and coupled using hydroxybenzotriazole and benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphinium hexafluorophosphate.

Purification was carried out on a Vydac $C_{18}$ column (5 × 30 cm) using a gradient of $CH_3CN$ in TEAP buffer at pH 5.30 (6–21%/60 min; 100 ml/min). Fractions were collected and screened for purity by analytical HPLC on a Vydac $C_{18}$ column (0.46 × 25 cm) eluted isocratically with 16.2% $CH_3CN$ in 0.1% trifluoroacetic acid (2 ml/min). Samples were also analyzed by capillary electrophoresis at 20 kV, using 0.1 M sodium phosphate at pH 2.50 and a fused silica capillary (50 cm × 75 μm). Fractions of the highest purity were pooled and repurified/desalted on the same preparative column, using a gradient of $CH_3CN$ in 0.1% trifluoroacetic acid (6–36%/60 min; 100 ml/min).

*Electrophysiology*—Wire electrodes were used to record extracellular synaptic currents from populations of endplates in frog cutaneus pectoris preparations that had been treated with α-bungarotoxin to irreversibly attenuate the synaptic response to a subthreshold level as described previously (10).

*Biological Assays*—Intraperitoneal injections into goldfish and 10–14-day-old Swiss Webster mice, and intracranial injection into mice were performed as described previously (11).

*Binding Assays*—Binding assays were performed using a membrane fraction enriched in nAChR prepared from *Torpedo californica* electroplax (Pacific Bio-Marine, Venice, CA). All procedures were performed at 0–4 °C. Fifty ml of buffer (0.25 M NaCl, 0.02 M HEPES, 2 mM EDTA, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 2 μM leupeptin were added to 50 g of frozen *Torpedo* tissue, followed by processing in a Sorvall Omnimixer (30 s on highest setting, 30 s off, for a total of 6 min) and 5 stroke cycles in a Dounce homogenizer. Large particulate matter was removed by centrifugation at 3000 × *g* for 10 min. Membrane fragments were collected by centrifugation of the supernatant at 17000 × *g* for 50 min. The resulting pellet was resuspended in 10 ml of buffer and stored in small aliquots at −70 °C. *Torpedo* receptors (0.38 pmol of α-bungarotoxin binding sites/mg of protein, 2 μg protein/0.1 ml assay) were 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 either unlabeled α-bungarotoxin or with various concentrations of synthetic PIVA, followed by a 15-min incubation with α-[$^{125}$I]bungarotoxin (Amersham Corp., 2000 Ci/mmol, 12.5 pmol/assay). Unbound α-bungarotoxin was separated from receptor by centrifugation in a microcentrifuge for 3 min at ~15,000 × *g*. The pellet was washed with 0.1 ml of 0.02 M HEPES, pH 7.4, and 5 mM EDTA, followed by centrifugation. Radioactivity in pellet and supernatant was determined using a Packard Multi-Prias γ counter. Nonspecific binding was typically less than 2.5% of total binding, determined by preincubation with 0.01 mM unlabeled α-bungarotoxin.

## RESULTS

*Toxin Purification and Characterization*—A chromatographic profile of crude milked venom is shown in Fig. 3*A*. *Arrows* indicate three fractions that caused paralysis upon injection into goldfish, and that proved to be proline-hydroxylation isoforms of a single peptide. Further purification of peptide A, in which all three proline residues were hydroxylated, is shown in Fig. 3, *B* and *C*. Peptides B and C were similarly purified.

*Amino Acid Sequences*—Sequencer analysis of reduced and alkylated peptide A is reported in Table I. A single unambiguous sequence was obtained for 25 cycles. Attempts to obtain LSIMS mass spectra were initially unsuccessful because of limited amounts of material; all of these peptides have consistently given a 20–50-fold lower sensitivity than is typical for peptides in this size range. Lys-C digestion of pyridylethylated toxin yielded two peptides. One of these corresponded in mass to that expected for the amidated C-terminal fragment (residues 18–25: monoisotopic $MH^+$ observed, 1045.48; theoretical, 1045.45). No successful analysis was achieved with the other. The deduced linear structure of the toxin is as follows: A = PIVA, GCCGSYONAACHOCSCKDROSYCGQ-$NH_2$, where O represents *trans*-4-hydroxyproline. By a similar methodology, peptides B and C were shown to be the under-hydroxylated peptides: B = [$Pro^{13}$]PIVA, GCCGSYONAACHPCSCKDROSYCGQ-$NH_2$; C = [$Pro^{7,13}$]PIVA, GCCGSYPNAACHPCSCKDROSYCGQ-$NH_2$.

After completion of the synthesis of peptides A and C (below), and completion of the biological characterization, more snails became available, allowing reisolation of all three isoforms. LSIMS analysis was successfully carried out on intact peptides, and the monoisotopic molecular ions $MH^+$ were in agreement with those predicted from the above sequences: A (observed, 2648.0; theoretical, 2647.93); B (observed, 2631.9; theoretical, 2631.93); C (observed, 2615.9; theoretical, 2615.94).

*Disulfide Bridge Analysis*—This was carried out on 10 nmol

TABLE I
*Sequence analysis of αA-conotoxin PIVA*

This amino acid sequence above was obtained for peak A from Fig. 3*A*, using procedure 1 described under "Materials and Methods." The data for the other peaks (*B* and *C* in Fig. 3*A*) are not shown.

| Cycle | Assigned residue | Yield |
|---|---|---|
| | | *pmol* |
| 1 | Gly | 102.4 |
| 2 | Cys | 99.9 |
| 3 | Cys | 115.8 |
| 4 | Gly | 104.1 |
| 5 | Ser | 28.6 |
| 6 | Tyr | 61.4 |
| 7 | Hyp | 110.0 |
| 8 | Asn | 90.4 |
| 9 | Ala | 83.1 |
| 10 | Ala | 88.3 |
| 11 | Cys | 80.4 |
| 12 | His | 41.8 |
| 13 | Hyp | 95.0 |
| 14 | Cys | 86.2 |
| 15 | Ser | 14.6 |
| 16 | Cys | 68.6 |
| 17 | Lys | 49.4 |
| 18 | Asp | 46.7 |
| 19 | Arg | 23.4 |
| 20 | Hyp | 59.0 |
| 21 | Ser | 8.9 |
| 22 | Tyr | 33.9 |
| 23 | Cys | 34.1 |
| 24 | Gly | 32.4 |
| 25 | Gln | 28.2 |

of native peptide isolated from venom. Reaction with 10 mM TCEP at pH 3.0 (2 min, 61 °C) gave a suitable distribution of partially reduced peptides (Fig. 4*A*). Three major intermediates (intermediates 1–3) are evident, and others are indicated by broadening of the original peptide peak N. Intermediates 1 and 3 were purified and alkylated with iodoacetamide to label free thiols; residual bridges were then reduced and alkylated with 4-vinylpyridine. Sequencer analysis of intermediate 1 released Cys(cam) at cycles 3 and 11, while Cys(pe) was released at cycles 2, 14, 16, and 23. Analysis of intermediate 3 released Cys(cam) at cycles 2, 3, 11, and 16 and Cys(pe) at cycles 14 and 23. All other amino acids were identical to those shown in Table I, fully confirming the original sequence assignment. Thus the bridging pattern is [$Cys^2$-$Cys^{16}$; $Cys^3$-$Cys^{11}$; $Cys^{14}$-$Cys^{23}$], and the two intermediates lie on the reduction pathway shown in Fig. 4*B*. This particular disulfide topology has not previously been found in conotoxins.

*Peptide Synthesis*—Peptides A and C were chosen as synthetic targets to provide sufficient material for more detailed biological study. Peptide A was constructed using Fmoc chemistry, linear peptide was purified by HPLC, and disulfide bridges were allowed to form in the presence of a glutathione redox buffer. Under these conditions greater than 50% of the product eluted at the position corresponding to natural peptide. The misfolded material was recycled to obtain more of the correct form. After further HPLC purification in the trifluoroacetic acid system the peptide was judged to be 97–98% pure by analytical HPLC in TEAP buffers. Co-elution experiments confirmed identical chromatographic behavior of natural and synthetic products. LSIMS analysis of synthetic peptide indicated the correct monoisotopic $MH^+$ (observed, 2647.9; theoretical, 2647.93). As with the natural peptide, ionization was very inefficient, and relatively large amounts of sample were required.

Peptide C was constructed using *t*-butyloxycarbonyl chemistry. Crude linear peptide was air-oxidized by stirring at pH 7.0; the reaction was judged complete after 4 days. Purification was

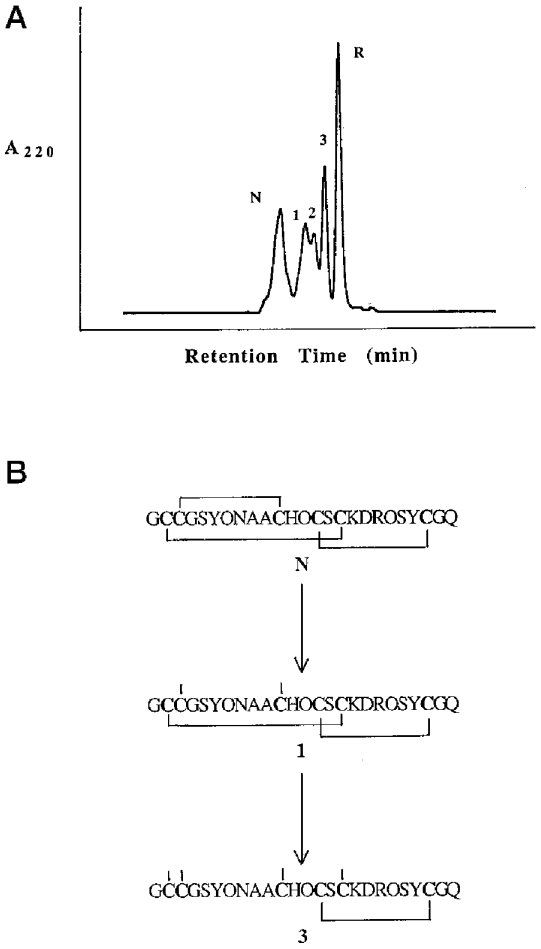


FIG. 4. *A*, reversed-phase HPLC chromatogram of αA-conotoxin PIVA, partially reduced by TCEP (10 mM, pH 3, 5 min at 61 °C). The absorbance peaks labeled *1*, *2*, and *3* correspond to the partially reduced peptides PR1, PR2, and PR3; *N* and *R* correspond to the native and completely reduced peptides, respectively. An analytical $C_{18}$ Vydac column was used for separating individual peaks using a gradient of $CH_3CN$ (6–30%) in trifluoroacetic acid (see "Materials and Methods"). *B*, disulfide bridge arrangements in native and partially reduced peptides.

carried out by adsorption onto Bio-Rex 70 cation exchanger, followed by preparative HPLC using TEAP buffers, with a final desalting and purification by HPLC in the trifluoroacetic acid system. TEAP at pH 5.30 was found to be preferable to the usual TEAP, pH 2.25, since the latter did not yield high purity fractions or a good yield of purified peptide. The advantage of using TEAP at various pH values has been recognized previously in the purification of synthetic peptides (12). The resulting peptide, which co-eluted with material isolated from venom, appeared to be 99% pure as analyzed by analytical HPLC and capillary electrophoresis. LSIMS showed the expected molecular ion $MH^+$ (observed, 2616.0; theoretical, 2615.94).

The major refolded material for both synthetic peaks A and C proved to be identical to their native counterparts; a mixture of native and synthetic peptide in each case gave a single sharp peak upon HPLC analysis (see Fig. 5). Both synthetic peptides were found to be biologically active by the fish paralysis assay. The chemical synthesis of these two peptides therefore confirms the sequence assignments given above, including the C-terminal amidation.

Because these peptides, like the α-conotoxins, inhibit the nicotinic acetylcholine receptor (see below), we have designated peak A αA-conotoxin PIVA, where the Roman numeral indi-

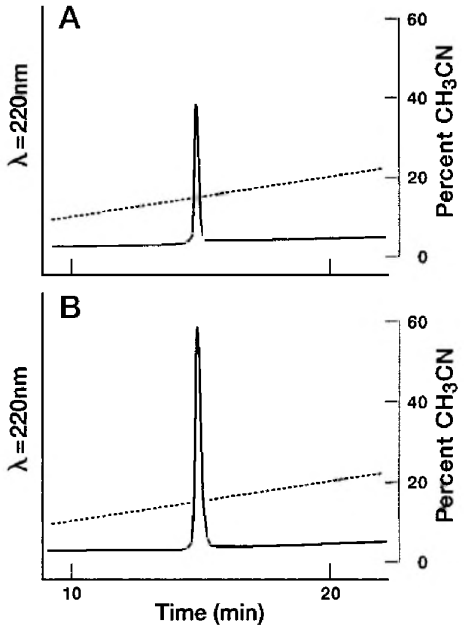


FIG. 5. *A*, reversed-phase HPLC chromatogram of synthetic αA-conotoxin PIVA (fully hydroxylated peptide). The peptide was eluted from a $C_{18}$ Vydac analytical column with a gradient of $CH_3CN$ (6–24%) in trifluoroacetic acid (see "Materials and Methods"). *B*, co-elution experiment. Equal amounts of the synthetic and native peptides were mixed and co-eluted using the same column, gradient, flow rate, and buffers as in *panel A*.

cates the new structural class.[2] Since peaks B and C are clearly under-hydroxylated derivatives of peak A, we designate them as [$Pro^{13}$]αA-conotoxin PIVA and [$Pro^{7,13}$]αA-conotoxin PIVA. However, the proportion of the under-hydroxylated forms (peaks B and C) relative to the fully hydroxylated toxin (peak A) varies from one milked venom preparation to the next. Although we previously detected under-hydroxylated forms of other conotoxins (see, for example Ref. 13), they have never been major species in any venom previously characterized. The under-hydroxylated forms may arise as a consequence of artificial aquarium conditions.

*αA-conotoxin PIVA Blocks the Nicotinic Acetylcholine Receptor*—The basis for the paralytic activity of αA-conotoxin PIVA was investigated by electrophysiological tests and binding experiments. The effect of the toxin on the frog neuromuscular junction is shown in Fig. 6*A*. The peptide clearly blocks the excitatory postsynaptic response. There was no difference between the time course of the partially blocked response and the control (Fig. 6*B*). The peptide blocked spontaneous miniature end plate potentials as well as the response to iontophoretically applied carbamylcholine without affecting the resting potential (results not shown), indicating that the peptide acts postsynaptically. These electrophysiological results strongly suggest that the peptide directly inhibits nicotinic acetylcholine

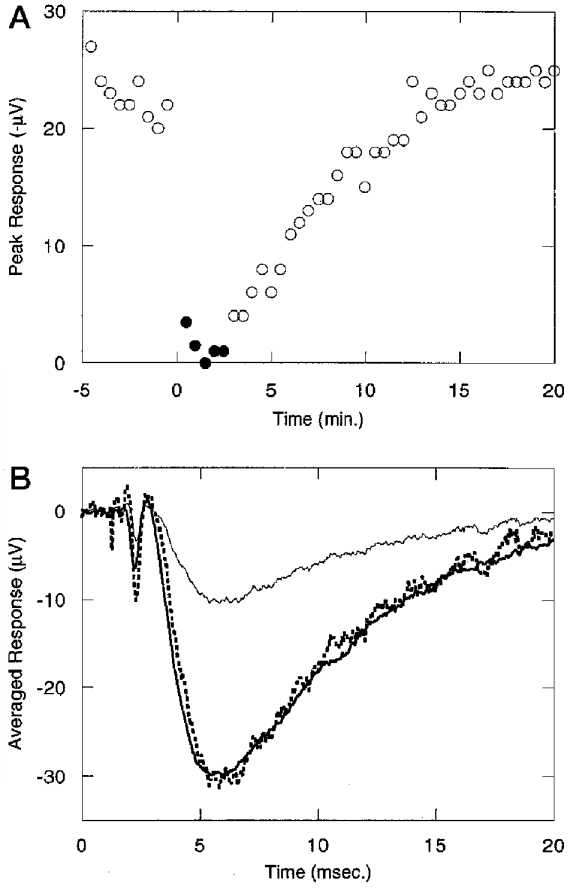


FIG. 6. *A*, exposure to 10 μM [$Pro^{7,13}$]αA-conotoxin PIVA reversibly blocks endplate currents. The motor nerve of a frog cutaneus pectoris muscle whose ACh receptors were partially blocked by α-bungarotoxin was stimulated every 30 s, and the evoked synaptic currents from a population of endplates was recorded extracellularly (see "Materials and Methods"). Peak amplitudes of the evoked synaptic currents are plotted as a function of time. [$Pro^{7,13}$]αA-conotoxin PIVA was applied at time 0. *Open circles*, responses before exposure to, and during washout of, toxin. *Closed circles*, responses in the presence of toxin. The response was rapidly blocked when the peptide was introduced and recovered relatively slowly upon peptide washout. *B*, exposure to 1 μM [$Pro^{7,13}$]αA-conotoxin PIVA reduces the endplate current amplitude 3-fold without affecting its time course. *Bold solid trace*, control response in the absence of toxin. *Finer solid trace*, response in the presence of toxin. *Dotted trace*, response in the presence of toxin normalized with respect to the control response by 3-fold expansion of its *vertical axis*. The normalized trace of the response in toxin coincides with the control trace, indicating that the toxin attenuates the postsynaptic response without altering its kinetics. Each trace represents the average of eight responses obtained under each condition. Rapid transients at t ≅ 2 ms are stimulus artifacts.

receptors.

This possibility was supported by competition binding experiments with α-[$^{125}$I]bungarotoxin used as a reporter for receptor occupancy of high affinity sites in the *Torpedo* electric organ, which are well-established to be on the nicotinic acetylcholine receptor. The results of binding experiments with αA-conotoxin PIVA and the $Pro^{7,13}$ derivative are shown in Fig. 7. Both peptides competitively inhibit α-bungarotoxin binding, indicating that these peptides target the macrosite that α-bungarotoxin binds to on the *Torpedo* receptor.

A survey of the *in vivo* biological activity of these peptides is shown in Table II.

## DISCUSSION

The results described above establish that the major paralytic toxin in *C. purpurascens* venom targeted to nAChRs, αA-conotoxin PIVA, has a strikingly different amino acid se-

[2] The peptides in this report define both a new *Conus* peptide family (αA), as well as a novel structural class. As we will detail elsewhere, we use the Roman numeral IV for all *Conus* peptides with the Cys framework –C$C X_n C X_2 C X C X_n$C–. We have characterized other conotoxin families of the same structural class but with different pharmacological specificities; these have the same disulfide framework but are not targeted to nicotinic acetylcholine receptors. All members of a given *Conus* peptide family share both a conserved Cys framework as well as a common pharmacological mechanism. We retain the Greek letter α for all *Conus* peptide families that inhibit the nAChR by binding to the ligand site α; to distinguish the new peptides from the α-conotoxins, we designate the former as αA-conotoxins. An additional distinction is that the distance between the first pair of cysteines and the next cysteine residue in the primary sequence is 7 amino acids in αA-conotoxin PIVA but 3–4 amino acids in all α-conotoxins.

quence from all other nAChR-targeted peptides previously characterized from *Conus* venoms. Despite the striking *structural* divergence, the mechanism by which this peptide causes paralysis is nevertheless similar to the well-characterized α-conotoxins from other fish-hunting *Conus* species, *i.e.* the peptide blocks the ACh binding site of the nAChR at the neuromuscular junction.

Comparison of the sequence of this new toxin, αA-conotoxin PIVA, with previously characterized α-conotoxins is shown in Table III. With one exception (α-conotoxin SII), the latter toxins have two disulfide bonds; in contrast, αA-PIVA has three. Furthermore, all paralytic α-conotoxins from fish-hunting *Conus* share the following conserved patterns of Cys and non-Cys amino acids, *i.e.* $CCX_3CX_5C$. The α-conotoxins from non-fish-hunting *Conus* species that have been described so far (5, 6), while having spacing different from that for fish-hunters as indicated above, nevertheless have the same Cys framework. In contrast, the new peptide, αA-PIVA, not only has a different Cys framework, but the spacing between the first pair of Cys residues and the third Cys is 7 amino acids instead of the 3 or 4 amino acids found in the nine characterized α-conotoxins. Thus, the new peptide is the first member of a new family of nAChR-targeted *Conus* peptides. Homologs of the *C. purpurascens* peptide will be designated αA-conotoxins (because of the divergence in the Cys framework, peptides belonging to this structural class will be given Roman numeral IV, as opposed to the regular α-conotoxins, which are always numbered I or II). In addition, *Conus* peptides with a Cys framework similar to that of PIVA but which are *not* targeted to the nAChR (and are therefore not αA-conotoxins) have also been characterized.[3]

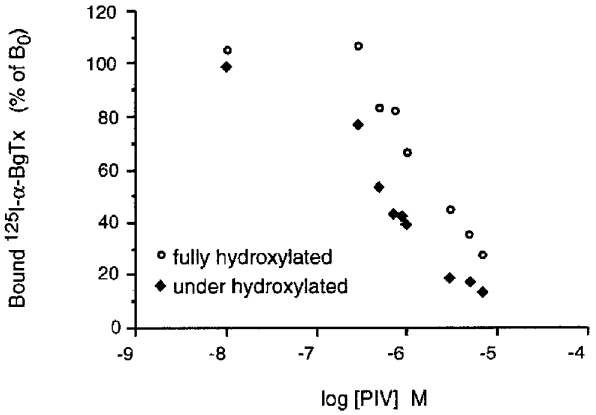


FIG. 7. **Competition binding of PIVA versus α-[$^{125}$I]bungarotoxin.** Competition binding with *Torpedo* electroplax membrane was performed as described under "Materials and Methods." Data points are the mean of three determinations at each concentration. $B_0$ is the amount of α-[$^{125}$I]bungarotoxin bound in the absence of competing peptide. The *open circles* and *closed triangles* are points for αA- and [Pro$^{7,13}$]αA-conotoxin PIVA, respectively.

TABLE II
*Bioassay of αA-conotoxin PIVA*

Peptide was injected intraperitoneally at a concentration of 0.5 nmol/gram of body mass. Weakness was defined for fish as loss of ability to swim against a weak current and for mice as loss of ability to cling to a wire mesh.

| Peptide | Time after injection | |
|---|---|---|
| | Weakness | Paralysis/death |
| | *min* | |
| αA-PIVA in fish | 2.7 | 4.3 |
| [Pro$^{7,13}$]αA-PIVA in fish | 3.2 | 5.0 |
| αA-PIVA in mice | 4.5 | 6.5 |
| [Pro$^{7,13}$]αA-PIVA in mice | 4.3 | 7.2 |

Thus, αA-PIVA is both the first member of a new family of *Conus* peptides, the αA-conotoxins, and the first representative of a new structural class of *Conus* peptides. The availability of a new group of nAChR-targeted *Conus* peptides that have significantly diverged from the α-conotoxin series provides new opportunities for probing the nAChR. Previously, reporter groups were attached to α-conotoxins at specific loci (14); such an approach can in principle be used to map the topology of the nAChR (15). Since *Conus* peptides are extensively cross-linked by disulfide bonds and are, therefore, fairly rigid, they provide a structurally discrete probe, which can be used to pinpoint the locations of residues in the receptor. Some α-conotoxins have been analyzed by multidimensional NMR techniques (16, 17); sufficient amounts of αA-conotoxin PIVA have been synthesized, and a structural analysis of these peptides by NMR methods is presently being carried out. Once the structural work is complete, αA-PIVA could be a useful probe for the nAChR based on an entirely different structural framework from that of the α-conotoxins.

The degree of under-hydroxylation of proline residues in αA-conotoxin PIVA deserves comment. Different samples of milked *C. purpurascens* venom show considerable variation in the degree of under-hydroxylation; in some venom samples, the two under-hydroxylated species described above are present at higher levels than the completely hydroxylated αA-PIVA (in contrast to the venom sample shown in Fig. 3*A*). Small differences in $IC_{50}$s shown in Fig. 7 are seen reproducibly, raising the possibility that the different hydroxylated forms have functional biological significance. We think that at least some of the under-hydroxylation observed may be an artifact of maintaining *C. purpurascens* in aquaria for extended periods of time. We have observed that several different *Conus* species become increasingly susceptible to pathology both in the periostracum and in laying down new shell if kept in aquaria in artificial sea water. Thus, it is possible that some under-hydroxylated forms observed in the milked venom are present at lower levels in *C. purpurascens* under natural conditions and that the under-hydroxylation observed may be a biochemical manifestation of a progressive pathology that occurs in aquaria. It is possible that some factors necessary for proline hydroxylation may be-

[3] G. Zafaralla, J. M. McIntosh, M. Grilley, L. J. Cruz, and B. M. Olivera, unpublished results.

TABLE III

| Conotoxin | Sequence[a] | Source | Reference |
|---|---|---|---|
| αA-PIVA | GCCGSYONAACHOCSCKDROSYCGQ* | *C. purpurascens* | This work |
| α-GI | ECCNPACGRHYSC* | *C. geographus* | 1 |
| α-MI | GRCCHPACGKNYSC* | *C. magus* | 2 |
| α-SI | ICCNPACGPKYSC* | *C. striatus* | 3 |
| α-SII | GCCCNPACGPNYGCGTSCS* | *C. striatus* | 4 |
| From non-fish-hunting *Conus* | | | |
| α-ImI | GCCSDPRCAWRC* | *C. imperialis* | 6 |
| α-PnIA | GCCSLPPCAANNPDYC* | *C. pennaceus* | 18 |

[a] The asterisk indicates C-terminal amidated, and O indicates *trans*-4-hydroxyproline.

come limiting. The pattern of under-hydroxylation suggests that the Pro residues are not equivalent as substrates for the hydroxylation enzyme, and that the ease of hydroxylation is in the order: $Pro^{20} > Pro^{7} > Pro^{13}$. In any case, the results in Fig. 7 were unexpected and surprising, and further studies investigating functional effects of proline hydroxylation are clearly desirable.

*C. purpurascens* is believed to be the only fish-hunting *Conus* species in the eastern Pacific marine geographic province. It has probably been isolated from fish-hunting Indo-Pacific species for an extended period of time. Its closest relative is thought to be *C. ermineus*, the major fish-hunting *Conus* species in the Atlantic marine province. An αA-conotoxin that differs significantly in sequence from αA-conotoxin PIVA has recently been identified in this species.[4] The discovery of the nAChR-targeted peptides in *C. purpurascens* that are so divergent from those found in other *Conus* species raises the intriguing possibility that fish-hunting may have evolved more than once in the Conidae. It will be of interest to determine which *Conus* species use the αA-conotoxin family as nAChR ligands instead of members of the α-conotoxin family.

*Acknowledgments*—We thank Dr. Robert Schackmann of the Utah Regional Cancer Center and Ron Kaiser of the Salk Institute for contributions to the sequencing and synthesis of peptides and Ben Johnson for milking the *C. purpurascens* specimens.

[4] J. M. McIntosh and J. Martinez, unpublished results.

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