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

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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:

\[
\text{GCGSYONACHCSDLCDVQCQ-NH}_2
\]

where O represents trans-4-hydroxyproline. The two other peptides purified from C. purpurascens venom are the under-hydroxylated derivatives, [Pro\textsuperscript{13}]αA-conotoxin PIVA and [Pro\textsuperscript{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)\textsuperscript{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 XCC(H/N)PACGXX(Y/F)XIC. 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\textsubscript{1}CC\textsubscript{2}C\textsubscript{3}C\textsubscript{4}C\textsubscript{5}C\textsubscript{6}.

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. 1B). 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...
The 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 storaxmuscarum*, *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 C18 Vydac preparative column (22 × 250 mm; 20 ml/min) with a guard column (22 × 50.8 mm).

As a secondary purification, a C18 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% CH3CN (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 C18 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 αA-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 pyridyethylated 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 αA-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 J MS HX310 double-focusing spectrometer, fitted with a Cs+ ion gun operated at +30 kV.

Solid Phase Peptide Synthesis—αA-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.
of analytical C18 Vydac column. A small portion of
and Methods. Peaks A, B, and C with arrows correspond to three forms of α
conotoxin PIVA. Panel B, further purification of peak A with an
analytical C18 Vydac column. A small portion of peak A was purified
using a gradient of CH₃CN (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.

![Graph](image)

**Fig. 3.** Panel A, reversed-phase HPLC chromatogram of C. purpurea
smilk. 0.5 ml of milked venom was injected into a C18
Vydac preparative column, and peptides were eluted with a gradient of
CH₃CN (0–36%) in trifluoroacetic acid as described under "Materials
and Methods." Peaks A, B, and C with arrows correspond to three forms of
α-conotoxin PIVA. Panel B, further purification of peak A with an
analytical C18 Vydac column. A small portion of peak A was purified
using a gradient of CH₃CN (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.

Amino acids were from Bachem (Torrance, CA) with side-chain protec-
tion, synthetic methodology, cleavage, limited amounts of material; al-
lofthesepeptideshaveconsist-
ent activity in pellet and supernatant was determined using a Packard
Multi-Priac γ counter. Nonspecific binding was typically less than
2.5% of total binding, determined by preequilibration with 0.01 mM
unlabeled α-bungarotoxin. Unbound α-bungarot-
oxin was separated from receptor by centrifugation in a microcentri-
fuge 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.
Radio-
activity in pellet and supernatant was determined using a Packard

**RESULTS**

Toxin Purification and Characterization—A chromato-
graphic profile of crude milked venom is shown in Fig. 3A. Arrows indicate three fractions that caused paralysis upon
injection into goldfish, and that proved to be proline-hydroxy-
lation isoforms of a single peptide. Further purification of pep-
tide 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—Sequence analysis of reduced and
alkylated peptide A is reported in Table I. A single unambigu-
ous sequence was obtained for 25 cycles. Attempts to obtain
LSIMS mass spectra were initially unsuccessful because of
limited amount of material; all of these peptides have consist-
ent activity in pellet and supernatant was determined using a Packard

**New Conus Peptide Family**
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. 4A). 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 [Cys2-Cys16; Cys3-Cys11; Cys14-Cys23], and the two intermediates lie on the reduction pathway shown in Fig. 4B. 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 and capillary electrophoresis. LSIMS showed the expected molecular ion MH$^+$ (observed, 2647.9; theoretical, 2647.94). 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 $\alpha$-conotoxins, inhibit the nicotinic acetylcholine receptor (see below), we have designated peak A $\alpha$A-conotoxin PIVA, where the Roman numeral indi-
The basis for the paralytic activity of \textit{tor}—cates the new structural class.\textsuperscript{2} Since peaks B and C are clearly work–CC use the Roman numeral IV for all artificial aquarium conditions. under-hydroxylated forms may arise as a consequence of artifical species in any venom previously characterized. They other conotoxins (see, for example Ref. 13), they have never previously detected under-hydroxylated forms of although we were. The 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 conditions.

\textit{\textalpha}A-conotoxin PIVA Blocks the Nicotinic Acetylcholine Receptor—The basis for the paralytic activity of \textit{\textalpha}A-conotoxin PIVA was investigated by electrophysiological tests and binding experiments. The effect of the toxin on the frog neuromuscular junction is shown in Fig. 6A. 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. 6B). 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 receptors.

\textsuperscript{2} The peptides in this report define both a new Conus peptide family (\textit{\textalpha}A), 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: \textit{CCX\textsubscript{2}CX\textsubscript{2}CCXX\textsubscript{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 \textit{\textalpha} for all Conus peptide families that inhibit the nAChR by binding to the ligand site \textit{\textalpha}; to distinguish the new peptides from the \textit{\textalpha}-conotoxins, we designate the former as \textit{\textalpha}A-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 \textit{\textalpha}A-conotoxin PIVA but 3–4 amino acids in all \textit{\textalpha}-conotoxins.

\textit{\textalpha}A-conotoxin PIVA was applied at \textit{\textalpha}M [Pro\textsuperscript{7,13}]a-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 \textit{\textalpha}M [Pro\textsuperscript{7,13}]a-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 \textit{t} = 2 ms are stimulus artifacts.

This possibility was supported by competition binding experiments with \(\textit{\textalpha}{\textsuperscript{[125I]}bungarotoxin binding, 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 \textit{\textalpha}A-conotoxin PIVA and the Pro\textsuperscript{7,13} derivative are shown in Fig. 7. Both peptides competitively inhibit \(\textit{\textalpha}-bungarotoxin binding, indicating that these peptides target the macosite that \(\textit{\textalpha}-bungarotoxin binds to on the Torpedo receptor.

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

\textbf{DISCUSSION}

The results described above establish that the major paralytic toxin in \textit{C. purpurascens} venom targeted to nAChRs, \textit{\textalpha}A-conotoxin PIVA, has a strikingly different amino acid se-
sequence 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, α-A-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, α-A-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. CXX3CXX3C. 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, α-A-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 α-A-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 α-A-conotoxins) have also been characterized.3

Thus, α-A-PIVA is both the first member of a new family of Conus peptides, the α-A-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 α-A-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, α-A-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 α-A-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 α-A-PIVA (in contrast to the venom sample shown in Fig. 3A). Small differences in IC50s 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-

![Graph](Image)

**Fig. 7. Competition binding of PIVA versus α-[125I]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. B0 is the amount of α-[125I]bungarotoxin bound in the absence of competing peptide. The open circles and closed triangles are points for α-A- and [Pro7,13]α-A-conotoxin PIVA, respectively.

| Peptide          | Time after injection (min) | Weakness | Paralysis/death |
|------------------|-----------------------------|----------|-----------------|
| α-A-PIVA in fish | 2.7                         | 4.3      | 4.3             |
| [Pro7,13]α-A-PIVA in fish | 3.2                         | 5.0      | 5.0             |
| α-A-PIVA in mice | 4.5                         | 6.5      | 6.5             |
| [Pro7,13]α-A-PIVA in mice | 4.3                         | 7.2      | 7.2             |

**Table III**

| Conotoxin | Sequence | Source | Reference |
|-----------|----------|--------|-----------|
| α-A-PIVA  | GCCSYONACIOCSKXDKOSYCGQ* | C. purpurascens | This work |
| α-GI      | ECCNPACGRHYSC* | C. geographus | 1 |
| α-MI      | GRCCHPACGKNYSC* | C. magus | 2 |
| α-SI      | IECNPACGPKYSC* | C. striatus | 3 |
| α-SII     | GCCNPACGNYGQGTSQC* | C. striatus | 4 |
| From non-fish-hunting Conus | | | |
| α-1ml     | GCCSDPRCAWRC* | C. imperialis | 6 |
| α-PnL     | GCCSLPPCAANNPDYC* | C. penncus | 18 |

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*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: $\text{Pro}^{20} > \text{Pro}^{7} > \text{Pro}^{13}$. 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. eminens, the major fish-hunting Conus species in the Atlantic marine province. An $\alpha$A-conotoxin that differs significantly in sequence from $\alpha$A-conotoxin PIVA has recently been identified in this species. 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 $\alpha$A-conotoxin family as nAChR ligands instead of members of the $\alpha$-conotoxin family.

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