Phylogenetic Specificity of Cholinergic Ligands: α-Conotoxin SI†

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ABSTRACT: The α-conotoxins are small peptide neurotoxins from the venom of fish-hunting cone snails which block nicotinic acetylcholine receptors (nAChRs). We describe the purification, characterization, and chemical synthesis of a new α-conotoxin from *Conus striatus*, α-conotoxin SI. In contrast to other AChR ligands, α-SI discriminates between different vertebrate nAChRs. The sequence of α-conotoxin SI is Ile-Cys-Cys-Asn-Pro^5-Ala-Cys-Gly-Pro-Lys^{10}-Tyr-Ser-Cys-NH_2. This sequence was confirmed by chemical synthesis. A des-Ile-α-SI derivative was also synthesized and is biologically active. Although α-conotoxin SI is highly homologous to previously described α-conotoxins, it has one noteworthy sequence feature which may account for its novel biological specificity. In all other α-conotoxins, there is a positively charged amino acid at residue 9; in α-conotoxin SI, this is replaced by proline. The discovery that different α-conotoxins can vary by orders of magnitude in their apparent affinity for different vertebrate receptors demonstrates that α-conotoxins will be useful probes for investigating phylogenetic differences between vertebrate nAChRs.

Acetylcholine receptors are pharmacologically divisible into two general classes, nicotinic and muscarinic. The nicotinic acetylcholine receptor (nAChR) directly controls a cation channel at the postsynaptic terminus; muscarinic receptors act on different ion channels through a second messenger system, presumably with a G protein intermediate. The nicotinic acetylcholine receptor from the *Torpedo* electric organ is probably the best understood of any receptor in an excitable tissue. Much of the recent progress achieved in understanding nicotinic acetylcholine receptors has been made possible by the availability of α-bungarotoxin (Conti-Tronconi et al., 1982), a polypeptide toxin from the venom of an elapiid snake (the Formosan Krait).

Our laboratories have characterized the α-conotoxins, which are found in the venoms of fish-hunting cone snails (genus *Conus*). These are peptides of 13–15 amino acids which block synaptic transmission by inhibition of the nAChR at the neuromuscular junction. In *Conus geographus* venom, the α-conotoxins, acting together with the ω-conotoxins (which block presynaptic voltage-sensitive Ca channels) and the μ-conotoxins (which directly block muscle sodium channel potentials), are the major agents which cause rapid paralysis of fish (Olivera et al., 1985). These 3 classes of paralytic peptides are probably largely responsible for the over 20 human fatalities that can be attributed to the stings of *Conus* species.

We previously described four molecular forms of α-conotoxins from the venoms of *Conus geographus* and *Conus magus* (Cruz et al., 1978; Gray et al., 1981; McIntosh et al., 1982). Like other antagonists of nicotinic acetylcholine receptors (curare, α-bungarotoxin), the previously characterized α-conotoxins did not show much phylogenetic discrimination between the various vertebrate receptors tested. In order to understand differences between vertebrate receptors, it is desirable to have molecular ligands that discriminate between different acetylcholine receptors. In this report, we demonstrate that the α-conotoxins have potential for providing such probes. We describe the purification and biochemical characterization of a new α-conotoxin from the venom of *Conus striatus*, the striated cone, which unlike previously characterized α-conotoxins is orders of magnitude less effective on the AChRs of mammals. We have correlated this specificity change to a novel sequence feature of this α-conotoxin.

Experimental Procedures

Materials. *Conus striatus* specimens were collected in the Philippines, the venom ducts were dissected from freshly killed specimens, and the venom was immediately lyophilized and stored at −20 °C. All other materials, reagents, and solvents used were of reagent or HPLC grade.

Purification of Conotoxin SI. Fraction A. Lyophilized venom from *C. striatus* (400–700 mg) was extracted with 1.1% HOAc as previously described (McIntosh et al., 1982). The extract was applied to a Sephadex G-25 column (110 × 2.5 cm) and eluted with 1.1% HOAc at a flow rate of 0.27 mL/min. Three major absorbance peaks were obtained (276 nm). When fractions from all peaks were bioassayed by intraperitoneal injection into fish, similar behavioral patterns were observed: angular swimming, jerking, slight body rigidity, and lateral repose prior to death. Further purification was carried out on material from the peak shown in Figure 1A (fraction A).

Fraction B. Fraction A was chromatographed using a semipreparative HPLC column (Ultropac TSK ODS-120T C18, 7.8 × 300 mm, 10 μm, fully end-capped) eluted with a gradient of CH_3CN in 0.1% TFA (0–48% CH_3CN in 30 min) at a flow rate of 2 mL/min. The peak indicated by an arrow in Figure 1B caused paralysis and death on intraperitoneal injection in fish (fraction B).

Fraction C. Fraction B was rechromatographed on an analytical VYDAC C18 HPLC column (4.6 × 250 μm, 5 μm, end-capped) using as eluant a gradient of CH_3CN in 0.1% TFA (12–36% CH_3CN in 20 min) at a flow rate of 1 mL/min. The major peak (↓) was found to be lethal to fish on intraperitoneal injection (fraction C).

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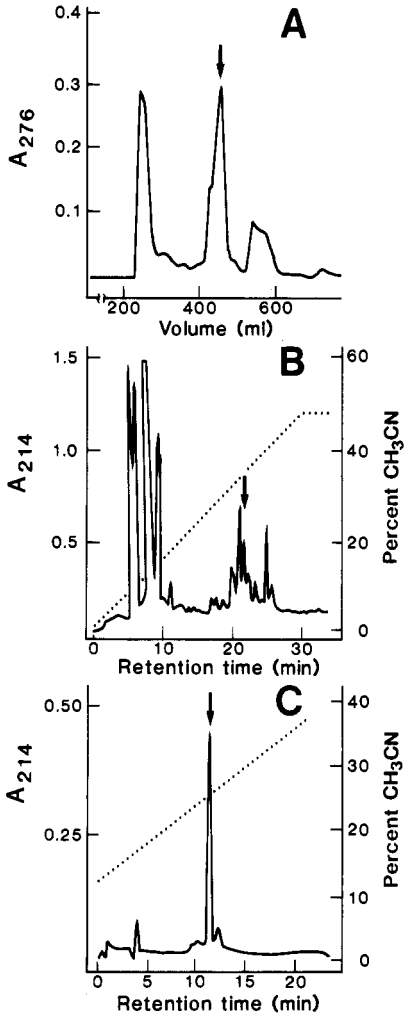

FIGURE 1: Purification of α-conotoxin SI. Elution profiles on Sephadex G25 (A), a semipreparative C18 HPLC column (B), and an analytical VYDAC C18 HPLC column (C) are shown; detailed procedures are given under Experimental Procedures. The arrows in the two top panels show fractions A and B, which were sequentially applied to the next column; fraction C was the material analyzed in Tables I and II. The dotted line shows the acetonitrile gradient that was applied.

Sequence Analysis of Conotoxin SI. Peptide from fraction C was reduced and carboxymethylated and analyzed in a spinning-cup sequencer as described previously (Edman & Begg, 1967).

Synthesis of Conotoxin SI and Des-Ile-SI. Synthesis was carried out by the solid-phase procedure of Merrifield (1963), following the general protocol of Gray et al. (1983). Methylbenzhydrylamine resin (2 g) was used as the starting support (Bachem Inc.; 0.55 mequiv/g substitution), and Boc-amino acids were purchased from Bachem. All, except Boc-Gly, were of the L configuration. Side chains were protected as Cys(Mob), Lys(2-Cl-Z), Tyr(Cl_2-Bzl), and Ser(Bzl). With one exception, couplings were carried out in dichloromethane and were mediated by diisopropylcarbodiimide (DICC). Asparagine was coupled without side-chain protection, using DMF as solvent, DICC as coupling agent, and 2 equiv of hydroxybenzotriazole to minimize side reactions. Coupling was repeated when Pro residues made the ninhydrin test (Kaiser et al., 1970) an unreliable guide for completeness of reaction.

Peptide was deprotected and removed from the resin as the C-terminal amide, using the low–high HF procedure of Tam et al. (1983). After lyophilization from 5% acetic acid, the peptide was reduced with DTT (10 mM in 6 M guanidinium chloride, pH 8; peptide concentration 57 mg/mL). The reduction mixture was then diluted to a final peptide concentration of 1.4×10^{-4} M with 0.05 M NH_4HCO_3, pH 8, and allowed to air-oxidize for 2 days at 25 °C. At this time, the Ellman test for –SH (Ellman, 1959) was negative, and a major component of the reaction mixture had the same mobility on HPLC as that of authentic material. This component was purified by preparative HPLC on Vydac C18, using the TEAP 5.4 and 0.1% TFA systems of Rivier et al. (1984).

Biological Activity. Biological activity was tested on mice, frogs, and fish by intraperitoneal and intracranial injection as described previously (Olivera et al., 1984).

Table I: Amino Acid Analysis of α-Conotoxin SI

amino acid	nmol	mole ratio
Asp	1.39	0.95 (1)[a]
Glu	0.34	0.23
Ser	1.62	1.12 (1)
Gly	1.71	1.18 (1)
Ala	1.33	0.92 (1)
Pro	2.98	2.05 (2)
Tyr	1.26	0.87 (1)
Cys[b]	3.24	2.23 (4)
Ile	1.08	0.74 (1)
Lys	1.78	1.22 (1)

[a] Values in parentheses indicate the number of residues found by sequence analysis. [b] Cysteine residues were unmodified, resulting in low yields.

Table II: Sequence Analysis of α-Conotoxin SI

step	assigned residue	yield (nmol of PTH-amino acid)[a]
1	Ile	3.10
2	Cys	0.84
3	Cys	1.29
4	Asn	1.54
5	Pro	1.16
6	Ala	1.02
7	Cys	0.73
8	Gly	1.04
9	Pro	0.51
10	Lys	0.97
11	Tyr	0.94
12	Ser	0.42
13	Cys	0.04

[a] Uncorrected for extraction efficiency or carryover.

RESULTS

Purification of α-Conotoxin SI. The purification of conotoxin SI is shown in Figure 1; details of the purification are given under Experimental Procedures. Crude venom was prepared from *Conus striatus* and applied to a Sephadex G25 column to separate the peptides from larger proteins (Figure 1A). The α-conotoxin SI was detected in one of the major peaks of the Sephadex G25 column eluate; when the biologically active G25 fraction was applied to a reverse-phase column, the profile in Figure 1B was obtained. A different HPLC column was used to purify the peptide to homogeneity (Figure 1C). The purified α-conotoxin SI gave the amino acid composition shown in Table I.

Sequence Analysis of α-Conotoxin SI. Upon reduction and carboxymethylation of homogeneous α-conotoxin SI, the peptide was analyzed by using an automatic spinning-cup sequencer. A single clear sequence was obtained through cycle 13 (Table II). The poor recovery of the C-terminal Cys is suggestive that this peptide may be amidated at the C-terminus; a blocked C-terminus was found in all previously characterized α-conotoxins.

Table III: Biological Activity of Some α-Conotoxins[a]

dose	α-conotoxin tested			
	GI	MI	SI	des-Ile-SI
test animal: mice				
0.2 nmol	death, 7.9	death, 5.9 min		
2.0 nmol	death, 5.7 min	death, 5.30 min	no effect	
10.0 nmol		death, 2.9 min	no effect	no effect
30 nmol				no effect
test animal: fish				
0.5 nmol		paralysis, 16–23 min; recovered	death, 12.4 min; 15.2 min	paralysis, 4–6 min; recovered
2 nmol		death, 8 min	death, 4.4 min; 5.5 min	death, 7.5 min; 10.3 min
test tissue: *Torpedo* electric organ				
IC_{50} (μM)	0.55	0.30	0.42	

[a] The test for biological activity on mice and fish was carried out as described under Experimental Procedures. The determination of an IC_{50} for α-conotoxins on α-bungarotoxin binding to AChRs using *Torpedo* electric organ as the test tissue was carried out as follows: frozen *Torpedo* electric organ was homogenized in 5 mL of SHT buffer (0.32 M sucrose and 5 mM HEPES brought to pH 7.4 with Trizma base) and centrifuged 35 min in a Beckman 75 TI rotor (45 000 rpm). The pellet was suspended in 2 mL of SHT plus 2% CHAPS by stirring for 30 min on ice. This suspension was centrifuged at 41 300 rpm for 35 min in an SW50.1 rotor, and the supernatant was recovered and stored at −70 °C. The α-bungarotoxin binding assays were carried out as described by Schneider et al. (1985). The *Torpedo* receptors were preincubated for 30 min either with 0.215 mL of SHT buffer containing lysozyme (1 μg) or with buffer containing unlabeled bungarotoxin or various concentrations of α-conotoxins; ^{125}I-α-bungarotoxin was then added for 15 min. After incubation, unbound α-bungarotoxin was separated from receptor by centrifugation for 3 min in a Biogel P100 column using a clinical centrifuge. The IC_{50} is the effective concentration of α-conotoxin GI, MI, and SI which displaces 50% or ^{125}I-labeled α-bungarotoxin binding under these conditions.

The sequence was confirmed by using fast atom bombardment (FAB) mass spectrometry. The value obtained for α-conotoxin SI (MH^+ 1353.49) confirms that the carboxyl terminus is amidated and indicates that all Cys residues are present as disulfides.

The sequence assignment in Table II was further confirmed by chemical synthesis of α-conotoxin SI as described under Experimental Procedures. The synthetic peptide was compared to the native peptide using analytical HPLC; synthetic and native peptides coelute if analyzed with the TFA/acetonitrile solvent system. A single sharp peak is obtained when the native/synthetic toxin mixture is analyzed.

The synthetic peptide gave the expected amino acid composition. FAB mass spectrometry of synthetic peptide showed the correct molecular ion (MH^+ 1353.44). Furthermore, the synthetic peptide was biologically active, with the same specific activity on fish as the native toxin (see below).

The complete chemical synthesis of biologically active toxin not only confirms the sequence assignment given above but also makes available significantly more material than can be readily obtained from natural sources.

Biological Effects of α-Conotoxin SI. α-Conotoxin SI was tested for biologiccal activity. In fish and amphibians, the effect of the peptide is similar to the biological effects of two previously characterized α-conotoxins, MI and GI. Thus, a 1-nmol injection of any of the three α-conotoxins into goldfish (1–1.5 g) causes obvious paralytic effects within 10 min, and death between 40 and 60 min. Indeed, α-conotoxin SI appeared to be slightly more effective as a paralytic toxin than α-conotoxin MI or GI. Paralysis was routinely seen when 0.1 nmol of toxin was used to inject goldfish; effects were sporadically observed even with 10-pmol injections. Binding experiments using ^{125}I-α-bungarotoxin as a probe demonstrated that all three conotoxins (GI, MI, and SI) were capable of displacing all specific α-bungarotoxin binding to the *Torpedo* receptor and had very similar apparent affinities for the *Torpedo* AChR; the IC_{50} values are all in the range of 0.3–0.5 μM using the α-bungarotoxin displacement assay (see Table III).

In contrast to the high efficacy of the toxin in fish, α-conotoxin SI is much less effective in mice. Injection of either α-conotoxin MI or α-conotoxin GI routinely causes death of mice in less than 10 min when 0.2 nmol is injected; surprisingly, intraperitoneal injection of α-conotoxin SI into mice showed little if any effect at doses from 2 to 10 nmol. The same general conclusion was reached from intracerebral injection of mice; if α-conotoxin SI is injected at levels of up to 30 nmol, the biological effects observed are minimal. In contrast α-conotoxin MI and GI cause death at levels <1 nmol. Since previously characterized α-conotoxins have been active in all vertebrate systems tested, we had anticipated that α-conotoxin SI would have generally similar activity on all vertebrate AChRs. The discovery that different α-conotoxins might have similar activity on one receptor (i.e., *Torpedo* AChR) and yet vary by orders of magnitude on a different AChR was unexpected.

Table IV: α-Conotoxin Sequences[a]

GI	Glu Cys Cys Asn Pro Ala Cys Gly Arg His Tyr Ser Cys-NH_2
GIA	Glu Cys Cys Asn Pro Ala Cys Gly Arg His Tyr Ser Cys Gly Lys-NH_2
GII	Glu Cys Cys His Pro Ala Cys Gly Lys His Phe Ser Cys-NH_2
MI	Gly Arg Cys Cys His Pro Ala Cys Gly Lys Asn Tyr Ser Cys-NH_2
SI	**Ile Cys Cys Asn Pro Ala Cys Gly Pro Lys Tyr Ser Cys-NH_2**
des-Ile-SI	Cys Cys Asn Pro Ala Cys Gly Pro Lys Tyr Ser Cys-NH_2

[a] α-Conotoxins GI, GIA, and GII are found in *Conus geographus* venom, α-conotoxin MI is from *Conus magus*, and α-conotoxin SI is from *Conus striatus*; the des-Ile-SI derivative is a synthetic α-conotoxin SI analogue with the sequence shown.

Sequence Features Important in Phylogenetic Discrimination. As shown in Table IV, α-conotoxin SI has two potentially unique features compared to all other previously characterized α-conotoxins. At the N-terminus, it has a hydrophobic residue, Ile, instead of the charged residues present at the N-termini of the *C. geographus* and *C. magus* peptides. A priori, the Ile substitution seems unlikely to be responsible for the phylogenetic discrimination; it was previously shown that removal of the N-terminal amino acid in α-conotoxin GI did not significantly change biological activity. The second unusual sequence feature is the substitution of Pro-Lys in positions 9 and 10; this is Arg-His in α-conotoxin GI and Lys-Asn asparagine in α-conotoxin MI. The substitution of proline for a positive amino acid at position 9 would be expected to result in a significant conformational change in the peptide chain.

In order to experimentally narrow down the sequence feature responsible for the change in affinity for mammalian AChRs, the biological activity of α-conotoxin SI lacking the N-terminal Ile was assessed. Synthetic α-conotoxin SI lacking the N-

terminal Ile was synthesized as described under Experimental Procedures; the peptide was tested for biological activity on fish. Its activity was compared to α-conotoxin SI, as well as α-conotoxin MI. These results are shown in Table III. It is clear that although all three peptides are active on fish, neither α-conotoxin SI nor the SI analogue lacking the N-terminal isoleucine is active on mice. This evidence is consistent with the hypothesis that it is the proline substitution at position 9 which may account for the phylogenetic discrimination exhibited by the SI-type peptides.

Discussion

Conus striatus is the third piscivorous cone venom from which α-conotoxins have been isolated. SI can be firmly placed in the α-conotoxin class because of its ability to compete with α-bungarotoxin, and because of its obvious sequence homology with the other α-conotoxins. The sequences of all natural α-conotoxins which have been characterized to date are shown in Table IV.

The peptide from *Conus striatus* has a unique feature: residue 9, which is Arg or Lys in all previously characterized α-conotoxins, is not occupied by a positively charged residue in α-conotoxin SI, but by a proline. However, the next residue (Lys) is positively charged. It has been shown previously that some biological activity is retained even if the positively charged amino acid is replaced by norleucine (Nishiuchi & Sakakibara, 1985) and that the amino terminus is also quite tolerant of substitution or deletion (Gray et al., 1984; Nishiuchi & Sakakibara, 1985).

Previously characterized α-conotoxins have been active in all vertebrate systems tested. The discovery that different α-conotoxins might have similar activity on one receptor (i.e., *Torpedo* AChR and presumably the teleost AChR) and yet vary by 2 orders of magnitude on a different (mammalian) AChR was quite unexpected. The data are consistent with the hypothesis that changes at residues 9 and 10 are responsible for the ability of α-conotoxin SI to discriminate between *Torpedo* (and teleost) AChRs vs the mammalian neuromuscular AChR. Additional analogues which combine sequence features of SI with those of conotoxins MI and GI are currently being synthesized, and their biological activity will be assessed. A major advantage of the α-conotoxins for specificity studies of this type is that they are small and relatively easy to synthesize and predictions of how sequence changes lead to a change in activity can be directly tested. The α-conotoxin/AChR system may be an optimal ligand/receptor pair to study at a molecular level how sequence changes in the ligand can lead to a different receptor binding specificity.

It is quite possible that these sequence differences in the α-conotoxins not only will result in phylogenetic discrimination but also will lead to different AChR subtype specificity. We have preliminary experimental results which suggest that α-conotoxins may be useful for subtyping neuronal AChRs (R. Karlstrom, unpublished results). Thus, the α-conotoxins should prove to be a valuable tool for defining AChR subtypes and for investigating phylogenetic changes in the receptor.

Added in Proof

A referee has pointed out to us a report that short-chain α-neurotoxins of snake venoms discriminate between human and murine AChR receptors (Ishikawa et al., 1985).

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