α-Conotoxin EI, a New Nicotinic Acetylcholine Receptor Antagonist with Novel Selectivity†

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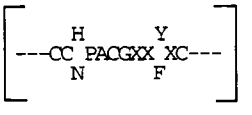
ABSTRACT: We report the isolation and characterization of a novel nicotinic acetylcholine receptor (nAChR) ligand. The toxin is an 18 amino acid peptide and is the first reported α-conotoxin from an Atlantic fish-hunting *Conus*. The peptide was purified from the venom of *Conus ermineus* and is called α-conotoxin EI. The sequence diverges from that of previously isolated α-conotoxins. We demonstrate that this structural divergence has functional consequences. In *Torpedo* nAChRs, α-conotoxin EI selectively binds the agonist site near the α/δ subunit interface in contrast to α-conotoxin MI which selectively targets the α/γ agonist binding site. In mammalian nAChRs α-conotoxin EI shows high affinity for both the α/δ and α/γ subunit interfaces (with some preference for the α/δ site), whereas α-conotoxin MI is highly selective for the α/δ ligand binding site. The sequence of the peptide is: Arg-Asp-Hyp-Cys-Cys-Tyr-His-Pro-Thr-Cys-Asn-Met-Ser-Asn-Pro-Gln-Ile-Cys-NH_2, with disulfide bridging between Cys4−Cys10 and Cys5−Cys18, analogous to those of previously described α-conotoxins. This sequence has been verified by total chemical synthesis. Thus, α-conotoxin EI is a newly-available tool with unique structure and function for characterization of nAChRs.

Nicotinic acetylcholine receptors (nAChRs)[1] in skeletal muscle and the electric organ of *Torpedo* are heteropentameric ligand-gated cation channels formed by four subunits in the stoichiometry $(\alpha_1)_2\beta_1\gamma\delta$. Several small molecule toxins isolated from plants, coral, and gastropods as well as polypeptide toxins from predatory organisms have been isolated which target nAChRs [for review see Chiappinelli (1993)]. The availability of these toxins has played a critical role in the progressive understanding of the structure and function of the nicotinic receptor. The nAChR requires two molecules of acetylcholine to bind two separate sites for channel opening. These nonequivalent binding sites are located at the α/γ and α/δ subunit interfaces (Blount & Merlie, 1989). Curariform antagonists bind with 1−2 orders of magnitude higher affinity to the α/γ site than to the α/δ site of both mammalian muscle and *Torpedo* receptors (Pedersen & Cohen, 1990; Kreienkamp et al., 1992; Sine, 1993).

The venoms of fish-hunting cone snails have proven to be an abundant source of small disulfide-rich peptides which target with high affinity and specificity to receptors and ion channels in the neuromuscular system of the prey (Olivera et al., 1985). Many of these peptides have become useful ligands in neuroscience, both as pharmacological tools and as biochemical probes for investigating receptor structure (Olivera et al., 1990). Among the prominent conotoxins found in the venoms of fish-hunting cone snails are a family of *Conus* peptides, the α-conotoxins, which target to the nicotinic acetylcholine receptor at the neuromuscular junction. From three different fish-hunting cone snail venoms, seven α-conotoxins with the following consensus sequence



have been purified and sequenced (Myers et al., 1993).

Two of these peptides, α-conotoxins MI and GI, have been shown to preferentially target the α/δ site by 10^4-fold over the α/γ site in mammalian muscle nAChRs (Kreienkamp et al., 1994; Groebe et al., 1995). In *Torpedo*, however, α-conotoxins MI and GI selectively target the α/γ binding site (Hann et al., 1994; Utkin et al., 1994; Groebe et al., 1995). Studies using chimeric subunits and site-directed mutagenesis have identified three specific amino acid differences in the δ and γ subunits that account for the differential α-conotoxin MI affinities in mouse muscle (Sine et al., 1995). One of the three amino acids that confers high affinity for the mouse δ subunit (Tyr 113) is absent from

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[1] Abbreviations α-BTX, α-bungarotoxin; α-CTx, α-conotoxin; HPLC, high-performance liquid chromatography; Hyp, *trans*-4-hydroxyproline; nAChR, nicotinic acetylcholine receptor; MTBE, methyl *tert*-butyl ether; NMP, *N*-methylpyrrolidone; TBTU, 2-(1*H*-benzotriazol-l-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TCEP, tris(2-carboxyethyl)phosphine; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid.

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the *Torpedo* δ subunit but present in the *Torpedo* γ subunit. This amino acid may account for MI's different selectivities for mouse vs *Torpedo*. Other differences between mouse muscle and *Torpedo* electric organ nAChRs, e.g., glycosylation (Kreienkamp et al., 1994; Keller et al., 1995), might also play a role.

α-Conotoxins MI and GI were isolated from *Conus magus* and *Conus geographus*, relatively large Indo-Pacific species collected in Philippine waters. In this study, we investigated the venom of *Conus ermineus*, the only known fish-hunting *Conus* found in the Atlantic Ocean. *C. ermineus* occurs over a wide geographic range, from the Caribbean to the West African coast. We describe the identification, purification, and biochemical characterization of an α-conotoxin from this *Conus* species.

In contrast to previously described α-conotoxins from fish-hunting *Conus* venoms, the *C. ermineus* peptide does not share the consensus sequence described above, and very significant sequence differences are seen between this peptide and the other previously characterized α-conotoxins. This structural divergence has significant functional consequences. α-Conotoxin EI in contrast to other α-conotoxins and curare, selectively targets the α/δ acetylcholine binding site in *Torpedo*.

EXPERIMENTAL PROCEDURES

Materials. Crude venom was obtained from milkings of *C. ermineus* kept in aquaria. The venom was subsequently stored at −70 °C until use. [^{125}I]α-BTX was from DuPont NEN; β-mercaptoethanol was from Pierce Chemical Co.; dithiothreitol was from Boehringer-Mannheim; TFA (sequencing grade) was from Aldrich; acetonitrile (UV grade) was from Baxter; iodoacetamide was from Sigma; tris(2-carboxyethyl)phosphine (TCEP) was synthesized by the method of Burns et al. (1991). Metocurine (also known as dimethyl-*d*-tubocurarine) was a gift from Lilly.

Venom Preparation. Individual milkings of *C. ermineus* venom collected from 10 snails were pooled together (final volume 1.5 mL) and concentrated by lyophilization to 0.5 mL. This concentrated mixture was then combined with 3 mL of 0.1% TFA immediately prior to injection onto the HPLC column.

Peptide Isolation and Sequencing. HPLC Purification. The HPLC apparatus consisted of HPXL pumps and either a Dynamax model UVI or UV-DII detector (Rainin, Woburn, MA). All columns were also purchased from Rainin. For isolation of peptide from venom and all subsequent purifications, buffer A consisted of 0.1% TFA and buffer B was 0.1% TFA and 60% acetonitrile. Initial purification of α-conotoxin EI from milked venom was accomplished using a semipreparative C_{18} Vydac column (10 mm × 25 cm, 5 μm particle size); flow rate was 5.0 mL/min. Subsequent purification steps of α-conotoxin EI utilized an analytical C_{18} Microsorb or C_{18} Vydac column (4.6 mm × 25 cm, 5 μm particle size); flow rate was 1.0 mL/min.

Sequence Analysis. Peptide from the final purification was stored at −20 °C in the 0.1% TFA/acetonitrile elution buffer. Purified peptide solution (approximately 250 pmol in 80 μL) was combined with 24 μL of 0.5 M sodium phosphate, pH 8.0, and 24 μL of reducing buffer (50 mM EDTA, 25 mM dithiothreitol, and 25% glycerol). The reaction vessel was flushed with argon and the reaction mixture was incubated at 65 °C for 15 min. After the vessel had cooled to room temperature, 3 μL of 4-vinylpyridine was added and the solution reacted in the dark for 25 min at room temperature. This solution was then diluted 3-fold with 0.1% TFA, and the alkylated peptide was purified on a C4 Microsorb MV column (4.6 mm × 25 cm, 5 μm particle size) with the gradient program (5%−40% B/70 min, followed by 40%−100% B/12 min). Sequencing was performed on an Applied Biosystems model 477A protein sequencer at the Protein/DNA Core Facility at the University of Utah Cancer Center.

Mass Spectrometry. Liquid secondary ionization mass spectra were measured using a Jeol HX110 (JEOL, Tokyo, Japan) double-focusing mass spectrometer operated at 10 kV accelerating voltage and a nominal resolution of 3000. The sample (in 0.1% aqueous TFA and 25% acetonitrile) was mixed in a glycerol/3-nitrobenzyl alcohol matrix (1:1) and analyzed with an electric field/accelerating voltage scan over a narrow mass range. The mass accuracy of the HX110 instrument under these conditions was typically better than 50 ppm.

Peptide Synthesis and Disulfide Analysis. The peptide was synthesized by solid-phase methods, employing standard protocols for fmoc-based chemistry on an ABI model 430A peptide synthesizer. Construction of the peptide-resin was accomplished on a Rink amide resin, using single couplings with TBTU. All amino acid derivatives were purchased from Bachem (Torrance, CA) and manually loaded into cartridges for the instrument. Side-chain protection was *tert*-butyl (Asp, Hyp, Ser, Thr, Tyr), trityl (Asn, Gln, His, Cys), or pentamethylchromansulfonyl (Arg). After completion of the synthesis, the terminal fmoc was removed from the peptide resin *in situ*, and the resin was washed with dichloromethane and methanol and dried under vacuum.

Deprotection and cleavage was carried out by mixing for 2 h at 25 °C, using 10 mL of reagent K (TFA:phenol:H_2O:ethanedithiol:thioanisole, 90:7.5:5:2.5:5 by volume) for 600 mg of peptide resin. The peptide solution was filtered rapidly into MTBE previously cooled to −10 °C to remove resin particles and to precipitate the peptide. A flocculent precipitate was obtained which was collected by centrifugation and washed three times with MTBE. The pellet was dissolved in sufficient B buffer (0.1% TFA in 60% acetonitrile) to give a one-phase solution and diluted extensively with A buffer (0.1% TFA). The linear peptide was purified by reversed-phase HPLC on a preparative C_{18} Vydac column (2.5 cm × 30 cm) using a gradient of 10%−50% B in 40 min.

Oxidative closure of the disulfide bridges was accomplished by stirring the peptide solution in an open Erlenmeyer flask, after dilution to 2 L, and adjustment to pH 7.5 using solid Tris. Progress of the oxidation was monitored by analytical HPLC. Once it was judged to be complete (16 h), the bicyclic peptide isomers were isolated by purification using a gradient of acetonitrile (6%−36%/40 min) in 0.05 M triethylamine phosphate, pH 2.25. Three isomers were obtained, which were desalted by rechromatography in the TFA system described above.

Disulfide Analysis. The partial reduction strategy of Gray (1993) was used. Synthetic peptide, 15 nmol, as eluted from HPLC, was incubated at room temperature with 10 mM TCEP in 0.1 M citrate, pH 3. Following a 3 min incubation, the reaction mixture was diluted with 0.1% TFA and injected onto a C_{18} Vydac HPLC column. Peptide fractions, as

identified by UV absorbance, were collected manually into 1.5 mL polypropylene centrifuge tubes. Partially reduced intermediates (monocyclic) were alkylated with 100 mg of iodoacetamide using the rapid alkylation method (Gray, 1993). Labeled peptides were submitted for sequence analysis to determine the locations of *S*-carboxamidomethyl-L-cysteine residues and hence the disulfide connectivity.

Bioassay. Swiss Webster mice (approximately 24 grams) were injected intraperitoneally (i.p.) with 150 μL of 150 mM NaCl containing varying amounts of toxin. Mice were observed for symptoms of paralysis, and death time was determined by monitoring heart beat with a stethoscope.

Goldfish (4−5 cm) were injected either i.p. or intramuscularly (i.m.) with 7 μL of 150 mM NaCl with or without toxin using a Hamilton syringe. Fish were observed for paralysis by placing them in a 500 mL beaker and stirring the water with a glass rod (approximately 120 revolutions per minute). Unaffected fish were able to maintain their position in the beaker by swimming against the current. As fish became progressively paralyzed they increasingly drifted with the current.

Inhibition of [^{125}I]α-BTX Binding to Nicotinic Receptors on Intact BC_3H-1 Cells. BC_3H-1 cells were maintained and prepared for experiments as previously described (Groebe & Abramson, 1995). Cells were incubated in 250 μL of assay buffer (140 mM KCL, 25 mM HEPES, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgSO_4$, 0.06 mg/mL BSA, pH 7.4) containing metocurine, α-conotoxins EI or MI, or combinations of the three drugs. [^{125}I]α-BTX (10 μL of 520 nM for a final concentration of 20 nM) was added and the reaction was incubated for an additional 15 min. Cells were then washed twice with 2.0 mL of assay buffer to remove unbound ligands, and receptor-bound [^{125}I]α-BTX was removed from the wells with two 0.5 mL washes of 1% Triton X-100 in water and counted in a γ counter. Nonspecific binding was determined from cells previously exposed to 100 nM α-BTX for 30 min. The total density of [^{125}I]α-BTX binding sites was determined from a 60 min incubation in the absence of competing drug. The total density of specific [^{125}I]α-BTX binding sites was 170 ± 14 fmol/well (n = 12) and 65% ± 1.5% were labeled by [^{125}I]α-BTX in 15 min. All assays were performed at room temperature in triplicate.

Inhibition of [^{125}I]α-BTX Binding to Nicotinic Receptors on Membranes from Torpedo Electric Organ. Membranes containing nAChRs were prepared from *Torpedo* electric organ and assayed as previously described (Valenzuela et al., 1992; Groebe et al., 1995). *Torpedo* nicotinic receptors (0.3−2.0 nM [^{125}I]α-BTX binding sites) were incubated in 108 μL of assay buffer (10 mM sodium phosphate, pH 7.4, 1.0 mM EDTA, 1.0 mM EGTA, 0.1% Triton X-100) containing metocurine, α-conotoxin EI or MI, or combinations of the three drugs. [^{125}I]α-BTX (12 μL of 10−100 nM) was added, and the reaction was incubated for 30 s. Aliquots of the reaction (50 μL) were spotted onto DE81 ion-exchange filters (Whatman), absorbed for 30 s, and then washed for at least 10 min in each of two successive 600 mL stirred baths of wash buffer (10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100). The filters were blotted between paper toweling, and bound [^{125}I]α-BTX was counted. Nonspecific binding was determined from receptors previously exposed to 100 nM α-BTX for 30 min. The total concentration of [^{125}I]α-BTX binding sites was determined from a 60 min incubation in the absence of competing drug, and less than 80% of the sites were labeled by [^{125}I]α-BTX in 30 s. All assays were performed at room temperature in duplicate.

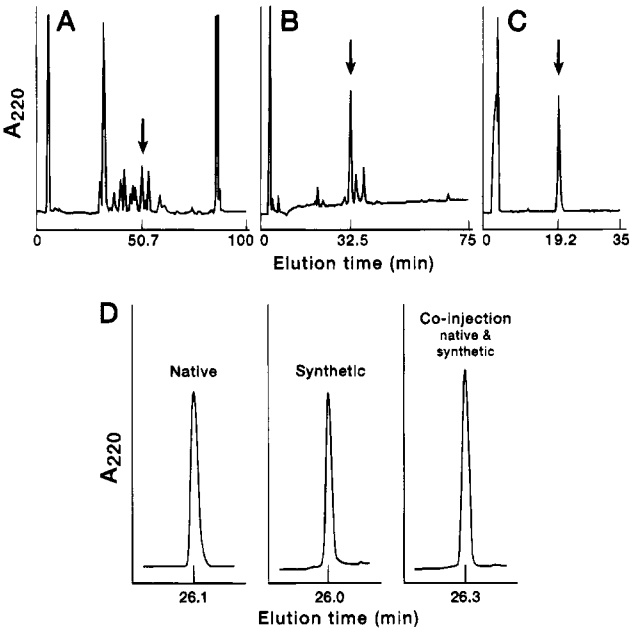


FIGURE 1: Purification of α-conotoxin EI from *C. ermineus* venom. Buffer A = 0.1% TFA; Buffer B = 0.1% TFA, 60% acetonitrile. Panel A, milked venom (see text) was applied to a semipreparative C_{18} Vydac column. Gradient program 1 was 0%−15% B/5 min, 15%−40% B/74 min, 40%−75% B/3 min, followed by 75%−100% B/14 min; flow rate was 5 mL/min. Panel B, material eluting at 50.7 min (arrow, panel A) was rerun on an analytical C_{18} Microsorb MV column with gradient program 2 (15%−25% B/10 min; 25%−40% B/75 min; 40%−100% B/2 min); flow rate was 1.0 mL/min. Panel C, material eluting at 32.5 min (arrow, panel B) was rerun on an analytical C_{18} Vydac column with gradient program 2 to obtain the final purified product. Time in min is shown on the *X* axis. Panel D, comparison of natural and synthetic α-conotoxin EI by HPLC. Analysis was carried out on a C_{18} Vydac column. The gradient began at 10% B and increased at 1% B/min.

The data for the interaction of the α-conotoxins and metocurine with nicotinic receptors were analyzed by nonlinear regression using functions describing the competitive binding of a ligand to a single site or two independent sites (Groebe et al., 1995). The best fit function to the data was determined by performing an *F*-test on the sum-of-squares of the residuals (Abramson & Molinoff, 1986). A more complex model (a model with more unknown parameters) was accepted only when a value of $p < 0.05$ was obtained. Nonlinear regression analysis was performed on a Northgate 386 personal computer using the software program Prism (GraphPad).

RESULTS

Isolation of α-conotoxin EI. Crude venom was initially fractionated by reversed-phase HPLC (see Figure 1A). The UV absorbance pattern is relatively simple compared to that of other crude cone venoms; this may reflect that milked venom is being used. In *Conus striatus*, for instance, it has been found that milked venom lacks most of the higher molecular weight components found in venom obtained from dissected venom ducts (Hopkins et al., 1995). It is hypothesized that the venom obtained from milkings is highly processed and therefore lacks some of the high molecular

weight components which may be present in precursor form. However, as we did not have access to crude venom from dissected *C. ermineus* ducts, a similar comparison cannot be made with this species.

Individual fractions from the initial purification step were assayed by i.m. injections into fish and i.p. injections into mice. Numerous active components were identified; the arrow in Figure 1A represents the pool containing α-conotoxin EI. This fraction was rechromatographed on a C_{18} Microsorb MV column, which we have found has slightly different selectivity from the C_{18} Vydac column (Figure 1B). The active fraction was further purified using a C_{18} Vydac column. Note the significantly different elution time with the Vydac vs the Microsorb column using the identical elution program (Figure 1C).

Biochemical Characterization of α-Conotoxin EI. Purified peptide was reduced, alkylated, and sequenced as described under Experimental Procedures. The sequence is Arg-Asp-Hyp-Cys-Cys-Tyr-His-Pro-Thr-Cys-Asn-Met-Ser-Asn-Pro-Gln-Ile-Cys-NH_2. Like other α-conotoxins, the peptide is cysteine rich, with Cys representing four of 18 residues. Liquid secondary ionization mass spectrometry of native peptide indicated that Cys residues are present as disulfides and that the C-terminus is amidated [monoisotopic MH^+ (Da): calcd, 2092.79; obsd, 2092.9].

The sequence was independently verified by the preparation of synthetic peptide as described in Experimental Procedures. Air oxidation of the linear synthetic peptide gave a mixture of three isomers, with relative yields of 35:60:5, of which the latter two separated very poorly using 0.1% TFA/acetonitrile as the eluent. These two were readily resolved by chromatography using 0.05 M TEAP, pH 2.25 (Rivier, 1978). The major isomer coeluted with natural peptide on HPLC (Figure 1D) and proved to be the biologically active peptide. The other isomers were inactive upon i.p. injection of approximately 10 nmol into mice (data not shown). To determine the disulfide bond arrangement of α-conotoxin EI, partial reduction of the synthetic peptide by TCEP was performed. Two monocyclic peptides, R1A and R1B, were purified by HPLC (Figure 2) and alkylated with iodoacetamide. Sequence analysis of the modified peptides showed that R1A was alkylated exclusively at Cys4 and Cys10 and that R1B was alkylated exclusively at Cys5 and Cys18. This disulfide pattern (Cys4−Cys10; Cys5−Cys18) is analogous to that of other α-conotoxins (see Table 1). A similar analysis was undertaken with the inactive isomer which coelutes with the active form in 0.1% TFA, and it proved to have a different Cys connectivity (Cys4−Cys5; Cys10−Cys18).

Biological Activity of α-Conotoxin EI. α-Conotoxin EI was isolated by screening fractions for paralytic activity in fish and mice. In fish, 1 nmol of toxin injected i.m. is sufficient to cause paralysis. We sought to quantify the activity in mice by comparing death time to dose (Cruz et al., 1978; Gray et al., 1983). Intraperitoneal injection of α-conotoxin EI into mice induces muscular weakness, followed by flaccid paralysis and ultimately death, presumably from immobilization of the skeletal muscle diaphragm. The elicited symptoms are quite typical of nicotinic acetylcholine receptor antagonists. Data were plotted according to the linear equation $Y = A + BX$, where Y is death time in min and X is the reciprocal dose in nmol. Least squares regression analysis yielded $A = 2.1$ min, $B = 49.7$ min·nmol

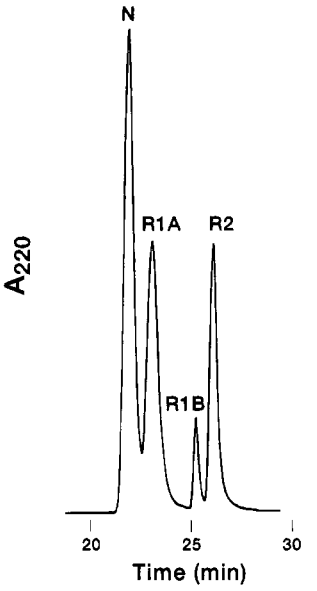


FIGURE 2: Partial reduction of synthetic α-conotoxin EI. Partially reduced peptide was analyzed via reversed-phase HPLC as described under Experimental Procedures. Peptides corresponding to the absorbance peaks were identified as follows; N, unreduced; R2, fully reduced; R1A and R1B, monocyclic intermediates.

Table 1[a,b]

α-CTx	sequence	source	ref
EI	RDOCCYHPTCNMSNPQIC*	*C. ermineus*	*c*
PNIA	GCCSLPPCAANNPDYC*	*C. pennaceus*	*d*
PNIB	GCCSLPPCALSNPDYC*	*C. pennaceus*	*d*
GI	ECCNPACGRHYSC*	*C. geographus*	*e*
MI	GRCCHPACGKNYSC*	*C. magus*	*f*
SI	ICCNPACGPKYSC*	*C. striatus*	*g*
	disulfide bonding	CC—C—C	

[a] O = hydroxyproline; * denotes C-terminal amide. [b] Note that the disulfide bonding pattern has not been determined for PNIA or PNIB. [c] This paper. [d] Fainzilber et al., 1994. [e] Gray et al., 1981. [f] McIntosh et al., 1982; Gray et al., 1983. [g] Zafaralla et al., 1988.

with a coefficient of determination (r^2) of 0.96. Data were linearly normalized by mouse weight with a 20 g mouse used as standard. Death time is linearly and positively related to the reciprocal of the amount of toxin injected. The specific activity of α-conotoxin EI is 0.36 units/nmol, where the unit of activity is defined as the quantity of material needed to cause death of a 20 g mouse in 20 min (Cruz et al., 1978). This compares to specific activities of 4.0 units/nmol for α-conotoxin MI and 1.5 units/nmol for α-conotoxin GI (Gray et al., 1983).

Inhibition of [^{125}I]α-BTX Binding to Nicotinic Receptors. The ability of α-conotoxins EI and MI to inhibit the binding of [^{125}I]α-BTX to nicotinic receptors from BC_3H-1 cells and *Torpedo* electric organ was determined (Figure 3). α-Conotoxins EI and MI both inhibited the association of [^{125}I]α-BTX to BC_3H-1 receptors with two apparent affinities (Figure 3, upper panel; Table 2). α-Conotoxin EI distinguished between the two acetylcholine binding sites on BC_3H-1 receptors by approximately 30-fold. In comparison, α-conotoxin MI distinguished between the two acetylcholine binding sites by greater than 10 000-fold, similar to previous observations (Kreienkamp et al., 1994; Groebe et al., 1995). α-Conotoxins EI and MI both inhibited the association of

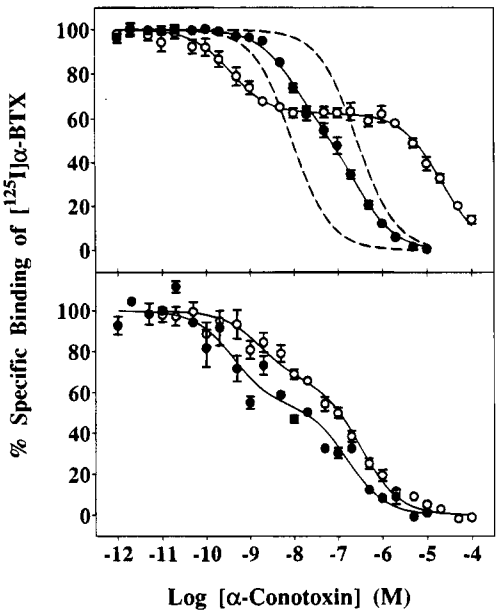


FIGURE 3: Inhibition of [^{125}I]α-BTX association to nicotinic receptors by α-conotoxins. α-Conotoxins EI (●) and MI (○) were incubated with nicotinic receptors for 2.0 h prior to the addition of [^{125}I]α-BTX. Upper panel, inhibition of [^{125}I]α-BTX association to nicotinic receptors on BC_3H-1 cells. Lower panel, inhibition of [^{125}I]α-BTX association to nicotinic receptors from *Torpedo* electric organ. The data shown are the mean ± SEM of three or four experiments. Solid lines through the data are for the best fit equation (two independent sites) determined by a statistical comparison of functions. The dashed lines represent simulated dose−response curves using a single-site model and affinities obtained from the two-site analysis of the α-conotoxin EI data.

Table 2: Affinities of α-Conotoxins EI and MI for Nicotinic Acetylcholine Receptors

α-conotoxin	receptor source	IC_{50}^1 (nM)	IC_{50}^2 (μM)	% high-affinity sites	IC_{50}^2/IC_{50}^1
EI	BC_3H-1	9.40 ± 1.20	0.28 ± 0.03	44 ± 5.6	30
MI	BC_3H-1	0.42 ± 0.15	23.00 ± 4.10	37 ± 2.5	55 000
EI	*Torpedo*	0.41 ± 0.09	0.19 ± 0.02	48 ± 1.5	460
MI	*Torpedo*	4.50 ± 1.60	0.48 ± 0.10	41 ± 4.3	110

[^{125}I]α-BTX to *Torpedo* receptors with two apparent affinities (Figure 3, lower panel; Table 2). α-Conotoxin EI distinguished between the two acetylcholine binding sites on *Torpedo* receptors by approximately 460-fold, demonstrating a significantly greater selectivity between the two acetylcholine binding sites of *Torpedo* receptors than between those of BC_3H-1 receptors. α-Conotoxin MI distinguished between the two acetylcholine binding sites in *Torpedo* by over 100-fold, consistent with previous observations (Hann et al., 1994; Groebe et al., 1995).

d-Tubocurarine and metocurine have been shown to have higher affinity for the acetylcholine binding site near the α/γ subunit interface of nicotinic receptors from both BC_3H-1 cells and *Torpedo* electric organ (Blount & Merlie, 1989; Pedersen & Cohen, 1990; Sine & Claudio, 1991; Kreienkamp et al., 1992; Sine, 1993). α-Conotoxin MI also has higher affinity for the acetylcholine binding site near the α/γ subunit interface of *Torpedo* receptors (Myers et al., 1991; Hann et al., 1994; Utkin et al., 1994; Groebe et al., 1995). However, α-conotoxin MI has higher affinity for the α/δ site of BC_3H-1 receptors (Kreienkamp et al., 1994; Groebe et al., 1995). To identify the higher-affinity α-conotoxin EI binding site of

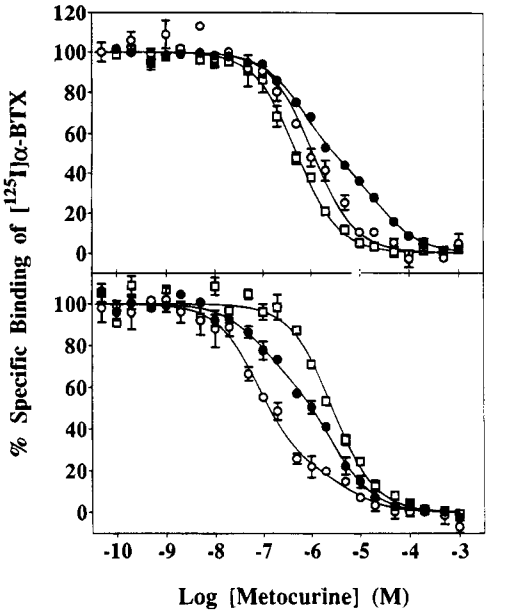


FIGURE 4: Inhibition of [^{125}I]α-BTX association to nicotinic receptors by metocurine in the absence and presence of α-conotoxins. Upper panel, nicotinic receptors on BC_3H-1 cells were incubated with metocurine in the absence (●) and presence of 100 nM α-conotoxin EI (○) or MI (□) for 30 min prior to the addition of [^{125}I]α-BTX. Preincubation of receptors with α-conotoxin for up to 2 h before addition of metocurine yielded similar results. In the absence of metocurine, α-conotoxin EI inhibited 61% ± 3.7% of the total [^{125}I]α-BTX binding sites and α-conotoxin MI inhibited 53% ± 1.7% of the total [^{125}I]α-BTX binding sites. Lower panel, nicotinic receptors from *Torpedo* electric organ were incubated with metocurine (●) for 2.0 h prior to the addition of [^{125}I]α-BTX. Receptors were incubated for 2.0 h with 5 nM α-conotoxin EI (○) or 100 nM MI (□) prior to a 2.0 h incubation with metocurine. In the absence of metocurine, α-conotoxin EI inhibited 49% ± 2.0% of the total [^{125}I]α-BTX binding sites and α-conotoxin MI inhibited 45% ± 1.3% of the total [^{125}I]α-BTX binding sites. The data shown are the mean ± SEM of three to five experiments. Lines through the data are for the best fit equation determined by a statistical comparison of functions.

Table 3: Affinities of metocurine in the absence and presence of α-conotoxins EI and MI[a]

receptor source	α-conotoxin	IC_{50}^1 (μM)	IC_{50}^2 (μM)	% high-affinity sites
BC_3H-1	none	0.75 ± 0.17	27 ± 1.5	56 ± 2.7
BC_3H-1	EI	1.1 ± 0.05	na	na
BC_3H-1	MI	0.51 ± 0.06	na	na
Torpedo	none	0.07 ± 0.02	3.0 ± 1.0	32 ± 4.9
Torpedo	EI	0.07 ± 0.03	4.7 ± 1.9	71 ± 13
Torpedo	MI	na	2.7 ± 0.12	na

[a] A second affinity was not applicable (na) when the data did not fit significantly better to a two-site model ($p > 0.05$).

BC_3H-1 receptors, the ability of metocurine to inhibit the association of [^{125}I]α-BTX was determined in the absence and presence of a constant concentration of α-conotoxin EI. At 100 nM, α-conotoxin EI would be expected to occupy approximately 91% of its higher-affinity site and approximately 26% of its lower-affinity site. At the same concentration, α-conotoxin MI would be expected to occupy greater than 99% of its higher-affinity site and less than 0.01% of its lower affinity site. In the absence of α-conotoxin, metocurine displayed two affinities for BC_3H-1 receptors (Figure 4 upper panel; Table 3). However, in the presence of either α-conotoxin EI or MI, metocurine displayed only a single high affinity for the receptor (Figure 4

upper panel; Table 3). These results demonstrate that both α-conotoxins EI and MI selectively block the lower-affinity metocurine binding site near the α/δ subunit interface. Thus, α-conotoxin EI, like α-conotoxin MI, has a higher affinity for the acetylcholine binding site near the α/δ subunit interface of receptors from BC_3H-1 cells.

Similar experiments were performed to identify the higher-affinity α-conotoxin EI binding site on *Torpedo* nicotinic receptors. The ability of metocurine to inhibit the association of [^{125}I]α-BTX to *Torpedo* receptors was determined in the absence and presence of a constant concentration of α-conotoxin EI or MI. At 5 nM, α-conotoxin EI would be expected to occupy approximately 92% of its higher-affinity site and less than 3% of its lower-affinity site. At 100 nM, α-conotoxin MI would be expected to occupy approximately 97% of its higher-affinity site and 16% of its lower-affinity site. In the absence of α-conotoxin, metocurine displayed two affinities for *Torpedo* receptors (Figure 4 lower panel; Table 3). In the presence of α-conotoxin EI, the proportion of sites with higher affinity for metocurine increased significantly. In additional experiments the fraction of remaining low-affinity metocurine sites could be reduced further by incubation with a higher concentration (20 nM) of α-conotoxin EI (data not shown). Thus, α-conotoxin EI displayed higher affinity for the α/δ site of *Torpedo* receptors. In the presence of α-conotoxin MI, however, metocurine displayed only a single low affinity for the receptor, consistent with α-conotoxin MI's higher affinity for the α/γ site (Figure 4, lower panel; Table 3).

Results with metocurine indicate that α-conotoxins EI and MI both have a higher affinity for the α/δ site of BC_3H-1 receptors. However, unlike α-conotoxin MI, α-conotoxin EI also has a higher affinity for the α/δ site in *Torpedo* receptors. To confirm these observations, the affinity of α-conotoxin MI was determined in the presence of a constant concentration of α-conotoxin EI. In the presence of 100 nM α-conotoxin EI, α-conotoxin MI displayed only a single low affinity for BC_3H-1 receptors, confirming that α-conotoxins EI and MI both have higher affinity for the α/δ site in BC_3H-1 receptors (Figure 5, upper panel). Similar experiments were performed with *Torpedo* receptors. In the presence of 5 nM α-conotoxin EI, the proportion of sites with high affinity for α-conotoxin MI increased significantly (Figure 5, lower panel). In additional experiments, the fraction of remaining low-affinity α-conotoxin MI sites could be reduced further by incubation with a higher concentration (20 nM) of α-conotoxin EI (data not shown). These results demonstrate that α-conotoxins EI and MI have higher affinity for different sites on *Torpedo* receptors. Thus, unlike α-conotoxin MI, α-conotoxin EI has higher affinity for the α/δ sites on both BC_3H-1 and *Torpedo* receptors.

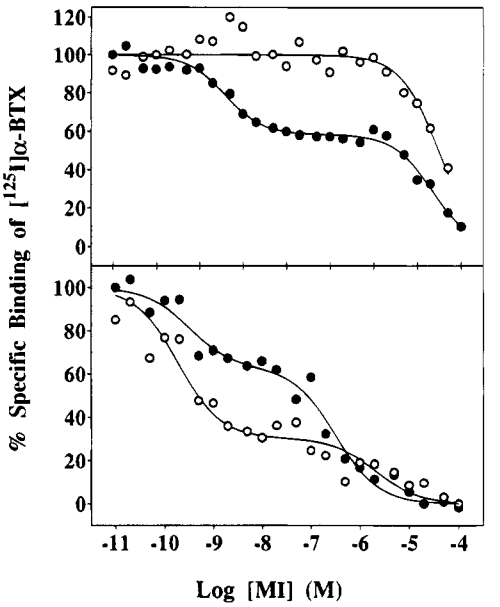
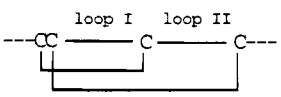


FIGURE 5: Inhibition of [^{125}I]α-BTX association to nicotinic receptors by α-conotoxin MI in the absence and presence of α-conotoxin EI. Nicotinic receptors were incubated with α-conotoxin EI for 2.0 h prior to a 2.0 h incubation with α-conotoxin MI. Upper panel, inhibition of [^{125}I]α-BTX association to nicotinic receptors on BC_3H-1 cells by α-conotoxin MI in the absence (●) and presence (○) of 100 nM α-conotoxin EI. Lower panel, inhibition of [^{125}I]α-BTX association to nicotinic receptors from *Torpedo* electric organ by α-conotoxin MI in the absence (●) and presence (○) of 5 nM α-conotoxin EI. The data shown are representative of three similar experiments.

DISCUSSION

The data presented above establish that α-conotoxin EI from *C. ermineus* is a nicotinic acetylcholine receptor antagonist. α-Conotoxin EI is a potent paralytic in fish, and it is apparently active over a wide phylogenetic spectrum of vertebrates, causing paralysis in mice as well. The presence of a venom component targeted to vertebrate nicotinic acetylcholine receptors was entirely expected, given the piscivorous habit of *C. ermineus*. A more surprising result was the amino acid sequence of α-conotoxin EI, which is significantly different from all other α-conotoxins previously described. With the exception of α-conotoxin SII, all known α-conotoxins belong to the so-called "two-loop" class of conotoxins and typically have two disulfide bonds (α-conotoxin SII has an additional disulfide bond, but nevertheless conserves the basic consensus sequence given in the Introduction) (Ramilo et al., 1992). The cysteine residues in all α-conotoxins can be aligned. If we represent an α-conotoxin schematically as shown below, where loop I consists of the amino acids between the second and third cysteines and loop II consists of the residues between the third and fourth cysteines,



then all previously characterized α-conotoxins from fish-hunting cone venoms have three amino acids in loop I and five amino acids in loop II. In contrast, α-conotoxin EI has four amino acids in loop I and seven amino acids in loop II (for convenience, we refer to these arrangements as the α3/5 and the α4/7 groups of α-conotoxins, respectively).

In this respect, α-conotoxin EI is similar to α-conotoxins PNIA and PNIB from *Conus pennaceus*, a snail-hunting species, which also belong to the α4/7 group (see Table 1). However, the phylogenetic specificity of these α4/7 peptides is different: α-conotoxin EI shows a broad spectrum of activity across vertebrates, while α-conotoxins PNIA/B, which are active in molluscs, appear to be inactive in vertebrate muscle systems (Fainzilber et al., 1994; Groebe et al., 1995). Thus, α-conotoxin EI has a phylogenetic specificity much like the other α-conotoxins from fish-hunting cone snails

such as α-conotoxin GI and α-conotoxin MI, and, although α-conotoxin EI and α-conotoxin PnI both belong to the α4/7 group, they clearly have different specificity. The activity of α-conotoxin EI in molluscan systems, however, has not been examined.

Structural divergence in α-conotoxins can lead to significant functional effects. We recently reported the isolation of α-conotoxin ImI which differs substantially from all reported α-conotoxins. This α-conotoxin is unique in that it blocks mammalian neuronal instead of muscle nAChRs (McIntosh et al., 1994). Similarly, the structural divergence of α-conotoxin EI from α-conotoxins GI and MI has important functional consequences. This is most dramatically illustrated in *Torpedo* nAChRs. In *Torpedo*, α-conotoxins MI and EI both distinguish between the two ligand binding sites by more than 100-fold. Their selectivities for each site, however, are opposite. α-Conotoxin MI preferentially binds the acetylcholine binding site located at the α/γ subunit interface vs the α/δ interface. This is consistent with the findings of other investigators (Hann et al., 1994; Groebe et al., 1995). α-Conotoxin GI also preferentially binds the α/γ site (Hann et al., 1994; Utkin et al., 1994). The α/γ interface is also the high-affinity site for curariform drugs (Sine, 1993). In contrast, in this study we demonstrate that α-conotoxin EI selectively binds the α/δ site. To our knowledge, α-conotoxin EI is the only nicotinic ligand with this specificity in *Torpedo*. It is now possible to selectively block either acetylcholine binding site of *Torpedo* receptors using either α-conotoxin MI or EI.

The functional effects of α-conotoxin EI vs MI also differ in mammalian nAChRs. When α-conotoxin MI is used to compete with [^{125}I]α-BTX for binding to receptors in BC_3H-1 cells, a broadly biphasic displacement curve is generated with over 4 orders of magnitude difference in dissociation constants for the two ligand binding sites. In contrast, α-conotoxin EI has intermediate affinity for both ligand binding sites, though one site is still preferred.

Although both toxins prefer the α/δ site of BC_3H-1 cell receptors, their relative affinities for the two acetylcholine binding sites are significantly different. At the α/δ site, α-conotoxin MI is 20-fold more potent than α-conotoxin EI. At the α/γ site, the situation is reversed with α-conotoxin EI 80 times more potent than α-conotoxin MI (Table 2). Thus, the structural differences between these peptides results in substantially different affinities for the nonequivalent acetylcholine binding sites. It will be of interest to determine which residues in the receptor interact with α-conotoxin EI. Presumably they are different from those residues which make contact with α-conotoxin MI (Sine et al., 1995).

Injection of α-conotoxin EI into mice and fish causes muscular paralysis consistent with nicotinic antagonist activity. Since the muscle nicotinic receptor requires acetylcholine to bind at both sites for channel activation, antagonists acting at either site will result in functional block of the receptor. Thus, α-conotoxin MI potently causes paralysis in mice presumably through its high-affinity binding to the acetylcholine site located near the α/δ subunit interface. In mice, α-conotoxin MI is 11-fold more potent than α-conotoxin EI. This compares well with the 20-fold greater affinity of α-conotoxin MI vs α-conotoxin EI for the α/δ binding site of mouse BC_3H-1 receptors. Thus, it appears that the *in vivo* activities of these toxins correlate with their affinities for the α/δ binding site. It should be noted, however, that in adult mouse muscle an ϵ subunit substitutes for the neonatal γ subunit. Thus, the *in vivo* results in mice [$(\alpha_1)_2\beta_1\epsilon\delta$ receptors] vs the *in vitro* results in BC_3H-1 cells [$(\alpha_1)_2\beta_1\gamma\delta$ receptors] are not strictly comparable.

We also note that, although the modified amino acid *trans*-4-hydroxyproline has been found in a number of other conotoxins (notably the ω-conotoxins and the μ-conotoxins), this modification has never previously been described in an α-conotoxin. In α-conotoxin EI, one of the proline residues has been hydroxylated while the two other proline residues remain unmodified. The functional significance of hydroxylation of prolines has not been further investigated. α-Conotoxin EI is unique among known conotoxins in having both hydroxylated and unmodified prolines in its sequence.

Although α-conotoxin EI is unusual when compared to other nAChR-targeted conotoxins from fish-hunting *Conus* venoms, the α4/7 group to which it belongs may in fact be the stem group of the α-conotoxin family. An analysis of cDNAs from venom ducts of several other *Conus* species which are not large Indo-Pacific fish-hunters has recently been carried out (A. Santos, B. Olivera, and D. Hillyard, unpublished results). Over a dozen peptides that are homologous with the α-conotoxins have been identified, and a preponderance of members of the α4/7 group was found. It would appear that the α3/5 group may in fact be restricted to a subset of fish-hunting cone snails. Because the two groups of α-conotoxins are significantly divergent, it will be of interest to determine the distribution of the α4/7 and the α3/5 α-conotoxin groups among the ~70 different fish-hunting *Conus* species and further assess their receptor subtype selectivities.

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