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α-Conotoxins, Small Peptide Probes of Nicotinic Acetylcholine Receptors†

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ABSTRACT: α-Conotoxins, a family of small peptides from the venoms of the *Conus* marine moluscs, are selective, snake α-neurotoxin-competitive antagonists of the nicotinic acetylcholine receptor. A new α-conotoxin, SIA, has been purified, sequenced, and synthesized. Cross-linking with bivalent reagents and photoaffinity labeling of the acetylcholine receptor with α-conotoxin yield covalent adducts. Surprisingly, cross-linking to other subunits is considerably more efficient than to the α subunit. The relative efficiency of photoactivatable cross-linking to different subunits of the receptor is a function of placement of the photoactivatable group on the toxin. Since the structures of α-conotoxins can be solved by 2D NMR [see Pardi et al. (1989) *Biochemistry 28*, 5494-5508; Kobayashi et al. (1989) *Biochemistry 28*, 4853-4860], this family of toxins should provide a set of new ligands for probing the acetylcholine receptor with considerable precision.

The fish-hunting cone snails (*Conus*) use venoms to paralyze their faster moving prey. These venoms are complex mixtures of peptides, each targeted to a particular macromolecular receptor (Olivera et al., 1989, 1990). The α-conotoxins, the subject of this report, are paralytic peptides found in the venoms which target to the nicotinic acetylcholine receptor (Gray et al., 1981; Olivera et al., 1990). Other characterized peptides in *Conus* venoms target to presynaptic calcium channels (the ω-conotoxins; Olivera et al., 1984), muscle sodium channels (the μ-conotoxins; Cruz et al., 1987), and

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glutamate receptors of the NMDA subtype (the conantokins; Olivera et al., 1990; Haack et al., 1990; Mena et al., 1990).

The α-conotoxins are the smallest of the major paralytic conotoxins found in fish-hunting *Conus* venoms. Typically, they are 13–15 amino acids in length. Five α-conotoxins have been purified and sequenced to date from the venoms of three different species, *Conus geographus*, *Conus striatus*, and *Conus magus*. Those characterized so far comprise a clearly homologous set of peptide sequences.

A large homologous set of α-conotoxin peptides may be particularly useful for probing nicotinic acetylcholine receptors because of their intermediate size between small cholinergic ligands (such as carbamylcholine) and the larger α-neurotoxins (snake venom polypeptides such as α-bungarotoxin and cobratoxin). They are the smallest peptidergic ligands for the acetylcholine receptor presently known and can be conveniently synthesized by chemical methods. In each α-conotoxin, there are a number of functional groups which can be derivatized without loss of biological activity. These are potential sites to which various reporter groups are attached. Structures of α-conotoxins have been determined by using 2D NMR methods (Pardi et al., 1989; Kobayashi et al., 1989); the position of any reporter groups can therefore be precisely predicted. Compared to the larger toxins (e.g., α-bungarotoxin) which have correspondingly greater surface areas which can interact with the receptor, interactions with the smaller α-conotoxins are more restricted. Thus, in principle, different sites on the ligand peptide can be localized viz à viz receptor sites by using cross-linking methods.

In order to use α-conotoxins as effective probes for the nicotinic acetylcholine receptor, the set of sequence variants available should be expanded. In addition, derivatives of each α-conotoxin, with reporter groups in different vectorial orientations, need to be prepared. In this report, we describe results which advance both of these objectives. A new α-conotoxin from *Conus striatus* venom is characterized, thereby expanding the set of natural sequences available. In addition, we used both bivalent and photoactivatable cross-linking methods to study the interaction of α-conotoxins with the well-characterized nicotinic ACh receptor from the *Torpedo* electric organ, perhaps the most thoroughly studied membrane receptor [for reviews, see McCarthy et al. (1986), Hucho (1986), and Popot and Changeux (1984)]. Two different photoactivatable derivatives of the same α-conotoxin were prepared and shown to give different labeling patterns. The results indicate that the α-conotoxins provide a set of excellent biochemical probes for exploring the nicotinic acetylcholine receptors, and should provide a better definition of ligand binding sites.

Materials and Methods

Acetylcholine Receptor. AChR were prepared from *Torpedo californica* electroplax obtained frozen from Pacific Bio-Marine (Venice, CA). Enriched postsynaptic membranes were prepared by the method of Elliott et al. (1980) employing the alkaline wash of Neubig et al. (1979). Affinity-purified AChR was prepared from crude membranes derived essentially from the initial purification steps, including osmotic wash, of Elliott et al. (1980). The membranes were extracted with 3% Triton X-100 [in 40 mM sodium phosphate (pH 7.6), 50 mM NaCl, 8 mM EDTA, and 0.08% sodium azide] for 2 h on ice and centrifuged at 100000*g* for 3 h. Agarose-immobilized acetylcholine was prepared from bromoacetylcholine bromide [esterified per Damle et al. (1978)] and Affi-Gel 401 (Bio-Rad), and AChR was affinity-purified from the extract as described by Karlin et al. (1976). AChR preparations were quantitated as ^{125}I-α-bungarotoxin binding sites (Schmidt & Raftery, 1973).

Purification of α-Conotoxin SIA. Conotoxin SIA was purified from the same Sephadex G-25 fraction of *Conus striatus* venom as that from which α-conotoxin SI was isolated (Zafaralla et al., 1988). This fraction was resolved by reversed-phase HPLC on a semipreparative C_{18} column (see Figure 1A) into numerous peaks, each one with a characteristic biological activity. α-Conotoxin SIA was purified from the peak indicated (Figure 1A) by HPLC on an analytical VYDAC C_{18} column (4.6 × 250 mm, 5 μm), eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA). α-Conotoxin SI was previously purified (Zafaralla et al., 1988) from the peak indicated by the lighter arrow in Figure 1A.

Sequence Analysis of Conotoxin SIA. The peptide was reduced and carboxymethylated and then analyzed in a spinning-cup sequencer as described previously (Edman & Begg, 1967).

Synthesis of Conotoxin SIA. Synthesis was carried out by the solid-phase procedure of Merrifield (1963), following the general protocol of Gray et al. (1983). Methylbenzhydrylamine resin (1.0 g) was used as the starting support (0.67 mmol/g substitution), together with Boc-amino acids (L configuration, where applicable) which were purchased from Bachem, CA. Cys^2 and Cys^7 were protected as Cys(Mob) and Cys^3 and Cys^{13} as Cys(Acm). Other side chains were protected as Tyr(Cl_2-Bzl), His(Tos), Lys(2-Cl-Z), and Asp(Bzl). With one exception, couplings were carried out in dichloromethane and were mediated by diisopropylcarbodiimide (DICC). Asparagine was coupled without side-chain protection, using dimethylformamide as solvent, DICC as coupling agent, and 2 equiv of 1-hydroxybenzotriazole to minimize side reactions. Coupling was repeated for the His residue since the ninhydrin test (Kaiser et al., 1970) is an unreliable guide for completeness of reaction onto Pro.

The peptide was deprotected and cleaved from the resin as the C-terminal amide, using the low–high HF procedure of Tam et al. (1983). Following ether extraction and lyophilization from 5% acetic acid, the peptide (500 mg in 10 mL of 0.2 M ammonium bicarbonate, pH 8.0) was reduced with dithiothreitol (28.5 mM) at 37 °C for 1.5 h. The reaction mixture was diluted, and acetic acid was added to a final concentration of 1 M; the final volume was 20 mL. This solution and 20 mL of 1 N NaOH were added simultaneously to 100 mL of potassium ferricyanide (1 mol/mol of SH) over a 0.5-h period. The reaction mixture containing the monocyclic peptide was lyophilized, taken up in 0.1% TFA, and run on a preparative reversed-phase C_{18} column (Prep Pak, Waters) eluted with a gradient of acetonitrile in 0.1% TFA. The peptide was located by amino acid analysis of peaks, and the sequence was confirmed before proceeding to the next step. For deblocking and oxidation of Cys^3 and Cys^{13}, 25 mg of peptide was dissolved in 160 mL of 3.75 N HCl in 30% acetic acid. Iodine (500 mg in 5 mL of methanol) was added dropwise while the solution was stirred. After 10 min, the reaction was quenched with 1 M ascorbic acid until the solution became clear, then 50% more ascorbic acid was added. The mixture was diluted 10-fold with 0.1% TFA before being pumped directly onto the preparative reversed-phase HPLC column. The amino acid composition of the peptide peak was checked, and the synthetic SIA was cochromatographed with the native peptide to verify identity. The biological activities were compared by intraperitoneal injection in mice.

Other α-conotoxins were similarly synthesized and purified by reversed-phase HPLC (Gray et al., 1983, 1984). GIA was

a gift from Jean Rivier of the Salk Institute.

Iodination of α-Conotoxin MI. α-Conotoxin MI was iodinated to high specific activity by using a modified Iodogen (Pierce) procedure (Salacinski et al., 1981). The reaction mixture was desalted on a Sephadex G-15 column with lysozyme carrier, and the product was purified by reversed-phase HPLC. ^{125}I-Conotoxin MI was stable for several months when stored at −20 °C in 40% methanol.

Binding Assay. Saturation binding of ^{125}I-α-conotoxin MI to AChR membranes was determined as follows. Aliquots of crude receptor membranes (40–100 μg of protein each) in *Torpedo* Ringers [20 mM Na-HEPES (pH 7.4), 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, and 0.02% NaN_3] were incubated with various concentrations of radioactive toxin in equal final volumes for 30 min at 0 °C. The reaction was stopped by centrifugation for 5 min in a Beckman Airfuge (80000*g*), and the supernatants were aspirated with a micropipet. The well-consolidated pellets were quickly rinsed with cold Ringers without resuspension and counted in a Packard Multi-Prias 1 γ counter with an 80% counting efficiency. Nonspecific binding was defined as that binding observed following preincubation with excess nonradioactive toxin.

^{125}I-Conotoxin–Acetylcholine Receptor Cross-Linking. To purified receptor membranes (ca. 10 μg of protein) in 0.5-mL Eppendorf tubes was added an equal volume of "cross-linking buffer" [10 mM sodium phosphate (pH 7.8), 250 mM NaCl, and 5 mM Na_2-EDTA, with 0.02% NaN_3, to which was added phenylmethanesulfonyl fluoride (1.0 mM) and iodoacetamide (5 mM) in a minimum volume of dry dimethyl sulfoxide just prior to use]. ^{125}I-α-Conotoxin MI, in the same buffer but without inhibitors, was added to each tube of membranes to 0.5 μM final concentration and incubated for 30 min at 0 °C. Bound toxin was then cross-linked to receptors with bifunctional agents of different tether lengths, viz, disuccinimidyl tartrate (DST), disuccinimidyl suberate (DSS), and ethylene glycol bis(succinimidyl succinate) (EGS), all from Pierce. Five microliters of 20 mM cross-linker in dry dimethylformamide (stored over 4-Å sieves) was added with continued incubation for 10 min on ice. Excess succinimides were quenched for 10 min by addition of 100 μL of 0.2 M Tris (pH 7.8). The samples were then pelleted for 5 min in an Eppendorf microfuge, then resuspended, and washed in a second volume of Tris, and the pellets were analyzed by electrophoresis.

Radioiodination of NHS-ASA. N-Hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA, Pierce; this and its derivatives are handled in subdued light) was radioiodinated by a modification of the method of Ji et al. (1985). To 10 μL of 0.5 M sodium phosphate (pH 7.0) in a 0.5-mL Eppendorf tube were sequentially added the following: 20 μL of 0.4 mM chloramine T (8 nmol) in acetonitrile, dimethylformamide (9:1), 5 μL of sodium [^{125}I]iodide (1 mCi, ca. 0.5 nmol, carrier-free in 0.1 M NaOH; Amersham), and 30 μL of 0.37 mM NHS-ASA (11 nmol) in acetonitrile. The mixture was vortexed and incubated 2–3 min. One hundred microliters of ethyl acetate was then added, and the product was washed with 2 × 100 μL of 10% NaCl. [This step is conveniently done by vortexing, briefly centrifuging, then drawing the mixture into a 200-μL miocropipet, where the phases quickly separate, and then disposing of the lower (aqueous) phase, while quantitatively recovering the product in the organic phase.] The extract was dried under a stream of dry nitrogen and used immediately to minimize radiolysis.

Nonradioactive Iodination of NHS-ASA. Larger, nonradioactive iodinations were performed for preparative-scale (trace radiolabel) experiments, for bioassay, and for chemical characterization. NHS-ASA is refractory to gentle iodination compared to other phenolics, e.g., tyrosine, while the azido group and succinimide are labile under the typically harsh conditions (reflux in mineral acid) of the more rigorous procedures. The following method, adapted from that of Woolett and Johnson (1943), produced virtually quantitative yield in minutes under gentle conditions. One milligram of NHS-ASA (3.7 μmol) was dissolved in 80 μL of iodine monochloride [75 μmol (Aldrich), 4 μL in 76 μL of glacial acetic acid] with vigorous mixing, incubated 8 min at room temperature, and then dried in vacuo. The dark red residue was twice washed of excess ICl by redissolution in 1-mL volumes of ethyl acetate with drying in vacuo. A sample of the resulting white residue was analyzed by electron-impact mass spectrometry, verifying the NHS-I-ASA identity of the product. The reversed-phase HPLC radiochromatogram of trace ^{125}I-labeled material revealed coelution of radioactivity only with the main product peak. The nonradioactive adduct was found to be completely stable in the dark at room temperature in the acidic column effluent (pH 2), which is typically strongly hydrolytic to succinimides. Advantage of this feature has afforded the opportunity of purification prior to condensation with ligand. However, derivatization with the unpurified NHS-I-ASA reaction product proved equally valuable (cf. below).

NHS-I-ASA Coupling to α-Conotoxin GIA. It was found that either the α-amino or lysine ε-amino functions can be derivatized with high selectivity, apparently owing to differences in the pK_a's and solvation of the two amines. Selective α-amino derivatization was done in neutral aqueous buffer. Excess (10 nmol) conotoxin GIA was dissolved in 20 μL of 0.1 M sodium phosphate (pH 7.4), transferred to NHS-^{125}I-ASA (200 pmol) in 2 μL of dry dimethyl sulfoxide, and incubated 15 min at room temperature. Selective ε-amino derivatization was done in anhydrous organic solvent: 10 nmol of conotoxin GIA was dissolved in 10 μL of 3.5 mM diisopropylethylamine in dry dimethylformamide and transferred to NHS-^{125}I-ASA, in which it was incubated 3–5 min at room temperature. In each case, product was purified by reversed-phase HPLC, and underivatized peptide was recovered (Figure 3). Except that NHS-I-ASA was present in thousandfold excess over that of the small-scale preparations, the otherwise same volumes and proportions were used for large-scale preparations. In the latter case, a preliminary purification was required in which the reaction was first stopped with 400 μL of 5% acetic acid, excess reagent was extracted with 2 × 700 μL volumes of ethyl acetate (as described under Radioiodination of NHS-ASA), and the aqueous phase was concentrated in a Speedvac (Savant). The structures of both isomers were confirmed by fast atom bombardment (FAB) mass spectrometric analysis (Dr. T. Lee of the Beckman Research Institute of the City of Hope) by which the correct molecular ions were observed at MH^+ 1911.34 [cf. Yazdanparost et al. (1986) for the apparent 2 mass unit discrepancy]. Radiosequence analysis revealed either extraction of radioactivity from the cup at the C-terminus or that the N-terminus was blocked to Edman degradation without extraction of radioactivity, for the N^ϵ and N^α adducts, respectively. Both products were biologically active as determined by intraperitoneal injection into mice (Gray et al., 1983).

The reversed-phase HPLC chromatogram of crude NHS-I-ASA has a characteristic profile of product and ancillary peaks, each of which are apparently reactive succinimides. Peptide derivatization with this heterogeneous preparation gave

a similar profile of peptide adducts, but with increased retention times. The product peak was therefore easily identified with reference to this profile and the desired product isolated.

Photoaffinity Labeling of the AChR. Aliquots of ^{125}I-ASA-α-conotoxin GIA (≥5 μCi) were dried in vacuo, each was redissolved in 2 μL of dimethyl sulfoxide to which were transferred either 100 μL of crude *Torpedo* membranes (20 μg of protein, ca. 9.6 pmol of α-bungarotoxin sites) or affinity-purified AChR (0.2 nmol of α-bungarotoxin sites). Samples were incubated 30 min on ice and, with continued magnetic stirring, were irradiated for ca. 5 min with a UV lamp (Model UVG-11 Mineralight) at a 2-cm distance. Reaction of extant nitrenes (ketenimines) was allowed to proceed in the dark for another 10 min on ice. Initially, however, nitrenes were quenched immediately following irradiation to minimize nonspecific incorporation (2% β-mercaptoethanol or 10 mM potassium *p*-aminobenzoate, final concentrations), but with no appreciable differences in labeling observed. Membranes were rinsed twice with buffer by resuspension and centrifugation, whereas solubilized receptor was trichloroacetic acid precipitated and the pellets of each were analyzed by electrophoresis and autoradiography. Larger samples were similarly prepared with ca. 100 pmol of pure AChR and 4 nmol of ^{125}I-ASA-α-conotoxin GIA and purified on preparative gels.

Selective Subunit Fragmentation. Electrophoretically purified subunits were dissolved in 100 mL of 70% formic acid, and a 10^3-fold molar excess of cyanogen bromide over methionine residues was added in 10 mL of 70% formic acid. After a 24-h incubation in the dark at room temperature, the reaction was stopped by a 10-fold dilution with distilled water, and the mixture was lyophilized. Cyanogen bromide digested peptide samples were submitted to a 2–5-day incubation in 70% formic acid at 37 °C, and then lyophilized (Piszkiewicx et al., 1978), to hydrolyze the labile Asp–Pro peptide linkage.

SDS–Polyacrylamide Gel Electrophoresis. Whole receptor and cyanogen bromide digestion products were dissolved in 3% SDS sample buffer with 5-min boiling, and electrophoresed on 7% and 11% polyacrylamide gels, respectively (Laemmli, 1970). Dilute-acid hydrolysates were resolved on Swank and Munkres gels (1971). Radioactive bands of the Coomassie-stained and dried gels were revealed autoradiographically by exposure of X-ray film (Cronex 7, Dupont) between twin intensifier screens (Cronex Quanta III, Dupont) for 1–20 h at −70 °C. Photoaffinity-labeled subunits from larger samples (ca. 100 pmol of pure AChR labeled with 4 nmol of ^{125}I-ASA-α-conotoxin GIA) were precipitated with trichloroacetic acid, washed with acetone, and purified on 7% polyacrylamide preparative gels (32 × 14 × 0.3 cm; Laemmli, 1970). Bands were briefly (15 min) Coomassie-stained, the wet gel was wrapped in Saran wrap, an X-ray film (Cronex 7, Dupont) was sandwiched between the gel and a single intensifier screen (Cronex Lightning Plus, Dupont), and radioactive bands were revealed by a 15-min exposure on ice. With the autoradiogram aligned, bands were cut from the gel and electroeluted (Hunkapillar et al., 1983). The electroeluate was dialyzed against several changes of distilled water (24 h at 4 °C) and dried in vacuo, and SDS was ion-pair-extracted (Koningsberg & Henderson, 1983). Purity was verified on analytical gels; recovery of radioactivity from the electroeluate was 90–95%.

Results

Purification and Characterization of α-Conotoxin SIA. A peptide fraction paralytic to mice was detected upon HPLC fractionation of *Conus striatus* venom. This paralytic activity eluted close to α-conotoxin SI (see Figure 1A), the major α-conotoxin in *Conus striatus* venom, which is relatively inactive in mammalian systems. The paralytic peptide was separated from α-conotoxin SI by rechromatography on an analytical VYDAC C_{18} HPLC column using an acetonitrile–TFA gradient (Figure 1B).

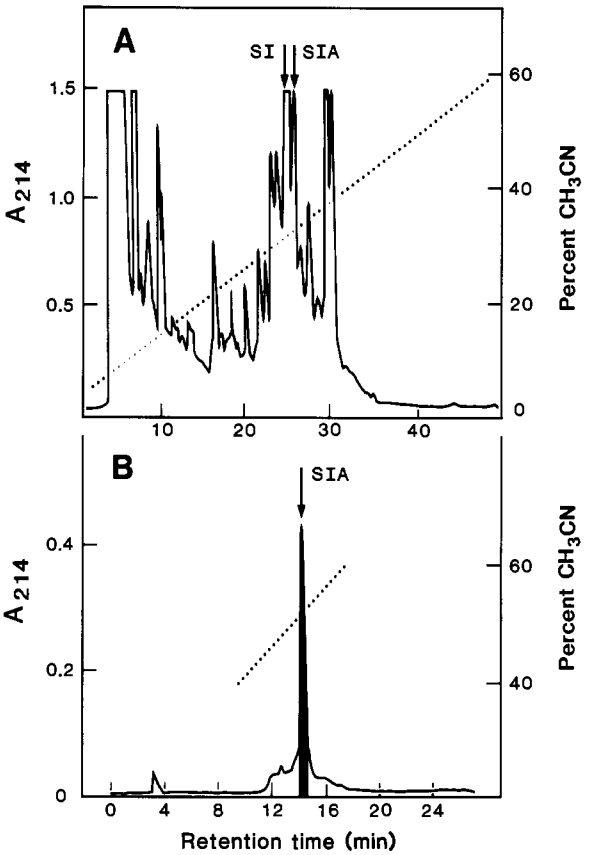


FIGURE 1: Purification of α-conotoxin SIA. Reversed-phase HPLC elution profiles of the Sephadex fraction on a semipreparative C_{18} column (A) and subsequent rerun of purified α-conotoxin SIA on an analytical VYDAC C_{18} column (B); detailed procedures are given under Materials and Methods. Also indicated is the peak corresponding to α-conotoxin SI which was previously characterized (Zafaralla et al., 1988).

Table I: Amino Acid Analysis of α-Conotoxin SIA (Native)

amino acid	nmol	mole ratio
Asp	1.64	1.88 (2)[b]
Gly	0.95	1.09 (1)
His	0.73	0.84 (1)
Ala	0.77	0.88 (1)
Pro	1.11	1.27 (1)
Tyr	0.93	1.07 (1)
Cys[a]	3.11	3.57 (4)
Phe	0.86	0.99 (1)
Lys	0.99	1.14 (1)

[a] Cysteines were analyzed as $CysSO_3H$ following performic acid oxidation of toxin or as the carboxymethyl derivatives. [b] Values in parentheses indicate the number of residues found by sequence analysis.

The purified material was subjected to amino acid analysis; the results were consistent with a 13 amino acid peptide containing 4 residues of cysteine (Table I). Sequence analysis of this material revealed an amino acid sequence that was highly homologous to other α-conotoxins, but with some distinctive differences (see Table III); all of the amino acids in the amino acid analysis were accounted for in the sequence. Since all other α-conotoxins are blocked at the C-terminal residue with an amide group, it was presumed that this was probably true of α-conotoxin SIA as well. This was confirmed by FAB mass spectrometry; with the observed molecular ion

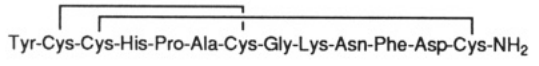
Table II: Sequence Analysis of α-Conotoxin SIA

step	assigned residue	yield (pmol of PTH amino acid)
1	Tyr	339
2	Cys	145
3	Cys	152
4	His	156
5	Pro	140
6	Ala	152
7	Cys[a]	132
8	Gly	117
9	Lys	162
10	Asn	98
11	Phe	153
12	Asp	76
13	Cys	38

[a] Analyzed as the carboxymethyl adduct.

of 1455.26; the calculated MH^+ for the C-terminally amidated peptide is 1455.51.

Further confirmation of the sequence determination above for α-conotoxin SIA (Table II) was obtained by chemical synthesis; detailed methods are described under Materials and Methods. The synthetic toxin was biologically active, and coeluted with the native toxin. The peptide was specifically synthesized with a disulfide linkage between Cys^2 and Cys^7, and Cys^3 and Cys^{13}. Thus, α-conotoxin SIA has the same disulfide linkages as were previously established for α-conotoxin GI from *Conus geographus*. These data establish that the complete covalent structure of α-conotoxin SIA is



In contrast to α-conotoxin SI, α-conotoxin SIA exhibits significant biological activity in mice. However, SIA is not quite as potent in mice as α-conotoxins GI or MI. When an intraperitoneal injection was carried out, 1 nmol of α-conotoxin GI caused death in 7.8 min; 1 nmol of α-conotoxin MI caused death in 9.6 min while 1 nmol of α-conotoxin SIA caused only paralysis but not death; 1.6 nmol of α-conotoxin SIA caused death in 90 min, while 5 nmol caused death between 5.4 and 6 min (two trials). When an intracranial assay was used, 1 nmol of α-conotoxin GI caused death in 2.3 min; 1 nmol of α-conotoxin SIA caused death in 5.7 min. Thus, α-conotoxin SIA is paralytic and lethal to mice, but somewhat less active than other α-conotoxins from *Conus magus* and *Conus geographus* venoms. However, it is much more active on higher vertebrates than the major α-conotoxin from *Conus striatus*, SI, which is inactive in mice at the dose range tested (Zafaralla et al., 1988).

Cross-Linking of ^{125}I-α-Conotoxin MI to the Torpedo Acetylcholine Receptor. A preliminary characterization of covalent coupling reactions of radiolabeled α-conotoxin to the *Torpedo* acetylcholine receptor using bivalent succinimide cross-linkers was carried out. α-Conotoxin MI was radiolabeled with ^{125}I at the single tyrosine residue. In these experiments, cross-linkers of 4-, 8-, and 12-atom tether lengths were used (thus yielding nominal radii of 0.65, 1.1, and 1.6 nm, respectively). The actual cross-linkers employed were disuccinimidyl tartrate (DST), disuccinimidyl suberate (DSS), and ethylene glycol bis(succinimidyl succinate) (EGS). The results are shown in Figure 2. The covalent attachment of radiolabeled α-conotoxin to receptor subunits was assayed by SDS–polyacrylamide electrophoresis after cross-linking, and the radiolabeled bands were detected by autoradiography.

The acetylcholine receptor subunit bands which were radiolabeled corresponded to relative molecular weights of ca.

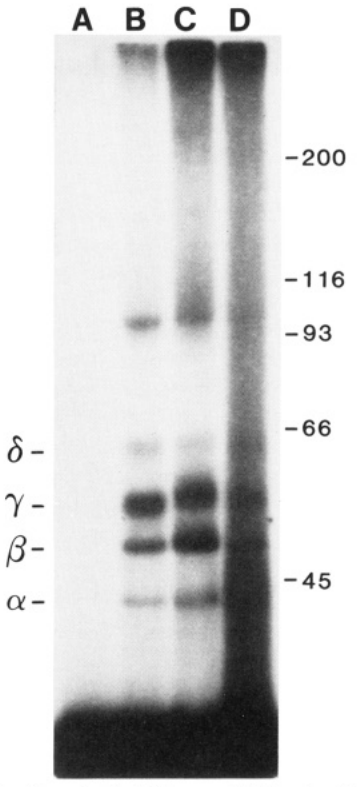


FIGURE 2: ^{125}I-α-Conotoxin MI cross-linking to the AChR with divalent succinimides. Autoradiogram of a 7% Laemmli (1970) gel following resolution of ^{125}I-α-conotoxin MI labeled AChR subunits bound via DST (lane B), DSS (lane C), and EGS (lane D), corresponding to 4-, 8-, and 12-atom cross-linker lengths, respectively. Lane A is control without addition of divalent cross-linker. Equal aliquots of "pure" AChR membranes (ca. 5 pmol of α-bungarotoxin sites) were first incubated with ^{125}I-α-conotoxin MI (0.5 μM final concentration) at 0 °C for 30 min; then to each was added cross-linkers (each with the same final concentration) with continued incubation for 10 min on ice, and quenched with excess Tris. (In the present example, membranes were quickly pelleted and resuspended to remove free label just prior to cross-linking. No appreciable differences in labeling were observed, however, when this step was omitted.)

42K, 52K, 62K, and 65K, approximately 2K greater than the Coomassie-stained counterparts. This is presumably due to the additional mass of the affinity ligand. When long tether lengths are used, the protein of 96 kDa is also prominently labeled, which has been identified as the Na^+/K^+-ATPase α subunit (Lindstrom et al., 1979). The unexpected feature of the results shown in Figure 2 is the increasingly selective labeling of the non-α subunits with decreasing cross-linker length. Labeling with the 12-atom cross-linker EGS was relatively nonspecific, and all 4 subunits appear to be labeled. However, with the shortest cross-linker, DST, there appears to be preferential labeling of β and γ subunits.

Photoaffinity Labeling of Membrane-Bound Acetylcholine Receptor Using N^{α}-^{125}I-ASA–α-Conotoxins. Because cross-linking with bivalent cross-linkers as described above is dependent on the local availability of free amines on both the ligand and receptor, specific labeling of β and γ subunits in the previous experiment could be due to accessibility of amino groups in these subunits (and the concomitant lack of such groups on the α subunit, for example). The use of photoactivatable cross-linkers minimizes this possibility, and therefore the bulk of our cross-linking experiments were carried out with such derivatives. We prepared ^{125}I-azidosalicylate (ASA) derivatives of α-conotoxin GIA. Since the sequence of α-conotoxin GIA is identical with that of α-conotoxin GI except for a Gly-Lys extension at the C-terminus, it seemed likely

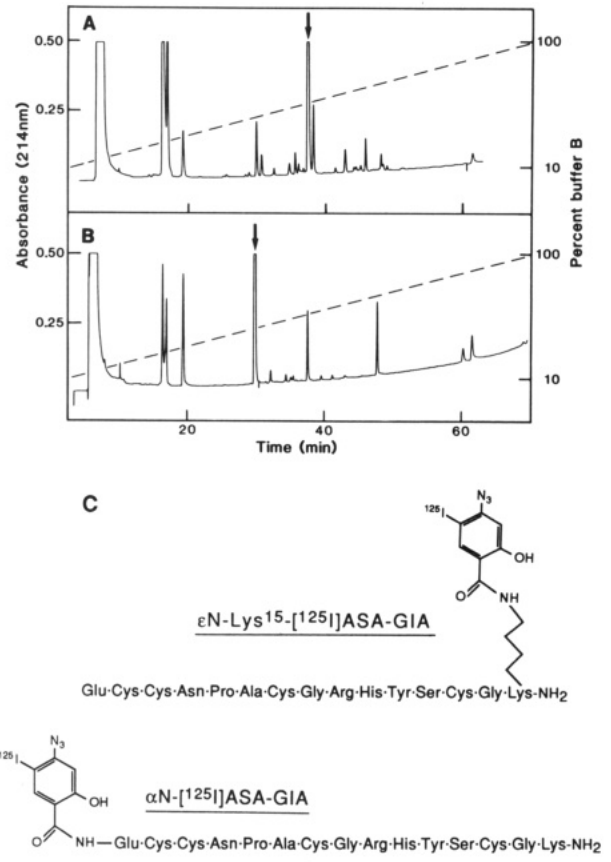


FIGURE 3: Selective ^{125}I-ASA labeling of α-conotoxin GIA. Reversed-phase HPLC purification of (A) N^{ϵ}-Lys^{15}-^{125}I-ASA–α-conotoxin GIA and (B) N^{α}-^{125}I-ASA–α-conotoxin GIA reaction mixtures (products indicated by arrows) on an analytical VYDAC C_{18} column (0.46 × 25 cm, 5-μm particle size, 300-Å pore size) with a linear gradient of 10–100% buffer B for 75 min, monitoring at 214 nm at 0.5 AUFS (A = 0.1 trifluoroacetic acid, B = 0.1% trifluoroacetic acid in 60% acetonitrile). Aliquots of NHS-^{125}I-ASA were reacted with α-conotoxin GIA either in 3.5 mM diisopropylethylamine in dry dimethylformamide or in phosphate buffer, yielding the N^{ϵ} and N^{α} adducts, respectively. Following reaction, mixtures were usually diluted with 5% acetic acid and washed of excess reagent with 2 volumes of ethyl acetate followed by concentration of the aqueous phase in vacuo, prior to chromatography. (C) Structures of the N^{ϵ} and N^{α} adducts.

that the C-terminal lysine residue of GIA was dispensable, and could be derivatized without losing biological activity. Accordingly, ^{125}I-ASA–GIA derivatives at both the ε-amino of the C-terminal lysine and the α-amino N-terminal glutamate were prepared as described under Materials and Methods. Selective reaction conditions for obtaining either the α-amino derivative or the ε-amino derivative were used; in the former case, a neutral aqueous buffer was employed, and in the latter a basic, aprotic solvent (see Materials and Methods and Figure 3A,B). The structures of the isomers were confirmed by FAB mass spectrometry and sequence analysis; structures are shown in Figure 3C. The biological activity of both ^{125}I-ASA–α-conotoxin GIA derivatives was qualitatively bioassayed by intraperitoneal injection into mice; both derivatives were biologically active. However, because of the instability of radiolabeled photoactivatable derivatives, these were routinely used immediately after preparation.

Figure 4 shows photoaffinity labeling of acetylcholine receptor membranes using either the N^{α}- or the N^{ϵ}-^{125}I-ASA–α-conotoxin GIA isomers. The photoaffinity label clearly is covalently bound mainly to the β and γ subunits of the acetylcholine receptor with less labeling of the δ subunit for the N^{α}-ASA derivative and almost no detectable labeling of the α subunit. In contrast, the ε-amino derivative of α-conotoxin

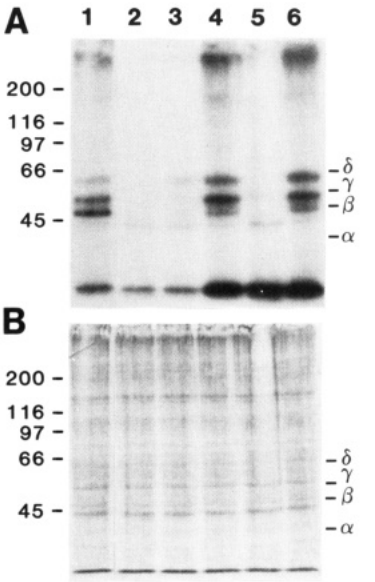


FIGURE 4: SDS–polyacrylamide gel electrophoresis and autoradiography of crude *Torpedo* membranes (ca. 0.6 pmol of α-bungarotoxin sites) photoaffinity labeled with α-conotoxin GIA analogues. Shown are an autoradiogram (A) and a Coomassie-stained gel (B). Labeling by N^{α}-^{125}I-ASA- and N^{ϵ}-Lys^{15}-^{125}I-ASA-derivatized GIA (lanes 1–3 and 4–6, respectively) was performed in the absence of cold ligand (lanes 2 and 4) or in the presence of excess cold ligand: α-conotoxin MI (lane 2), *d*-tubocurarine (lane 3), α-conotoxin GIA (lane 5), or ω-conotoxin GVIA (lane 6).

GIA shows more intense labeling of the γ and δ subunits. The major labeling observed in Figure 4 is clearly specific. No labeling occurs if the receptor is preincubated with an excess of α-conotoxins, α-bungarotoxin, or *d*-tubocurarine; however, normal labeling was observed if the receptor was preincubated with ω-conotoxin GVIA, a presynaptic calcium channel specific ligand.

Photoaffinity Labeling of Detergent-Solubilized Acetylcholine Receptor. The acetylcholine receptor was solubilized by using Triton X-100 and then purified by affinity chromatography. Figure 5 shows an experiment in which the solubilized receptor was photolabeled with the N^{α}-^{125}I-ASA–α-conotoxin GIA derivative. The striking result is that detergent extraction apparently modulates either α-conotoxin binding to the receptor or the efficiency of photolabeling such that all photolabeling of the β subunit is abolished and the γ subunit is labeled exclusively under these conditions.

We have not definitively identified the sites of photoaffinity cross-linking on the receptor. However, by monitoring the behavior of the trace radiolabel, a preliminary characterization was possible. The γ-subunit photolabeled with N^{α}-^{125}I-ASA–α-conotoxin GIA was purified and cleaved with cyanogen bromide, and a single radiolabeled band was obtained with an apparent molecular weight of 19–22K. This is consistent only with photolabeling of the γCB2 fragment corresponding to the amino acid sequence γ121–303. The γCB2 fragment could be cleaved further in 70% formic acid at a single Asp–Pro pair (residues γ183–184), and a single major radioactive band of molecular weight 10K was observed which was rather polydisperse (i.e., likely glycosylated). This probably corresponds to the amino-terminal fragment of γ121–183, which is of estimated molecular weight 9.8K (6.3K peptide, 1.5K polysaccharide at Asn^{145}, plus 2K affinity label; the C-terminal fragment of the cyanogen bromide peptide would have a predicted molecular weight of 15K, and no label appeared at this position). These data are consistent with the cross-linking site on the γ subunit being between amino acids γ121 and 183,

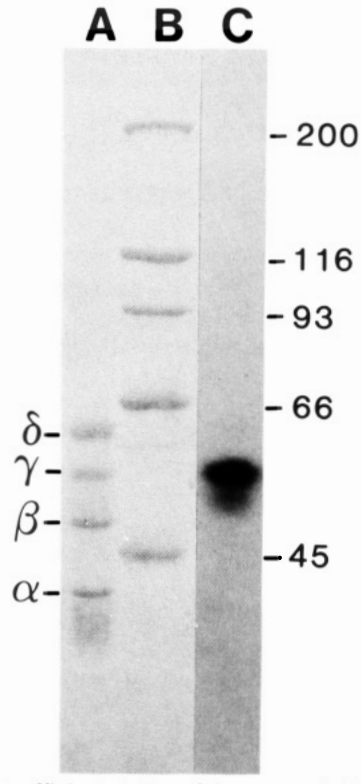


FIGURE 5: Photoaffinity labeling of detergent-solubilized affinity-purified AChR (0.2 nmol of α-bungarotoxin sites). Coomassie stain (lane A), molecular weight markers (lane B), and autoradiogram of receptor of lane A photoaffinity labeled with N^{α}-^{125}I-ASA–α-conotoxin GIA (Lane C), resolved on a 7% polyacrylamide Laemmli (1970) gel. Notice the exclusive labeling of the γ subunit.

although this assignment should be considered tentative. A similar analysis of the β subunit derived from photolabeled membranes is consistent with the major cross-link on the β subunit occurring within β149–225 [detailed data are presented in Myers (1989)].

Discussion

The most generally accepted model for the nicotinic acetylcholine receptor from *Torpedo* electric organ locates the sites for acetylcholine binding on the two α subunits. Considerable evidence has been presented from many laboratories in support of this model [reviewed in Popot and Changeux (1984) and Hawrot et al. (1988)]. A variety of agonists and antagonists such as carbamylcholine, *d*-tubocurarine, and α-bungarotoxin are also believed to bind at this site, albeit with different sized "footprints" on the receptor. Because carbamylcholine and curare prevent binding of α-conotoxin to the receptor, and excess α-conotoxin abolishes all specific α-bungarotoxin binding, it seemed reasonable to assume that the binding site of α-conotoxins would be at these classical cholinergic sites. Thus, our expectation was for α-subunit-specific cross-linking of α-conotoxins to the acetylcholine receptor.

It was therefore a surprise to find that in a variety of experimental protocols designed to covalently attach toxin to the receptor subunit to which it was bound, no preferential labeling of the α subunits was observed. Under most conditions, the α subunit was the most poorly labeled of the four different subunits of the *Torpedo* nicotinic acetylcholine receptor. Indeed, as the possible cross-linking radius was progressively decreased (i.e., using the shortest bivalent cross-linker, as well as relatively constrained photoactivatable derivatives), increased specificity for the β and γ subunits became evident. If detergent-solubilized, purified receptor were used, only the γ subunit was labeled by the photoactivatable derivative ^{125}I-ASA–α-conotoxin GIA. Clearly, therefore, the simple expectation that cross-linking should be highly biased toward and perhaps even occur exclusively on the α subunits of the acetylcholine receptor was not met in these experiments. The specificity of cross-linking was established by the fact that unlabeled excess α-conotoxins and *d*-tubocurarine, but not ω-conotoxin, abolished cross-linking by the radiolabeled ligand (see Figure 4).

These results, opposite to those that might be a simplistic expectation from present models of cholinergic sites, clearly indicate that a further exploration of α-conotoxin/receptor interactions is warranted. The results are consistent with at least two general classes of hypotheses. The binding sites for the cholinergic ligands may not be restricted to the α subunits (Conti-Tronconi & Raftery, 1986); thus, the non-α subunits may be specifically cross-linked in the experiments shown above because strong binding sites for α-conotoxins exist on the other subunits. This raises the question why α-conotoxins and standard ligands believed to be α-specific displace each other. A second possibility is that the relevant binding occurs at sites near the junctions of subunits (such as at the α–γ interface) and that the groups which can be cross-linked with bivalent or photoactivatable cross-linkers are facing away from the α-subunit determinants of the binding site, and toward other subunits. A similar hypothesis has been proposed on the basis of photoaffinity labeling using *d*-[^{3}H]tubocurarine (Pedersen & Cohen, 1990). At this time, we cannot distinguish between these (and other) hypotheses. However, the observed absence of photolabeling at the α subunits would not have been the straightforward expectation from either one of the hypotheses above.

The α-conotoxins have many chemical groups which can potentially be modified; because these peptides are small, preparing specific derivatives is relatively straightforward. In this work, two homologous photoactivatable derivatives of α-conotoxin GIA were made. In one homologue, the reporter group is attached to the N-terminus; in the other derivative, to an ε-amino group near the C-terminus. We demonstrated that the relative efficiency of cross-linking to different subunits of the acetylcholine receptor was a function of placement of the photoactivatable group on the toxin.

This initial work suggests that the set of α-conotoxins in cone snail venoms should be an unparalleled set of tools for exploring the surface of the acetylcholine receptor. Work on α-conotoxins so far leads to the expectation that each of the ca. 50 different fish-hunting *Conus* venoms will have its own distinctive set of α-conotoxins; our laboratories have purified approximately 100 peptides (of all types) from 10 *Conus* venoms, and not a single sequence has been found in more than 1 venom. The cone snails have probably developed special genetic mechanisms for generating hypervariability in their toxins, while stringently conserving a few disulfide bonding patterns [see Olivera et al. (1990)]. For this reason, widely differing sequences in these relatively small homologous toxins are available. For example, if the 2 major α-conotoxins used in this study are compared (i.e., α-conotoxin GIA versus SIA), the cysteine residues are completely conserved (4 out of 4 are in identical positions), but only 2 out of the 11 non-cysteine amino acids in GIA are found in SIA. Combining 2D NMR work (such as was recently published for α-conotoxin GI) with the type of position-specific cross-linking results we obtained should reveal the orientation of the toxin with respect to the receptor. The availability of a large homologous set of peptides of highly variable sequence provides great flexibility in or-

Table III: α-Conotoxin Amino Acid Sequences

toxin	sequence	charge	ref
GI	ECCNPACGRHYSC-NH_2	+1.5	a
CIA	ECCNPACGRHYSCGK-NH_2	+2.5	b
GII	ECCHPACGKHFSC-NH_2	+2	c
MI	GRCCHPACGKNYSC-NH_2	+3.5	d
SI	ICCNPACGPKYSC-NH_2	+2	e
SIA	YCCHPACGKNFDC-NH_2	+1.5	this work
Disulfide bridging	** * * / * *		f

[a] From *Conus geographus*: cf. Gray et al. (1988). [b] From *Conus geographus*: cf. Gray et al. (1988). [c] From *Conus geographus*: cf. Gray et al. (1988). [d] From *Conus magus*: cf. Gray et al. (1988). [e] From *Conus striatus*: cf. Zafaralla et al. (1988). [f] Gray et al., 1984; Nishiuchi & Sakakibara, 1982.

ienting toxin reporter groups on the receptor. For example, there are clearly amino groups in α-conotoxins MI and SIA which when derivatized would provide a different reporter group orientation from the two derivatives of α-conotoxin GIA used in this study.

The sequences shown in Table III come from only 3 out of the ca. 50 fish-hunting cone species. In addition, there are hundreds of cone species which prey on invertebrates of various types; since most of their invertebrate prey have cholinergic transmission at the neuromuscular junction, it is reasonable to suppose that many of their venoms will have AChR-targeted peptides as well. Thus, the α-conotoxins which have been sequenced are only a small subset of the total natural α-conotoxin sequence diversity present in cone snail venoms. With this work, we have only begun to tap the enormous potential of this family of peptides as ligands for probing acetylcholine receptors.

Registry No. α-Conotoxin SIA, 135190-31-7; α-conotoxin MI, 88217-10-1; α-conotoxin GIA, 129510-84-5; DST, 77658-91-4; DSS, 68528-80-3; EGS, 70539-42-3; εN-Lys^{15}-ASA-GIA, 135190-32-8; αN-ASA-GIA, 135190-33-9; αN-Lys^{15}-[^{125}I]ASA-GIA, 135190-34-0; εN-[^{125}I]ASA-GIA, 135224-96-3; *N*-hydroxysuccinimidyl-4-azidosalicylic acid, 96602-46-9.

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