α-Conotoxin OmlA Is a Potent Ligand for the Acetylcholine-binding Protein as Well as α3β2 and α7 Nicotinic Acetylcholine Receptors

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Abstract

The molluskan acetylcholine-binding protein (AChBP) is a homolog of the extracellular binding domain of the pentameric ligand-gated ion channel family. AChBP most closely resembles the α-subunit of nicotinic acetylcholine receptors and in particular the homomeric α7 nicotinic receptor. We report the isolation and characterization of an α-conotoxin that has the highest known affinity for the Lymnaea AChBP and also potently blocks the α7 nAChR subtype when expressed in Xenopus oocytes. Remarkably, the peptide also has high affinity for the α3/β2 nAChR indicating that α-conotoxin OmlA in combination with the AChBP may serve as a model system for understanding the binding determinants of α3β2 nAChRs. α-Conotoxin OmlA was purified from the venom of Conus omaria. It is a 17-amino-acid, two-disulfide bridge peptide. The ligand is the first α-conotoxin with higher affinity for the closely related receptor subtypes, α3/β2 versus α6/β2, and selectively blocks these two subtypes when compared with α2/β2, α4/β2, and α1/β1δε nAChRs.

Nicotinic acetylcholine receptors (nAChRs)2 are found in the neuromuscular junction, peripheral nervous and central nervous systems of both invertebrates and vertebrates. These receptors play essential roles in mediating synaptic transmission and modulating the release of a variety of neurotransmitters. Different molecular forms of the nAChR are comprised of homopentameric (α7 and δ9) and heteropentameric (e.g. α3/β2, α4/β2, and α1/β1γε) arrangements of subunits that have discrete anatomical locations and distinct physiological functions. Dysfunction or dysregulation of nAChRs is implicated in a variety of

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2The abbreviations used are: nAChR, nicotinic acetylcholine receptors; GABA, γ-aminobutyric acid; GABA_A, γ-aminobutyric acid type A; GABA_C, γ-aminobutyric acid type C; HPLC, high performance liquid chromatography; Fmoc, 9-fluorenylmethoxycarbonyl; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
neuropsychiatric disease states including schizophrenia, Parkinson, Alzheimer, depression and nicotine addiction (1). Several drug discovery programs aim to develop specific drugs that selectively act on subtypes of nAChRs.

The AChBP, synthesized in molluscan glial cells, is proposed to function as a modulator of synaptic ACh transmission. ACh, acting on a glial nAChR, induces cellular release of AChBP. AChBP, in turn, binds presynaptically released ACh, acting as a synaptic buffer to dampen synaptic transmission (2). AChBP has sequence similarity (15–28% identity) with subunits of the cysteine loop ligand-gated ion channel family that includes nAChRs, GABA\textsubscript{A}, GABA\textsubscript{C}, 5-hydroxytryptophan type 3, and glycine receptors (3). These receptors assemble as heteromeric or homomeric pentamers of subunits, and each subunit has an NH\textsubscript{2}-terminal extracellular ligand-binding domain and four COOH-terminal transmembrane spans that serve as the channel pore-forming domain. AChBP is homologous to the extracellular domain of the cysteine-loop family. The crystal structure of AChBP has provided a structural template for examining ligand recognition in nAChRs by spectroscopic analysis of conjugated fluorophores, structural modeling, dynamics of deuterium/hydrogen exchange, computational docking of ligands, and direct monitoring of ligand occupancy by changes in intrinsic Trp fluorescence (4–8).

Despite several conserved features of the ACh-binding site among different nAChR subtypes and the AChBP, certain ligands are able to discriminate between nAChRs of different subunit composition. The structural basis of nAChR subtype selectivity and the sequence determinants governing specificity are subjects of intense investigation.

\textit{α}-Conotoxins are a family of small peptides used by carnivorous marine snails to envenomate their prey. These peptides are small, disulfide-linked, conformationally constrained antagonists of nAChRs. They generally target the ligand-binding site of these receptors. Although the fold of their peptide backbone is highly conserved, differences in amino acid side chains lead to a remarkable degree of receptor subtype specificity (9, 10).

Recently, \textit{α}-conotoxin ImI (11) and an analog of \textit{α}-conotoxin PnIA (PnIA (A10L,D14K)) (12) have been crystallized bound to the AChBP from \textit{Aplysia californica}. Both of these \textit{α}-conotoxins preferentially target the \textit{α}7 nAChR. In contrast, \textit{α}-conotoxin MII (13), a ligand that binds with high affinity to the \textit{α}3/\textit{β}2 and \textit{α}6/\textit{β}x nAChRs, had only approximately micromolar affinity for the \textit{Lymnaea} and \textit{Aplysia} AChBPs (12). In this report we describe the purification of a novel peptide from the venom of the molluscivorous \textit{Conus omaria}. The peptide not only avidly binds AChBP from \textit{Bulinus}, \textit{Lymnaea}, and \textit{Aplysia}, but also selectively blocks neuronal nAChRs with greatest antagonism appearing for the \textit{α}3/\textit{β}2 subtype.

**EXPERIMENTAL PROCEDURES**

**Venom Extraction**

Crude venom from dissected ducts of \textit{C. omaria} was collected in the Philippines, lyophilized, and stored at –70 °C until use. All reagents were pre-cooled to, and extraction procedures were carried out, at 4 °C. Thirty ml of 0.1% trifluoroacetic acid was added to
each of two tubes containing 250 mg each of crude venom. The mixture was vigorously vortexed for 30 min and then centrifuged at 17,000 × g for 20 min. The resulting supernatant was stored and the pellet resuspended in 0.1% trifluoroacetic acid and the above procedure was repeated twice. All resulting supernatants were combined and vacuum passed through a 0.45-mm filter. The filtered material was diluted to 800 ml with 0.1% trifluoroacetic acid for loading onto a preparative HPLC column as described in the legend to Fig. 1.

Chemical Synthesis

The peptide was synthesized, 0.45 mmol/g, on a Fmoc amide resin using Fmoc chemistry and standard side protection except on cysteine residues. Cys residues were protected in pairs with either S-trityl on Cys$^{1}$ and Cys$^{3}$ (the first and third Cys), or S-acetamidomethyl on Cys$^{2}$ and Cys$^{4}$. The peptide was removed from the resin and precipitated. A two-step oxidation protocol was used to -fold selectively the peptides as described previously (14). Briefly, the disulfide bridge between Cys$^{2}$ and Cys$^{8}$ was closed by dripping the peptide into an equal volume of 20 mM potassium ferricyanide, 0.1 M Tris-HCl, pH 7.5. The solution was allowed to react for 30 min, and the monocyclic peptide was purified by reverse-phase HPLC. Simultaneous removal of the S-acetamidomethyl groups and closure of the disulfide bridge between Cys$^{3}$ and Cys$^{13}$ was carried out by iodine oxidation. The monocyclic peptide and HPLC eluent was dripped into an equal volume of iodine (10 mM) in H$_{2}$O:trifluoroacetic acid:acetonitrile (78:2:20 by volume) and allowed to react for 10 min. The reaction was terminated by the addition of ascorbic acid, diluted 20-fold with 0.1% trifluoroacetic acid, and the bicyclic peptide was purified by HPLC on a reverse-phase C$_{18}$ Vydac column using a linear gradient of 0.1% trifluoroacetic acid, 0.092% trifluoroacetic acid, 60% acetonitrile, and the remainder H$_{2}$O.

Mass Spectrometry and Sequencing

Measurements were performed at the Salk Institute under the direction of Jean Rivier. Liquid secondary ionization mass spectrometry and matrix-assisted laser desorption ionization time-of-flight mass spectrometry were utilized. Reduction and alkylation of Cys residues and chemical sequencing was performed as previously described (13).

Reduction and Equilibrium Oxidation of  α-Conotoxin OmIA

Twenty nmol of synthetic α-conotoxin OmIA prepared by the two-step oxidation protocol described above was reduced, HPLC purified, and then reoxidized using glutathione. Reduction was carried out in a 1-ml volume at room temperature for 1 h. Reducing solution was 0.1 M Tris, pH 8.7, 1 mM EDTA, and 50 mM dithiothreitol. Eighty μl of formic acid was added to the solution at the end of the reaction. Reduced peptide was purified by HPLC and lyophilized. Six nmol of reduced α-conotoxin OmIA was dissolved in 30 μl of 0.01% trifluoroacetic acid. This was added to a solution that at a final volume of 300 μl was 0.1 M Tris, pH 8.7, 1 mM EDTA, 1 mM oxidized glutathione, and 1 mM reduced glutathione. The solution was reacted at room temperature for 2 h and terminated by the addition of 24 μl of formic acid.
Expression and Purification of AChBPs

AChBPs from *Lymnaea stagnalis* (Ls), *Aplysia californica* (Ac), and *Bulinus truncatus* (Bt) were expressed using cDNAs synthesized from oligonucleotides engineered for mammalian codon usage, as previously described (6, 15, 16). Briefly, the AChBP gene was inserted into a FLAG-CMV-3 expression vector (Sigma) with the aminoglycoside phosphotransferase II gene, to confer aminoglycoside resistance, and a preprotrypsin leader peptide followed by a NH₂-terminal FLAG epitope. AChBP-transfected HEK-293 cells were selected with G418 to generate stably expressing cell lines. Dulbecco’s modified Eagle’s medium (MediaTech CellGro) containing 3% fetal bovine serum was collected at 3-day intervals from multitier flasks for up to 4 weeks. Adsorption onto anti-FLAG M2 affinity gel followed by elution with the FLAG peptide (both from Sigma) yielded purified protein in quantities between 0.5 and 5 mg/liter of media. Purity and assembly of subunits as a pentamer were assessed by SDS-PAGE and fast protein liquid chromatography.

Two AChBP homologs (Bt-AChBP and Bt-AChBP-2) that differ by eight residues have been identified in the tissue of *B. truncatus* (17). Our initial attempts expressing Bt-AChBP showed low expression in our system. During the process of converting Bt-AChBP to Bt-AChBP-2 by mutagenesis, we observed that the single mutation, F149L, results in comparatively robust expression of a soluble pentameric entity. We used the F149L construct for this investigation.

Radioligand Competition Assays

An adaptation of a scintillation proximity assay was used to determine the apparent $K_d$ as reported previously (16). Briefly, AChBP (final concentration ~500 pM binding sites), polyvinyltoluene anti-mouse SPA scintillation beads (0.1 mg/ml, Amersham Biosciences), monoclonal anti-FLAG M2 antibody from mouse (Sigma), and $[^3]$H]epibatidine (5 nM final concentration for Ls and Bt, 20 nM for Ac) were combined in phosphate buffer (0.1 M, pH 7.0) with increasing concentrations of competing ligand in a final volume of 100 μl. Total binding was determined in the absence of competing ligand, and nonspecific binding was measured by adding a saturating concentration (15 μM) of methyllycaconitine. The resulting mixtures were allowed to equilibrate at room temperature for a minimum of 2 h and measured on a LS 6500 liquid scintillation counter (Beckman Scientific). The data obtained are normalized, fit to a sigmoidal dose-response curve (variable slope), and the $K_d$ calculated from the observed EC₅₀ (18) using GraphPad Prism version 4.02 for Windows. A minimum of three independent experiments, performed in duplicate, were used to determine the $K_d$ values reported. This procedure required less than 10 μg of each binding protein for this study, including initial compound screening and preliminary assays.

Electrophysiology

Oocytes were harvested and injected with cRNA encoding nAChR subunits as described previously (13). All clones were from rat, except for the muscle subtype that was from mouse. The rat $\alpha 6$ subunit does not express with the rat $\beta 2$ subunit (19). We therefore used a chimera that contains the NH₂-terminal extracellular $\alpha 6$ subunit sequence linked to the remaining portion of the $\alpha 3$ subunit protein as a model of activity at $\alpha 6$ subunit-containing nAChRs. The chimeric $\alpha 6/\alpha 3$ nAChR consists of amino acids 1–237 of the rat $\alpha 6$ subunit.
protein linked to amino acids 233–499 of the rat α3 subunit protein. The chimeric junction is located at the paired arginine residues immediately preceding the M1 transmembrane segment of the α3 subunit. The subunit is as previously described except that a valine to arginine change at amino acid 278 in the original chimeric construct was corrected; all other subunits were as previously described (20).

A 30-μl cylindrical oocyte recording chamber fabricated from Sylgard was gravity-perfused with ND96A (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 1 μM atropine, 5 mM HEPES, pH 7.1–7.5) at a rate of ~2 ml/min. All toxin solutions also contained 0.1 mg/ml bovine serum albumin to reduce non-specific adsorption of peptide. Toxin was preapplied for 5 min. ACh-gated currents were obtained with a two-electrode voltage clamp amplifier (model OC-725B, Warner Instrument, Hamden, CT), and data were captured as previously described (21). The membrane potential of the oocytes was clamped at ~70 mV. To apply a pulse of ACh to the oocyte, the perfusion fluid was switched to one containing ACh for 1 s. This was automatically done at intervals of 1–5 min. The shortest time interval was chosen such that reproducible control responses were obtained with no observable desensitization. This time interval depended on the nAChR subtype being tested. ACh was diluted in ND96A for tests of all nAChR subtypes except α7, in which case the diluent was ND96 (no atropine) (22). For control responses, the ACh pulse was preceded by perfusion with ND96 (for α7) or ND96A (all others). No atropine was used with oocytes expressing α7, because it has been demonstrated to be an antagonist of these receptors.

The concentration of ACh was 10 μM for trials with α1/β/δε; 200 μM for α7, and 100 μM for all other nAChRs. Toxin was bath applied for 5 min, followed by a pulse of ACh. Thereafter, toxin was washed away and subsequent ACh pulses were given every 1 min, unless otherwise indicated. All ACh pulses contain no toxin, for it was assumed that little if any bound toxin washed away in the brief time (less than 2 s rise time to peak). In our recording chamber, the bolus of ACh does not project directly at the oocyte but rather enters tangentially, swirls and mixes with the bath solution. The volume of entering ACh is such that the toxin concentration remains at a level >50% of that originally in the bath until the ACh response has peaked (<2 s). The average peak amplitude of three control responses just preceding exposure to toxin was used to normalize the amplitude of each test response to obtain a “% response” or “% block.” Each data point of a dose-response curve represents the average value ± S.E. of measurements from at least three oocytes. Dose-response curves were fit to the equation: % response = 100/[1 + ([toxin]/IC₅₀)^nH], where nH is the Hill slope with Prism software (GraphPad Software).

**Molecular Modeling**

Molecular modeling was done on Silicon Graphic Indigo O₂ computer using the programs Discover and Insight II (Accelrys, San Diego). The three-dimensional structure of Lymnaea AChBP was built with the Homology module of Insight II using as templates two molecules; α-cobratoxin bound Lymnaea AChBP (Protein Data Bank code 1YI5) (23), and for the C-loop (residues 183–192), the structure of Aplysia AChBP bound to α-conotoxin ImI (PDB code 2BYP) (11). In the case of α-conotoxin OmIA, the NMR derived solution conformation was used as the conotoxin template (PDB code 2GCZ) (24). The modeled
structure was then minimized for 10,000 iterations of steepest descent \textit{in vacuo}, using the distant-dependent dielectric constant, with the program Discover.

Both structures were superimposed and \( \alpha \)-conotoxin OmIA was located in the position where its backbone atoms were superimposed with homologous atoms of \( \alpha \)-conotoxin in \textit{Aplysia} AChBP. The systems containing AChBP and \( \alpha \)-conotoxin underwent 800 iterations of steepest descent minimization \textit{in vacuo} with fixed Ca trace atoms of AChBP using the distant-dependent dielectric constant, with the program Discover. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (25).

\section*{RESULTS}

\subsection*{Purification and Sequence Determination of \( \alpha \)-Conotoxin OmIA}

A crude extract of lyophilized \textit{C. omaria} venom was initially fractionated using reversed phase chromatography. Because many conotoxins are positively charged, we subsequently utilized cation exchange chromatography to achieve further purification (Fig. 1). Final purification and desalting were carried out and the product was reduced, alkylated, and sequenced as described under “Experimental Procedures.” The sequence is GCCSHPACNVNNPHICG. Mass spectrometry indicated that Cys residues are present as disulfides and that the COOH-terminal \( \alpha \)-carboxyl group is amidated (monoisotopic MH\(^+\): calculated 1720.66, observed 1720.7). The sequence was additionally confirmed though total chemical synthesis (see below). Based on the structure and function (detailed below), the peptide was named \( \alpha \)-conotoxin OmIA according to the proposed nomenclature (26).

\subsection*{Chemical Synthesis}

The peptide was synthesized using solid phase Fmoc chemistry as described under “Experimental Procedures.” The disulfide connectivity was assumed to be that of other venom-derived \( \alpha \)-conotoxins, that is Cys\(^1\)–Cys\(^3\) and Cys\(^2\)–Cys\(^4\). Orthogonal protection was used on Cys pairs to direct the folding. Liquid secondary ionization mass spectrometry confirmed full oxidation of the peptide product (monoisotopic MH\(^+\): calculated 1720.66, observed 1720.5). On reversed phase HPLC, the fully folded synthetic peptide elutes at a time that corresponds to that of the native peptide. When the native and synthetic peptides were mixed together, they co-eluted, indicating that the native peptide has Cys\(^1\)–Cys\(^3\) and Cys\(^2\)–Cys\(^4\) connectivity (Fig. 2). This was further confirmed by experiments showing that the disulfide isomers may be resolved on HPLC. Fully oxidized synthetic peptide was reduced with dithiothreitol, and chromatographed. The reduced peptide elutes later and is well separated from the native peptide (Fig. 3, A and B). Equilibrium folding of the reduced peptide using glutathione gave three major products corresponding to the three theoretical disulfide arrangements; each had a mass consistent with that of fully folded peptide. Synthetic peptide prepared by directed folding co-elutes on HPLC with the second eluting of the three equilibrium folding products (Fig. 3, C–E). Given that disulfide forms 1 and 2 from equilibrium folding of the peptide are not well resolved on HPLC, directed folding is the synthetic procedure of choice.
Activity at AChBP and nAChRs

AChBPs from the fresh water snails, L. stagnalis and B. truncatus and the sea slug A. californica were expressed from the respective cDNAs. OmIA was tested for its ability to displace the binding of $[^3H]$epibatidine or $[^{125}I]\alpha$-bungarotoxin to the three AChBPs as measured by a scintillation proximity assay. The $K_d$ values determined using $[^3H]$epibatidine are consistent with those using $[^{125}I]\alpha$-bungarotoxin and indicate that OmIA has high affinity for AChBP from all three species (Fig. 4 and Table 1).

We next tested activity on rat nAChRs expressed in Xenopus oocytes. AChBPs have considerable sequence identity with the homomeric $\alpha 7$ nAChR. OmIA effectively blocks the ACh-induced response on homomeric $\alpha 7$ nAChRs at 100 nM. By contrast, it is inactive on the $\alpha 1/\beta 1/\delta/e$ subtype of nAChR at 10 $\mu$M, indicating a high selectivity for this neuronal subtype versus adult muscle subtype (Fig. 5). Concentration-response testing on multiple subtypes of nAChRs indicated that the peptide has highest affinity for the $\alpha 3/\beta 2$ nAChR, yet is inactive at $\alpha 2/\beta 2$ and $\alpha 4/\beta 2$ nAChRs (Fig. 5 and Table 2). To our knowledge, OmIA is the only reported $\alpha$-conotoxin that potently and preferentially targets $\alpha 3/\beta 2$ nAChRs and also has high affinity for the AChBP. In contrast, for example, $\alpha$-conotoxin MII, which blocks $\alpha 3/\beta 2$ nAChRs expressed in Xenopus oocytes with an IC$_{50}$ of 2.2 nM (27), has an IC$_{50}$ of only 780 nM for L. stagnalis AChBP and 4,100 nM for A. californica AChBP (12).

Docking Studies

Because the OmIA conotoxin has high affinity for all three AChBPs, our docking studies compared OmIA versus $\alpha$-conotoxin ImI, a toxin with a high degree of selectivity for Aplysia and Bulinus over Lymnaea. The $\alpha$-conotoxin ImI-Aplysia complex has been resolved at high resolution (PDB code 2BYP, 2.07 Å). The docked structures show a close fit of both OmIA and ImI at the subunit interface and under the C-loop flap (Fig. 6). The additional mass of the OmIA toxin at its COOH-terminal end (17 versus 12 amino acids) is accommodated in the binding site region at the outer perimeter of the complementary face and partially exposed to solvent.

DISCUSSION

In this report we describe the purification of a new $\alpha$-conotoxin from the venom of C. omaria. To our knowledge, this is the first characterization of a venom peptide from this species. C. omaria is found in both the Pacific and Indian Oceans in shallow, subtidal waters to a depth of up to 100 m. It is also found on coral reefs and in reef lagoons, in sand and rubble. Its shell ranges in size from 45 to 86 mm. This carnivore feeds on other gastropods including Cypraeidae, Olividae, and Strombidae. The presence of an $\alpha$-conotoxin in the venom of C. omaria suggests that cholinergic transmission plays an important role in the molluskan prey of this cone snail.

The isolated peptide, $\alpha$-conotoxin OmIA, is a 17-amino acid peptide with an amidated COOH terminus. It is similar in the spacing of its Cys residues to peptides isolated from several other species of cone snail (Table 3). Co-elution studies with synthetic peptide indicate that the disulfide connectivity of the Cys residues is the same as that determined for
all previous venom-derived α-conotoxins, that is Cys\textsubscript{1}–Cys\textsubscript{3}, Cys\textsubscript{2}–Cys\textsubscript{4}. Although OmIA potently blocks all three of the AChBPs and the α7 nAChR, it lacks the Leu\textsuperscript{10} side chain that appears to serve as a critical binding anchor for other α-conotoxins such as PnIB and MII with high affinity for α7 (12, 28–30). This suggests that OmIA may represent an important new probe for AChBP and neuronal nAChR-binding sites. Its three-dimensional structure determined by NMR provides a valuable template for structural comparisons (24). OmIA is also structurally unique among isolated α-conotoxins in having an additional glycine after the most COOH-terminal Cys residue.

OmIA potently displaces the competitive antagonist, α-bungarotoxin, and the agonist, epibatidine, from their mutually exclusive binding sites on Lymnaea, Bulinus, and Aplysia AChBPs. This indicates that OmIA acts at the ligand-recognition site of AChBP and, based on homology, is a competitive antagonist of nAChRs. The prey of cone snails may be broadly divided into three types, fish, mollusks, and worms. Some species of cone snail specialize in one particular prey type, whereas others are omnivorous. Peptides from the fish-hunting cone snails, α-conotoxin GI from C. geographus and α-conotoxin MII from C. magus, are comparatively weak ligands for the AChBP (12). α-Conotoxin ImI, from the worm-hunting C. imperialis, potently binds to the Aplysia and Bulinus AChBPs, but not Lymnaea AChBP (Table 1) (11, 15). OmIA from the mollusk-hunting C. omaria is potent on AChBP from both freshwater and marine mollusks. α-Conotoxin ImI selectively blocks subtypes of invertebrate nAChRs in Aplysia and Lymnaea neurons (31, 32). Similarly, C. omaria may target molluskan nAChRs homologous to molluskan AChBP.

Docking of the ImI and OmIA conotoxins to the α7 receptor and the Lymnaea AChBP reveals two regions of the AChBP-binding site that appear accountable for the loss of ImI affinity for the Lymnaea-binding protein. The primary one arises from electrostatic repulsion near the apical position of the bound conotoxin where Arg\textsuperscript{11} of the toxin is proximal to Arg\textsuperscript{148} and His\textsuperscript{146} of the Lymnaea AChBP (Fig. 6). Residues in the α7 nAChR homologous to His\textsuperscript{146} and Arg\textsuperscript{148} in Lymnaea AChBP are two glycines (Figs. 6 and 7). In Aplysia and Bulinus AChBPs fewer cationic side chains are found in this region (Fig. 7). A secondary site of repulsion may arise from interaction of Arg\textsuperscript{7} of the ImI toxin with Lys\textsuperscript{139} of the Lymnaea AChBP, but those cationic charges appear to be spaced further apart and somewhat compensated for by nearby anionic residues. OmIA contains an Ala at the 7 position, reducing the potential for electrostatic repulsion.

A second AChBP-conotoxin complex has been resolved crystallographically in PnIA (A10L,D14K), a 16-amino acid peptide (12). This toxin contains an Asn residue at the position homologous to Arg\textsuperscript{11}. Both PnIA (A10L,D14K) and OmIA contain a potentially cationic side chain near the COOH terminus at position 14. However, the COOH terminus has an external exposure so as to minimize electrostatic repulsion.

The multiplicity of subunits composing the nAChRs lead to a large combinatorial diversity of nAChRs. Receptor subtypes identified to date have unique anatomical distributions and physiological properties. To identify the anatomical location, subunit composition, and functional roles of the multiple nAChRs, additional selective ligands are needed as pharmacological and structural probes (1, 33).
α-Conotoxin OmIA has potent activity on mammalian nAChRs as well, suggesting that critical binding determinants may be shared between the AChBP and mammalian nAChRs. However, these sites do not appear to be universally conserved among the nAChRs. Whereas α-conotoxin OmIA potently blocks α3β2 nAChRs, it is inactive at 10 μM concentration at α2β2 and α4β2 nAChRs. Because the β2 subunit is present in each of these instances, this activity profile suggests that important toxin binding interactions occur at sites present on the α3 subunit, but lacking in the α2 and α4 subunits. In addition, there is a ~400-fold difference in IC50 between α3β2 and α3β4 nAChRs suggesting a β2 to β4 subunit preference.

The nAChR α3 and α6 subunits are closely related evolutionarily with ~80% identity in the ligand binding extracellular domain (34). A peptide related to α-conotoxin OmIA, α-conotoxin MII, shows a slight (~5-fold) preference for α6/α3β2β3 versus α3β2 nAChRs. This slight preference was successfully exploited by serial Ala mutations to create α-conotoxin-based ligands with up to 2000-fold selectivity for α6/α3β2β3 versus α3β2 nAChRs (27). In contrast, the new peptide, OmIA, shows an 18-fold preference for α3β2 versus α6/α3β2β3.

The structural templates developed from crystallographic data of the AChBPs and modeling with homologous nAChR structures provide a means of ascertaining the structural determinants giving rise to receptor selectivity for the α-conotoxin family. Lewis and colleagues (35, 36) have shown that high affinity binding to α3β2 receptors for the α-conotoxins carrying the large side chain at residue 10 requires a relatively small side chain at the subunit interface in apposition with residue 10. This interaction involves the complementary subunit interface, in this case the β subunit, not containing the C loop.

Using a strategy similar to that described above for α-conotoxin MII in conjunction with crystallographic templates, α-conotoxin OmIA may serve as a lead for producing ligands to target α3β2 nAChRs even more selectively. The α3β2 subtype appears to be present in human brain and may be involved in dopaminergic modulation (37). Hence, selective ligands for the α3β2 nAChR subtype, as shown by the OmIA lead compound, may prove to have substantial therapeutic utility.

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FIGURE 1. Purification of \( \alpha \)-conotoxin OmIA by HPLC

A, supernatant from the crude venom extract (see “Experimental Procedures”) was diluted to 800 ml with ice-cold 0.1% trifluoroacetic acid and loaded on a Vydac reversed phase C18 column (10-\( \mu \)m particle size; 22-mm inner diameter \( \times \) 25-cm length) and eluted with a linear gradient that beginning at 98% buffer A, 2% buffer B and increased to 100% buffer B over 98 min. Flow rate was 20 ml/min. B, the indicated material (arrow, panel A) was lyophilized and then dissolved in 4 ml of 0.1% trifluoroacetic acid. The solution was loaded onto a Vydac reversed phase C8 column (5-\( \mu \)m particle size; 10-mm inner diameter \( \times \) 25-cm length) and eluted with a linear gradient that beginning at 100% buffer A, 0% buffer B and increased to 35% buffer A, 65% buffer B over 65 min. Flow rate was 5 ml/min. C, ~15% of
the indicated material (arrow, panel B) was lyophilized and dissolved in 1 ml of buffer A and loaded onto a Vydac protein-SCX (strong cation exchange) column (0.75 × 5 cm) and eluted using a linear gradient from 100% buffer A to 35% buffer A, 65% buffer B over 65 min. Flow rate was 1 ml/min. D, material indicated (arrow, panel C) and material from additional purifications of the remaining material shown in panel B were combined and desalted using a reversed phase Vydac C18 column (5-μm particle size, 4.6 mm inner diameter × 25-cm length) using a linear gradient that beginning at 100% buffer A and increased to 40% buffer A, 60% buffer B over 60 min. Flow rate was 1 ml/min. Buffer A was 0.1% trifluoroacetic acid; buffer B was 0.092% trifluoroacetic acid, 60% acetonitrile, the remainder was H₂O in panels A, B, and D. In panel C, buffer A was 10 mM NaH₂PO₄, 50% acetonitrile, pH 2.5, and buffer B was the same as buffer A with the addition of 250 mM NaCl. The absorbance was monitored at 220 nm in panels A, B, and D and 280 nm in panel C.
FIGURE 2. Co-elution of native and synthetic α-conotoxin OmIA

A, HPLC of ~400 pmol of α-conotoxin OmIA. B, HPLC of ~350 pmol of native α-conotoxin OmIA. C, co-elution of ~400 pmol of synthetic and 350 pmol of native α-conotoxin OmIA. The peptides were run on a C18 Vydac reversed phase column (5-μm particle size, 4.6 mm inner diameter × 25-cm length) using a linear gradient that began at 100% buffer A and increased to 40% buffer A, 60% buffer B over 60 min. Buffer A was 0.1% trifluoroacetic acid; buffer B was 0.092% trifluoroacetic acid, 60% acetonitrile, the remainder H₂O. Flow rate was 1 ml/min. The absorbance was monitored at 220 nm.
FIGURE 3. Co-elution of α-conotoxin OmIA synthesized by directed folding versus equilibrium folding
A, HPLC of 60 pmol of α-conotoxin OmIA synthesized with two-step oxidation of disulfide bonds as described under “Experimental Procedures.” B, synthetic α-conotoxin OmIA after reduction by dithiotheitol (see “Experimental Procedures”). C, glutathione oxidation of reduced α-conotoxin OmIA (see “Experimental Procedures”). D, co-elution of 60 pmol of α-conotoxin OmIA synthesized with a two-step oxidation and 60 pmol of peak 1 of glutathione-oxidized α-conotoxin OmIA (see panel C). E, co-elution of 60 pmol of α-conotoxin OmIA synthesized with two-step oxidation and 60 pmol of peak 2 of glutathione-
oxidized α-conotoxin OmIA (see panel C). The peptides were run on a C18 Vydac reversed phase column with conditions as described in the legend to Fig. 2.
Concentration-response curves were determined for α-conotoxin OmIA displacement of radioligand binding to AChBP from *L. stagnalis* and *A. californica* as described under “Experimental Procedures.” A, competition with [125I]α-bungarotoxin. B, competition with [3H]epibatidine. Calculated $K_d$ values are shown in Table 1. $n = 6$ for all experiments.

FIGURE 4. α-Conotoxin OmIA binding to AChBP
FIGURE 5. Effect of α-conotoxin OmIA on nAChR subtypes

A, α-conotoxin OmIA selectively blocks α7 versus muscle nAChR. nAChRs were expressed in Xenopus oocytes as described under “Experimental Procedures.” α-Conotoxin OmIA at 500 nM (α7) or 10 μM (muscle subtype) was added to the oocyte solution for 5 min prior to the addition of ACh. A single experiment is shown. Similar results were obtained in two additional experiments utilizing different oocytes in each study. B, α-conotoxin OmIA effect on α7, α3β4, and muscle receptor α1β1δε. C, α-conotoxin OmIA effect on β2-containing nAChRs. IC50 values are shown in Table 2. Error bars are the S.E. Data are from 3–6 oocytes.

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FIGURE 6. Docking of \( \alpha \)-conotoxins OmIA and ImI to the \( L. \) stagnalis acetylcholine-binding protein and to a homology model of the human \( \alpha 7 \) nicotinic receptor

The \( \alpha \)-conotoxin-AChBP complexes are based on the crystal structure (PDB code 1Y15) (23), whereas the human \( \alpha 7 \) receptor structures were constructed as described under “Experimental Procedures.” A and C, \( \alpha \)-conotoxin OmIA and ImI, respectively, docked to \( Lymnaea \) AChBP. B and D, \( \alpha \)-conotoxin OmIA and ImI, respectively, docked to a model of the human \( \alpha 7 \) nAChR. The yellow bonds show the two \( \alpha \)-conotoxin disulfide linkages (in ImI, Cys\(^2\)–Cys\(^8\) and Cys\(^3\)–Cys\(^12\); in OmIA, Cys\(^2\)–Cys\(^8\) and Cys\(^3\)–Cys\(^16\)) and their close apposition to the vicinal cysteines in loop-C of the AChBP structure (Cys\(^{187}\) and Cys\(^{188}\)) and \( \alpha 7 \) model (Cys\(^{189}\) and Cys\(^{190}\)). All side chains and amide linkages on the \( \alpha \)-conotoxins are shown with blue and red depicting nitrogen and oxygen, respectively. Side chains in
Lymnaea showing potential repulsive interactions with Arg^{11} in ImI and the homologous side chains in α7 are numbered.
FIGURE 7. Structural alignment of the human α7 nicotinic acetylcholine receptor and the crystallographic coordinates of the acetylcholine-binding proteins from *L. stagnalis*, *A. californica*, and *B. truncatus*

Conserved residues are shown on a black background with bold denoting points of structural identity from an alignment of the crystallographic coordinates of the following complexes: *L. stagnalis* with nicotine (PDB code 1UW6), *A. californica* with epibatidine (PDB code 2BYQ), and *B. truncatus* with CHAPS (PDB code 2BJ0). The plus (+) and minus (−) denote residues within the 4.0-Å radius of interaction at the subunit interface. Residues that are within 5 Å of all three molecules present in the binding site are italicized.
TABLE 1

$K_d$ of $\alpha$-conotoxins OmIA and ImI for AChBPs

| AChBP  | Radioligand | $\alpha$-OmIA | $\alpha$-ImI |
|-------|-------------|---------------|--------------|
|       | $K_d \pm$ S.E.$^{a}$ | $n_H \pm$ S.E.$^{b}$ | $K_d \pm$ S.E.$^{c}$ | $n_H \pm$ S.E.$^{c}$ |
| **Lymnaea** | $[^3]$H]Epibatidine | 1.7 ± 0.1 | 1.01 ± 0.02 | >10,000 |
|       | $^{125}$I-Labeled $\alpha$-Bgtx | 2.0 ± 0.2 | 0.99 ± 0.05 |
| **Aplysia** | $[^3]$H]Epibatidine | 6.7 ± 0.3 | 1.10 ± 0.06 | 2.1 ± 0.1 | 0.96 ± 0.15 |
|       | $^{125}$I-Labeled $\alpha$-Bgtx | 7.7 ± 0.1 | 1.06 ± 0.02 |
| **Bulinus** | $[^3]$H]Epibatidine | 18.5 ± 1.6 | 0.95 ± 0.09 | 10.2 ± 1.3 | 1.21 ± 0.09 |

$^a$Calculated from observed IC$_{50}$ of six replicate measurements as described under “Experimental Procedures.”

$^b$n$_H$ denotes the Hill slopes as described under “Experimental Procedures.”

$^c$Calculated from observed IC$_{50}$ of three replicate measurements as described under “Experimental Procedures.”
### TABLE 2

IC$_{50}$ of $\alpha$-conotoxin OmIA for nAChRs

| mAChR    | IC$_{50}$ | CI$_{a}$ | n$_{H}$$_{b}$ | CPA |
|-----------|-----------|----------|---------------|-----|
| $\alpha$/δε | >10,000   |          |               |     |
| $\alpha$2/δ | >10,000   |          |               |     |
| $\alpha$3/δ | 11.0      | 7.1–17   | 0.60          | 0.41–0.8 |
| $\alpha$4/δ | >10,000   |          |               |     |
| $\alpha$6/$\alpha$3/δ/β | 201       | 101–401  | 0.74          | 0.41–1.1 |
| $\alpha$3/δ/β | >10,000   |          |               |     |
| $\alpha$7 | 27.1      | 18.4–39.6| 1.15          | 0.64–1.7 |

$^a$ 95% confidence interval.

$^b$ n$_{H}$ denotes the Hill Slope as described under “Experimental Procedures.”
TABLE 3

Receptor subtype selectivity of the α-conotoxins

Residues are numbered using the NH$_2$-terminal glycine in several of the α-conotoxins as residue I. Symbols represent: #, amidated COOH terminus; ^, free carboxyl; *, when combined with additional subunits.

| α-CTx | Residues | nACHR targets | Reference |
|-------|----------|---------------|-----------|
| OmlA  | GCCSHPCNVMNHICG# | α3β2<α7>α6β2β3 | This work |
| BuI A | GCCSTPPCAVY---C# | $k_{off}$ β2*> $k_{off}$ β4* | 20 |
| PeI A | GCCSHPAVNHPELC# | α9α10<α3β2<α7 | 38 |
| GIC   | GCCSHPAAGNNHICG# | α3β2=6β3β3 | 39 |
| GID   | IRDXCCSNACPVRVNOHVC^ | α7=α3β2>α4β2 | 40 |
| PLA   | RDPCSSQPVCTVHNPQIC# | α6*>α3β2 | 19 |
| Iml   | GCCSDFRCAMR------C# | α7<α9 | 41,42 |
| ImlL  | ACCSDFRCCR------C# | α7 | 43 |
| AulB  | GCCSYPPCFATNPD-C# | α3β4 | 21 |
| PaI A [A10L] | GCCSLPPCALNPPDYC# | α7>α3β2 | 28,29 |
| MII   | GCCSNPVCHLEHSNLC# | α6<α3β2 | 13,27 |
| GI    | ECC-NPACGRHYS--C# | α1β1γ8 | 41 |

$^a$X is gamma carboxyglutamate; O is 4-hydroxyproline.