α-Conotoxins ImI and ImII

SIMILAR α7 NICOTINIC RECEPTOR ANTAGONISTS ACT AT DIFFERENT SITES*

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A novel conotoxin, α-conotoxin ImII (α-CTx ImII), identified from Conus imperialis venom ducts, was chemically synthesized. A previously characterized C. imperialis conotoxin, α-conotoxin ImI (α-CTx ImI), is closely related; 9 of 12 amino acids are identical. Both α-CTx ImI and α-CTx ImII functionally inhibit heterologously expressed α7 nAChR with similar IC50 values. Furthermore, the biological activities of intracranially applied α-CTx ImI and α-CTx ImII are similar over the same dosage range, and are consistent with α7 nAChR inhibition. However, unlike α-CTx ImI, α-CTx ImII was not able to block the binding of α-bungarotoxin to α7 nAChRs. α-Conotoxin ImI and α-bungarotoxin-binding sites have been well characterized as overlapping and located at the cleft between adjacent nAChR subunits. Because α-CTx ImI and α-CTx ImII share extensive sequence homology, the inability of α-CTx ImII to compete with α-BgTx is surprising. Furthermore, functional studies in oocytes indicate that there is no overlap between functional binding sites of α-CTx ImI and α-CTx ImII. Like α-CTx ImI, the block by α-CTx ImII is voltage-independent. Thus, α-CTx ImII represents a probe for a novel antagonist binding site, or microsite, on the α7 nAChR.

Marine snails in the genus Conus have venoms that contain a remarkable number of small peptide neurotoxins. Many of these peptides, the conotoxins, are rich in cysteine residues and are highly disulfide-bonded. Known conotoxins may be divided into families based on shared features (reviewed in Refs. 1 and 2). Members of a given conotoxin family have a characteristic number and spacing of cysteines, a conserved disulfide connectivity, and similar receptor targets. However, the toxins in a given family show great variability in their intercysteine sequence, and this accounts for the high degree of receptor subtype specificity within a toxin family. For example, the α-conotoxins are inhibitors of nicotinic acetylcholine receptors (nAChRs), but individual α-conotoxins show a high degree of selectivity for different nAChR subtypes including the neuromuscular subtype and various neuronal subtypes (3). Minor changes in the sequence of the non-Cys residues of conotoxins can profoundly change their receptor subtype specificity. For example, the conotoxin α-CTx PtIA preferentially targets the α7 nAChR and α-CTx PtIB preferentially targets the α3β2 nAChR despite the fact that the toxins only vary in two of 16 amino acids (3).

In this report, we describe the discovery of a novel α-conotoxin, α-conotoxin ImII (α-CTx ImII) from the worm-hunting snail, Conus imperialis. This molecule is very similar to the previously characterized C. imperialis toxin α-conotoxin ImI (α-CTx ImI) (it is identical in 9 of 12 identical). Like α-CTx ImI, α-CTx ImII inhibits the α7 nAChR, and both toxins display very similar potencies against this receptor. Unlike α-CTx ImI, however, α-CTx ImII does not compete with α-bungarotoxin (α-BgTx), a classical competitive inhibitor of the α7 nAChR. Additionally, we show that α-CTx ImI and α-CTx ImII share little, if any, overlap in their functional binding sites on the receptor. The discovery of α-CTx ImII thus illustrates that not only can small changes in intercysteine amino acids alter subtype specificity, but they can also result in toxins that target the same receptor subtype at different sites.

EXPERIMENTAL PROCEDURES

Materials—Puregene reagents were purchased from Gentra (Minneapolis, MN); PCR and molecular biology reagents were from Invitrogen (Carlsbad, CA); salts, acetylcholine, and α-BgTx were from Sigma; 3H-α-BgTx (>200 Ci/mmol) was from Amersham Biosciences; rat brains minus cerebellum were from Zivic Miller (Zelionople, PA); HEK293 cells, Duboce’s modified Eagle’s media, and fetal bovine serum were from ATCC (Manassas, VA); and all other cell culture reagents were from Sigma. The plasmid pPE803-α7 cRNA-H5-HT3 was a gift from Dr. N. S. Miller (4). The plasmid for generation of rat α7 nAChR RNA was a gift from Dr. J. Boulter.

Discovery of α-CTx ImII—The sequence of α-CTx ImII was obtained as part of a systematic analysis of α-conotoxin sequences, using PCR amplification of both cDNA and genomic DNA (5–7). The specimen of C. imperialis analyzed was collected in the Philippines, and hepatopancreas and venom duct tissue was isolated and stored at –70 °C. The cDNA was prepared from venom duct as described previously (8), and genomic DNA was extracted from hepatopancreas using Puregene reagents and the marine invertebrates protocol provided by the manufacturer (Gentra).

Peptide Synthesis and Folding—Linear α-CTx ImI was synthesized and oxidized to form disulfide bridges (folded) as described previously (9). Linear α-CTx ImII was synthesized by standard Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry, using an ABI model 430A peptide synthesizer at the University of Utah core facility. The peptide was folded to give the correct disulfide connectivity (first Cys to third Cys and second Cys to fourth Cys) using orthogonal Cys protection. The first and third Cys residues had stable Cys(S-acetomidomethyl) protection, whereas the second and fourth Cys residues had acid-labile Cys(S-trityl) protection. A previously described folding scheme (3) that se-

Ringer’s solution; 5-HT, 5-hydroxytryptamine (serotonin); 5-HT3 receptor, type 3 serotonin receptor.

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vitrogen) according to the manufacturer’s instructions for HEK293 AC I linearized plasmid. Oocytes were injected two times.

pendence of inhibition of rat α7 nAChRs, toxin was applied using a static bath method. That is, the ACh pulses and ND96 flow were halted, and conotoxin was applied to the bath. The bath was allowed to equilib­rate for 5 min before the ND96 flow was resumed at the same time as the ACh pulse was applied. ACh pulses and ND96 flow continued until stable ACh-evoked currents were re-established. To determine the inhibition at different conotoxin concentrations, the peak current elicited by the first ACh pulse following toxin exposure was normalized to the peak current elicited following controls where ND96 alone, instead of toxin, were applied.

To show that the 5-min exposure to toxin was sufficient for toxin/receptor binding to reach equilibrium, 5- and 10-min exposures of 1, 0.35, and 0.005 µM α-CTx Imll or 1, 0.35, and 0.1 µM α-CTx Imll were carried out, and no difference in percentage inhibition was seen at the two times.

To investigate the voltage dependence of α-CTx Imll inhibition, the block caused by 1 µM α-CTx Imll was measured as described above, but at a range of holding potentials randomly altered between −110, −90, −70, −50, −30, and −10 mV.

Preparation of Cells Expressing Rat α7-5-HT1A Chimera—The plasmid pGKSV2-α7-5-HT1A-HT was a gift from Dr. N. S. Miller (4). It encodes a chimeric receptor that has the N-terminal ACh-binding do­main from α-CTx Imll, the C-terminal domain of the C. imperialis 5-HT receptor. The chimera was expressed in HEK293 cells, which are null for endogenous α-BgTx binding. HEK293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 100 µg/ml streptomycin, and 100 units/ml penicillin. Cells were transfected with pGKSV2-α7-5-HT1A-HT, using LipofectAMINE (In­vitrogen) according to the manufacturer’s instructions for HEK293 cells. After 48 h, the cells were washed with ice-cold potassium Ringer’s (PR) solution (140 mM KCl, 5.4 mM NaCl, 1.7 mM MgCl2, 25 mM HEPES, and 30 µM NaHCO3), and stored at −70 °C until use.

Preparation of Crude Rat Brain Membranes—Crude rat brain membranes were prepared as described previously (3) except that membranes were frozen and stored in PR.

Comparison Binding Assays—Each assay (300 µl total volume) consisted of the following in PR: 200 µl of thawed cells or crude rat brain membranes, a final concentration of 4 nM 3-125I-α-BgTx (Amersham Bioseiences) and various concentrations of α-CTx Imll, α-CTx Imll, or 100 µM MLA (to determine nonsaturatable binding). α-CTx Imll, α-CTx Imll, or MLA were preincubated with cells for 30 min prior to the addition of 3-125I-α-BgTx (applied in a volume of 4 µl). The radioactivity was then added to bind for 15 min during which its association with receptor was linear with time (data not shown). The assays were quenched with 500 µl of ice-cold d-tubocurarine (2400 µM). The cells were harvested (using a Brandell cell harvester) through Whatman GF-B filters pretreated with 4% nonfat dry milk. The filters were washed three times with about 800 µl of PR and were counted using a γ-counter (Packard). Nonsaturatable binding determined in assays containing 100 µM MLA was subtracted from all readings and the resulting specific 3-125I-α-BgTx binding was normalized as a percentage of specific binding in the absence of toxin. Assays were done at 25 ± 2 °C.

Data Analysis—Data were analyzed and plotted using PRISM soft­ware (Graphpad). Competition binding dose response curves were fit to the equation: % binding = 100/(1 + ([ toxin]/EC50)α), and electrophysi­ological dose response curves were fit to: % response = 100(1 + ([ toxin]/IC50)β).

PCRD-based Discovery of α-CTx Imll—Members of a conotoxin family, both from a given Conus species as well as from different species, share conserved sequence elements in their gene structure (12, 13). Thus, PCR strategies can amplify fragments of conotoxin genes that include sequence encoding the mature toxin. PCR was used to amplify a-conotoxin gene fragments from C. imperialis genomic DNA and cDNA. The heter­ogeneous pools of PCR product were cloned and independent clones were sequenced; sequences encoding two closely related peptides, α-CTx Imll and α-CTx Imll, were found (Fig. 1). The α-CTx Imll peptide had previously been purified from C. imperialis venom (9) and is a potent and specific competitive inhibitor of rat α7 nAChRs (14, 15). Based on the predicted sequence from the clone, α-CTx Imll was chemically synthesized and folded to form disulfide bonds (see “Experimental Procedures”), and the synthetic peptide was then used to evaluate potential interactions with α7 nAChRs.

The Biological Activity of α-CTx Imll Is Similar to That Seen for the α7 nAChR-targeting Toxins α-CTx Imll and α-BgTx—α-CTx Imll and α-BgTx have been shown to cause complex seizures when introduced intracranially into rats (14). This behavior is believed to be because of inhibition of α7 nAChRs. To see if α-CTx Imll caused similar effects, intracranial injec­tions of α-CTx Imll and α-CTx Imll were made in young mice. As can be seen in Table I, the effects of both toxins were generally similar and are consistent with both toxins acting on the neuronal α7 subtype of the nAChR.

α-CTx Imll, Like α-CTx Imll, Inhibits ACh-gated Currents in Rat α7 nAChRs—The ability of α-CTx Imll to inhibit ACh-gated currents through rat α7 nAChRs heterologously expressed in X. laevis oocytes was determined as described under “Experimental Procedures.” α-CTx Imll inhibits these currents; the concentration dependence of inhibition is shown in Fig. 2. As previously reported (14), α-CTx Imll was also found to be an inhibitor of oocyte-expressed rat α7 nAChRs. Using the protocol described under “Experimental Procedures,” α-CTx Imll and α-CTx Imll were found to have similar IC50 values (191 nm for α-CTx Imll and 441 nm for α-CTx Imll).

Block by α-CTx Imll, Like That by α-CTx Imll, Is Voltage-independent—The functional inhibition of oocyte-expressed rat α7 nAChRs by 1 µM α-CTx Imll was measured at different holding potentials. As can be seen from Fig. 2C, the percent block was independent of holding potential indicating that the activity of α-CTx Imll is not voltage-dependent. Block by α-CTx Imll is also voltage-independent (15).

α-CTx Imll, Unlike α-CTx Imll, Does Not Inhibit α-BgTx Binding to Rat α7 nAChRs—α-BgTx is a classical competitive inhibitor of some nAChR subtypes, including the α7 subtype. Therefore, the abilities of α-CTx Imll and α-CTx Imll to inhibit 3-125I-a-bungarotoxin binding to different rat α7 nAChR preparations were assessed.

The ability of α-CTx Imll and α-CTx Imll to compete with 3-125I-α-BgTx for binding to crude rat brain membranes was determined as described under “Experimental Procedures.” As shown in Fig. 3A, α-CTx Imll is unable to significantly inhibit

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A

DNA sequences and predicted amino acid sequences of toxin precursors

α-ImII

\[\text{AGA GCA TGG TGT TCC GAT CTT CGC TGT AGA TGG TGT GGT TGA...}\]

R A C C S D R R W R C G

α-ImI

\[\text{AGA GGA TGG TGT TCC GAC CTT CGC TGT GCC TGG AGA TGT GGT TGA...}\]

R G C C S D P R C A W R C G

B

Mature toxin sequences

α-ImII

\[\text{ACCSDRRCRWC#}\]

α-ImI

\[\text{GCCSDPRCWRWC#}\]

Fig. 1. A, fragments of the α-CTx ImII gene were PCR amplified from genomic DNA and cDNA prepared from C. imperialis tissue collected in the Philippines. The nucleotide sequence in the vicinity of the region encoding mature toxin is shown with the predicted translation product. The putative mature peptide sequence is in bold letters. The N-terminal of the mature toxin is deduced by the presence of an Arg (R) in the larger precursor molecule that can act as a cleavage site for the release of the mature toxin. The C terminus is deduced from the presence of a stop codon; for the mature peptide we assume that the C-terminal Gly (G) is post-translationally removed, leaving Cys-12 amidated (amidation is represented by #). Fragments of the α-CTx ImI gene were also found in the pool of PCR products. A fragment of the α-CTx ImI gene in the vicinity of the region encoding mature toxin is shown, as is the known amino acid sequence of mature α-CTx ImI (9). B, the mature toxin sequences of α-CTx ImI and α-CTx ImII. The residues of α-CTx ImII that differ from α-CTx ImI are underlined.

Table I

<table>
<thead>
<tr>
<th>Amount of conotoxin</th>
<th>Number of affected animals/total</th>
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<tbody>
<tr>
<td>nmol</td>
<td>α-CTx ImII</td>
</tr>
<tr>
<td>1.0</td>
<td>1/4</td>
</tr>
<tr>
<td>5.0</td>
<td>2/5</td>
</tr>
<tr>
<td>10</td>
<td>4/6</td>
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<tr>
<td>25</td>
<td>3/3</td>
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3-125I-α-BgTx binding, whereas α-CTx ImI inhibits all specific 3-125I-α-BgTx binding. This contrasts with the functional inhibition of receptors expressed in oocytes, where both conotoxins exhibited roughly equal IC50 values (compare Fig. 3A with Fig. 2A).

The 5-HT3 receptor is highly homologous to the α7 nAChR, and the N-terminal Ach-binding domain of the α7 nAChR has been used to replace the N-terminal 5-HT-binding domain from the 5-HT3 receptor (4, 16, 17). The resulting chimera can be expressed in HEK293 cells such that α-BgTx-binding sites are produced at a level ~1000-fold higher than when the native α7 receptor is used (17). In addition, the chimera retains the pharmacology of the wild-type receptor with respect to many cholinergic agonists and antagonists (16, 17). The ability of α-CTx ImII and α-CTx ImI to inhibit 3-125I-α-BgTx binding to rat α7-5-HT3 chimera was tested as described under “Experimental Procedures.” As shown in Fig. 3B, the same pattern of inhibition was seen with the chimera as with the native α7 receptor. Again, α-CTx ImII was unable to significantly block 3-125I-α-BgTx binding, but α-CTx ImI inhibited all specific binding of the radiolabel.

Competition for Functional α-BgTx-binding Sites—It was previously shown using rat hippocampal neurons (15) that preincubation of α7 nAChRs with α-CTx ImI prevents the very slowly reversible functional block by α-BgTx. We have used a similar approach to investigate the functional binding sites of α-CTx ImII and α-CTx ImI on oocyte-expressed rat α7 nAChRs. It was found that a 5-min bath application of 100 μM α-BgTx is sufficient to block about 95% of ACh-gated current in oocytes expressing rat α7 nAChRs. Because of the very slow off-rate of α-BgTx, no significant recovery was observed after washing toxin from the oocyte bath (Fig. 4A).

However, when oocytes were pretreated for 5 min with 100 μM α-CTx ImI and then subjected to a 5-min co-application of α-BgTx and α-CTx ImI, very rapid and essentially full recovery was observed after washing out the toxins. This result is consistent with α-CTx ImI binding preventing the slowly reversible block by α-BgTx, i.e., that the two toxins compete for the same functional site. However, a much more limited ability to protect against block by α-BgTx (Fig. 4C) is achieved by a similar preincubation with α-CTx ImII. Note that 5 min of bath application of α-CTx ImI and α-CTx ImII is sufficient for both to reach equilibrium with receptor (see “Experimental Procedures”).

Preincubation with a High Concentration of α-CTx ImII Does Not Inhibit Binding of α-CTx ImII to Oocyte-expressed Rat α7 nAChRs—The ability of α-CTx ImII to bind to oocyte-expressed receptor was tested with and without pre-equilibration of oocytes with a high concentration of α-CTx ImI. As can be seen in Fig. 5A, a 5-min bath application of α-CTx ImI (100 μM) or α-CTx ImII (10 μM) is sufficient to completely inhibit ACh-gated ion currents in oocyte-expressed rat α7 nAChRs. Subsequent washout results in full recovery for both toxins; however, α-CTx ImII has a noticeably slower off-rate than α-CTx ImI. Although the differences are subtle, they are highly reproducible and a diagnostic functional difference between the toxins.

When 100 μM α-CTx ImI was bath-applied to oocytes expressing rat α7 nAChRs for 10 min, the characteristic fast off-rate of α-CTx ImI was observed (Fig. 5C). However, when 100 μM α-CTx ImI was bath-applied for 5 min and 10 μM α-CTx ImII was then added, giving 5 min of co-application of α-CTx ImI and α-CTx ImII, the characteristic slow off-rate for α-CTx ImII was observed, and the result was not detectably different from that of the control experiment in Fig. 5B (no toxin was applied for 5 min, 10 μM α-CTx ImII was then added for 5 min).

This suggests that 100 μM α-CTx ImI does not inhibit α-CTx ImII binding to rat α7 nAChRs despite this concentration being
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Fig. 2. A, concentration dependence of conotoxin inhibition of ACh-gated currents through \textit{Xenopus} oocyte-expressed rat \( \alpha_7 \) nAChRs. Curves were determined as described under “Experimental Procedures.” The size of ACh-gated currents at a given conotoxin concentration are plotted as the percent of no-toxin controls. \( \alpha \)-CTx ImI (squares) and \( \alpha \)-CTx ImII (triangles) have very similar curves (\( \alpha \)-CTx ImI, \( IC_{50} = 191 \text{nM} \), \( n_H = 0.879 \); \( \alpha \)-CTx ImII, \( IC_{50} = 441 \text{nM} \), \( n_H = 1.195 \)). B, representative traces showing block of the ACh-gated currents by \( \alpha \)-CTx ImII. Pulses of ACh (1 s at 1-min intervals) gate currents in oocytes expressing rat \( \alpha_7 \) nAChRs. \( \alpha \)-CTx ImII applied in a static bath (see “Experimental Procedures”) results in a concentration-dependent reduction of the peak height of ACh-gated currents obtained simultaneously with the resumption of buffer flow. C, filled circles, the amplitudes of ACh-gated currents through oocyte-expressed rat \( \alpha_7 \) nAChR at different holding potentials are normalized such that the average amplitude of responses gated at \(-70 \text{ mV}\) is \(-1 \). Open circles, the amplitudes of ACh-gated currents at different holding potentials following 5-min applications of 1 \( \mu \text{M} \) \( \alpha \)-CTx ImII are normalized such that the \(-70 \text{ mV}\) response after toxin application is equal to the \(-70 \text{ mV}\) response in the absence of toxin (i.e., \(-1 \)). Data points and error bars, mean \( \pm \) S.E. for 3 to 6 measurements.

Analogs of \( \alpha \)-Conotoxins ImI and ImII—The peptides \( \alpha \)-CTx ImI and \( \alpha \)-CTx ImII are identical in 9 of 12 amino acids. Because they appear to target different sites on the \( \alpha_7 \) nAChR, we performed a structure/function study to identify which amino acids were critical for the difference in targeting. Of the three differences, those at positions 6 (Pro \textit{versus} Arg) and 9 (Ala \textit{versus} Arg) seem the most striking. At position 1 (Gly \textit{versus} Ala), the two residues differ only by a methyl group. Additionally, the absence of a first loop Pro is very unusual in \( \alpha \)-conotoxins (see Table II). The two analogs shown in Table II were thus synthesized (see “Experimental Procedures”) and characterized.

Both analogs are significantly less functionally potent than the corresponding native peptides as determined by electrophysiological characterization of \( \alpha_7 \) nAChR inhibition (data not shown). Nevertheless, what is clearly indicated by the data is that the presence of a proline residue at position 6 is the major determinant of whether a peptide will compete with radiolabeled \( \alpha \)-bungarotoxin for binding to the \( \alpha_7 \) receptor (Fig. 6). Thus, R6P \( \alpha \)-CTx ImII is better at displacing \( \alpha \)-bungarotoxin...
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**Fig. 3.** Inhibition of 3-125I-α-BgTx binding to crude rat brain membranes (A) and α7-5-HT3 chimera (B). α-CTx ImII (squares) and α-CTx ImI (circles) were added to compete with radiolabeled α-bungarotoxin as described under “Experimental Procedures.” The specific binding of 3-125I-α-BgTx at each conotoxin concentration is normalized to that obtained in the absence of conotoxins. For α-CTx ImI, the EC50 is 1,560 nM (nH = 0.59) on crude membranes and 407 nM (nH = 0.71) on α7-5-HT3 chimera. The α-CTx ImII data were fit to a curve and the α-CTx ImI data were fit to a straight line as described under “Experimental Procedures.” Data points and error bars, mean ± S.E. for 3 to 6 measurements.

than native α-CTx ImI. In contrast, replacement of the Pro-6 residue in α-CTx ImII with Arg results in failure to displace α-bungarotoxin even at a concentration of 100 μM (compared with an EC50 for native α-CTx ImI of 407 nM). Thus, the presence or absence of proline at position 6 determines whether or not these peptides preferentially bind to a site that overlaps with the α-bungarotoxin-binding site or another site.

**DISCUSSION**

We report the discovery and characterization of α-CTx ImII that has high sequence identity (9 of 12 amino acids) to α-CTx ImI (Fig. 1); both peptides are from the venom ducts of *C. imperialis* (9). α-CTx ImI is a specific competitive inhibitor of the α7 nAChR subtype (14, 15). Given the close sequence similarity of α-CTx ImI and α-CTx ImII, it was not surprising that α-CTx ImII was also found to inhibit the α7 nAChR. However, most unexpectedly, the two closely related peptides appear to cause their similar functional effects by binding to different sites on the α7 nAChR.

α-CTx ImII was found to be similar to α-CTx ImI in the behavioral effects observed when injected intracranially into mice; both peptides elicited complex seizures, weakness, tremors and, at higher doses, death. Similar behavior was also observed following intracerebral-ventricular injection of α-BgTx, another α7 nAChR inhibitor, into rats (14). In view of its homology to α-CTx ImI and the characteristic symptoms observed when it was injected into the central nervous system, α-CTx ImII was tested for its ability to inhibit ACh-gated currents in *Xenopus* oocytes expressing rat α7 nAChRs.

**Fig. 4.** Functional competition between α-BgTx and α-conotoxins. *Xenopus* oocytes expressing rat α7 nAChRs were voltage-clamped as described under “Experimental Procedures” and their responses to 1-s ACh pulses at 1-min intervals were recorded. The peak heights are all normalized to the average of 5 peaks recorded prior to toxin application. A, bath perfusion was paused for 11 min. After 1 min, 1 μl of ND96 was added; after 6 min, α-BgTx was added (black bar) in 1 μl of ND96 to a final concentration of 100 nM; after 11 min, ND96 flow and ACh pulses were resumed. B, bath perfusion was paused for 11 min. After 1 min, α-CTx ImI in 1 μl of ND96 was added (striped bar) to a final concentration of 100 μM; after 6 min, α-BgTx was added (black bar) in 1 μl of ND96 to a final concentration of 100 nM; after 11 min, ND96 flow and ACh pulses were resumed. C, the same protocol as in B was used except that α-CTx ImII was added (white bar) instead of α-CTx ImI. Data points and error bars, mean ± S.D. for 4 repetitions.
Fig. 5. Functional competition between α-CTx ImI and α-CTx ImII. Xenopus oocytes expressing rat α7 nAChRs were voltage-clamped as described under “Experimental Procedures” and their response to brief ACh pulses at 1-min intervals were recorded. The peak heights are all normalized to the average of 5 peaks recorded prior to toxin application. A1, bath perfusion was paused for 6 min. After 1 min, α-CTx Iml in 1 μl of ND96 was added to a final concentration of 100 μM (striped bar); after 6 min, ND96 flow and ACh pulses were resumed. A2, the same protocol as in A1 was applied except that α-CTx ImII was added to a final concentration of 10 μM (white bar). B, bath perfusion was paused for 11 min. After 1 min, 1 μl of ND96 was added; after 6 min, α-CTx Iml in 1 μl of ND96 to a final concentration of 10 μM (white bar); after 11 min, ND96 flow and ACh pulses were resumed. C, bath perfusion was paused for 11 min. After 1 min, α-CTx Iml was added in 1 μl of ND96 to a final concentration of 100 μM (striped bar); after 6 min, 1 μl of ND96 was added; after 11 min, ND96 flow and ACh pulses were resumed. D, bath perfusion was paused for 11 min. After 1 min, α-CTx Iml was added in 1 μl of ND96 to a final concentration of 100 μM; after 6 min, α-CTx ImII was added in 1 μl of ND96 to a final concentration of 10 μM; after 11 min, ND96 flow and ACh pulses were resumed. Data points and error bars, mean ± S.D. for four repetitions for B, C, and D. A1 and A2 are representative traces.

α-CTx ImII was found to inhibit the receptor with an IC₅₀ similar to that of α-CTx ImI (Fig. 2) when the toxins were tested using identical protocols.

The first surprising result was obtained when α-CTx ImII was tested in a competition assay with 3₁₂₅I-α-BgTx. As had been previously demonstrated by others (18), we found that α-CTx ImI competed with α-BgTx for binding to the receptor. In contrast, α-CTx ImII did not appreciably displace α-BgTx binding in the concentration range tested. These results for α-CTx ImI and α-CTx ImII were obtained with both rat brain α7
nAChRs (Fig. 3A) as well as rat α7-5-HT₃ chimeras (Fig. 3B). Furthermore, experiments using rat α7 nAChRs expressed in oocytes (Fig. 4) demonstrated that preincubation with α-CTx ImII prevents α-BgTx from binding to its functionally relevant site, a result consistent with competitive antagonism, and previously shown by others (15). On the other hand, α-CTx ImII had only a very weak effect on α-BgTx inhibition of oocyte-expressed receptor, consistent with a different site.

The binding site for competitive antagonists of nAChRs is located at the interfaces between subunits that make up the receptor (reviewed in Refs. 19 and 20). The site includes contacts in three conserved loops from one subunit (loops A, B, and C) that make up the + face, and four loops from an adjacent subunit (loops I to IV) that make up the − face. The binding of α-CTx ImI to the α7 nAChR is affected by mutations in or near loops A, B, and C, and II and III (17, 18). The α-BgTx site on the α7 nAChR has also been mapped to the A, B, and C loops (21), and a loop II mutation causes a minor reduction in α-BgTx affinity (22). The data in Fig. 5 suggest that α-CTx ImI and α-CTx ImII do not bind to the same site at a subunit interface. Assuming a potential five identical subunit interfaces in the α7 nAChR pentamer, and that occupation of even one site by α-CTx ImI results in inhibition of the receptor, then the concentration of α-CTx ImI that occupies half the potential sites, Kₐ, is related to the functional IC₅₀ by IC₅₀Kₐ = 0.15 (3), i.e. Kₐ = IC₅₀ × 6.67. The IC₅₀ of α-CTx ImI on the α7 nAChR is 191 nM (Fig. 2). Therefore, 100 μM α-CTx ImI (see Fig. 5) would clearly occupy most subunit interface-binding sites on the α7 nAChR (assuming these are identical) and should significantly reduce binding of α-CTx ImII if α-CTx ImI and α-CTx ImII share a binding site. The α-CTx ImII-binding site awaits definitive characterization; however, several possibilities are outlined below.

Because the primary structures of α-CTx ImI and α-CTx ImII are so similar, and because they share the characteristic α-conotoxin disulfide framework, it seems possible that α-CTx ImII also binds to the interface between α7 subunits. In this case, the inability of α-CTx ImII to compete with α-BgTx or α-CTx ImI might be explained by the following models.

One potential explanation for the results is that α-CTx ImI and α-CTx ImII can simultaneously bind at a single subunit interface by positioning differently within the cleft at different microsites. In fact, α-BgTx appears to make more contacts with the + face than with the − face of the α7 nAChR subunit interfaces (21, 22). It is possible, for example, that α-CTx ImII binds predominantly to the + face and is thus unable to displace α-BgTx, whereas α-CTx ImI, because of many contacts in the + face, disrupts many α-BgTx-receptor interactions, and is thus able to compete with this toxin.

An alternative explanation is based on the work of Green and co-workers (23), who have shown that despite amino acid sequence identity, the subunits of a functional α7 nAChR receptor are not identical. Evidence was presented that the functional α7 nAChR complex requires a mixture of α7 subunits that are in at least two states that differ in their N-terminal domain conformation and the oxidation state of Cys residues (23). A direct consequence of this nonidentity is that putative ligand-binding sites located between subunits become distinguishable. One possibility is that one type of interface between α7 subunits is the α-CTx ImI and α-BgTx-binding site, whereas another type of subunit interface does not bind α-BgTx, but is the α-CTx ImII target site. Bertrand and co-workers (24) have shown that for the competitive α7 nAChR antagonist MLA there are five identical binding sites. This is not necessarily incompatible with a heterogeneous interface model. MLA may recognize structural elements at interfaces that are unaffected by the state of flanking subunits. However, other ligands might...
be sensitive to the state of flanking subunits and thus have distinguishable interface-binding sites. In fact, there is evidence to support the notion of nonhomogeneous a-BgTx-binding sites on a7 nAChRs (25). Additionally, in mouse brain, some [3H]MLA-binding sites are resistant to competition by a-BgTx (26); because the resistant fraction does not appear to be because of a distinct MLA receptor, a simple explanation could be that MLA binds to all five subunit interfaces but a-BgTx, even at high concentrations, cannot.

Although a-CTx ImI and a-CTx ImII show extensive sequence homology, it is possible that a-CTx ImII binds to a nonsubunit-interface site on the receptor. For example, it might bind extracellular regions of the receptor that are not in the N-terminal ACh-binding domain, i.e., the extracellular loop that occurs between two transmembrane helices of the a7 nAChR or the C-terminal extracellular region. a-CTx ImII could also potentially bind to nonsubunit interface regions on the N-terminal ACh-binding domain or the channel pore; however, because a-CTx ImII block is not voltage dependent, this supports the model that it is not an open channel blocker.

The experiments with analogues suggest that although a-CTx ImI and a-CTx ImII have very similar sequences, the amino acid residue at position 6 (Pro in a-CTx ImI, Arg in a-CTx ImII) is critical in determining where they bind on the a7 nAChR. Relative to wild-type a-CTx ImI, P6R a-CTx ImI is a very poor competitor of a-BgTx binding to a7-5-HT3 chimera. In contrast, R6P a-CTx ImII has an enhanced ability to compete with a-BgTx compared with wild-type a-CTx ImII. Because the two native toxins apparently target different sites, a key determinant for selectivity is which amino acid is present at position 6.

Additional information about interactions of a-CTx ImI and a-CTx ImII with their distinct binding sites can be derived from the analog toxin data if one assumes the initial Gly and Ala residues in the two toxins are functionally equivalent. In this case, the P6R a-CTx ImI analog is equivalent to R9A a-CTx ImII and the R6P-aCTx-ImII analog is equivalent to A9R a-CTx-ImII. Because P6R a-CTx ImI does not compete with a-BgTx for binding to the a7 nAChR, this strongly suggests that R9A a-CTx ImII would be like a-CTx ImII and also not compete with a-BgTx. Because R6P a-CTx ImII has some ability to compete a-BgTx but is not as potent as a-CTx ImII, this strongly suggests that A9R a-CTx ImII would compete with a-BgTx for binding to the a7 nAChR but would be a less potent competitor than a-CTx ImII. Taken together, these observations imply that the residues at position 9 in a-CTx ImI and a-CTx ImII are not critical in determining whether the a-CTx ImI or a-CTx ImII site is targeted, but are important for optimal affinity of a-CTx ImI and a-CTx ImII for their respective sites.

The discovery of a-CTx ImII reveals that Conus imperialis has two toxins that inhibit the rat a7 nAChR, and that these act at different sites. Although caution must be applied when extrapolating this observation to the native prey, it suggests that Conus imperialis may target marine worms with both a-CTx ImI and a-CTx ImII, which may bind to different sites on an "a7-like" receptor in native prey. This would represent a second example of some snail venom containing two distinct antagonists of the same nAChR. It was previously demonstrated that Conus purpurascens produces two structurally unrelated nAChR antagonists, a competitive a-conotoxin and a noncompetitive 3H-conotoxin (reviewed in Ref. 1). The present case is different, however, in that the toxins are both a-conotoxins that are very closely related to each other in sequence. A caveat that must be applied to this model is that natural, venom-derived a-CTx ImII may possess post-translational modifications that were not incorporated in the synthetic peptide used in this study. The native toxin may thus differ from the synthetic molecule in its functional properties, i.e., it may not target an a7-like receptor at all. On the other hand, post-translational modification in a-conotoxins isolated from venom have so far been limited to C-terminal amidation and tyrosine sulfation (a-CTx ImI and a-CTx ImII lack tyrosine residues).

Previously, it has been shown that very minor changes in the intercysteine amino acid sequences of conotoxins can drastically affect their specificity. The toxins a-CTx PnIA and a-CTx PnIB from Conus penancens are different in only 2 of 16 amino acids, but preferentially block a3p2 and a7 nAChRs, respectively (3). The discovery of a-CTx ImII illustrates that in Conus imperialis, minor differences between two toxins result in molecules that target, not distinct receptor subtypes, but distinct sites on a single nAChR subtype.

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REFERENCES