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 ImII and α-CTx ImI functionally inhibit heterologously expressed rat α7 nAChRs 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 as part of a systematic analysis of conotoxins. Type specificity within a toxin family. For example, the sequence, and this accounts for the high degree of receptor 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; 3,125I-α-BgTx (>200 Ci/mmol) was from Amersham Biosciences; rat brains minus cerebellum were from Zivic Miller (Zelienople, PA); HER293 cells, Dubecco’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 pZe8SV2-α7(Cys2→Gly)-HT was a gift from Dr. N. S. Millar (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-acetimidomethyl) protection, whereas the second and fourth Cys residues had acid-labile Cys(S-trityl) protection. A previously described folding scheme (3) that se-

This paper is available on line at http://www.jbc.org
quently closed the second Cys to fourth Cys bridge and then the first Cys to third Cys bridge was used to generate toxin. The analogs [P4R]-

Bioassay—Biological activity of synthetic α-conotoxins was tested by intracranial injection into young mice as described previously (10). Electrophysiology—Complementary RNA encoding rat α7 nAChR was transcribed from the T7 promoter of pBluescript II SK(-) into Xenopus laevis oocytes as described previously (11). The RNA was generated by in vitro transcription using a plasmid that was a gift from Dr. J. Boulter. The plasmid carries a rat α7 nAChR cDNA clone (accession number M85273) inserted into the EcoR I site of pBS SK(-). RNA was transcribed from the T7 promoter of Smal linearized plasmid. Oocytes were injected 1–2 days after harvesting and used for voltage clamping 1–7 days after injection.

RESULTS

PCR-based Discovery of α-CTx ImII—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 α-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 ImI and α-CTx ImII, were found (Fig. 1). The α-CTx ImI 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 ImII 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 ImI Is Similar to That Seen for the α7 nAChR-targeting Toxins α-CTx ImI and α-BgTx—α-CTx ImI 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 ImII caused similar effects, intracranial injections of α-CTx ImI and α-CTx ImII 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.
Similar α7 nAChRs Antagonists Act at Different Sites

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A

DNA sequences and predicted amino acid sequences of toxin precursors

α-ImI

AGA GCA TGC TGT GCC TGG AGG TGT GGT TGA...
R A C S D R R W C G *

α-ImII

AGA GGA TGC TGT GCC GCT TGG AGT GGT TGA...
R G C S D P R C A W R C G *

B

Mature toxin sequences

α-ImI ACCSDRRCRWRC#

α-ImII GCCSDPRC#RWRC#

Fig. 1, A, fragments of the α-CTx ImI 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 pools 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 and α-CTx ImII. The residues of α-CTx ImI that differ from α-CTx ImI are underlined.

Table I

Dose dependence of effects of α-CTx ImI and α-CTx ImII on mice

Intraocular injections were made in young mice as described under “Experimental Procedures.” The behavior of the mice was observed for at least 1 h. The numbers of animals exhibiting clearcut effects divided by the total number injected are shown for each dose. The effects seen were fine to coarse tremors, circling, weak gait and at the higher doses, rolling and death.

| Amount of conotoxin injected (μmol) | Number of affected animals/total |
|-------------------------------------|----------------------------------|
| 1.0                                 | 1/4                              |
| 5.0                                 | 2/5                              |
| 10                                  | 4/6                              |
| 25                                  | 3/3                              |

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 HEEK293 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 ImI and α-CTx ImII 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 ImI was unable to significantly block 3,125I-α-BgTx binding, but α-CTx ImII 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 nM 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 ImII, very rapid and essentially full recovery was observed after washing out the toxins. This result is consistent with α-CTx ImII 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 (100 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 ImII 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 ImII does not inhibit α-CTx ImII binding to rat α7 nAChRs despite this concentration being...
about 520 times greater than the functional IC\textsubscript{50}. These, as well as the previous data, are consistent with the conclusion that H\textsubscript{9251}-CTx ImI and H\textsubscript{9251}-CTx ImII have little if any overlap in their high affinity binding sites on the H\textsubscript{9251}7 receptor. Nevertheless, occupancy of the two different sites by each H\textsubscript{9251}-conotoxin leads to functional block of the receptor.

Analogs of H\textsubscript{9251}-Conotoxins ImI and ImII—
The peptides H\textsubscript{9251}-CTx ImI and H\textsubscript{9251}-CTx ImII are identical in 9 of 12 amino acids. Because they appear to target different sites on the a7 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 versus Arg) and 9 (Ala versus Arg) seem the most striking. At position 1 (Gly versus Ala), the two residues differ only by a methyl group. Additionally, the absence of a first loop Pro is very unusual in H\textsubscript{9251}-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 a7 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 a-bungarotoxin for binding to the a7 receptor (Fig. 6). Thus, R6P a-CTx ImII is better at displacing a-bungarotoxin.
than native. In contrast, replacement of the Pro-6 residue in \( \alpha-CTx \) ImI with Arg results in failure to displace \( \alpha-CTx \) ImI of 100 nM (compared with an EC\(_{50} \) for native \( \alpha-CTx \) 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 \( \alpha-CTx \)-bungarotoxin-binding site or another site.

**DISCUSSION**

We report the discovery and characterization of \( \alpha-CTx \) ImII that has high sequence identity (9 of 12 amino acids) to \( \alpha-CTx \) ImI (Fig. 1); both peptides are from the venom ducts of \( C. imperialis \) (9). \( \alpha-CTx \) ImI is a specific competitive inhibitor of the \( \alpha-7 \) nAChR subtype (14, 15). Given the close sequence similarity of \( \alpha-CTx \) ImI and \( \alpha-CTx \) ImII, it was not surprising that \( \alpha-CTx \) ImII was also found to inhibit the \( \alpha-7 \) nAChR. However, most unexpectedly, the two closely related peptides appear to cause their similar functional effects by binding to different sites on the \( \alpha-7 \) nAChR.

\( \alpha-CTx \) ImI was found to be similar to \( \alpha-CTx \) 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 \( \alpha-BgTx \), another \( \alpha-7 \) nAChR inhibitor, into rats (14). In view of its homology to \( \alpha-CTx \) ImI and the characteristic symptoms observed when it was injected into the central nervous system, \( \alpha-CTx \) ImII was tested for its ability to inhibit ACh-gated currents in \( Xenopus \) oocytes expressing rat \( \alpha-7 \) nAChRs.
$\alpha$-Ctx ImII was found to inhibit the receptor with an IC$_{50}$ similar to that of $\alpha$-Ctx ImI (Fig. 2) when the toxins were tested using identical protocols. The first surprising result was obtained when $\alpha$-Ctx ImII was tested in a competition assay with 3-125I-$\alpha$-BgTx. As had been previously demonstrated by others (18), we found that $\alpha$-Ctx ImI competed with $\alpha$-BgTx for binding to the receptor. In contrast, $\alpha$-Ctx ImII did not appreciably displace $\alpha$-BgTx binding in the concentration range tested. These results for $\alpha$-Ctx ImI and $\alpha$-Ctx ImII were obtained with both rat brain $\alpha$7

![Diagram](https://via.placeholder.com/150)

**Fig. 5.** Functional competition between $\alpha$-Ctx ImI and $\alpha$-Ctx ImII. *Xenopus* oocytes expressing rat $\alpha$7 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, $\alpha$-Ctx ImI in 1 $\mu$l of ND96 was added to a final concentration of 100 $\mu$M (striped bar); after 6 min, ND96 flow and ACh pulses were resumed. A2, the same protocol as in A1 was applied except that $\alpha$-Ctx ImII was added to a final concentration of 10 $\mu$M (white bar). B, bath perfusion was paused for 11 min. After 1 min, 1 $\mu$l of ND96 was added; after 6 min, $\alpha$-Ctx ImII was added in 1 $\mu$l of ND96 to a final concentration of 10 $\mu$M (white bar); after 11 min, ND96 flow and ACh pulses were resumed. C, bath perfusion was paused for 11 min. After 1 min, $\alpha$-Ctx ImI was added in 1 $\mu$l of ND96 to a final concentration of 100 $\mu$M (striped bar); after 6 min, 1 $\mu$l 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, $\alpha$-Ctx ImI was added in 1 $\mu$l of ND96 to a final concentration of 100 $\mu$M; after 6 min, $\alpha$-Ctx ImII was added in 1 $\mu$l of ND96 to a final concentration of 10 $\mu$M; 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.
All the native sequences except α-CTx ImII are from a recent review (1). O, hydroxy proline; #, C-terminal amidation. Y15 is sulfated in EpII.

α-Conotoxins have the disulfide connectivity; however, several possibilities are outlined below.

ImII also binds to the interface between

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 octopylexpressed receptor, consistent with a different binding 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 I 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). This and other evidence are consistent with α-CTx ImI-binding sites in α7 nAChRs overlapping with ACh and α-BgTx-binding sites, and being at subunit interfaces.

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 is $K_D = 0.15$ (3), i.e. $K_D = IC_{50} \times 6.67$. The $IC_{50}$ of α-CTx ImI on the α7 nAChR is 191 nM (Fig. 2). Therefore, 100 mM 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 ImI or α-BgTx to the α7 nAChR subunit.

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-BgTx 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

![Fig. 6. Competition binding of α-conotoxin analogs.](http://www.jbc.org/)

- **A**. Inhibition of $3^{-125}$I-α-BgTx binding to α7-5-HT$_3$ chimera by α-CTx ImI (squares) and P6R α-ImI (circles). The specific binding of $3^{-125}$I-α-BgTx at each conotoxin or mutant toxin concentration is normalized to specific $3^{-125}$I-α-BgTx binding in the absence of peptide. The α-CTx ImI data was fit to a curve and the P6R α-CTx ImI data was fit to a straight line as described under “Experimental Procedures.” For α-CTx ImI, the $EC_{50}$ is 407 nM and the $n_H$ is 0.71. B, inhibition of $3^{-125}$I-α-BgTx binding to rat α7-5-HT$_3$ chimeras by α-CTx ImII (circles) and P6R α-CTx ImII (squares) was determined as described under “Experimental Procedures.” The specific binding of $3^{-125}$I-α-BgTx at each conotoxin or mutant toxin concentration is normalized to specific $3^{-125}$I-β-BgTx binding in the absence of peptide. The P6R α-CTx ImII data was fit to a curve and the α-CTx ImII data was fit to a straight line as described under “Experimental Procedures.” For P6R α-CTx ImII, the $EC_{50}$ is 19.3 μM and the $n_H$ is 0.78.

Data points and error bars, mean ± S.E. for 3 to 6 measurements.

In fact, α-BgTx appears to make more contacts with the face than with the face at 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 at subunit interfaces 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 α-BgtX-binding sites on α1 nAChRs (25). Additionally, in mouse brain, some [3H]MLA-binding sites are resistant to competition by α-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 α-BgtX, even at high concentrations, cannot.

Although α-CTx ImI and α-CTx ImII show extensive sequence homology, it is possible that α-CTx ImI 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 α7 nAChR or the C-terminal extracellular region. α-CTx ImII could also potentially bind to nonsubunit interface regions on the N-terminal ACh-binding domain or the channel pore; however, because α-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 α-CTx ImI and α-CTx ImII have very similar sequences, the amino acid residue at position 6 (Pro in α-CTx ImI, Arg in α-CTx ImII) is critical in determining where they bind on the α7 nAChR. Relative to wild-type α-CTx ImI, P6R α-CTx ImI is a very poor competitor of α-BgtX binding to α7-5HT3 chimera. In contrast, R6P α-CTx ImII has an enhanced ability to compete with α-BgtX compared with wild-type α-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 α-CTx ImI and α-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 α-CTx ImI analog is equivalent to R9A α-CTx ImI and the R6Pα-CTx-ImII analog is equivalent to A8R α-CTx ImI. Because P6R α-CTx ImI does not compete with α-BgtX for binding to the α1 nAChR, this strongly suggests that R9A α-CTx ImI would be like α-CTx ImI and also not compete with α-BgtX. Because α-CTx ImII has some ability to compete α-BgtX but is not as potent as α-CTx ImII, this strongly suggests that A9R α-CTx ImII would compete with α-BgtX for binding to the α7 nAChR but would be a less potent competitor than α-CTx ImI. Taken together, these observations imply that the residues at position 9 in α-CTx ImI and α-CTx ImII are not critical in determining whether the α-CTx ImI or α-CTx ImII site is targeted, but are important for ensuring optimal affinity of α-CTx ImI and α-CTx ImII for their respective sites.

The discovery of α-CTx ImII reveals that C. imperialis has two toxins that inhibit the rat α7 nAChR, and that these act at different sites. Although caution must be applied when extrapolating this observation to the native prey, it suggests that C. imperialis may target marine worms with both α-CTx ImI and α-CTx ImII, which may bind to different sites on an “α7-like” receptor in native prey. This would represent a second example of cone 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 α7-conotoxin and a noncompetitive φ-conotoxin (reviewed in Ref. 1). The present case is different, however, in that the toxins are both α-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 α-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 α7-like receptor at all. On the other hand, post-translational modification in α-conotoxins isolated from venom have so far been limited to C-terminal amidation and tyrosine sulfation (α-CTx ImI and α-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 α-CTx PnIa and α-CTx PnIB from Conus panaceus are different in only 2 of 16 amino acids, but preferentially block α3β2 and α7 nAChRs, respectively (8). The discovery of α-CTx ImII illustrates that in C. 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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