Structural Elements in α-Conotoxin ImI Essential for Binding to Neuronal α7 Receptors*

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The neuronal-specific toxin α-conotoxin ImI (CTx ImI) has the sequence Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH2, in which each cysteine forms a disulfide bridge to produce a constrained two-loop structure. To investigate the structural basis for bioactivity we mutated individual residues in CTx ImI and determined bioactivity. Bioactivity of the toxins was determined by their competition against 125I-labeled α-bungarotoxin binding to homomeric receptors containing α7 sequence in the major extracellular domain and 5HT-3 sequence elsewhere. The results reveal two regions in CTx ImI essential for binding to the α7/5HT-3 receptor. The first is the triad Asp-Pro-Arg in the first loop, where conservative mutations of each residue diminish affinity by 2–3 orders of magnitude. The second region is the lone Trp in the second loop, where an aromatic side chain is required. The overall results suggest that within the triad of the first loop, Pro positions the flanking Asp and Arg for optimal interaction with one portion of the binding site, while within the second loop, Trp stabilizes the complex through its aromatic ring.

α-Conotoxins are small, cysteine-rich peptides isolated from the venom of marine cone snails (1). They competitively inhibit nicotinic acetylcholine receptors (AChRs)1 and include various subtypes which specifically target either muscle or neuronal AChRs. The four cysteines in α-conotoxins form two intramolecular disulfide bridges to produce a conformationally constrained two-loop structure (Fig. 1). Muscle-specific α-conotoxins include MI, GI, and SI, while neuronal-specific toxins include ImI (CTx ImI) and MII, which target α7 and α7β2 receptors, respectively (1–3). Owing to their specificity for muscle and neuronal acetylcholine receptors, α-conotoxins are valuable probes of binding sites of nicotinic AChR subtypes (4–7).

Specificity of a particular α-conotoxin likely reflects structural differences in the various AChR binding sites. Binding sites of nicotinic AChRs are formed at interfaces between pairs of α and non-α subunits (reviewed in Ref. 8). In the muscle AChR, the binding sites are formed by α1−δ, α1−γ, and α1−ε subunit pairs, whereas in the homomeric α7 AChR the binding sites are formed by pairs of identical subunits, α7−α7. Thus across the various AChR subtypes, the different binding site interfaces contribute different residues which are recognized by the various α-conotoxins.

All α-conotoxins contain two disulfide bridges, proline in the first loop, and basic and aromatic residues in the second loop. However, each α-conotoxin targets a particular binding site through differences in its number and type of residues. For example, CTx ImI contains four residues in the first loop and three in the second, whereas muscle-specific conotoxins contain three residues in the first loop and five in the second. Furthermore, unlike muscle-specific conotoxins, CTx ImI contains both positively and negatively charged residues in the first loop. Thus structural differences in α-conotoxins likely reflect structural differences at the various AChR binding site interfaces.

Competitive antagonists are potential probes of binding site structure that can identify residues of close approach which are distant in the linear sequence or contained in different protein subunits. When the antagonist is structurally constrained it can also serve as a molecular caliper for estimating distances between these residues. For example, previous work showed that the conformationally restricted antagonist dimethyl-d-tubocurarine bridges the α and γ subunits in the muscle receptor through interaction between its two quaternary nitrogens and tyrosines in each subunit (9, 10). Given the distance between quaternary nitrogens in dimethyl-d-tubocurarine, the two tyrosines in the α and γ subunits are estimated to be 11 Å apart. Similarly, α-conotoxins are potential probes of the binding sites of muscle and neuronal AChRs. Solution and crystal structures of α-conotoxins reveal a triangular structure with positive charges at two vertices separated by 15 Å (11–13). Thus, by identifying active residues in CTx ImI and the α7 binding site interface, residues of close approach can be identified, and their separation can be estimated.

We recently identified residues of the α7 binding site that confer neuronal specificity of CTx ImI (23). The present paper continues our work characterizing the α7 binding site by constructing a series of CTx ImI mutants and measuring binding affinity of the mutant toxins. The results reveal two key regions in CTx ImI essential for bioactivity.

EXPERIMENTAL PROCEDURES
Materials—125I-Labeled α-bungarotoxin (α-Bgt) was purchased from NEN Life Science Products, d-tubocurarine chloride from ICN Pharmaceuticals, α-conotoxins MI and GI from American Peptide Co., 293 human embryonic kidney cell line (293 HEK) from the American Type Culture Collection, and α-conotoxin SI, unlabeled α-Bgt, and DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)) from Sigma. Human α7 and rat 5HT-3 subunit cDNAs were generously provided by Drs. John Lindstrom and William Green. Sources of the human acetylcholine receptor subunit cDNAs were as described previously (15).

Synthesis and Purification of Conotoxin ImI—Mutant and wild type α-conotoxin ImI were synthesized by standard Fmoc (9-fluorenyl methoxycarbonyl) chemistry on an Applied Biosystems 431A peptide synthesizer. During synthesis, cysteine protecting groups (S-triphenylmethyl) were incorporated at cysteines 3 and 12, and acetalimidomethyl
α-Conotoxin ImI Binding Determinants for α7 Receptors

**FIG. 1.** Comparison of neuronal-specific α-conotoxins ImI and MII with muscle-specific α-conotoxins MI, GI, and SI.

α-Conotoxin ImI

α-Conotoxin MI

α-Conotoxin GI

α-Conotoxin SI

α-Conotoxin MII

α-Conotoxin GI

α-Conotoxin MI

α-Conotoxin SI

α-Conotoxin MII

**TABLE I**

| Conotoxin       | Observed \(M_r\) | Calculated \(M_r\) | %Abs |
|-----------------|------------------|-------------------|------|
| Non-oxidized CTx ImI | 1352.0 | 1351.5 | 3.3 |
| CTx MII         | 1395.0 | 1393.6 | 5.2 |
| Wild type CTx ImI | 1324.0 | 1323.5 | 3.9 |
| Acetylated G1   | 1335.0 | 1335.6 | 3.8 |
| S4A             | 1350.7 | 1352.6 | 3.2 |
| D5R             | 1364.0 | 1365.6 | 3.7 |
| D5K             | 1365.0 | 1364.7 | 4.0 |
| P6G             | 1311.5 | 1311.6 | 3.1 |
| P6A             | 1325.0 | 1326.5 | 2.7 |
| P6V             | 1352.5 | 1353.6 | 2.9 |
| R7Q             | 1324.0 | 1323.5 | 3.9 |
| R7K             | 1324.0 | 1323.6 | 3.9 |
| R7E             | 1324.0 | 1324.5 | 3.9 |
| A9S             | 1367.0 | 1367.6 | 3.1 |
| W10F            | 1312.0 | 1312.6 | 2.7 |
| W10T            | 1287.0 | 1286.5 | 2.3 |
| R11Q            | 1324.0 | 1323.5 | 3.8 |
| D5R + R7D       | 1350.0 | 1351.5 | 4.3 |

\[1 - Y = 1/1 + ([\text{ligand}]/K_{\text{app}})^{n}\] (Eq. 1)

\[1 - Y = \text{fract}_1(1/1 + ([\text{ligand}]/K_1)) + (1 - \text{fract}_1)(1/1 + ([\text{ligand}]/K_2))\] (Eq. 2)

**RESULTS**

α₇/5HT-3 Receptor Assay—Studies of the homomeric α₇ binding site have been limited by low expression of native α₇ in mammalian cells (18). To increase expression, we and others constructed α₇/5HT-3 chimeras containing the extracellular domain of α₇ joined to the M1 junction of the rat 5HT-3 receptor (19, 20, 23). We showed that the extracellular domain of human α₇ maintained the ligand recognition properties of wild type human α₇, but that the presence of 5HT-3 sequence greatly enhanced expression (23). Thus, to determine affinities of the CTx ImI mutants described in the present study, we measured binding to α₇/5HT-3 receptors expressed in 293 HEK cells.

Structures of Wild Type and Mutant CTx ImI—Wild type and mutants of CTx ImI were synthesized as described under "Experimental Procedures." Molecular weights of each toxin were determined by mass spectrometry and compared with calculated molecular weights (Table I). The close agreement between measured and calculated molecular weights supports the amino acid compositions and formation of the two intramolecular disulfide bonds. To further confirm that both disulfide bonds formed, we assayed for free sulfhydryls using the colorimetric reaction of DTNB. Whereas the linear, nonoxidized CTx ImI reacted strongly with DTNB, neither the commercially available CTx MI nor any of the CTx ImI mutants reacted (Table I). Thus, the mass spectrometry data combined with DTNB assay confirm that the wild type and mutant conotoxins are fully oxidized.

Neuronal Specificity of CTx ImI—To establish that CTx ImI shows the correct neuronal specificity for our α₇/5HT-3 chimeras, we measured binding to 5HT-3 cDNAs using calcium phosphate precipitation as described (10). We included 100 µg of each conotoxin was dissolved in 200 µl of 0.1 mM phosphate buffer, 4 µl of DTNB was added, the mixture was incubated at room temperature for 30 min for color development, and absorbance at 405 nm was measured. Reactivity of each synthetic CTx ImI mutant is expressed relative to that obtained for 100 µg of non-oxidized CTx ImI (Table I).

Construction of α₇/5HT-3 Chimera and Expression in 293 HEK Cells—Acetylcholine receptor subunit cDNAs were subcloned into the cytomegalovirus-based expression vector pRBG4 (10). The α₇/5HT-3 chimera (α₇/200/5HT-3) was constructed by bridging a 58-base pair synthetic oligonucleotide from a T7 polymerase site in human α₇ to an EcoRV site in rat 5HT-3. The nucleotide sequence of the chimera was confirmed by dideoxy sequencing. HEK cells were transfected with muscle or α₇/5HT-3 cDNAs using calcium phosphate precipitation as described (10). Two days after transfection, intact cells were harvested by gentle agitation in phosphate-buffered saline containing 5 mM EDTA for ligand binding measurements.

Ligand Binding Measurements—Ligand binding to intact cells was measured by competition against the initial rate of 115I-[labeled] Bgt binding (10). The cells were briefly centrifuged, resuspended in potassium Ringer’s solution containing 140 mM KCl, 5.4 mM NaHCO₃, 1.7 mM MgCl₂, 25 mM HEPES, and 30 mg/ml bovine serum albumin, to adjust to a pH of 7.4 with 10 mM NaOH. Specified concentrations of ligand were added 30 min prior to addition of 3.75 nM 115I-Bgt, which was allowed to bind 15 min to occupy approximately half of the surface receptors. Binding was terminated by addition of 2 ml of potassium Ringer’s solution containing 600 µM d-tubocurarine chloride. All experiments were performed at 24 ± 2 °C. Cells were harvested by filtration through Whatman GF-B filters using a Brandel Cell Harvester and washed three times with 3 ml of potassium Ringer’s solution. Prior to use, filters were soaked in potassium Ringer’s solution containing 4% skim milk. Nonspecific binding was determined in the presence of 10 nM α-Bgt and was typically 3% of the total number of binding sites. The total number of binding sites was determined by incubation with toxin for 120 min. The initial rate of toxin binding was calculated as described (17) to yield the fractional occupancy of competing ligand. Binding measurements were analyzed according to either the monophasic Hill equation (Equation 1) or the sum of two distinct binding sites (Equation 2),
mera, we compared binding of CTx ImI with that of the muscle-specific conotoxins by competition against the initial rate of \(^{125}\)I-labeled conotoxins in binding to human adult muscle receptors. Whereas CTx MI, GI, and SI bind with high affinity to muscle receptors, CTx ImI binds with much lower affinity (Fig. 2, bottom panel and Table II). In addition, conotoxins GI and SI select between the two sites of the muscle receptor, with the two-site fit revealing dissociation constants different by 70- to 100-fold, respectively (Table II). By contrast, the monophasic binding of conotoxins ImI and MI indicate similar affinities for both binding sites of the muscle receptor; the two-site fit reveals dissociation constants different by only 5- and 10-fold for CTx ImI and MI, respectively.

Thus, our \(\alpha_7\)-H5-HT-3 receptor and CTx ImI show the expected neuronal specificity.

**Mutagenic Scan of CTx ImI**—We introduced conservative substitutions for each non-cysteine residue in CTx ImI, and measured binding of each mutant toxin to \(\alpha_7\)-H5-HT-3 receptors. The results reveal two key regions in CTx ImI essential for high affinity binding (Fig. 3 and Table III). The first region is the triad Asp-Pro-Arg in the first loop, which individual mutations decrease affinity by 70–500-fold. The second region is the single tryptophan in the second loop, which when mutated to threonine decreases affinity by 30-fold. On the other hand, mutating the four remaining non-cysteines in CTx ImI does not significantly alter affinity for \(\alpha_7\)-H5-HT-3 receptors. These mutations include acetylation of the amino-terminal glycine, inability to bind at position 5, and correct side chain length are required at position 5.

**Side Chain Specificity of the Active Residues**—To determine the chemical nature of the contributions of each of the four essential residues in CTx ImI, we introduced a systematic series of side chains at each position and measured binding of each mutant toxin to \(\alpha_7\)-H5-HT-3 receptors (Fig. 4). Beginning with aspartic acid at position 5, neutralization by substituting asparagine decreases affinity by 100-fold, as described above (Fig. 3). However, replacement with glutamic acid, which lengthens the side chain but maintains the negative charge, decreases affinity even more. Introducing the positively charged lysine produces the greatest decrease of affinity at position 5. These results demonstrate that a negative charge and correct side chain length are required at position 5, suggesting an interaction with a focal electron acceptor in the receptor binding site.

We next examined the remaining charged residue of the triad, arginine at position 7. Surprisingly, maintaining the positive charge by mutation to lysine decreases affinity 100-fold, similar to the decrease observed by mutation to the neutral glutamine (R7Q in Fig. 3). Introducing the negatively charged glutamic acid produces the greatest decrease of affinity at position 7. Thus the structural requirements at position 7 are analogous to those at position 5. A positive charge with particular size is required, suggesting interaction with an electron-rich subsite in the receptor.

We considered the possibility that Asp-5 and Arg-7 form an intramolecular salt bridge essential for activity of CTx ImI. Thus we switched the positions of the two charged residues with the mutation D5R + R7D, with the goal of maintaining the salt bridge of the native toxin. The double mutant toxin decreases affinity by 2000-fold, the greatest decrease observed, indicating that either the salt bridge is not formed, or a salt

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

**Fig. 2. Neuronal specificity of CTx ImI. Top panel.** 293 HEK cells were transfected with the \(\alpha_7\)-H5-HT-3 subunit cDNA and binding of CTx ImI, GI, MI, and SI to the resulting surface receptors was determined as described under “Experimental Procedures.” The curves through the data are fits to either the Hill equation (Equation 1) with mean and S.E. of the fitted parameters given in Table II. **Bottom panel.** 293 HEK cells were transfected with human muscle \(\alpha_\beta\) subunit cDNAs and binding of CTx ImI, GI, MI, and SI was determined as described under “Experimental Procedures.” The curves through the data are fits to either the Hill equation (Equation 1) or the two-site equation (Equation 2). Mean and S.E. of the fitted parameters are given in Table II.

|                | \(K_{app}\) \(\mu M\) | \(n_H\) \(10^{-3}\) | \(K_A\) \(\mu M\) | \(K_B\) \(\mu M\) | \(n\) |
|----------------|-------------------------|----------------------|-------------------|-------------------|-----|
| Human \(\alpha_7\)-H5-HT-3 |                         |                      |                   |                   |     |
| CTx ImI        | 2.38 ± 0.5              | 0.85 ± 0.02          |                   |                   | 10  |
| CTx GI         | 685 ± 37                | 0.88 ± 0.04          |                   |                   | 2   |
| CTx MI         | 86 ± 4.3                | 0.89 ± 0.03          |                   |                   | 2   |
| CTx SI         | 634 ± 22                | 0.89 ± 0.08          |                   |                   | 2   |
| Human \(\alpha_\beta\)10-3 |                         |                      |                   |                   |     |
| CTx ImI        | 119 ± 4.0               | 1.1 ± 0.06           | 54.4 ± 0.9        | 364 ± 12          | 3   |
| CTx GI         | 0.02 ± 0.003            | 0.58 ± 0.05          | 0.14 ± 0.005      | 0.002 ± 0.0001    | 5   |
| CTx MI         | 0.03 ± 0.001            | 0.85 ± 0.02          | 0.09 ± 0.009      | 0.009 ± 0.001     | 5   |
| CTx SI         | 0.7 ± 0.07              | 0.49 ± 0.05          | 0.08 ± 0.014      | 8.31 ± 1.2        | 5   |

**TABLE II**

Apparent affinities of conotoxins ImI, GI, MI, and SI for \(\alpha_7\)-H5-HT-3 and human \(\alpha_\beta\) receptors

Values are least squares fits to the two-site (Equation 2) or Hill Equation (Equation 1) from the series of experiments shown in Fig. 2. \(K_{app}\) is the apparent dissociation constant, \(n_H\) is the Hill coefficient, and \(n\) is the number of independent experiments. \(K_A\) and \(K_B\) are the dissociation constants for each site obtained from a two-site fit with the fractional contribution of each site set to 0.5.
bridge between Asp-5 and Arg-7 is not the basis for high affinity.

The third member of the triad, proline at position 6, is conserved in all α7-conotoxins described to date. The large decrease in affinity produced by the mutation P6G (Fig. 3) suggests that proline orients the side chains of Arg-7 and Asp-5 for optimal interaction with the receptor binding site. Because glycine contributes only a hydrogen side chain, we constructed P6A and P6V to increase side chain size to approach that of proline. Both P6A and P6V mutations decrease affinity similar to P6G, suggesting that conformational restriction by proline at position 6 is required for high affinity binding to the α7 receptor.

The second essential region is the lone tryptophan in the second loop at position 10, which when mutated to threonine decreases affinity by 30-fold (Fig. 3). To determine whether aromaticity is required at position 10, we exchanged tryptophan for phenylalanine. Affinity of the W10F mutant decreases only 3-fold compared with CTx ImI, indicating that an aromatic side chain is required at position 10.

The overall results reveal active residues in both loops of CTx ImI. The first loop contains a triad of residues that requires specific charge, side chain length, and conformational restriction. The second loop contains a tryptophan which contributes its aromatic ring to stabilize the α7-CTx ImI complex.

**DISCUSSION**

To investigate the basis of specificity of CTx ImI for neuronal α7 receptors, we used α7/5HT-3 chimeras to express α7 binding sites and to measure binding affinity of a series of CTx ImI mutants. The results reveal two regions of CTx ImI that confer specificity for human neuronal α7 receptors. The first region is the conformationally sensitive triad Asp-Pro-Arg within the first loop of CTx ImI. Subtle changes in side chain lengths of the aspartic acid and arginine reduce affinity, and their side chains appear to be held in place by the intervening proline. Thus, the triad must maintain a specific conformation to fit properly into a specific and focal counterpart in the α7 binding site. The second region is the single tryptophan within the second loop of CTx ImI. Studies of side chain specificity at

| CTx ImI mutants | $K_{\text{app}}$ ($\mu$M) | $n_H$ | $n$ |
|-----------------|--------------------------|------|-----|
| Wild type CTx ImI | 2.38 ± 0.5 | 0.85 ± 0.02 | 10 |
| Acetylated G1 | 4.25 ± 0.2 | 0.95 ± 0.04 | 4 |
| S4A | 3.77 ± 0.2 | 0.87 ± 0.01 | 4 |
| D5N | 262 ± 10 | 0.85 ± 0.03 | 4 |
| D5E | 616 ± 39 | 0.88 ± 0.04 | 4 |
| D5K | 1890 ± 67 | 0.92 ± 0.05 | 4 |
| P6G | 140 ± 16 | 0.86 ± 0.06 | 4 |
| P6A | 120 ± 9 | 0.96 ± 0.07 | 4 |
| P6V | 250 ± 13 | 1.00 ± 0.01 | 4 |
| R7Q | 924 ± 54 | 0.95 ± 0.05 | 4 |
| R7K | 290 ± 13 | 0.95 ± 0.04 | 4 |
| R7E | 1650 ± 105 | 0.87 ± 0.06 | 4 |
| A9S | 1.90 ± 0.2 | 0.89 ± 0.05 | 4 |
| W10F | 9.90 ± 1.0 | 0.96 ± 0.07 | 4 |
| W10T | 63.8 ± 5.5 | 1.00 ± 0.09 | 4 |
| R11Q | 3.78 ± 0.4 | 0.97 ± 0.07 | 4 |
| D5R + R7D | 4540 ± 239 | 0.88 ± 0.09 | 4 |

**TABLE III**

Binding parameters of conotoxin ImI mutants for α7/5HT-3 receptors

Values are least squares fits to the Hill equation from the series of experiments shown in Figs. 3 and 4. $K_{\text{app}}$ is the apparent dissociation constant, $n_H$ is the Hill coefficient, and $n$ is the number of independent experiments.

**FIG. 3.** Mutagenic scan of CTx ImI. Dissociation constants of the CTx ImI mutants are expressed as the log ratio relative to wild type CTx ImI. The affinity of wild type CTx ImI for α7/5HT-3 receptors is shown by the vertical bold line, and the error bars indicate ± S.D. The drawings to the right are schematic representations of the mutant toxins, with the mutant residues highlighted. Mean and S.E. of the fitted parameters are given in Table III.

**FIG. 4.** Side chain specificity of the determinants of CTx ImI affinity. For each CTx ImI mutant, affinity for α7/5HT-3 receptors is expressed as in Fig. 3. The affinity of wild type CTx ImI for α7/5HT-3 receptors is shown by the vertical bold line, and the error bars indicate ± S.D. Mean and S.E. of the fitted parameters are given in Table III. In the schematic representations to the right, X indicates the mutant residue.
position 10 indicate the requirement of an aromatic ring.

Mutagenesis and site directed labeling studies establish that each ligand binding site in muscle and neuronal AChRs contains contributions from both α and non-α subunits. Residues of the α portion of the binding site, termed the (+) face, are located in three regions well separated in the primary sequence, suggesting a three-loop model of the (+) face of the binding site (reviewed in Refs. 8 and 22). Similarly, residues of the non-α portion of the binding site, termed the (−) face, are located in four separate regions of the primary sequence, suggesting a four loop model for the (−) face of the binding site (8, 22). Unlike the two binding sites of the muscle AChR, which are formed at interfaces between α1–δ and either α1–γ (fetal) or α1–ε (adult) subunit pairs, binding sites of the homo-oligomeric αγ receptor are formed at interfaces between pairs of identical subunits, αγ–αγ. Consequences of a homo-oligomeric pentamer include the potential for five binding sites and formation of both the (+) and (−) faces by a single αγ subunit.

α-CTx ImI is a competitive antagonist of neuronal αγ receptors. It contains two disulfide bonds which hold the toxin in a constrained two-loop structure. The solution and crystal structures of members of the α-conotoxin family reveal a compact triangular structure with two positive charges separated by 15 Å, similar to the rigid structure and 11 Å separation of quaternary nitrogens of curariform antagonists. Previous work showed that curariform antagonists bridge the interface between the α and γ subunits of the muscle receptor through quaternary-aromatic interactions (9). Similarly, the two loops of CTx ImI likely bridge the (+) and (−) faces of the αγ binding site. Because CTx ImI is small enough for structural determination at atomic resolution, it may be used as a molecular caliper to estimate distances between points of ligand contact at the αγ binding site.

Our studies reveal two distinct regions in CTx ImI essential for binding to neuronal αγ receptors. The first region is within the first loop of CTx ImI, the conformationally-sensitive triad Asp-Pro-Arg. Previous studies with CTx MI demonstrated the importance of proline in the first loop, where the mutation P6G reduced biopotency approximately 100-fold (14). Because our results with CTx ImI reveal a similar decrease in affinity with P6G, and because Pro-6 is conserved in all α-conotoxins, the proline likely contributes to structural rigidity along with the two disulfide bridges.

Aside from Pro-6, no other residue elements have been reported in the first loop of the α-conotoxins. For CTx IMI, we show that aspartic acid at position 5 and arginine at position 7 are essential for high affinity binding. Surprisingly, conservative substitutions of Asp-5 and Arg-7 markedly diminish activity of CTx ImI. The substitutions D5E and R7K, which alter side chain length by one methyl group while maintaining charge, reduce affinity more than 100-fold. In addition, introducing opposite charges at Asp-5 and Arg-7 reduces affinity approximately 1000-fold, suggesting repulsion by residues at the αγ binding site. The overall results of the Asp-5 and Arg-7 side chain experiments suggest that both charge and side chain length are important for the activity of CTx ImI. Together with residue Pro-6, Asp-5, and Arg-7 form a conformationally sensitive triad essential for CTx ImI affinity. Our results do not distinguish whether P6 contributes directly to CTx ImI binding or structurally stabilizes the first loop. However, the presence of a glycine mutation at position 6 likely allows rotational freedom around its α carbon that would affect the orientation of the side chains of Asp-5 and Arg-7.

The second essential region in CTx ImI is the single Trp at position 10 of the second loop. Mutation of the two remaining non-cysteines in the second loop fails to affect CTx ImI affinity. Similar to CTxs IMI, the muscle-specific conotoxins contain a conserved aromatic residue in the penultimate position of the second loop (Fig. 1). Tryptophan of CTx IMI occupies the position equivalent to that of tyrosine in CTx MI, GI, and SI. Replacement of L-tyrosine with D-tyrosine in CTx MI decreases bioactivity, indicating that the conformation of the tyrosine is essential (14). Our results show that converting tryptophan to threonine in CTx ImI reduces affinity 30-fold. However, converting tryptophan to phenylalanine, which maintains the aromatic side chain, decreases affinity only 3-fold. Thus an aromatic side chain at position 10 stabilizes the CTx ImI-αγ receptor complex.

Surprisingly, mutations similar to those that affect affinity of muscle α-conotoxins do not affect affinity of CTx ImI. For example, a cationic side chain in the second loop is critical for activity of the muscle-specific α-conotoxins MI and SI (6, 7). Our results with CTx IMI show no effect of the mutation R11Q. In addition, previous work suggested that the N-terminal amide stabilizes the toxin-receptor complex through a π-cation interaction (12). By contrast, acetylation of the amino-terminal glycine does not affect affinity of CTx IMI for αγ receptors.

The overall results reveal two structural motifs in CTx ImI that confer high affinity binding to αγ receptors. Knowledge of the precise contacts between CTx ImI and αγ awaits experiments that mutate residues in both the toxin and the receptor.

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REFERENCES

1. Olivera, B. M., Rivier, J., Scott, J. K., Hillyard, D. R., and Cruz, L. J. (1991) J. Biol. Chem. 266, 22067–22070
2. McIntosh, J. M., Yoshikami, D., Mahe, K., Nielsen, D. B., Rivier, J. E., Gray, W. R., and Olivera, B. M. (1994) J. Biol. Chem. 269, 16733–16739
3. Cartier, G. E., Yoshikami, D., Gray, W. R., Luo, S., Olivera, B. M., and McIntosh, J. M. (1996) J. Biol. Chem. 271, 7522–7528
4. Sine, S. M., Krienckamp, H.-J., Bren, M., Maeda, R., and Taylor, P. (1995) Neuro 15, 205–211
5. Harvey, S. C., McIntosh, J. M., Carrieri, G. E., Maddox, P. N., and Leutwy, C. W. (1997) Mol. Pharmacol. 51, 336–342
6. Hano, R. M., Pagan, O. B., Gregory, L. L., Jácome, T., and Eterovic, V. A. (1997) Biochemistry 36, 9051–9056
7. Groebe, D. R., Gray, W. R., and Abramson, S. N. (1997) Biochemistry 36, 6469–6474
8. Princey, R. J., and Sine, S. M. (1998) in The Nicotinic Acetylcholine Receptor: Current Views and Future Trends (Barrantes F. J., ed.) pp. 31–59, Landes Bioscience, TX, in press
9. Fu, D., and Sine, S. M. (1994) J. Biol. Chem. 269, 26152–26157
10. Sine, S. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 9436–9449
11. Pardi, A., Galdes, A., Florance, J., and Manicone, D. (1989) Biochemistry 28, 5494–5501
12. Kobayashi, Y., Ohkubo, T., Kyogoku, Y., Nishizumi, Y., Sakakibara, S., Braun, W., and Go, N. (1989) Biochemistry 28, 4853–4860
13. Gudell, L. W., Martin, J. A., Shan, L., Edmundson, A. B., and Gray, W. R. (1996) Biochemistry 35, 11329–11335
14. Hashimoto, K., Uchida, S., Yoshida, H., Nishizumi, Y., Sakakibara, S., and Yokari, K. (1985) Eur. J. Pharmacol. 118, 351–354
15. Ohsu, K., Wang, H.-L., Milene, M., Bren, N., Brengman, J. M., Nakano, S., Quiram, P. A., Pruitt, J. N., Sine, S. M., and Engel, A. G. (1996) Neuron 17, 157–170
16. Andrade, D., Albericio, F., Sóle, N. A., Munson, M. C., Ferrer, M. J., and Barany, G. (1994) in Methods in Molecular Biology: Peptide Synthesis Protocols (Pennington, M. W., and Dunn, B. M., eds) Vol. 35, pp. 139–140, Humana Press, Totowa, NJ
17. Sine, S. M., and Taylor, P. (1979) J. Biol. Chem. 254, 3315–3325
18. Cooper, S. T., and Millar, N. S. (1997) J. Neurochem. 68, 2140–2151
19. Eiselle, J. L., Bertrand, S., Galzi, J. L., Devillers-Thiéry, A., changeux, J. P., and Bertrand, D. (1993) Nature 366, 479–483
20. Rangwala, F., Dristel, R. C., Rakhlin, S., Koe, E., Alturi, P., Harkins, A. B., Fox, A. P., Salmon, S. B., and Green, W. N. (1997) J. Neurosci. 17, 8201–8212
21. Johnson, D. S., Martinez, J., Egg horny, A. B., Heine mann, S. F., and McIntosh, J. M. (1995) Mol. Pharmacol. 48, 194–199
22. Tsigelny, I., Sugiyama, N., Sine, S. M., and Taylor, P. (1997) Biophys. J. 73, 52–66
23. Quiram, P. A., and Sine, S. M. (1998) J. Biol. Chem. 273, 11001–11006