Hydrophobic Pairwise Interactions Stabilize α-Conotoxin MI in the Muscle Acetylcholine Receptor Binding Site*

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The present work delineates pairwise interactions underlying the nanomolar affinity of α-conotoxin MI (CTx MI) for the α-δ site of the muscle acetylcholine receptor (AChR). We mutated all non-cysteine residues in CTx MI, expressed the αβδ2 pentameric form of the AChR in 293 human embryonic kidney cells, and measured binding of the mutant toxins by competition against the initial rate of 125I-α-bungarotoxin binding. The CTx MI mutants P6G, A7V, G9S, and Y12T all decrease affinity for αβδ2 pentamers by 10,000-fold. Side chains at these four positions localize to a restricted region of the known three-dimensional structure of CTx MI. Mutations of the AChR reveal major contributions to CTx MI affinity by Tyr-198 in the α subunit and by the selectivity determinants Ser-36, Tyr-113, and Ile-178 in the δ subunit. By using double mutant cycles analysis, we find that Tyr-12 of CTx MI interacts strongly with all three selectivity determinants in the δ subunit and that δSer-36 and δIle-178 are interdependent in stabilizing Tyr-12. We find additional strong interactions between Gly-9 and Pro-6 in CTx MI and selectivity determinants in the δ subunit, and between Ala-7 and Pro-6 and Tyr-198 in the α subunit. The overall results reveal the orientation of CTx MI when bound to the α-δ interface and show that primarily hydrophobic interactions stabilize the complex.

Recent studies have used protein toxins to probe active sites of ligand- and voltage-gated ion channels (1–6). By identifying multiple pairwise interactions, these studies define dimensions of the active site according to the known structure of the toxin. The studies also establish the underlying basis for molecular recognition in high affinity protein complexes. Here we probe the muscle AChR with the peptide toxin α-conotoxin MI and use double mutant cycles analysis to identify pairs of residues that confer the nanomolar affinity of the complex.

Mutagenesis and site-directed labeling studies establish that the ligand binding sites of the muscle AChR are formed at interfaces between αi and either δi, εi, or γ subunits (7, 8). Residues on the αi face of the binding site are found in three well separated regions of the primary sequence, termed loops A, B, and C. Using the numbering system for the mouse α1 subunit, key residues in these loops include Tyr-93 in loop A, Trp-149 in loop B, and Tyr-190 and Tyr-198 in loop C. Similarly, residues on the non-α face of the binding site are found in four well separated regions of the primary sequence, termed loops I through IV. Using the numbering system for the mouse δ subunit, key residues in these loops include Ser-36 in loop I, Trp-57 in loop II, Tyr-113 in loop III, and Ile-178 in loop IV. The observation that these seven loops converge to form a localized binding site has led to a multi-loop model of the major extracellular domain of the AChR (8).

α-Conotoxins are small, disulfide-rich peptides that competitively inhibit muscle and neuronal nicotinic AChRs (9). All α-conotoxins have a conformationally constrained two-loop structure formed by two disulfide bridges. However, the various α-conotoxins differ by the number and type of residues in each loop, allowing specific targeting of receptor subtypes. α-Conotoxins specific for muscle AChRs include MI, GI, and SI, and contain three residues in the first loop and five in the second (Fig. 1). Muscle-specific α-conotoxins can be further subdivided according to their ability to select between the two AChR binding sites; CTx MI and GI select between the two binding sites by 10,000-fold, whereas CTx SI selects between the sites by 100-fold (10–12). Moreover, CTx MI binds to the α-δ site of the muscle AChR with nanomolar affinity and stays bound for more than 6 h (13). Their site selectivity and exceedingly high affinity make CTx MI and GI powerful probes of the structure of the muscle AChR binding site.

Residues from both α and non-α faces of the AChR binding site stabilize bound CTx MI and include residues from four of the seven loops. The α face contributes Tyr-198 and Tyr-190 from loop C (14), whereas the δ face contributes Ser-36 from loop I, Tyr-113 from loop III, and Ile-178 from loop IV (10). Selectivity of CTx MI for the two AChR binding sites owes to residue differences in δ and γ subunits at these three positions and can be transferred from one binding site to the other by exchanging residues at these key positions. That both α and non-α subunits contribute to CTx MI binding suggests that the toxin bridges the subunit interface, whereas the modular exchangeability across γ and δ subunits suggests the key residues contribute directly to CTx MI binding.

By mutating residues in both the AChR and CTx MI, the present work further tests the hypothesis that the toxin bridges the binding site interface. We use double mutant cycles analysis to distinguish interacting from non-interacting pairs...
of residues in the complex. We find that CTx MI interacts with the α-δ site of the AChR through four hydrophobic residues in its N- and C-terminal loops. Furthermore, the key side chains in CTx MI localize in a hydrophobic cluster that interacts with hydrophobic and aromatic residues from both the α and δ subunits.

**EXPERIMENTAL PROCEDURES**

**Materials—**α-Conotoxin MI was purchased from American Peptide Company; 293 human embryonic kidney cell line (293 HEK) and BOSC 23 HEK cell line were from the American Type Culture Collection; 125I-tubocurarine chloride was from ICN Pharmaceuticals; and 5,5'-dithiobis-2-nitrobenzoic acid was from Sigma.

**Synthesis and Purification of Conotoxin MI—**Wild type and mutant α-conotoxin MI were synthesized by standard Fmoc (N-(9-fluorenyl)methoxy carbonyl) chemistry on an Applied Biosystems 431A peptide synthesizer. Cysteine protecting groups (S-triphenylmethyl) were incorporated during synthesis at cysteines 4 and 14, and aceticamidomethyl protecting groups (ACM) were incorporated at cysteines 3 and 8. The linear peptide was purified by a reversed-phase high performance liquid chromatography using a Vydac C18 preparative column with trifluoroacetic acid/acetoniitrile buffer. Two disulfide bridges were formed as follows: the cysteine S-triphenylmethyl-protecting groups of cysteines 4 and 14 were removed during trifluoroacetic acid cleavage of the linear peptide from the support resin, and the peptide was oxidized by molecular oxygen to form the 4-14 disulfide bond by stirring in 50 mM ammonium bicarbonate buffer, pH 8.5, at 25 °C for 24 h. The peptide was lyophilized and then the second bridge was formed as follows: the ACM-protecting groups on cysteines 3 and 8 were removed oxidatively by iodine as described (15), except the peptide/iodine reaction was allowed to progress for 16 h prior to carbon tetrachloride extraction. Residual iodine was separated from the pure product by high performance liquid chromatography. The purified product was verified by mass spectrometry (Table I). The CTx MI mutants are named as follows: the first letter and number refer to the wild type residue and position, and the following letter is the substituted residue at that position.

**Confirmation of Disulfide Bond Synthesis by Ellman’s Analysis—**To confirm disulfide bond formation, we compared reactions with Ellman’s reagent for linear, non-oxidized CTx MI, commercially available CTx MI, and all of our synthetic CTx MI mutants. For each conotoxin, 100 μg was dissolved in 200 μl of 0.1 M phosphate buffer; 4 μl of 5,5'-dithiobis-2-nitrobenzoic acid was added, and the mixture was incubated at room temperature for 30 min. The absorbance at 405 nm was measured. Reactivity for each synthetic mutant is expressed relative to that obtained for the non-oxidized, linear CTx MI (Table I).

**Expression Receptor Mutations and in 293 HEK Cells—**Mouse AChR subunit cDNAs were subcloned into the cytomegavirus-based expression vector pBBo4 (16). All mutations of the α subunit were constructed as described previously (17). The single point mutations S36K, Y113S, and δ178F, as well as the double mutant δ/S66K/δ1178F and triple mutant δ/S66K/δ/113S/δ178F were constructed as described (10). The double mutant δ/S66K/δ/113S was constructed by ligation of the 400-bp P/MIF-P/MIF fragment containing δ113S to the 5100-bp P/MIF-P/MIF fragment containing the mutation S66K. The double mutant δ/S113S/δ1178F was constructed by ligation of the 400-bp P/MIF-P/MIF fragment containing δ113S with the 5100-bp P/MIF-P/MIF fragment containing δ178F. All constructs were confirmed by dideoxy sequencing.

Human embryonic kidney cells (293 HEK) were transfected with mutant or wild type subunit cDNAs using calcium phosphate precipitation as described previously (16). BOSC 23 HEK cells were used in some experiments with low expressing mutant AChRs. AChR subunit cDNAs were combined in the ratio 2:1:2 for α, β, and δ subunits, respectively. After 24 h at 37 °C, the transfected cells were incubated at 31 °C for an additional 48 h. Three days after transfection, intact cells were harvested by gentle agitation in potassium phosphate-buffered saline containing 5 mM EDTA.

**Ligand Binding Measurements—**α-Conotoxin MI binding to intact cells was measured by competition against the initial rate of 125I-labeled α-bungarotoxin (18). After harvesting, the cells were briefly centrifuged, resuspended in potassium Ringer’s solution, and divided into aliquots for α-conotoxin binding measurements. Potassium Ringer solution contains 140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl2, 1.7 mM MgCl2, 25 mM HEPES, and 30 mg/liter bovine serum albumin adjusted to pH 7.4 with 10 mM NaOH. Specified concentrations of α-conotoxin

**RESULTS**

**Mutagenesis of CTx MI—**Mutations of CTx MI were generated by standard peptide synthesis methods as described under “Experimental Procedures.” Molecular weight and the presence of two disulfide bonds were verified by mass spectrometry and by negative reaction with Ellman’s reagent (Table I). Because CTx MI binds 10,000-fold more tightly to the α-δ than to the α-γ interface of the AChR (10), we measured CTx MI binding to the α-βδ pentameric form of the AChR expressed on the surface of 293 HEK cells. As measured by competition against the initial rate of 125I-labeled bungarotoxin binding, our synthetic CTx MI bound with a Kd of 0.94 nM (Table II) and was indistinguishable

![Fig. 2. Mutagenic scan of CTx MI. Dissociation constants for the mutant α-conotoxins are expressed as the log ratio relative to wild type CTx MI. The relative dissociation constant for wild type CTx MI for αβδ pentamers is shown by the vertical bold line. The drawings are schematic representations of the mutant α-conotoxins, with the mutant residues in bold. Means and S.E. of the fitted parameters are given in Table II.](http://www.jbc.org/)
We mutated all non-cysteines in CTx MI and measured binding of each mutant conotoxin to $\alpha_2\beta_2$ pentamers. Our mutagenic scan of CTx MI reveals four residues essential for high affinity binding as follows: Ala-7 and Pro-6 in the N-terminal loop and Tyr-12 and Gly-9 in the C-terminal loop (Fig. 2; Table II). Within the N-terminal loop, mutation of Ala-7 to valine decreases affinity nearly 10,000-fold, whereas mutation to serine decreases affinity by 50-fold (Table II), indicating that position 7 requires a side chain that is both small and hydrophobic. Mutation of the adjacent Pro-6 to glycine decreases affinity nearly 10,000-fold, whereas mutation to alanine or valine decreases affinity 400- and 800-fold, respectively, suggesting the need for both restricted rotation of the peptide backbone and hydrophobic contributions at position 6.

Within the C-terminal loop of CTx MI, mutation of Tyr-12 to threonine decreases affinity 10,000-fold, whereas mutation to phenylalanine maintains high affinity (Table II), indicating a purely aromatic contribution at position 12. Mutation of Gly-9 to alanine decreases affinity nearly 10,000-fold, whereas mutation to alanine or valine decreases affinity 400- and 800-fold, respectively, suggesting the need for both restricted rotation of the peptide backbone and hydrophobic contributions at position 6.

The four bioactive residues of CTx MI map to a restricted region of its three-dimensional structure (Fig. 3; Ref. 19), indicating that the contact surface at the ligand binding site is likely to be small and complementary to the active region of the toxin. Side chains of the bioactive residues occupy corners of an irregular trapezoid 6.4 to 8.0 Å long and 3.9 to 6.6 Å wide, creating a hydrophobic patch in an otherwise hydrophilic peptide. The ring structures of Pro-6 and Tyr-12 stack parallel to each other, whereas Ala-7 protrudes at right angles to Pro-6, and the methylene $\alpha$-carbon of Gly-9 leaves a pronounced cavity rimmed by hydrophobic side chains. Thus bioactivity of CTx MI owes to a three-fingered hydrophobic structure at one end of the toxin.

Contractions of the $\alpha$ Subunit to CTx MI Binding—Aromatic residues in the AChR $\alpha$ subunit are widely recognized to contribute to ligand affinity (7). We therefore examined the key aromatic residues, Tyr-93, Tyr-190, Tyr-198, Trp-149, and Tyr-151, as potential points of interaction with CTx MI. The mutation $\alpha$Y198T decreases affinity 1000-fold, whereas $\alpha$Y190T decreases affinity 30-fold (Fig. 4; Table III). These results, obtained in symmetric $\alpha_2\beta_2$ receptors, are similar to those described for the $\alpha$-site in asymmetric $\alpha_2\beta_2\gamma$ receptors (14). Mutation of the remaining aromatic residues, $\alpha$Y93T, $\alpha$Trp-149, and $\alpha$Tyr-151, only weakly affects affinity. Thus $\alpha$Tyr-198 and $\alpha$Tyr-190 are candidates for interaction with CTx MI.

Contributions of the $\delta$ Subunit to CTx MI Binding—Previous
work (10) showed that residue differences at three equivalent positions of the γ and δ subunits confer the 10,000-fold selectivity of CTx MI for the α-δ over the α-γ interface of the AChR. We therefore re-examined these selectivity determinants as potential points of interaction with CTx MI. Single point mutations of the selectivity determinants, δS36K, δY113S, and δI178F, produce only modest changes in affinity for CTx MI (Fig. 5; Table III). However, when S36K and I178F are combined into a single δ subunit, affinity for CTx MI decreases considerably more than with either mutation alone (Fig. 5), indicating that these residues are interdependent in stabilizing CTx MI (10, 14). The remaining combinations of double mutations, (S36K/Y113S) and (Y113S/I178F), produce roughly additive changes in affinity, indicating little interdependence of these selectivity determinants. When mutations of all three selectivity determinants are combined into a single δ subunit, CTx MI affinity falls 10,000-fold to that of low affinity α₂γ₂ pentamers (Fig. 5). Thus all three selectivity determinants in the δ subunit are candidates for interaction with CTx MI.

**Pairwise Interactions between CTx MI and the α-δ Site**—Thermodynamic double mutant cycles analysis has been widely used to identify noncovalent interactions between residues within a single protein and between residues joining different proteins (1–6, 20). To generate a mutant cycle for pairs of CTx MI and AChR mutations, dissociation constants are determined for the four possible combinations of wild type (W) and mutant (M) receptors (r) and conotoxins (t): WrWt, MrWt, WrMt, and MrMt. The resulting dissociation constants are then used to calculate a coupling coefficient Ω (20) shown in Equation 1,

\[
\Omega = \frac{K_d(WrWt) \cdot K_d(MrMt)}{K_d(WrMt) \cdot K_d(MrWt)} \quad (Eq. 1)
\]

If Ω equals unity the pair of residues does not interact, whereas if Ω deviates from unity the pair of residues interacts.

To identify pairs of interacting residues, we focus on residues in the AChR and CTx MI that significantly affect affinity of the complex and then apply double mutant cycles analysis to all

![Graphs showing binding curves for different combinations of wild type and mutant receptors with CTx MI](image)

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**Fig. 5. Mutagenesis of selectivity determinants in the δ subunit and effects on CTx MI binding.** Dissociation constants of the mutants for CTx MI are expressed as the log ratio relative to wild type α₂β₂ receptor. The affinity of the α₂β₂ receptor is shown by the vertical bold line, and for the α₂γ₂ receptor by the vertical dashed line. The error bars indicate ± S.E. Mean and S.E. of the fitted parameters are given in Table III.

**Fig. 6. Binding of wild type and mutant AChR and CTx MI pairs.** A, displays binding curves for the indicated pairs of interacting wild type (WT) and mutant CTx MI. The δMMM indicates the triple mutation δS36K/Y113S/I178F). Smooth curves are fits to the Hill equation with the following parameters: WT + CTx MI, K_app = 0.85 ± 0.05 nM and n_H = 0.66 ± 0.04 (open squares); WT + Y12T, K_app = 8.0 ± 0.3 μM and n_H = 1.04 ± 0.04 (filled squares); δMMM + CTx MI, K_app = 23.6 ± 1.3 μM and n_H = 0.93 ± 0.05 (open circles); δMMM + Y12T, K_app = 185 ± 13.8 μM and n_H = 0.77 ± 0.05 (filled circles). B, displays binding curves for the indicated pairs of non-interacting wild type and mutant AChR and CTx MI. Smooth curves are fits to the Hill equation with the following parameters: WT + CTx MI, K_app = 0.61 ± 0.13 nM and n_H = 0.69 ± 0.08 (open squares); WT + N11K, K_app = 15.3 ± 0.8 nM and n_H = 0.85 ± 0.03 (filled squares); αY198T + CTx MI, K_app = 1.29 ± 0.08 μM and n_H = 1.05 ± 0.06 (open circles); αY198T + N11K, K_app = 4.68 ± 0.23 μM and n_H = 0.99 ± 0.05 (filled circles).
possible pairs of receptor-conotoxin mutations. We find the strongest interaction between the triad of selectivity determinants in the δ subunit and Tyr-12 of CTx MI; binding curves for the corresponding mutant cycle are shown in Fig. 6A. Individually, mutations in either the receptor or the conotoxin decrease affinity by 10,000-fold. However when mutations in both the receptor and conotoxin are examined together, affinity decreases by 100,000-fold, which is 3 orders of magnitude less than predicted if the contributions were additive. Double mutant cycles analysis reveals a coupling coefficient of 1584 for the Y198T/P6G pair, corresponding to an interaction free energy of 4.3 kcal/mol (Table IV).

Whereas mutant cycles analysis can identify interacting pairs of residues, it can also identify non-interacting pairs of residues, as illustrated for the pair αY198T/N11K (Fig. 6B). The receptor mutation αY198T decreases affinity by 1000-fold, whereas the CTx MI mutation N11K decreases affinity by 30-fold. When the two mutations are examined together, affinity decreases by 30,000-fold, which is purely additive, demonstrating that αY198T and Asn-11 do not interact. Thus double mutant cycles analysis readily distinguishes interacting from non-interacting pairs of residues in the AChR-CTx MI complex.

Pairwise Interactions between CTx MI and the α Subunit—

Applied to the α subunit face of the AChR binding site, mutant cycles analysis reveals that Tyr-198 interacts significantly with the bioactive residues Ala-7, Pro-6, and Tyr-12 in CTx MI (Fig. 7 and Table IV). The coupling coefficient for the αY198T/A7V pair is 638 and corresponds to an interaction free energy of 3.8 kcal/mol. The αY198T/P6G pair exhibits a weaker coupling coefficient of 100, perhaps owing to reduction of a joint contact surface formed by Ala-7 and Pro-6 or to increased conformational flexibility of Ala-7 caused by the P6G mutation. The weaker coupling coefficient of 39 for the Y12T/αY198T pair is likely due to an indirect interaction in which the Y12T mutation produces global changes that propagate to either Ala-7 or Pro-6 (Fig. 3), both of which couple strongly to αY198T. Alternatively, the Y12T mutation may allow reorientation of the conotoxin due to loss of the interaction between Tyr-12 and the δ subunit (see Fig. 6A below). The fourth bioactive residue in CTx MI, Gly-9, shows a conspicuous lack of coupling to any of the conserved tyrosines in the α subunit. The rank order of coupling to αTyr-198, Ala-7 > Pro-6 > Tyr-12 >> Gly-9, suggests that CTx MI binds with Ala-7 opposing Tyr-198 of the α subunit.

Pairwise Interactions between CTx MI and the δ Subunit—

Applied to the δ subunit face of the AChR binding site, mutant cycles analysis reveals only weak coupling for the 24 pairs of receptor-conotoxin mutations (Fig. 8; Table IV). The weak coupling mirrors the relatively small changes in CTx MI affinity produced by mutations of individual selectivity determinants in the δ subunit (Fig. 5).

On the other hand, when all three selectivity determinants are mutated in the same δ subunit, we find strong coupling to Tyr-12 of CTx MI (Fig. 6A). To identify which of the three selectivity determinants are interdependent in coupling to Tyr-12, we paired double mutations in the δ subunit against the Y12T mutant. Pairing either of the double mutations, δ(S36K/Y113S) or δ(Y113S/I178F) against Y12T reveals weak or undetectable coupling (Fig. 9). However, pairing δ(S36K/I178F) against Y12T reveals significant coupling with δ equal to 200 (Figs. 6A and 9). Thus Ser-36 and Ile-178 jointly stabilize the tyrosine side chain at position 12 of CTx MI.

To look for additional interactions between CTx MI and the δ subunit, we paired each CTx MI mutation against the triple mutation δ(S36K/Y113S/I178F). Mutation of each of the four bioactive residues in CTx MI reveals significant coupling to the triad of selectivity determinants in the δ subunit, with the rank order Tyr-12 > Ala-7 > Gly-9 > Pro-6 (Fig. 10). The rank order of coupling suggests CTx MI binds with Tyr-12 opposing the δ subunit. The much weaker coupling between Tyr-12 and Tyr-198 of the α subunit (Fig. 7) further supports orientation of Tyr-12 toward the δ subunit.

For some residues in CTx MI, we detect strong coupling to residues in both α and δ subunits (Figs. 7 and 10). The conotoxin/receptor pairs, A7V/δ(S36K/Y113S/I178F) and A7V/αY198T, show equivalent coupling coefficients (Figs. 7 and 10; Table IV). That Ala-7 couples equivalently to the same residues in α and δ subunits suggests either close approach of αTyr-198 and the triad δ(Ser-36/Tyr-13/Ile-178) or interaction of these residues with a common residue that couples to Ala-7. We also...
find equivalent coupling coefficients for the pairs, P6G/Δ(S36K/Y113S/I178F) and P6G/Δ(Y198T). However, because proline in the conotoxin is mutated, the apparent equivalent coupling to α and δ subunits likely owes to global conformational changes in the conotoxin that prevent it from bridging the binding site interface. Finally, Gly-9 interacts with residues in the δ but not the α subunit, as the G9S/Δ(S36K/Y113S/I178F) pair shows strong coupling (Fig. 10), but the G9S/Δ(Y198T) pair shows weak coupling (Fig. 7). Thus Ala-7 in CTx MI couples strongly to residues in both α and δ subunits, but Tyr-12 and Gly-9 couple preferentially to residues in the δ subunit.

The overall results demonstrate that the three selectivity determinants in the δ subunit, Ser-36, Tyr-113, and Ile-178, together provide the major source of stabilization for CTx MI in the AChR binding site. Furthermore, these selectivity determinants in the δ subunit, together with Tyr-198 in the α subunit, produce the nanomolar affinity between CTx MI and the α-δ binding site.

**DISCUSSION**

The present work establishes the essential hydrophobic nature of the interaction between CTx MI and the α-δ site of the muscle AChR. Together with the known structure of CTx MI, the results place into close proximity key residues from both α and δ subunits. The four essential bioactive residues in CTx MI localize to a restricted region of its three-dimensional structure, creating a hydrophobic surface for presentation to the α-δ binding site. Three of the four bioactive residues in CTx MI interact with residues from both α and δ subunits, suggesting remarkably close association of the two subunits. The rank order of the strength of the interactions establishes the orientation of CTx MI in the binding site; Ala-7 orients toward the α subunit and Tyr-12 toward the δ subunit. Of the seven loops in the AChR known to converge to the binding site interface, four loops contain residues that interact with CTx MI as follows: Tyr-198 in loop C of the α subunit and Ser-36, Tyr-113, and Ile-178 in loops I, III, and IV in the δ subunit. The multiple focal interactions, together with the nanomolar affinity of the complex, suggest highly complementary surfaces of the toxin and receptor at the region of contact.

NMR studies have established the solution structure of CTx MI (19), which appears as a partially flattened tripod, with feet formed by side chains of Arg-2, His-5, and Lys-10 (Fig. 3). The space between the His-5 and Lys-10 appendages contains the bioactive residues, which extend their side chains outward from the convex side of the tripod. Side chains of the bioactive residues occupy corners of an irregular trapezoid, with the

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**TABLE I**

**Molecular weights and oxidation states of wild type and mutant α-conotoxins**

Molecular weight was determined by mass spectrometry and compared to the calculated molecular weight. The percent absorbance (% Abs) is the reactivity of each α-conotoxin to DTNB relative to that of linear, non-oxidized α-conotoxin as described under “Experimental Procedures.” Commerically available CTx MI is included for comparison.

| Conotoxin             | Observed Mr | Calculated Mr | % Abs |
|-----------------------|-------------|---------------|-------|
| Non-oxidized CTx MI   | 1356.0      | 1354.0        | 1.4   |
| CTx MI                | 1454.0      | 1453.0        | 1.8   |
| Acetylated G1         | 1454.0      | 1453.0        | 1.8   |
| R2Q                   | 1465.0      | 1465.0        | 1.4   |
| H5A                   | 1428.0      | 1430.0        | 1.9   |
| P6G                   | 1454.0      | 1453.0        | 1.8   |
| P6A                   | 1467.0      | 1467.0        | 3.2   |
| P6I                   | 1509.5      | 1510.0        | 1.8   |
| P6V                   | 1495.5      | 1497.0        | 2.1   |
| G9A                   | 1494.5      | 1498.3        | 2.6   |
| G9S                   | 1494.5      | 1497.2        | 2.9   |
| G9V                   | 1472.2      | 1474.4        | 2.7   |
| A7V                   | 1466.2      | 1467.6        | 2.2   |
| A7S                   | 1467.3      | 1468.5        | 2.2   |
| K10Q                  | 1495.0      | 1499.0        | 2.6   |
| K10Y                  | 1532.0      | 1535.0        | 2.1   |
| N11K                  | 1509.0      | 1509.0        | 1.7   |
| Y12T                  | 1432.0      | 1432.7        | 2.0   |
| Y12F                  | 1478.0      | 1478.0        | 3.2   |
| S13A                  | 1493.0      | 1493.0        | 2.1   |
| S13V                  | 1494.0      | 1495.0        | 2.2   |

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surface contour between the corners falling away into a pronounced cavity. The shape of the bioactive region is like that of an outstretched left hand with the outer two fingers closed to the palm. The Pro-6 and Tyr-12 side chains extend as fingers parallel to each other, the Ala-7 side chain extends as the thumb at right angles, and the a-carbon of Gly-9 recedes to form the cavity between the fingers and the palm. Thus positioned on the convex side of the tripod scaffold, the bioactive region presents three hydrophobic fingers to the a-d subunit interface.

Considerable work demonstrates that both a and non-a subunits form the AChR binding sites (7, 8). However, the pairwise coupling observed here demonstrates even closer association of a and d subunits than previously thought. The evidence comes from the very strong coupling between an individual residue in CTx MI and residues from both a and d subunits; Ala-7 and Pro-6 in CTx MI each couple strongly to Tyr-198 in the a subunit and to the triad of selectivity determinants in the d subunit. This interpretation of close association of subunits seems inseparable, whether Tyr-198 in the a subunit and the selectivity determinants in the d subunit contact CTx MI directly or whether they contact a third residue in the binding site that does.

In addition to the pairwise interactions detected here, the key binding site determinants in the AChR satisfy other criteria for direct interaction with CTx MI. First, these residues are exposed on the surface of the protein. Surface exposure of aTyr-198 was established by photoaffinity labeling by nicotine (21) and by interaction of aTyr-198 with one of the two quaternary nitrogens in the competitive antagonist dimethyl-d-tubocurarine (22). Similarly, aTyr-111 is equivalent to aThr-111 in the Torpedo AChR, which was photoaffinity labeled by [3H]d-tubocurarine (23), and is close to aThr-119 and aLeu-121, which were accessible to methanethiosulfonate reagents when mutated to cysteine (24). Also, aIle-178 neighbors aAsp-180, which was cross-linked by a bifunctional reagent tethered to the a subunit (25). Second, extensive mutagenesis studies have detected only these determinants as stabilizing CTx MI. Mutagenesis of loops A, B, and C in the a subunit revealed contributions of only Tyr-198 and Tyr-190 to CTx MI affinity (14). Likewise, screening the entire extracellular domains of a and d subunits by constructing chimeras revealed only aSer-36, aThr-113, and aIle-178 as contributors to CTx MI binding (10). The chimera studies thus exclude all other residues differing between a and d subunits as contributors to CTx MI binding. Whereas unexaminered residues in the a and d subunits remain as formal possibilities for directly contacting CTx MI, the large contributions to affinity and strong pairwise interactions we observe are best explained by direct contributions of aTyr-198, aSer-36, aThr-113, and aIle-178 to CTx MI binding.

Both the CTx MI and the a-d binding site contribute hydrophobic and aromatic residues to form the high affinity complex. The stabilization likely owes to exclusion of water from the predominantly hydrophobic surfaces and to hand-in-glove complementarity between surfaces of the toxin and the binding site that maximize van der Waal’s interactions. The pairwise coupling observed here, together with the structure of CTx MI, suggests the following picture of the complex. Isoleucine at position 178 of the d subunit fits into the hydrophobic cavity in CTx MI, interacting with all four bioactive residues (Fig. 3). The stabilization by aIle-178 depends on Ser-36 located on the same d subunit (Figs. 9 and 10), which may supply a hydrogen bond that positions aIle-178 or, due to its small size, may allow the isoleucine side chain to penetrate into the hydrophobic cavity of CTx MI. The third determinant of the d subunit, aTyr-113, may interact with the rim of the hydrophobic cavity of CTx MI, lodging closest to Ala-7 and Gly-9 (Fig. 10). The aromatic hydroxyl of aTyr-113 likely hydrogen bonds to an acceptor not yet identified, as its mutation to phenylalanine markedly decreases affinity of CTx MI. Finally, Tyr-198 of the a subunit completes the stabilization through hydrophobic interactions with Ala-7 and Pro-6 of CTx MI (Fig. 7). The aromatic hydroxyl of aTyr-198 is not essential for high affinity binding, as its mutation to phenylalanine is without effect. This picture of the complex represents a testable hypothesis for future mutagenesis and site-directed labeling studies.

The remaining portion of CTx MI is hydrophilic, comprising the three legs of the tripod structure, but does not interact with key residues in the a-d binding site. However, because the mutations R2Q, H5A, K10Q, N11K, and S13A decrease CTx MI affinity from 20- to 50-fold, these hydrophilic residues contribute importantly to the overall high affinity of the complex. Arg-2 and Lys-10 of CTx MI may partner in long range electrostatic interactions with anionic side chains in either a or d of CTx MI, lodging closest to Ala-7 and Gly-9 (Fig. 10).
subunits. The surface of CTx MI should also sterically accommodate the surface contour of the binding site interface. Thus CTxs MI likely provides a negative image of the surface of the binding site interface.

The CTx MI-AChR complex exhibits similar overall features to those found in other high affinity protein-protein complexes. The picomolar complex formed between fasciculin and acetylcholinesterase is held together largely by hydrophobic interactions between alkyl and aromatic side chains that closely complement surfaces of both partners of the complex (26). The complex formed between growth hormone and its receptor contains two strong hydrophobic contacts at its center, flanked by multiple weaker contacts mediated by charged groups (27). The binding interface was pointed out to be like that of a cross-section through a folded globular protein, with hydrophobic residues inside and hydrophilic residues outside. Analogously, the core of the CTx MI-AChR complex is strikingly hydrophobic, whereas the periphery is hydrophilic.

Hydrophobic contacts are the predominant source of the nanomolar affinity of the CTx MI-AChR complex. Free energy of burying hydrophobic residues has been estimated to be ~15 cal/mol per Å² of hydrophobic surface (28). The total accessible surface area of the seven side chains of the complex is 730 Å², giving a potential hydrophobic contribution of ~10.9 kcal/mol, which approaches ~12.2 kcal/mol of binding free energy expected for a complex with nanomolar affinity. Also, residues flanking the selectivity determinants in the δ subunit are particularly hydrophobic, with the following local sequences: VALSL (residues 33–37), LVY (111–113), and WIII (176–179). Thus residues flanking the selectivity determinants may introduce additional hydrophobicity at the δ subunit portion of the binding site interface. Hydrophobic contacts are therefore the predominant sources of the nanomolar affinity of the CTx MI-AChR complex.

Solution structural studies of CTx MI demonstrate slowly interconvertible major and minor conformations, with the major conformation representing approximately 80% of the total (19, 30). The structure of CTx MI depicted in Fig. 3 is that of the major conformation, but the structure of the minor conformation has not been reported. However, for CTx GI, which has a similar pharmacological fingerprint to CTx MI, atomic coordinates of both major and minor conformations have been reported (31). Comparison of all reported structures of CTx GI indicates that the major conformers of CTx MI and GI are structurally similar (31–34). If the two conformers for CTx GI are comparable to those for CTx MI, we can ask whether any of the four bioactive residues change positions between major and minor conformations. Side chains of Pro-6, Ala-7, and Gly-9 have similar coordinates in CTx MI and GI and change very little between major and minor conformations. However, to achieve the minor conformation of CTx GI, the peptide backbone between glycine 9 and serine 13 twists so the side chain of Tyr-12 moves out of the hydrophobic pocket to protrude on the opposite side of the toxin (31, 32). If the minor conformation is the one bound to the AChR, the α and δ subunits would be estimated to be farther apart than if the major conformer is bound. Additionally, when we mutate CTx MI one conformer may be favored over the other, potentially affecting the affinity of the complex. For example, glycine at position 9 may stabilize the peptide backbone of residues 9–13 to maintain the major conformation of the native structure, owing to its ability to accommodate unique φ and ψ bond angles. Mutation of glycine 9 to serine or valine could potentially destabilize the major conformation, allowing the bioactive Tyr-12 to move out of the hydrophobic pocket; affinity would increase or decrease, depending on which of the two conformers is bound in the high affinity complex. Additional studies are required to determine whether the minor conformation of CTx MI is similar to that of CTx GI and whether CTx MI changes conformation upon binding to the AChR.

Previous studies suggested that muscle-specific α-conotoxins interact with the AChR by presenting appropriately spaced positive charges to anionic loci in the binding site interface (11, 12, 29). Although α-conotoxins MI, GI, and SI each contain multiple positively charged nitrogenous, the present work clearly demonstrates that these positive charges are not the predominant source of high affinity of this class of α-conotoxins. On the contrary, each of the muscle-specific α-conotoxins contains the four hydrophobic residues we identify as essential for bioactivity: Pro-6, Ala-7, Gly-9, and Tyr-12 (Fig. 1). Like CTx MI, CTx GI selects between α-γ and α-δ binding sites by 10,000-fold, but CTx SI is not as selective and binds with only micromolar affinity to muscle AChRs (11). The proline at position 10 of CTx SI likely distorts the C-terminal loop such that Tyr-12 is no longer positioned to interact with its hydrophobic counterpart in the AChR binding site. Perhaps the natural target for CTx SI better accommodates Pro-10 and Tyr-12 in the C-terminal loop. Thus muscle-specific α-conotoxins rely on hydrophobic rather than polar or electrostatic interactions to achieve high affinity.

The overall results reveal the essential hydrophobic nature of the interaction between CTx MI and the α and δ subunits of the AChR binding site interface. High affinity of CTx MI is due to three hydrophobic fingers extending from an otherwise hydrophilic scaffold. Analogously, a hydrophobic pharmacophore may underlie bioactivity of other members of the α-conotoxin family (35). The region of contact at the AChR binding site comprises closely packed residues from both α and δ subunits. The pairwise interactions we identify provide spatial constraints to define our picture of the AChR binding site.

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