Site-specific Charge Interactions of α-Conotoxin MI with the Nicotinic Acetylcholine Receptor*

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We have tested the importance of charge interactions for α-conotoxin MI binding to the nicotinic acetylcholine receptor (AChR). Ionic residues on α-conotoxin MI were altered by site-directed mutagenesis or by chemical modification. In physiological buffer, removal of charges at the N terminus, His-5, and Lys-10 had small (2–4-fold) effects on binding affinity to the mouse muscle AChR and the Torpedo AChR. It was also demonstrated that conotoxin had no effect on the conformational equilibrium of either receptor, as assessed by the effects of the noncompetitive antagonist proadifen on conotoxin binding and, conversely, the effect of conotoxin on the affinity of phencyclidine, proadifen, and etidium. Conotoxin displayed higher binding affinity in low ionic strength buffer; neutralization of Lys-10 and the N terminus by acetylation blocked this affinity shift at the αδ site but not at the αγ site. It is concluded that Ctx residues Lys-10 and the N terminal interact with oppositely charged receptor residues only at the αδ site, and the two sites have distinct arrangements of charged residues. Ethidium fluorescence experiments demonstrated that conotoxin is formally competitive with a small cholinerigic ligand, tetramethylammonium. Thus, α-conotoxin MI appears to interact with the portion of the binding site responsible for stabilizing agonist cations but does not do so with a cationic residue and is, consequently, incapable of inducing a conformational change.

The muscle type nicotinic acetylcholine receptor (AChR) is a pentameric ligand-gated cation channel with a subunit stoichiometry of α2β2δ (see Ref. 1 for review). The homologous subunits surround a central pore that forms the ion conductive pathway. The AChR comprises two extracellular ligand-binding sites for ACh that lie at the interfaces between the α-γ and the α-δ-subunits (2, 3). Competitive antagonists and natural toxins often have different affinities for the two sites that arise from the distinct contributions of the γ and δ-subunits to each site (4). For example, d-tubocurarine binds the αγ sites of the Torpedo AChR with an affinity more than 100-fold greater than for the αδ site (5).

A similar characteristic was observed for the marine snail α-conotoxins, some of which bind the mouse muscle AChR sites with affinities that differ by more than 10,000-fold, where the αδ site is bound with higher affinity (see Ref. 6 for review). The differences in affinity have been attributed to three specific residues in mouse muscle AChR αγ-subunit (γ-Lys-34, γTyr-111, and γPhe-172) and the corresponding homologous residues in the δ-subunit (7). Recent work (8) has also identified α-subunit residues that affect binding of α-conotoxin MI (Ctx) in a site-selective manner, which suggests that Ctx may bind with distinct configurations at the two sites. Ctx residues Gly-9 and Pro-6 (see Table I) and residues on the δ-subunit and between Ala-7 and Pro-6 and binding site residues in the α-subunit appear to stabilize binding through hydrophobic interactions (9).

Significant affinity differences have also been observed for binding to the Torpedo AChR (10–12); however, in this case the αγ site is bound with higher affinity. Chiara et al. (12) showed that one AChR residue, γTyr-111, and its δ-subunit homolog, δArg-113, accounted for much of the affinity differences in Torpedo AChR. They further suggested that the affinity change arose from specific charge repulsion between δArg-113 and Ctx residue Lys-10. This Ctx residue is highly conserved among α-conotoxins; a cationic amino acid is present in this position (see Table I), with the exception of α-conotoxin SI, where it is in the adjacent position, or in SII where it is absent (Table I). The homologous residue in α-conotoxin G1 (Arg-9) was identified as being responsible for the high affinity binding of conotoxins to the αγ site; when it was substituted for a proline, affinity decreased dramatically for the αγ site (13). This observation was consistent with the low affinities of α-conotoxins SII and SII for the αγ site, which have a Pro at this position.

Several studies suggest that ionic interactions may not be the critical aspect of the role of this residue in binding. Analysis of pairwise interactions in the mouse muscle AChR failed to reveal strong interactions between δTyr-113 and Ctx Lys-10 (9). However, that analysis did not examine a charge-change at δTyr-113 to Arg, the amino acid present in the Torpedo AChR. Studies on analogs of α-conotoxin GI that changed the corresponding Arg-9 to alanine displayed affinity changes that appeared site-independent (14), suggesting that the charge per se was not important to binding.

Conotoxin binding appears to block competitively the bind-
ing of α-bungarotoxin and to block biological activity of AChRs. Yet, α-conotoxins do not appear to influence the conformation of the mouse AChR (15). The structural model of Unwin and co-workers (16, 17) suggests the presence of transverse tunnels leading to a buried site for ACh binding. But such tunnels appear unlikely to permit access of a ligand the size of Ctx to the inner recesses of the binding site. An alternative model is that Ctx binds at the ACh-binding site entrance to block access of smaller ligands but may not interact closely with residues critical for stabilizing agonist binding. However, Bren and Sine (9) measured interactions of Ctx with α-subunit residues Tyr-93, Tyr-190, and Tyr-198, residues thought to be critical in stabilizing agonist binding. The evidence that Ctx interacts with AChR-binding site residues appears incompatible with the structural model of the AChR and with a role for cationic residues in binding.

To examine to the role of charged residues in stabilizing binding of Ctx to the AChR and to examine whether Ctx interacts intimately with residues critical for agonist binding, we determined the binding properties of charge variants of Ctx, as well as several other Ctx mutants. We present data that the charge of Ctx Lys-10 and that of His-5 and the N terminus are relatively unimportant to the net binding affinity toward either the mouse muscle type AChR or the Torpedo electric organ AChR. We further demonstrate that binding of Ctx is conformationally insensitive. Nonetheless, the toxin is formally competitive with the ammonium moiety of ACh. This cationic toxin does not appear to take advantage of the innate charge stabilization the receptor confers on agonists and many competitive antagonists but nonetheless occupies the same steric space. Studies in low ionic strength, however, did reveal distinct contributions of charge interactions from the γ- and δ-subunits, but these likely arise from interactions with negatively charged residues on the AChR.

**EXPERIMENTAL PROCEDURES**

**Materials—**All the enzymes for molecular biology, the pMALp2 plasmid vector, and amylose resin were supplied by New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Genosys (The Woodlands, TX). Culture media, competent cells, and isopropylthio-β-galactoside were from Life Technologies, Inc. DNA was sequenced using a kit, version 2.0, from U. S. Biochemical Corp. Peptidylglycine α-amidation enzyme was from Takara (Beverley, CA), and synthetic Ctx was purchased from Sigma. AChR-rich membranes were prepared from fresh or frozen Torpedo californica electric organ as described previously (18).

**Synthesis and Modification of Conotoxins—**α-Conotoxin MI (Ctx) was prepared by peptide synthesis at the Baylor College of Medicine protein core facility. The reduced peptide was purified by preparative reversed phase HPLC (Vydac 250 × 22 mm, C18); elution was by a linear gradient of CH3CN containing 0.09% trifluoroacetic acid. The peptide was renatured by oxidation of cysteines to form disulfides by stirring in air according to the procedures described by Zafranal et al. (19) and by Myers (20). The renatured peptide was purified by reversed phase HPLC; it displayed the skewed peak profile characteristic of the conformational transition observed between two states (21). The elution profile was identical to that of a commercial preparation of Ctx (Sigma) and gave identical binding constants as assessed by inhibition of [3H]ACh binding on AChR-rich membranes from Torpedo (22).

Two variants of Ctx were also prepared by peptide synthesis, Ctx Ala-7 → Ser and Ctx Tyr-12 → Trp. These were purified as the linear peptide. Oxidation of the sulfhydryls to form the correct disulfide bonding was carried out by exposure in 1 mM GSSG. This redox buffer required only 30 min to 1 h for complete oxidation of the peptide, rather than the several days required for air oxidation. The peptides were then purified by preparative reversed phase HPLC as described above. These variants also displayed the skewed peak characteristic of the conformational transition observed for native Ctx. In all cases, the composition was confirmed by amino acid analysis.

Chemical modifications of Ctx at the N terminus and Lys-10 were carried out as follows. Reductive methylation of Ctx was carried out by the method of Jentoft and Dearborn (23). Briefly, 2 mg of Ctx was reacted in 1 ml of 50 mM NaOH containing 3 mCi of [14C]acetate. The Ctx was separated by preparative HPLC. The reaction yielded two peaks. Amino acid analysis and mass spectrometry revealed that the larger second peak contained the product with reaction of TNB at both the N terminus and Lys-10.

**Amino acid analysis** of the results of chemical reactions revealed loss of detectable Lys and one of the two Gly residues after methylation and after reaction with trinitrobenzoyl sulfonate. The analysis of the methyated Ctx was similar to that of unmodified toxin, as expected for an acid-labile adduct. Mass spectroscopy revealed parent ion masses of 1493, 1549, 1577, and 1915 for Ctx, Met-Ctx, Ac-Ctx, and TNB-Ctx respectively; these masses corresponded to those expected for each modification. The loss of primary amines in the modified toxins was verified by mass of reactivity to fluorescein. Fluorescence measurements showed that there were no amines available for reaction for each of the modified toxins, whereas Ctx showed reactivity that corresponded to the presence of 3 Lys residues.

**Design and Construction of Synthetic Gene for Conotoxin MI—**We have constructed a recombinant expression plasmid encoding a maltose-binding protein-Ctx fusion protein, using pMAL-p2 system (25). The system was chosen to direct secretion of the MBP fusion protein into the periplasmic space for proper disulfide bond formation. Codons for Ctx residues were chosen according to Echerichia coli codon usage (26). The Ctx was appended by a synthetic glycine amine (27). Two stop codons were added at the end of the gene followed by a Pil site for cloning the synthetic gene into pMALp2 at the XmnI and PstI site. The XmnI site included sequence encoding the protease factor Xa cleavage site, located just 5’ to the toxin sequence (25, 27) such that cleavage of the fusion protein by factor Xa liberates Ctx with no additional amino acids. Complementary oligonucleotides were purified by gel electrophoresis in 20% polyacrylamide containing 7% urea. They were then 5’-phosphorylated using polynucleotide kinase and annealed by combining equimolar amounts of each oligomer (300 pmol) in 100 μl of 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 50 mM NaCl, followed by heating to 95°C for 5 min and slowly cooling to room temperature. The duplex DNA was gel-purified, digested with PstI, and inserted into the expression plasmid pMALp2 at XmnI and PstI sites. Transformants containing the conotoxin M1 gene (pMAL-Cono) were verified by DNA sequencing (28).

**Expression and Purification of Recombinant Ctx—**Cells containing pMAL-Cono clone were grown at 37°C in LB broth containing 0.2% glucose and 100 μg/ml ampicillin to an absorbance of 0.5 at 600 nm, and then induced with 0.5 mM isopropylthio-β-galactoside for 3 h. A periplasmic fraction was prepared by osmotic shock (29), and the fusion for Ctx was purified by affinity chromatography on amylose resin (30) column (10 ml) that had been equilibrated previously with 10 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA. The column was washed with 10 volumes of this buffer, and the bound MBP-conotoxin fusion protein was then eluted with buffer containing 5 mM maltose. Fractions were analyzed by SDS-PAGE.

The purified fusion protein (MBP-conotoxin) was cleaved with factor Xa at a w/w ratio of 1% for 48 h at room temperature following
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SDS-PAGE analysis. The result showed about 50–70% release of Ctx. The recombinant Ctx was then purified by HPLC on a semi-preparative Vydac C8 column. Elution was by a linear gradient of 0–60% acetonitrile containing 0.09% trifluoroacetic acid at a flow rate of 3 ml/min. HPLC fractions were analyzed by Tricine-SDS-PAGE (31). To amido Ctx, the pooled fractions were treated with peptidylglycine alpha-amidating enzyme according to a standard procedure (32) for 4 h at 37 °C followed by repurification on HPLC as described above. Ctx mutants Lys-10 → His, Lys-10 → Pro, and His-5 → Glu were produced as described above for Ctx with appropriate codon replacements in the synthetic oligonucleotides. The yields of the Ctx analogs were lower than that of native Ctx because the extent of factor Xa digestion was lower. Because of the limited amount of material, duplicate experiments on Torpedo AChR were carried out just once.

**Binding Assays**—Binding of the various conotoxin derivatives was measured by competitive inhibition of the initial rate of bungarotoxin binding, as described previously (33). Binding to Torpedo AChR was measured using the assay described by Schmidt and Raftery (34) or by filtration over GF/F filters. For filtration the following procedure was followed. Samples of AChR-rich membranes and competitive ligand were preincubated in HTTPS (250 mM NaCl, 5 mM KCl, 3 mM CaCl2, 2 mM MgCl2, 0.002% NaN3, 20 mM Hepes, pH 7.0) containing 0.1% BSA for 30 min to reach equilibrium. The binding was initiated by addition of 125I-BgTx (0.5–3 nM), and the samples were allowed to incubate at ambient temperature for 45 min. Reactions were quenched by addition of 300 nM non-radioactive alpha-BgTx in HTTPS. The samples were then filtered over Whatman type GF/C filters that had been presoaked in 1% polyethyleneimine. The filters were then washed with 10 ml of HTTPS and counted in an γ-radiation counter (Beckman Instruments). For low ionic strength experiments, binding was carried out in 10 mM Hepes, pH 7.0, with 0.1% BSA. Binding reactions were quenched at 30 s to remain in the linear range of the initial association rate.

Binding to mouse muscle type AChR expressed on the surface of tissue culture cells (BC3H1) was carried out as described earlier (35) using a 24-well plate assay or, alternatively, by a filtration assay (4). The latter assay was carried out by suspending the cells in potassium-Ringer buffer (140 mM KCl, 5.4 mM NaCl, 12.6 mM CaCl2, 1.7 mM MgCl2, 25 mM Hepes, pH 7.5). The samples were incubated in 100 μM conotoxin and bound to the AChR, and the extent of binding reflects the extent of desensitization of the AChR when the AChR and ethidium concentrations are low relative to the Kd values for ethidium (～500 nM). Ethidium binding was measured by fluorescence enhancement, essentially as described by Lurtz and Pedersen (36), except that fluorescence was monitored through a filter (550 nm cut on, Orion model 59502) to obtain a higher signal to noise ratio. Binding to the NCA site of the mouse AChR was also measured by [3H]phencyclidine binding using a centrifugation assay as described previously (22). Binding was carried out in low concentrations of [3H]phencyclidine (～1 nM) and is reported as bound over free, which compensates for the change in free [3H]phencyclidine upon binding of competing ligand.

Binding was analyzed by nonlinear least squares fitting to Equation 1 for inhibition by competitive binding to two independent sites present in equal concentrations,

\[ B = B_{\text{max}}(1 + 1/K_{N1}) + 1/(1 + 1/K_{N2}) \]  
(Eq. 1)

where \( K_{N1} \) and \( K_{N2} \) represent the binding constants for the inhibitor, \( B_{\text{max}} \) the maximum amount of binding at each site, and I the concentration of inhibitor. In some cases, binding was fit to Equation 2 for the binding of two sites present in differing concentrations.

\[ B = B_{\text{max}}(1 + 1/K_{N1}) + B_{\text{max}}(1 + 1/K_{N2}) \]  
(Eq. 2)

This was necessary because 125I-BgTx binds with unequal rates to the two sites under some circumstances. Data for inhibition of Ctx binding to the mouse muscle AChR were generally fit with Equation 2, whereas data for inhibition of binding to Torpedo AChR could be fit with Equation 1. For the analysis of ethidium fluorescence, the enhancement of binding by agonists was fit to the Hill Equation 3.

\[ B = B_{\text{max}}/(1 + (K/L)^n) \]  
(Eq. 3)

or to the binding isotherm (the Hill equation with \( n = 1 \)). Inhibition of TMA binding by Ctx was fit to Equation 4 for inhibition at a single binding site.

\[ B = B_{\text{max}}(1 + K/L) \]  
(Eq. 4)

**RESULTS**

Like other α-toxins that interact with the nicotinic AChR, α-conotoxins are cationic. Ctx carries a net charge of +3.5, counting His-5 as ½ charge and including the N-terminal charge. To test whether these charged moieties play a significant role in the interaction of α-conotoxins with the AChR, we examined the binding energies of toxins that had been modified or mutated at the charged loci. One receptor residue that had been identified as constituting an electrostatically repulsive interaction was δArg-113 in the Torpedo AChR. The homologous residue in the mouse muscle AChR is a Tyr. Therefore, we compared affinities of toxins in both these species as a means of assessing the importance of charge repulsion between this residue and Ctx. We utilized two approaches to generate Ctx structural analogs, expression of recombinant Ctx in E. coli, and chemical modification of synthetic Ctx.

**Expression and Purification of Recombinant Ctx in E. coli**—Ctx could be expressed and refolded in E. coli by attaching a synthetic gene to the secreted maltose-binding protein. The fusion protein was isolated by amylose affinity chromatography and Ctx cleaved by factor Xa. Purification by HPLC, amidation, and a final HPLC purification yielded a product that was indistinguishable from commercial Ctx and from synthetic Ctx, as judged by migration SDS-polyacrylamide gel electrophoresis and by HPLC (Fig. 1, insets). The net yield of recombinant toxin was about 50 μg/liter of cell. Recombinant Ctx was compared with synthetic Ctx and with a commercial product (Sigma) for binding activity on mouse muscle AChR and on T. californica AChR by inhibition of 125I-α-BgTx binding as described under “Experimental Procedures.” The affinities of each material were indistinguishable (data not shown). Proper refolding and disulfide bond formation was apparent from comigration of recombinant and synthetic Ctx on HPLC and because it is required for biological activity (37, 38).

**Effects of Ctx Modifications on Binding Affinity**—Ctx was chemically modified by reductive methylation, by acetylation, or by trinitrobenzoxylation, using standard reaction methods (see “Experimental Procedures”). Each modified toxin was purified by reversed phase HPLC and shown to be quantitatively modified at Lys-10 and at the N terminus by mass spectrometry, by lack of reactivity to fluorescamine, and by amino acid analysis. Methylation will increase the bulk of the reacted amines but will not alter the charge; both the N-terminal amine and Lys-10 were found to be dimethylated. The effect of amine dimethylation on affinity was negligible (Fig. 2 and Table II), demonstrating that the simple size of these amines was relatively unimportant for binding. Acetylation of the amines resulted in formation of a neutral acetamide with more steric bulk than the methylation. Nonetheless, there was less than a 2-fold effect on affinity for the mouse AChR and a 3-fold decrease for the Torpedo AChR αγ site (Fig. 2 and Table II). The small affinity changes suggest that the charges of Lys-10 and the N terminus do not contribute substantially to the binding energy. The modification by addition of a picric acid moiety, (TNB-Ctx) does cause a significant shift of binding to the αδ site of Torpedo AChR, and the αδ site of the mouse AChR.
of 13–15-fold. The effects on mouse ad and Torpedo ad sites were smaller (Table II).

These results indicated that the Lys-10 side chain charge was relatively unimportant for binding, although added steric bulk could affect binding at some sites. We further tested the ability of histidine, an aromatic cationic residue, and proline to affect the overall affinity at this position. Proline occurs as a natural substitution in α-conotoxin SI and SII at this position; α-Conotoxin Lys-10 → Pro and Lys-10 → His were produced by expression in E. coli, and their affinities for the mouse AChR (A) and the Torpedo AChR (B) were compared with Ctx (C) as described in Fig. 2. Each symbol represents the average of two determinations, and the data are normalized to the initial rate of binding observed in the absence of competitor. The solid lines represent the best fits to a model for inhibition at two binding sites.

Both these toxins have substantially lower affinity for the AChR (13).

\[ \text{Log}_{10} [\text{competitor}] \]

\( \text{[M]} \)

- Ctx MI
- Methylated Ctx
- Acetylated Ctx
- TNB-Ctx
The cation associated with agonists and many competitive antagonists affects the conformational equilibrium of the AChR. Cationic moieties of Ctx bound the ACh-binding site in the same manner as the ammonium of ACh, it would be expected to affect the conformation as well.
Nonetheless, Ctx appears to be competitive with other toxins in the same space as that occupied by an agonist ammonium group. The attractive interactions at the TMA site must be mediated by charged moieties outside of the ammonium group, such as the acetate of ACh or the larger bulk of other toxins. To test whether Ctx directly interacts with the AChR in the most critical part of the ACh-binding sites, the portion that stabilizes ammonium binding, we tested whether Ctx was directly competitive with the small agonist TMA.

We also determined the affinity of acetyl-Ctx (Fig. 7A) to test whether neutralizing the N terminus and Lys-10 was important to the charge interactions observed in low ionic strength buffer. Acetyl-Ctx displayed clear two-site binding in low ionic strength, whereas binding was well fit by single site function in physiological buffer. Therefore, we could not, a priori, assign sites to the high and low affinity components. To determine unambiguously which site corresponded to the high affinity component, we carried out inhibition by acetyl-Ctx with the $\alpha\gamma$ site blocked by including $1 \mu M$ d-tubocurarine (Fig. 7B, A). The inhibition was well fit by a single site binding function with a $K_1$ value that corresponded to that of the low affinity component (Table III). This demonstrated that the high affinity component reflected acetyl-Ctx binding to the $\alpha\gamma$ site and that the lower affinity reflected binding to the $\alpha\delta$ site.

In low versus high ionic strength, the shift in affinity of acetyl-Ctx was similar to the shift observed for Ctx itself at the high affinity, $\alpha\gamma$ site, $-10$ to $-30$-fold. Acetylation, therefore, does not affect ionic interactions at the $\alpha\gamma$ site. The ionic strength-induced change in affinity must arise from charges other than the N terminus or Lys-10. The ionic strength shift of acetyl-Ctx affinity at the low affinity $\alpha\delta$ site was much smaller ($1$ to $3$-fold), particularly when compared with the shift of Ctx itself ($-300$-fold). Thus, the ionic strength change affects interactions between negatively charged residues at the $\alpha\delta$ site and Ctx residues Lys-10 or the N terminus or both. The attractive interactions at the $\alpha\gamma$ site must be mediated by other residues since the shift by ionic strength is unaffected by acetylation.

**Ctx Competes with a Small Agonist**—The data strongly suggested that charged moieties on Ctx do not participate in binding and that there was not a functional group that acted as the equivalent of the ammonium group present in most agonists and antagonists. In addition, Ctx did not affect the conformational state of the AChR as would be expected if it occupied the same space as that occupied by an agonist ammonium group. Nonetheless, Ctx appears to be competitive with other toxins and with ACh; we have shown that Ctx blocks $[^3H]ACh$ binding and that it blocks $[^3H]d$-tubocurarine binding (data not shown). However, it was possible that Ctx sterically blocked binding by interaction with the sites that stabilize moieties outside of the ammonium group, such as the acetate of ACh or the larger bulk of other toxins. To test whether Ctx directly interacts with the AChR, we utilized the observation that Ctx does not affect the conformation of the AChR, whereas TMA desensitizes the AChR. To assess the conformational state, we utilized the fluorescent enhancement of ethidium upon binding the AChR. Ethidium preferentially binds the desensitized conformation (22, 39). Conditions of relatively low ethidium and low AChR concentrations were chosen such that the fluorescence increase due to ethidium binding would reflect the increase in affinity due to desensitization. Fig. 8A shows the increases in fluorescence caused by titration of ethidium/AChR suspensions with carbamylcholine and with TMA. Both ligands caused concentration-dependent changes in fluorescence that correspond to the binding of ligand and concomitant desensitization of the AChR. Carbamylcholine increased fluorescence with a Hill coefficient of 1.5 and a $K_{obs}$ of 170 nM, which corresponds closely to the values for equilibrium binding (data not shown). The $K_{obs}$ for TMA was 3 $\mu$M with a Hill coefficient of 1.

To determine whether conotoxin competes with TMA binding, Ctx was titrated into suspensions of ethidium/AChR that contained varying concentrations of TMA. Example titrations with 0, 10, 20, and 100 $\mu$M TMA are shown in Fig. 8B. The curve with no TMA demonstrates that Ctx by itself has only a small effect on ethidium fluorescence. As titration with Ctx displaced TMA from the ACh-binding sites, the AChR displayed less ethidium binding and fluorescence as the desensitized conformation was not stabilized by Ctx. In each case the inhibition was well fit by a single site inhibition function with a background fluorescence approaching that found in the absence of TMA. The $K_{obs}$ was determined for each titration and replotted against the TMA concentration (Fig. 8C). There is a linear relationship between the TMA concentration and the $K_{obs}$, indicative of direct competition.

The results cannot be accounted for by inhibition through a ternary complex of AChR, TMA, and Ctx unless one ligand conformationally reduces the binding of the second ligand with a coupling energy of more than 3.4 kcal/mol. This value was determined from the maximum TMA concentration used.

**Table II**

| Compound   | Mouse AChR $K_1$ | Mouse AChR $K_2$ | Torpedo AChR $K_1$ | Torpedo AChR $K_2$ |
|------------|----------------|----------------|-------------------|-------------------|
|            | $nM$           | $\mu M$        | $nM$              | $\mu M$           |
| Ctx        | 3.6 ± 0.6      | 72 ± 16        | 93 ± 11           | 1.7 ± 0.3         |
| Acetyl-Ctx | 3.9 ± 0.7      | 170 ± 25       | 412 ± 130         | 2.5 ± 0.3         |
| Methyl-Ctx | 1.9 ± 0.4      | 110 ± 35       | 117 ± 12          | 10 ± 7            |
| TNBS-Ctx   | 40 ± 21        |                | 1400 ± 300        | 10 ± 7            |
| Ctx K10P   | 30,000 ± 10,000|                | 2400 ± 900        | 11.3 ± 10         |
| Ctx K10H   | 67 ± 3         | 520 ± 300      | 1.4 ± 1           |                   |
| Ctx H5E    | 19 ± 3         | 640 ± 130      | 10 ± 7            |                   |
| Ctx A7S    | 8 ± 4          | 100 ± 27       | 450 ± 170         | 7.0 ± 1.8         |
| Ctx Y12W   | 3.5 ± 0.8      | 100 ± 74       | 20 ± 8            | 1.5 ± 0.6         |
| Ctx + proadifen | 5.3 ± 3       |                | 57 ± 13           | 1.8 ± 0.8         |
| Ctx + tetracaine | 61 ± 16      |                | 1.6 ± 0.3         |                   |

*a* A dashed line indicates that the data were best described by a fit to Equation 4; a blank space indicates that the second $K$ value could not be determined.
mental Procedures." The value for the presence of excess BgTx to define background binding is also shown (A). The solid curves are the best fits to a model for inhibition at two binding sites (Equation 2). The IC50 values for this experiment are 11 and 5 nM in the absence and presence of proadifen, respectively. B, Torpedo AChR-rich membranes (3.4 nM ACh sites) were incubated with the indicated concentrations of Ctx (0.1 mg/ml) was determined in the absence of proadifen (C) or no added ligand (D). The initial rate of binding of 125I-α-BgTx was measured using the 24-well assay as described under "Experimental Procedures." The data points in both panels represent the average of two independent determinations that generally varied by less than 5% and are plotted as fractional binding of the maximum (f). The K values obtained from the fits are as follows: Ctx, K1 = 4.4 nM; acetyl-Ctx, K1 = 6.3 nM and K2 = 194 nM; acetyl-Ctx plus d-tubocurarine, K1 = 373 nM; d-tubocurarine, K1 = 48 nM and K2 = 17 μM.

**FIG. 7.** Ionic interactions are site-specific. A, the binding affinities of Ctx (○) and acetyl-Ctx (□) were measured by inhibition of the initial rate of 125I-α-BgTx binding to AChR-rich membranes in 10 mM Hepes, 0.1% BSA by filtration as described under "Experimental Procedures." The dotted and solid lines represent the best fits to a single site inhibition function or a two-site inhibition function, respectively. For reference are shown the fitted curves for Ctx (-) and acetyl-Ctx (---) inhibition in HTPS from parallel experiments. B, inhibition of 125I-α-BgTx binding by d-tubocurarine (dTC, ○) and by acetyl-Ctx (△) in the presence of 1 μM d-tubocurarine. The best fits to a two-site binding function with variable site stoichiometry and a single site binding function, respectively, are shown as solid curves. The data points in both panels represent the average of two independent determinations that generally varied by less than 5% and are plotted as fractional binding of the maximum (f). The K values obtained from the fits are as follows: Ctx, K1 = 4.4 nM; acetyl-Ctx, K1 = 6.3 nM and K2 = 194 nM; acetyl-Ctx plus d-tubocurarine, K1 = 373 nM; d-tubocurarine, K1 = 48 nM and K2 = 17 μM.

**TABLE III**

| Compound                  | K1     | K2     | n         |
|---------------------------|--------|--------|-----------|
| Ctx                       | 3 ± 1  |        | 3         |
| Acetyl-Ctx                | 12 ± 5 | 320 ± 120 | 3         |
| Acetyl-Ctx + 1 μM d-tubocurarine | 470 ± 140 | 2       |

**DISCUSSION**

We examined various charged Ctx residues to determine whether they were critical for high affinity binding to the AChR, in a manner similar to the cationic moiety of agonists. Elimination of charge at Lys-10 and the N terminus affected binding affinity 2–4-fold. A charge reversal at His-5 consistently changed affinity 6-fold at all sites. Such modest changes show that individual charge-charge interactions are not a highly significant component of stabilizing Ctx binding in physiological buffer, despite the generally conserved cationic nature of these toxins. Binding affinity to Torpedo AChR was, however, significantly modified upon neutralization of Lys-10 when examined at low ionic strength, and the effect was much stron-
We conclude that Lys-10 or His-5 do not interact with the AChR in the same manner as cationic groups of agonists that are required for channel activation. This is shown by the modest affinity changes upon modification and by the general failure of Ctx to modulate the conformational state of the AChR. Ctx does not appear to interact with other residues that influence the conformational equilibrium between the resting and desensitized states of the AChR. However, Ctx does formally compete with the binding of TMA, suggesting that steric hindrance prevents the simultaneous occupancy of both Ctx and TMA. Consequently, Ctx does interact with the portion of the binding site responsible for stabilizing agonist cations but does not do so with a cationic residue and is therefore incapable of inducing a conformational change.

Our binding data reveal affinities of Ctx for the Torpedo αγ site that differ from those observed by others (10, 11). Some of the larger differences can be accounted for by the distinct buffer conditions used as follows: Hann et al. (13) and Groebe et al. (10) used low ionic strength buffers to measure binding in the presence of detergents. However, the data of Chiara et al. (12) was carried out in physiological buffer that revealed affinities 5–10-fold higher than we observed. Our binding data on the mouse muscle AChR is in complete agreement with that of others (7). We also obtained similar binding data for commercial, synthetic, and recombinant Ctx and observed consistent inhibition of \([3H]ACh\) binding and of \([3H]\alpha\text{-tubocurarine}\) binding (22) (data not shown). The source of the difference remains unknown.

Ionic Interactions in Binding—It has long been argued that localized charges stabilize the binding of agonists and competitive antagonists. Cholinergic agonists universally carry a positively charged group (40), and for the AChR, a simple quaternary ammonium in the form of TMA is a completely competent agonist. Localization of several anionic residues (γAsp-180 and αAsp-152) in the vicinity of the ACh-binding sites has supported the idea of charge stabilization of ligand binding (41, 42), but it remains unclear whether these residues act as countercharges to the ligand ammonium. Experiments that examined the effect of ionic strength on ligand binding were more consistent with a diffuse charge distribution near the binding site than a single countercharge in close proximity (43). α-Toxins of snakes and marine snails are generally basic and have binding that is strongly dependent on ionic strength (34, 44), suggesting that ionic interactions constitute a significant component of the net binding energy.

Sine et al. (7) identified several residues critical for the site selectivity of Ctx binding to mouse muscle receptor, including residues γLys-34, γTyr-111, and γHis-172. The homologous residue to γTyr-111 in Torpedo δ-subunit is δArg-113. Chiara et al. (12) demonstrated that δArg-113 dramatically weakened binding of Ctx to the αδ site, and they inferred an electrostatic repulsion with Lys-10. At the Torpedo AChR δ-subunit, acetylation of Lys-10 does appear to cause an increase in affinity of 2–4-fold, consistent with lessened repulsion. However, this change is much smaller than the 1000-fold effect observed by Chiara et al. (12). Our data suggest that the δArg-113 interaction is not through electrostatic repulsion at the N terminus, His-5, or Lys-10 of Ctx. We cannot rigorously exclude an interaction through Ctx Arg-2, which we have not modified; however, it appears unlikely that Arg-2 is responsible because conotoxins GI and SIA bind with similar selectivity to the Torpedo AChR (13) and have a Glu and Tyr in the homologous positions, respectively (see Table I). We conclude that Lys-10 of Ctx and δArg-113 are not in close apposition because electrostatic repulsion is not alleviated by charge neutralization. This is in

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 8.** Ctx competes with the small agonist, TMA. Binding of 200 nM ethidium to 100 nM AChR was measured by fluorescence enhancement in HTTPS buffer as described under “Experimental Procedures.” A, ethidium binding was enhanced by increasing concentrations of carbamylcholine (○) or of TMA (△); controls are also shown in the presence of 20 μM propridin (□). The data for carbamylcholine were fit to the Hill equation (Equation 3) and yielded a $K_{app}$ of 170 nM with a Hill coefficient of $n = 1.5$. The data for TMA were fit to a single-site binding isotherm (Equation 4) and yielded a $K_{app}$ of 3.3 μM. B, inhibition of ethidium fluorescence by varying concentrations of Ctx was measured in the presence of no TMA (○), 10 μM TMA (□), 20 μM TMA (▲), and 100 μM TMA (▼). Panel C, the $K_{obs}$ for inhibition of TMA binding by Ctx was determined by titration of increasing concentrations of Ctx into suspensions of AChR, ethidium, and TMA as shown in B. The $K_{obs}$ for inhibition at various TMA concentrations are replotted versus the TMA concentration. The fit to a line yields a slope of 0.028 with an intercept of 0.088 μM.
agreement with the conclusions of Groebe et al. (14) who used a Arg-9 → Ala mutation of α-conotoxin GI to analyze binding to the Torpedo and mouse AChRs.

Ionic interactions become more apparent in low ionic strength, where we observed similar affinities of Ctx for both sites on the Torpedo AChR, indicating a larger change in affinity for the αδ site. This shift is consistent with stronger attraction among opposite charges. If charge repulsion among cationic residues had been significant, lower ionic strength should have exacerbated the repulsion, resulting in relatively lower affinity. The observation of higher affinity, rather than lower, at the αδ site, reinforces the conclusion that charge repulsion between Lys-10 and the δArg-113 of the Torpedo AChR is not a factor that causes site selectivity in binding.

In low ionic strength, reducing the charge on Ctx by acetylation of Ctx and the N terminus results in a larger shift on the Torpedo αδ-subunit than the γγ-subunit. This suggests that attractive forces are lost upon neutralization and that they are more significant for the αδ-subunit than the γγ-subunit. The results further suggest the presence of distinct charge distributions at the γγ and αδ sites. For the αδ site, binding of Ctx is enhanced 300–600-fold upon lowering ionic strength, and this is reduced to a 1–3-fold effect for diacetyl-Ctx. This suggests that the primary stabilizing ionic interactions occur from the αδ site to Lys-10 or the N terminus. In contrast for the γγ site, the binding affinity of Ctx is enhanced about 30-fold upon lowering ionic strength, and this is similar for diacetyl-Ctx. Thus, ionic strength effects are similar even when charge is neutralized at Ctx Lys-10 and the N terminus. This suggests that the weaker stabilizing ionic interactions at the γγ site occur through one or more of the remaining ionic residues on Ctx, Arg-2 or His-5. The His-5 → Glu mutation shows similar effects at both sites of Torpedo AChR, suggesting a role for Arg-2; however, those experiments will need to be conducted in low ionic strength.

Examination of the γ- and δ-subunit N-terminal domain sequences does not lead to immediate insights into the difference in amino acids that could account for the binding in low ionic strength. Since the ionic changes reflect attractive interactions, the difference in binding should reflect differences in the distribution of anionic residues near the Ctx-binding sites. There are nearly two dozen such possible sites, some near the binding site loops and many not. Unfortunately, it is also unclear whether such differences in charge distribution will be important for binding since charge changes have slight effects on binding in physiological ionic strength.

Conotoxin Binds Independently of Conformation—Our data shows that Ctx binding is affected less than 2-fold by noncompetitive antagonists on both Torpedo and mouse AChR and that Ctx affects the binding of NCAs on the Torpedo AChR less than 2-fold. Ctx, therefore, binds equivalently to the desensitized and resting forms of the AChR. The results are consistent with a similar finding of Prince and Sine (15) that Ctx binding to muscle AChR was insensitive to the presence of proadifen. It can be inferred that Ctx does not interact strongly with residues that mediate conformational changes even though Ctx competes for ACh binding.

An alternative interpretation is that Ctx acts as a cap to block an entrance to the binding site but does not intimately contact the residues that normally stabilize binding. A capping mechanism should have permitted us to observe simultaneous binding of Ctx and a small ligand, such as TMA. We tested this notion explicitly by examining competition of Ctx with TMA. The mutual inhibition was linear over a 300-fold range of TMA concentrations, which represents an interaction energy of >3.4 kcal/mol. Allosteric effects were unlikely to account for this interaction since we had shown Ctx binding to be insensitive to the fundamental conformational change of desensitization. The interaction most likely represents sterier repulsion. Such a conclusion suggests that Ctx inserts a moiety into the most critical part of the ACh-binding site and does not merely cap access to the site. The moiety, however, is clearly not one of the Ctx cationic residues because they affect binding only weakly.

Our results show that charge-charge interactions between Ctx and the ACh-binding sites are weak, at best, under physiological conditions but reveal distinct arrangements of charged residues at each site or, as suggested by Sugiyama et al. (8), that Ctx binds in distinct orientations at the two sites. They furthermore show intimate binding of Ctx with the ACh-binding site that is independent of cationic residues on Ctx and that do not affect the conformation of the AChR. The lack of effect on conformation may be a consequence of not placing a cation in the critical part of the binding site but seems remarkable nonetheless.

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