Orientation of d-Tubocurarine in the Muscle Nicotinic Acetylcholine Receptor-binding Site*

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Ligand modification and receptor site-directed mutagenesis were used to examine binding of the competitive antagonist, d-tubocurarine (dTC), to the muscle-type nicotinic acetylcholine receptor (AChR). By using various dTC analogs, we measured the interactions of specific dTC functional groups with amino acid positions in the AChR γ-subunit. Because data for mutations at residue γTyr117 were the most consistent with direct interaction with dTC, we focused on that residue. Double mutant thermodynamic cycle analysis showed apparent interactions of γTyr117 with both the 2N and the 13′-positions of dTC. Examination of a dTC analog with a negative charge at the 13′-position failed to reveal electrostatic interaction with charged side-chain substitutions at γ117, but the effects of side-chain substitutions remained consistent with proximity of γTyr117 to the cationic 2N of dTC. The apparent interaction of γTyr117 with the 13′-position of dTC was likely mediated by allosteric changes in either dTC or the receptor. The data also show that cation-π electron stabilization of the 2N-position is not required for high affinity binding. Molecular modeling of dTC within the binding pocket of the acetylcholine-binding protein places the 2N in proximity to the residue homologous to γTyr117. This model provides a plausible structural basis for binding of dTC within the acetylcholine-binding site of the AChR family that appears consistent with findings from photoaffinity labeling studies and with site-directed mutagenesis studies of the AChR.

The muscle nicotinic acetylcholine receptor (AChR) is a member of the ligand-gated ion channel superfamily. It is a pseudo-symmetric pentamer with a subunit stoichiometry of α₂βγδ. The agonist-binding sites, which are responsible for activating channel opening, lie at the interfaces between the α- and γ-subunits and between the α- and δ-subunits (1). The two sites are similar and share conserved features contributed by their respective α-subunits as well as the amino acids that remain constant among the homologous γ- and δ-subunits. The γ- and δ-subunits, however, also determine affinity differences of the two sites for various ligands. Affinity differences between the sites extend to both agonists, such as epibatidine (2) and carbamylcholine (3), and to competitive antagonists, such as d-tubocurarine (4).

Identification of binding site residues has been carried out by affinity and photoaffinity labeling, by cross-linking studies, by analysis of expressed chimeric receptors, and by site-directed mutagenesis (5). These approaches have found that the binding sites consist of residues from two subunits from several regions (also referred to as loops) that are distant in the linear subunit sequences. Loop c in the α-subunit includes residues α184–α198, which constitute a significant part of the binding determinant for α-bungarotoxin (6). It also includes an unusual, highly conserved vicinal disulfide bond between αCys192 and αCys193 (7) and several conserved tyrosine residues (αTyr190 and αTyr198). The two other regions within the α-subunit include the residues αTyr93 (8, 9) and αTyr149 (10), respectively. The latter residue appears particularly important for interaction with agonist ammonium moieties (11).

Studies addressing the binding site contributions from the γ- and δ-subunits have likewise found contributions from sequence-separated regions of amino acids. Amino acid γTyr55 (12) lies within one region. A second region includes residues γLeu186–γTyr117. The alternating pattern of residues contributing to binding in this region led to the proposal that the sequence makes a hairpin turn in their vicinity of the binding site (13). Several other γ-subunit residues dispersed in the sequence contribute to ligand binding, either directly or through allosteric effects. They include γLys34, γSer181, γPhe173, and γAsp179 (14–16).

The detailed interactions between ligands and this sizable set of amino acid residues are largely unknown. However, the prevalence of aromatic amino acids as well as detailed studies using unnatural amino acid substitution support the hypothesis that cation-π electron interaction are critical for stabilizing the ammoniums of agonists (11, 17). Such studies also yielded observations on the possible interactions of several other aromatic residues; Nowak et al. (17) showed that αTyr93 is likely to act as a hydrogen bond donor, whereas the aromatic ring of αTyr198 appears to interact with the quater-

α-bungarotoxin; CTX, α-conotoxin MI; dTC, d-tubocurarine; HPLC, high performance liquid chromatography; PEI, polyethyleneimine; PBS, phosphate-buffered saline; wt, wild type.
nary ammonium of acetylcholine.

Antagonists such as dTC have been utilized to study binding site interactions, partly because of the significant affinity difference between the two binding sites. dTC has 100–500-fold higher affinity for the αγ site than the αδ site (4). Nonetheless, α-subunit residues also affect binding of dTC. αTyr198 has a strong impact on dTC affinity; when mutated to αTyr198, the AChR displayed a 10-fold greater affinity for dTC, but the mutation had little effect on acetylcholine affinity or efficacy (18). This suggests a unique interaction between the αTyr198 hydroxyl and dTC. Because mutations at αTyr109 and γTyr117 had similar effects on metocurine (sometimes referred to as dimethyl-tubocurarine) affinity, it was proposed that these amino acids each stabilize one of the two quaternary ammoniums on metocurine through cation–π electron interactions (19).

The recent atomic resolution structure of the acetylcholine-binding protein from Lymnaea stagnalis (AChBP), a protein homologous to the N-terminal, ligand-binding domain of the AChR, shows that many of the residues implicated by the studies listed above are in proximity to a binding pocket that can contribute to stabilization of binding of agonists and antagonists (20). The structure included a solvent Heps molecule that indicates the likely binding locus. The structure is in accord with many of the prior observations regarding ligand-binding site structure and substantially refined the current thinking about ligand-receptor interactions. However, the details of the interactions with cholinergic ligands remain to be fully elucidated, as do the conformational changes that correlate with channel opening and desensitization.

Our previous studies (21, 22) have taken advantage of dTC analogs to examine the interaction of various functional groups to binding interactions and conformational transitions. The structures of the analogs are shown in Fig. 1. For both mouse and Torpedo AChRs, we demonstrated that the stereochemistry at the 1 carbon was important for high affinity binding but that the ammoniums at the 2- and 2'-positions need not be quaternary for high affinity binding (21). Furthermore, dTC ring D, which includes the 12- and 13'-positions, interacts in a site-selective manner, consistent with a possible interaction with the γ- and δ-subunits. 13'-Modification further altered the propensity of dTC to desensitize the Torpedo AChR, an observation that may serve as a clue for the structural changes that occur upon conformational shifts of the AChR. In order to pinpoint such interactions between a ligand functional group and a particular amino acid residue, ligand binding energies must be examined in concert with receptor mutagenesis.

In this study, we utilize a double mutant thermodynamic cycle analysis to analyze the interaction between our library of dTC analogs (21) and residues in the AChR-binding site, with particular emphasis on the interaction with residue γTyr117. We demonstrate that both the 2-N and the 13'-positions of dTC appear to interact with this amino acid but with a proximal interaction only to the 2-N. Based on this conclusion and using the structure of the AChBP, we present a plausible structural model for the orientation and position of dTC within the binding pocket.

**EXPERIMENTAL PROCEDURES**

**Materials**—The α-subunit cDNA clone of the mouse nicotinic AChR was a gift from Dr. Mike White (MCP Hahnemann University); the β-, γ-, and δ-subunit cDNAs were gifts from Dr. James Patrick (Baylor College of Medicine). Restriction enzymes were purchased from Invitrogen or from New England Biolabs (Beverly, MA). The QuickChange mutagenesis kit was obtained from Stratagene (La Jolla, CA), and Endo-free Mega and Giga kits were obtained from Qiagen (Valencia, CA). HEK293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). PEI was obtained either from Miles or from Aldrich. 125I-α-Bungarotoxin was obtained from Amersham Biosciences. Whatman GF/C filters were obtained through VWR Scientific (Houston, TX). All other chemicals were obtained from Sigma or from standard sources.

**Synthesis of d-Tubocurarine Analogues**—Synthesis of most of the d-tubocurarine analogs was described previously (21). Synthesis of several new analogs is described as follows. For 13-sulfo-d-tubocurarine (sulfo-dTC, 7,12'-dihydroxy-6,6'-dimethoxy-13'-sulfonate-2,2'-trimethylcurarine trifluoroacetate), 1 g of dTC was treated with 5 ml of concentrated H2SO4 on ice for 3 h. The product was precipitated by dilution with 500 ml of cold ether and collected by centrifugation. The product was purified by cation exchange chromatography over a CM-25 Sephadex column (Amersham Biosciences) and eluted with a gradient of 50–400 mM ammonium acetate, pH 6.8. Pure fractions were collected and lyophilized with a net yield of 25%. The material was desalted on a reversed phase Beckman C-18 Ultrapure preparative HPLC column (22 × 150 mm). The column was eluted with a gradient of 10–40% of 0.9% trifluoroacetic acid/CH3CN. The final product was lyophilized to a white powder. The identity was verified by mass spectrometry (m/z = 689 and 345, for the di-cation). For diacetyl-d-tubocurarine (7,12'-dihydroxy-6,6'-dimethoxy-2,2'-2'-trimethylcurarine dinitrate), 1.07 g of dTC was treated with 2.5 ml of acetic anhydride in 8 ml of glacial acetic acid and 874 mg of sodium acetate, essentially according to Dutcher (23). The product was isolated by crystallization of the nitrate salt with a yield of 86%. This product was 95% pure as judged by reversed phase HPLC, the remaining contaminant corresponded to a 1,10-diacetate compound. For diacetyl-d-chondocurarine (6,6'-dimethoxy-13'-ido-2,2'-2'-tetramethylcurarine trifluoroacetate), 74.5 mg was dissolved in 0.1M acetate buffer (pH 5.6, 100 ml) on ice. After 30 min of cooling, 0.5 ml of neat 2-n-mercaptoethanol was added to quench the excess ICI. The mixture was diluted to 250 ml, adjusted to pH 8.0 using NH4OH, and subjected to a CM-25 Sephadex cation exchange column chromatography. The column was eluted with a linear gradient from 50 to 300 mM NH4HCO3, pH 8.0. The fractions containing pure product were identified by reversed phase HPLC, pooled, and lyophilized. The dry salts were dissolved in 0.1% trifluoroacetic acid/H2O and applied to a 21.2 × 150-mm Ultraprep C18 reversed phase HPLC column (Beckman) in four batches and then eluted with a gradient of 20–50% CH3CN over 60 min. The pure fractions were pooled and lyophilized to a white powder (47.4 mg, 49 μmol, 25%). This product was judged pure by HPLC, UV-visible spectroscopy, 1H NMR spectroscopy (two aromatic protons were shifted to 8.76 and 6.72 compared with 8.76 and 6.06 of chondocurarine), and mass spectrometry (m/z = 749.2, 749.1 for the double ion). For di-demethyl-d-tubocurarine (6,6',7,7-dibromo-2,2'-2'-trimethylcurarine trifluoroacetate), dTC (10 mg) was heated to 160 °C in 300 μl of phosphoric acid. The reaction mixture was diluted with 0.1% trifluoroacetic acid/H2O and applied to a preparative reversed phase HPLC column. A major and a minor product were isolated, both essentially pure, and lyophilized. The product, to product corresponding to 6,6'-dimethoxy-2,2'-dimethylcurarine (verified by mass spectrometry (m/z = 581.2 and m/z = 291.2 for the double ion). The minor product was singly demethylated.

**Recombinant Plasmids**—For the expression studies, the cDNA of each subunit of the mouse muscle AChR (α, β, γ, and δ) was released from the original plasmid by using appropriate combinations of restriction enzymes and purified by agarose gel electrophoresis. The cDNA for each of the four subunits was subsequently cloned into the eukaryotic expression vector pcDNA3 (Invitrogen) to create the expression plasmids pCDNA3-NA-α, pCDNA3-NA-β, pCDNA3-NA-γ, and pCDNA3-NA-δ, respectively.

**Site-directed Mutagenesis**—The pCDNA3-NA-γ plasmid was digested with the restriction enzymes EcoRV and XhoI, and the resulting two DNA fragments were purified by agarose gel electrophoresis. The small DNA fragment (NA-γ-Y) contained the sites for introducing the desired mutations, and the large DNA fragment (pCDNA-NA-γ-L) contained the remainder of the γ-subunit cDNA attached to the pCDNA vector; it was saved for later use. The NA-γ-Y DNA fragment was subsequently cloned into the pBlueScript vector (Stratagene) that was pre-digested with the restriction enzymes EcoRV and XhoI. Point mutations were created by site-directed mutagenesis using appropriate pairs of mutagen-specific oligonucleotide primers and the QuickChange mutagenesis kit, following the manufacturer’s protocol. Subsequently, the plasmid DNA was transformed into the Escherichia coli XL-1 blue. The overnight cultures of three to four colonies from each transformation plate were grown, and plasmids were purified from these cultures using the Wizard plasmid purification kit (Promega, Madison, WI). The sequence of the entire DNA insert of each putative mutant plasmid was analyzed to confirm the desired mutation and to verify the sequence.
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fidelity of the rest of the DNA. The Na-γ-S DNA fragment containing a given mutation was then released from the plasmid using the restriction enzymes EcoRV and XhoI, purified by preparative agarose gel electrophoresis, and ligated with the previously purified pCDNA.NA-γ-L DNA fragment (see above). After verification by DNA sequencing, a recombinant plasmid containing the mutated full-length Na-γ-eDNA was selected for large scale purification. Large scale purifications were carried out using either the Endofree Plasmid Mega (for more than 500 ml of culture) or the Endofree Plasmid Giga (2,500 ml of culture) kit. This purification was necessary for optimal expression of AChRs.

Cell Culture and Transfection—HEK293 cells were grown to 70–90% confluence in 100-mm culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 unit/ml penicillin, and 0.1 μg/ml streptomycin. In overnight incubation, 250 μl of PEI stock was diluted with 1.5 ml of sterile PBS, and the DNA was then added and mixed. The PBS/PEI/DNA mixture was incubated for 20–45 min at ambient temperature before addition to the culture media. The old culture medium was then removed from the dish, and the PBS/PEI/DNA mixture was added. Cells were reseeded into a new dish and harvested 48–72 h after transfection. The DNA was then released from the plasmid using the restriction enzymes EcoRV and XhoI, purified by preparative agarose gel electrophoresis, and ligated with the previously purified pCDNA.NA-γ-L DNA fragment (see above). After verification by DNA sequencing, a recombinant plasmid containing the mutated full-length Na-γ-eDNA was selected for large scale purification. Large scale purifications were carried out using either the Endofree Plasmid Mega (for more than 500 ml of culture) or the Endofree Plasmid Giga (2,500 ml of culture) kit. This purification was necessary for optimal expression of AChRs.

Ligand Binding—[H]Acetylcholine binding and binding of 125I-labeled-BgTx to Torpedo AChR was carried out as described previously (21). For determination of binding constants to mutant mouse AChR expressed in tissue culture cells, the following protocol was observed. Cells were scraped into PBS, centrifuged, and resuspended in high potassium/Ringer's containing 140 mm KCl, 5.4 mm NaCl, 1.8 mm CaCl2, 1.7 mm MgCl2, 25 mm Hepes, pH 7.0, with 30 μg/ml bovine serum albumin (16). Samples were incubated with the indicated concentration of competing ligand for 30 min at ambient temperature before the addition of 0.1–0.3 nM 125I-labeled-BgTx (2000 Ci/mmol). A first order binding rate was observed for more than 2 h. Binding was stopped at 2 h with excess, cold, nonradioactive [3H]Acetylcholine and the samples were stored on ice. Samples were filtered and washed with PBS over GF/C filters that had been soaked cold overnight with 4% Carnation Instant Nonfat Dry Milk in 0.1 unit/ml penicillin, and 0.1 μg/ml streptomycin. Data were plotted and analyzed using SigmaPlot (Jandel, CA). Data were plotted and analyzed using SigmaPlot (Jandel, CA). Sequence analysis revealed that the site (25).

Results

In order to examine the proximal relationship of AChR amino acid residues to functional groups on dTC, we assessed the degree of interaction by double mutant thermodynamic cycle analysis. In general, the affinities of mutant and wild type AChRs were measured for a pair of dTC analogs. The four K values thus obtained were used to determine the value of the interaction coefficient, Ω (see "Experimental Procedures"). An initial goal was to identify amino acids that had specific interactions with dTC. Our previous work (21, 22) had shown that modification of dTC to metocurine or to iodo-dTC shifted the ligand affinities and that the magnitude of the shifts differed at the two binding sites. Comparison of dTC with these analogs would test for interactions at the 2-N ammonium and at the 7-, 12-, and 13-C positions. Therefore, our preliminary experiments utilized these two analogs, although not exclusively.

To determine amino acids likely to interact at these dTC sites, we focused on those γ-subunit residues that had been identified to contribute to site-selective binding. Because dTC binds with higher affinity to the γ1 site than to the δ site on both Torpedo AChR and mouse AChR, γ-subunit amino acids were initially mutated to those amino acids found in the homologous, aligned positions of δ- or ε-subunits of either Torpedo or mouse muscle AChR. We first examined those residues identified by chimeric constructs to contribute to site-selective dTC binding: γ1Le116, γ1Tyr117, and γ1Ser161 (16). Conservationary mutations at γ1116M and γ1116V displayed small, 2–4-fold affinity increases for metocurine over the wild type AChR and small affinity decreases for iodo-dTC. Mutations γ1117T and γ1117S displayed significant (10-fold) affinity changes for metocurine as well as for chondocurarine, a ligand that differs from dTC by a single 2-N methyl group (see Fig. 1), whereas smaller affinity changes were observed for dTC itself. Mutations γ1S161A, γ1S161M, and γ1S161K had more varied effects as follows: γ1S161A caused only small changes in iodo-dTC affinity; γ1S161M caused 5-fold lower affinities in several dTC derivatives; and γ1S161K affected neither dTC nor chondocurarine affinity but caused a 5-fold reduction of iodo-dTC affinity. Although there were clearly observed changes in affinity, further experiments with γ1Ser161 mutants failed to reveal interaction with any particular locus on dTC. Although the mutations we

was a package HyperChem (version 5.1, Hypercube Inc., Gainesville, FL) using the MM+ force field. The initial structure of dTC was taken from the coordinates of a crystal form (28) and was energy-minimized, which produced only minor changes in the structure. Initial simulations were sped up by using a reduced structure of the AChBP. Three of the five subunits were deleted; of the remaining two subunits, only the amino acids in immediate ligand contact plus one layer of surrounding amino acids were considered during energy minimization. For the initial manual alignment of dTC in the AChBP-binding site, dTC was placed in the binding site with the 2'-ammonium at the site of the ethanesulfonic acid-piperaziner nitrogen of Heps, a ligand found in the original structure of the binding site (20). dTC was energy-minimized in several rounds without allowing changes in protein structure. To resolve steric conflicts or trapping in local energy minima, occasional manual changes in dTC location were made. When a preliminary structure was obtained for bound dTC, it was then merged with two complete subunits of the acetylcholine-binding protein. Subsequent dTC energy minimization included minimization of all contact residues in the context of two complete AChBP subunits.

To mimic the structure of the mouse AChR αγ-binding site, the aligned, homologous mouse residues were substituted for the side chains of contact residues. The substituted side chains are shown in Fig. 8C. After each substitution, the side chain was minimized to conform to the surrounding protein environment. dTC was then energy-minimized in the context of the new structure. Two separate rounds of minimizations were performed. First, dTC was minimized while the protein was kept rigid. Second, after the approximate dTC conformation was found, dTC and 31 nearby residues were then all minimized collectively to yield the final structure.

RESULTS

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examined at residues γLe116, γTyr117, and γSer161 had the expected effects on metocurine affinity, according to the observations of Sine (16); only mutations at γTyr117 correlated with specific dTC interactions.

Next, we examined two residues found to be photoaffinity-labeled by dTC or by 4-benzoylbenzoyloxyline (13, 29), γLeu109 and γSer161. Neither conservative nor nonconservative mutations of γLeu109 produced substantial changes in the affinities for dTC or of several analogs. γS111Y caused small changes in affinity, whereas γS111R resulted in consistent 3–10-fold changes on all analogs tested. We further tested a residue implicated in the site-selective binding of α-conotoxin, γPhε172 (15). Mutation of γF172D produced little change in dTC affinity. γF172H caused only small changes in affinity for several analogs.

Of all the residues tested, mutations at γTyr117 yielded Ω values that most consistently correlated with structural changes in dTC. Therefore, subsequent experiments focused on this residue in more detail. This residue had been further examined by Fu and Sine (19) who proposed that stabilization of curare-type antagonist binding occurred through π-electron interactions to one of the two curare ammoniums. Our preliminary experiments were consistent with γTyr117 interactions at the 2-N ammonium but did not exclude other possible interaction loci.

γTyr117 Interactions with dTC—Two ligands, dTC and chondocurarine, were compared to test 2-N interaction with γTyr117. We initially examined two mutants, γY117F and γY117A, that represent a conservative change and a large size change in side-chain structures, respectively. The mutation γY117F resulted in higher affinity for both dTC and chondocurarine (Fig. 2B) with similar 10-fold changes in both ligand affinities. The calculated interaction coefficient Ω, therefore, was near 1. In contrast, the γY117A mutation resulted in higher affinity for dTC than for chondocurarine, yielding an average Ω value of 10 (Fig. 2A). This result suggested that there is an interaction between the 2-N and γTyr117 for large changes in side-chain structure, whereas the more conservative change from γY117F yielded higher affinity for both ligands but no apparent interaction. These two mutations, γY117A and γY117F, yielded similar affinities for dTC, despite the disparity in side-chain structure. The difference in size suggests that simple steric effects alone do not account for the interaction. Because alanine is a non-aromatic residue, cation–π-electron stabilization also appears not to be required for high affinity binding.

To assess whether the 2-N position was truly the predominant interaction locus and to assess the nature of the interaction, we examined the interaction between a broader selection of ligand pairs and γTyr117 substitutions. The γY117A substitution had such a strong effect on dTC affinity that we also examined γY117F as representative of a large, aliphatic amino acid. γY117D and γY117K were included to examine charge effects on binding. We chose ligand pairs to examine specific functional groups on dTC in matched experiments. The results of this series of experiments are presented in Fig. 3, and a summary of corresponding Kᵢ values obtained for wild type and mutant receptors is found in Table I.

Comparison of tubocurarine and dTC reflects a change of a methyl group at the 2’-N (Fig. 3). This change yielded uniformly low Ω values, indicating no interaction between the 2’-N functional group and γTyr117. Comparison of dTC with chondocurarine reflects a change of a methyl group at the opposite, 2-N ammonium. This change displayed an Ω value ranging from 1 to 20 that depended on the mutation that was analyzed. Examination of the 7’-hydroxyl, 12’-hydroxyl, the 7’,12’-dihydroxyl (as a pair), and 6,6’-methoxy groups (as a pair), generally yielded low interaction values. Comparison of dTC with its 13’-substituted analog, iodo-dTC, revealed interactions of similar magnitude to those observed for the 2-N substituted analog. Overall, these data suggest interactions of γTyr117 with the 2-N of dTC and with the 13’-position of dTC with the degree of interaction highly dependent on the particular side-chain substitution.

Electrostatic Interactions at the 13’-Position—The Reynolds

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**Table 1: Structures of d-Tubocurarine analogs.** This set of ligands includes several new derivatives: diacetyl-dTC, di-demethyl-dTC, iodo-chondocurarine, and sulfo-dTC.

| NAME | R₁ | R₂ | R₃ | R₆ | R₇ |
|------|----|----|----|----|----|
| d-Tubocurarine (dTC) | CH₃ | H | H | CH₃ | CH₃ |
| Acetyl-dTC | COCH₃ | CH₃ | H | CH₃ | CH₃ |
| Demethyl-dTC | H | CH₃ | H | CH₃ | CH₃ |
| Metocurarine | H | H | H | CH₃ | CH₃ |
| 7’-O-methyl-chondocurarine | CH₃ | CH₃ | CH₃ | CH₃ | CH₃ |
| 12’-O-methyl-chondocurarine | CH₃ | CH₃ | CH₃ | CH₃ | CH₃ |
| Iodo-dTC | CH₃ | H | H | CH₃ | CH₃ |
| Iodo-chondocurarine | CH₃ | H | H | CH₃ | CH₃ |
| Sulfo-dTC | CH₃ | H | H | CH₃ | SO₂CH₃ |

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**Fig. 2. Interaction of the dTC 2-N with γTyr117.** The binding constants of dTC (○, □) and chondocurarine (×, △) were determined for A, wild type AChR (○, △) and γY117A (×, △), and for B, wt and γY117F (○, △), in the presence of 80 nM CTX by inhibition of the initial rate of [¹²⁵I]γ-BgTx binding as described under “Experimental Procedures.” The solid lines indicate the best fits to a single binding site model. Each symbol represents the average of duplicate determinations. The following Kᵢ values are reported in nM, A, wt-dTC, 108; wt-chondocurarine, 122; γY117A-dTC, 6; γY117A-chondocurarine, 31; γY117F-dTC, 11; γY117F-chondocurarine, 7; Ω = 0.6.
and Palmer (28) crystal structure of dTC shows the distance between the 2-N and the 13′-position to be 7.4 Å, a distance that is near the longest dimensions of the amino acid side chains examined. Therefore, it seemed unlikely that there would be van der Waals contact between γTyr117 and both the 2-N and the 13′-positions. To determine whether one functional group represented a physically closer interaction than the other, we examined the binding of another 13′-derivative, sulfo-dTC. This compound bears a negatively charged sulfonate at the 13′-position (see Fig. 1). With this ligand, we could gauge proximity through charge attraction or repulsion to γY117 when that residue was mutated to charged side chains.

We measured affinities of three ligands, dTC, sulfo-dTC, and chondocurarine (Fig. 4, A–C, respectively) for the AChR with mutations to the ionic side chains γY117D and γY117K. For comparison, we also measured the affinities for their respective closest neutral congeners, γY117N and γY117M. Comparison of dTC affinities with sulfo-dTC affinities (Fig. 4, A and B) shows similar patterns of affinity shifts upon side-chain substitution. Sulfo-dTC had ∼100-fold lower affinity for each side chain substitution than dTC, indicating that the 13′-sulfonate moiety does decrease affinity. However, the affinity shifts were similar, regardless of the charge change on the γY117 side chain. This result indicated that 13′-sulfonate was not likely in proximity to γY117 side chains. To quantify the degree of interaction, we determined the corresponding Ω values for both changes, from neutral to cationic and from neutral to anionic side chains. Both Ω values were near 1 (Table II), values consistent with no electrostatic interaction.

For further comparison, measurement of iodo-dTC affinities for these mutated AChRs were intermediate between those of dTC and sulfo-dTC but retained a similar pattern of affinity shifts (Table II). This observation was also consistent with a lack of proximal interaction between the 13′-position and γY117.

**Fig. 3.** The 2-N and the 13′-positions interact with γTyr117. Ω values were determined for selected γTyr117 mutants and ligand functional groups by measuring binding constants for ligand pairs against wild type and mutant receptor pairs as described under “Experimental Procedures.” The ligand pairs used to determine interaction for each functional group were as follows: tubocurine versus dTC (2′N); dTC versus chondocurarine (2N); chondocurarine versus metocurine (7′ & 13′ OH); 7′-O-methyl-chondocurarine versus chondocurarine (7′ OH); 12′-O-demethyl-dTC versus chondocurarine (12′ OH); dTC versus di-demethyl-dTC (6′ & 6′ OH); dTC versus iodo-dTC (13′).

**Table I.** Binding of d-tubocurarine analogs to wild type and mutant mouse AChRs. ANOVA values for ligands were determined by inhibition of [3H]Baclofen binding to wild type and mutant mouse AChR as described under “Experimental Procedures.”

| Mutant    | Tubocurine (K<sub>i</sub>) | Chondocurarine (K<sub>i</sub>) | Metocurine (K<sub>i</sub>) | Iodo-dTC (K<sub>i</sub>) | Sulfo-dTC (K<sub>i</sub>) |
|-----------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Y117A     | 110 ± 40 (4)                | 500 ± 200 (4)               | 670 ± 120 (2)               | 2100 ± 200 (2)              | 6700 ± 500 (4)              |
| Y117F     | 150 ± 40 (4)                | 500 ± 200 (4)               | 670 ± 120 (2)               | 2100 ± 200 (2)              | 6700 ± 500 (4)              |
| Y117N     | 150 ± 40 (4)                | 500 ± 200 (4)               | 670 ± 120 (2)               | 2100 ± 200 (2)              | 6700 ± 500 (4)              |
| Y117T     | 150 ± 40 (4)                | 500 ± 200 (4)               | 670 ± 120 (2)               | 2100 ± 200 (2)              | 6700 ± 500 (4)              |
| Y117D     | 150 ± 40 (4)                | 500 ± 200 (4)               | 670 ± 120 (2)               | 2100 ± 200 (2)              | 6700 ± 500 (4)              |
| Y117K     | 150 ± 40 (4)                | 500 ± 200 (4)               | 670 ± 120 (2)               | 2100 ± 200 (2)              | 6700 ± 500 (4)              |

The errors listed are standard deviations from three independent determinations, as given in the parentheses.

* indicates not determined.
It remains unclear whether the consistently lower affinities observed for sulfo-dTC were due to the volume of the sulfonate ion, to its negative charge, or to both. When the neutral to charged amino acid changes were examined with chondocurarine, the dTC analog with an added methyl group on the 2-N, the pattern of side-chain effects on affinity was clearly distinct from that seen for dTC and sulfo-dTC (Fig. 4C). This was consistent with interaction between γ117 and the 2-N. Because we do not have an analog that neutralizes or reverses charge at the 2-N position, it was not possible to test electrostatic interactions by Ω values. Nonetheless, affinity shifts upon charge changes at γ117 were clearly consistent with cationic interactions; mutation of γY117K lowered affinity for all the ligands tested, whereas mutation of γY117D increased affinity (Table I and Table II), albeit to a lesser extent than the decrease caused by γY117K. These data suggest a proximal interaction between γTyr117 and the 2-N ammonium of dTC.

Interactions of Iodo-chondocurarine—An explanation for the apparent interaction of γTyr117 with both the 2-N position and the 13'-position, as seen in Fig. 3, is that the 13'-modifications shift the position or the conformation of dTC within the binding site such that interactions with the 2-N position are affected. Such a linkage in binding between the two dTC functional groups should be apparent in a thermodynamic cycle analysis of dTC analogs. To complete such an analysis, we synthesized a ligand that had been derivatized at both the 13'- and the 2-N positions, iodo-chondocurarine (Fig. 1).

**Table II**

| Receptor   | dTC  | 1-dTC | S-dTC       | K_{in,toc}/K_{toto} | Ω     |
|------------|------|-------|-------------|---------------------|-------|
| γTyr117 (wt)| 130±30 (8) | 163±9 (4) | 1710±230 (4) | 1.0                 |       |
| Y117D      | 11±7 (7)   | 333   | 900±90 (4)  | 84                  | 1.4   |
| Y117N      | 48±20 (7)  | 573±5 (2) | 5500±1800 (5)| 115                 |       |
| Y117K      | 6100±1600 (7) | 31000±4000 (2) | 600000±220000 (3) | 98                 | 1.0   |
| Y117M      | 53±22 (7)  | 670±120 (2) | 5240±1100 (5) | 99                  |       |

![Fig. 4. Electrostatic interactions between γTyr117 and dTC 13'-position.](Image)

**Fig. 4.** Electrostatic interactions between γTyr117 and dTC 13'-position. The affinities of AChR mutants γY117D (○), γY117N (△), γY117K (■) and γY117M (◆) for dTC (A), sulfo-dTC (B), and chondocurarine (C) were determined by incubating the appropriately transfected HEK293 cells with the indicated concentrations of the competing ligands. The initial rate of 125I-β-gtx binding was then measured in the presence of CTX as described under “Experimental Procedures.” Solid lines indicate the best fits to a single binding site model.

**Fig. 5.** Iodo-chondocurarine binding to Torpedo and to mouse AChR. A, binding constants for dTC (○), chondocurarine (◆), iodo-dTC (□), and iodo-chondocurarine (■) were determined by inhibition of the initial rate of 125I-α-Bgtx binding to Torpedo AChR-rich vesicles as described under “Experimental Procedures.” Data points are averages of duplicate determinations; the solid curves represent the best fits of the data to two-site binding models with a variable site ratio. B, K<sub>j</sub> values for iodo-chondocurarine binding to mouse AChR in the absence (○) and presence (□) of 40 nM CTX were determined by inhibition of the initial rate of 125I-α-Bgtx as described under “Experimental Procedures.” Data points are averages of triplicate determinations; the solid curves are the best fits to two-site and to one-site binding models, respectively. C, K<sub>j</sub> values for binding of dTC (○), iodo-chondocurarine (◆), and iodo-chondocurarine (■, ◆) to γY117K (○, ◆) and γY117T (□, ■) were determined in the presence of 40 nM CTX. The solid curves represent the best fits to a single site binding model.
Initial characterization of this compound by Torpedo AChR binding revealed substantially higher site selectivity between the αγ and αδ site affinities, when compared with dTC, chondocurarine, and iodo-dTC (Fig. 5A). Affinities for the mouse AChR sites were similarly affected with slightly enhanced affinity for the αγ site and 3–4-fold decreased affinity for the αδ site (Fig. 5B and Table III). Binding in the absence and presence of 40 nM α-conotoxin MI to block binding to the αδ site confirmed that iodo-chondocurarine bound with higher affinity to the αγ site of the AChR. The iodo-chondocurarine affinity for the αγ site was not significantly different (Table III) from the affinities of dTC, chondocurarine, and iodo-dTC (Table I), indicating no interaction between the 13′ and 2-N functional groups in the context of the native γTyr117 residue.

The affinity changes at the αδ site, however, were significant. Because the natural amino acid in the δ-subunits of both mouse and Torpedo AChR in the position homologous to γTyr117 is Thr and because the γY117T mutation caused large changes in affinity of chondocurarine (Table I), we examined the interactions of γY117T mutation with iodo-chondocurarine. For comparison, we also examined the closest congener, γY117S, the difference in side-chain structure being the loss of a methyl group. Binding measurements of dTC and iodo-chondocurarine show that dTC affinity was affected strongly by this change in side-chain structure, whereas the affinities for iodo-chondocurarine were insensitive to the change in side-chain structure but were substantially lower (Fig. 5C).

We constructed a set of linked thermodynamic cycles (Fig. 6) using the Kᵢ values in Tables I and III. The outer edges of the diagram (in black) constitute a cycle that represents stepwise ligand structural changes for the γY117T mutant (Ω = 3.7); the inner cycle (in gray) has the corresponding changes for the γY117S mutant (Ω = 1.4). The Ω values indicate that the interaction between 13′- and 2-N positions is stronger in the context of the mutation γY117T than γY117S. The corresponding Ω value for the native γTyr117 is 1.4, which is similar to that for γY117S. The four trapezoidally shaped cycles, which connect the inner and outer cycles, represent the interactions between the amino acids and stepwise changes in ligand functionality. Addition to dTC of either the 2-N-methyl or the 13′-iodine alone yielded low Ω values of 1–2. However, further addition of either second substituents produced higher interaction values (4.3 and 10); thus, interaction was only observed upon the second ligand modification. Examination of the affinities for the γY117T mutant shows that either single dTC modification resulted in decreased ligand affinity, but the second modification did not further affect affinity. In contrast, for γY117S, each modification decreased affinity independently. Whether interactions occur appears to be highly sensitive to the amino acid residue context at position γY117.

**TABLE III**

| Receptor          | Method               | αγ      | αδ     |
|-------------------|----------------------|---------|--------|
| Torpedo AChR      | ¹²⁵I-α-BgTx          | 48 ± 6  | 41,000 ± 2,000 |
| Torpedo AChR      | [³H]acetylcholine    | 23 ± 2  | 137,000 ± 12,000 |
| Mouse AChR, wt    | ¹²⁵I-α-BgTx          | 97 ± 46 | 6,500 ± 3,600   |
| Mouse AChR, Y117S | ¹²⁵I-α-BgTx          | 160 ± 90|         |
| Mouse AChR, Y117S | ¹²⁵I-α-BgTx          | 5,455 ± 730|   |
| Mouse AChR, Y117T | ¹²⁵I-α-BgTx          | 6,301 ± 2,700| |

* These experiments were carried out in the presence of 40 nM CTX to block binding to the αδ site.

**Fig. 6. Thermodynamic cycle analysis of the interaction of 13′-iodo and 2-N methyl groups with γY117.** The Ω, interaction coefficient values for the 6 possible cyclic combinations of four ligands, dTC, chondocurarine (C), iodo-dTC (dTC), and iodo-chondocurarine (ICC) binding to two AChR mutants, γY117S and γY117T, were determined from the averaged Kᵢ values in Tables I and III. Each Kᵢ value is represented in the figure by the ligand abbreviation. The outer black cycle represents the values for binding to γY117T. The inner gray cycle represents binding to γY117S. For each ligand, the values for binding the two AChR mutants are connected with an arrow that lies between the outer black cycle and the inner gray cycle. The four trapezoidally shaped cycles thus created also form thermodynamic cycles. The Ω values are shown inside each individual cycle, except for the γY117T, outer black cycle, where the value is given outside the diagram.
chain substitutions, without β-carbon branched groups, have slopes similar to those that do (e.g. γY117A, γY117N, and γY117D), indicating no formal interaction.

Modeling of dTC into the Structure of the AChBP—We computer-modeled dTC into the binding pocket of the AChBP to determine whether our observations on the interaction of dTC were consistent with the structure of this binding site. To mimic the conditions of the mutation experiments, we further changed the side chains of the residues in contact with dTC to those of the mouse AChR. We used the aligned α-subunit residues for those corresponding to the A-chain of the AChBP and the γ-subunit residues for those corresponding to the B-chain.

Energy minimization yielded a satisfactory structure of dTC in the binding pocket without bad contacts or unrealistic bond lengths and angles. The binding site residues moved only minimally in order to accommodate dTC (Fig. 8). Movement of A-Trp143 (homologous to AChR dTrp144) was critical for accommodating the 6'-hydroxymethyl moiety of dTC (compare Fig. 8, C and D). The displacement was primarily rotation about the tryptophan α-β carbon bond (48°) and the β-γ carbon bond (29°). Without this movement, rings A and B of dTC became severely distorted during minimization. There was also movement of A-Tyr192 (α′Tyr193) about its β-γ carbon axis to accommodate the movement of A-Trp143, lesser movement of A-Tyr195 (α′Tyr196) to accommodate the bulk of the 2′-quaternary ammonium, and of A-Tyr192 (α′Tyr198) due to steric interaction between its 4-hydroxy and dTC ring D.

This model is appealing because the dTC 2-N is clearly opposed to γTyr117, as suggested by our data, and the opposite, 2′-ammonium, is deep in the conserved binding pocket, which is also consistent with our data. dTC maintains close protein contact in the binding pocket without gross disturbance of the backbone configuration. Although it is possible that dTC can have distinct binding modes, the simplest alternatives appear unlikely. Rotation of dTC about its long axis places the 2′-N quaternary ammonium nearer dTrp149 and γTrp52; however, this part of the binding site is sterically too crowded to accommodate the 2′-ammonium without significant distortion of the A and B rings of dTC or substantial movement of the loop c region. Rotation of dTC about the axis normal to the structure in Fig. 8A yielded a plausible, energy-minimized structure with the 2′-N near γTyr117 and the 2-N deep in the conserved pocket. Although this orientation may constitute an alternative binding mode, it is inconsistent with the observed interactions between γTyr117 and the 2-N.

The conformation of bound dTC is distinct from the conformations observed by atomic resolution structures of curare compounds (28, 30–32) and from any of the low energy conformations of dTC found by Zhorov and Brovtsyna (33) using molecular modeling. The region of the 2-N and 1-C of dTC is relatively strained, and if dTC in its bound conformation is removed from the context of the protein and permitted to relax by energy minimization, this region changes structure. The bound conformation is, therefore, not a local minimum energy state of dTC but is stabilized by close interactions with the surrounding protein. The close contact with the binding site in the current model suggests that binding of distinct dTC conformers would require substantial movement of the protein to bind. Energy minimizations with dTC placed in the binding pocket in the same general orientation but with various other conformations often displaced α-subunit loop c (residues 188–199; data not shown) with concomitant disruption of contacts between loop c and the γ-subunit. If such dislocations of the protein occur naturally, this might permit alternative modes of dTC binding beyond those considered here.

**DISCUSSION**

Our goal was to determine the nature of interaction of dTC with the AChR. In addition, we tested whether a double-mutant thermodynamic cycle analysis could be applied to analysis of ligand-protein binding in a system where the ligand was substantially smaller than the peptide toxins typically employed (e.g. Refs. 34 and 35). During the course of the studies, predominantly weak interaction energies were observed; therefore, determination of proximal interactions required examination of the patterns from many mutations. γTyr117 apparently interacts with dTC at both the 13′ and the 2-N positions; however, the results from charge changes were inconsistent with proximity to the 13′-position. Therefore, the 13′-interactions are likely allosteric: changes in structure of either dTC or

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**Fig. 7.** The affinities of dTC and chondocurarine are affected by γ117 side-chain structure. $K_i$ values for inhibition of $^{125}$I-α-Bgtx binding to wild type and mutant mouse receptors were determined as described under "Experimental Procedures" and are plotted for all amino acids tested. For numerical data, see Table I. For each mutant, the two $K_i$ values for chondocurarine and for dTC are connected by a line.
AChBP.

The atomic resolution structures of dTC and the AChBP dTC in the putative acetylcholine-binding site as described in served as the starting points for generating a model for the binding of ligand binding and subsequent channel opening and desensitization.

Structure-Activity of d-Tubocurarine—Of particular interest to this study was mapping the AChR sites of interaction of the 12'- and 13'-dTC positions because we had shown these sites to affect site-selective binding and desensitization (21, 22). Whereas we observed interactions at the 13'-position to γTyr117, these were inconsistent with proximal interactions. This observation suggested that 13'-modifications invoke conformational changes of dTC, the protein, or both that produced the interaction energy or that dTC has an alternative mode of binding where the interactions are distinct, such as an orientation rotated to swap the 2-N and 2'-N positions. Protein allosteric effects might be consistent with the increased desensitization caused by 13'-modified dTC analogs and suggest a possible role for γTyr117 in desensitization of the AChR.

These observations also suggested an allosteric interaction between the 13' and the 2-N position of dTC, mediated either by changes in protein structure or dTC structure. We tested for that interaction by comparing binding of iodo-chondocurarine to dTC, iodo-dTC, and chondocurarine. Interaction between the functional groups was strongly dependent on the γ117 side-chain structure; for the native γTyr117 and for γY117S, there were no apparent interactions, whereas interaction was observed with γY117T. The mixed results may indicate compensatory structural changes in the ligand or protein upon binding. They also show that some side-chain structures can greatly reduce affinity of 13'-modified ligands. Clearly, curare binding is sensitive to such changes in structure, but full understanding of either direct or indirect interactions will require further experimentation.

The moderate interaction energies observed and the apparent allosteric interactions limited the scope of our conclusions. The largest affinity changes and interaction energies were usually observed upon charge changes, as seen in other examples utilizing thermodynamic cycle analysis (36). The modest changes in interaction energies seen otherwise may reflect the intrinsic flexibility of the ligand or the protein to compensate for structural changes. In this respect, dTC is known from crystal structures (28, 30, 31) and molecular modeling (33) to have several distinct, stable conformations. These concerns may limit the general applicability of this method to understanding binding of flexible ligands to allosteric proteins.

Molecular Modeling of dTC—We constructed a model for the binding of dTC to the AChR using the crystal structure of the AChBP (20). This model is consistent with much of the photoaffinity labeling data that indicated proximity between dTC and Torpedo residues γTyr190, αTyr198, αCys192 (37), δTrp57 (the primary site of labeling in the heterologous subunits and homologous to γTrp55), as well as with minor labeling of γTyr117 (13). dTC photoaffinity labeling of γTyr111 was minor, relative to other residues (13), and this residue does not contact the protein shown. C, dTC within structure of the putative binding site showing the 31 residues that were included in the minimization. Labels for several important residues are according to the mouse AChR sequence. D, the corresponding starting structure of the AChBP prior to amino acid substitution and minimization, from the protein data bank. Amino acid side chains corresponding to those in C are labeled according to the AChBP sequence.

Fig. 8. Conformational changes of dTC upon binding the AChBP. The atomic resolution structures of dTC and the AChBP served as the starting points for generating a model for the binding of dTC in the putative acetylcholine-binding site as described in “Experimental Procedures.” The side chains surrounding the binding pocket were changed to the aligned residues in the mouse α- and γ-subunits. Stereoscopic images of the following structures are shown. A, the starting structure of dTC based on the dTC crystal structure (28). B, the structure of dTC after energy minimization in the AChBP, with no
dTC in the model shown in Fig. 8. Mutagenesis experiments have shown that αY198F enhances dTC affinity about 10-fold (18). This observation is consistent with the close contact of the 4-OH group of αTYr117 with ring D of dTC (Figs. 1 and 8); removal of this OH group would clearly relieve steric strain. The type of interaction seen here is consistent with the aromatic-aromatic interaction suggested previously (17, 18). Mutagenesis of αTYr117, γTYr117, or δTYr117 has little effect on dTC affinity, whereas αY190F reduces dTC affinity 10-fold (8). The rationale for these observations was not clear from inspection of the model.

Of the three residues identified by analysis of chimeras (16) as being important for site-selective dTC binding, yLeu116, γTYr117, and γSer161 only γTYr117 appears to be in direct contact with dTC in our model. yLeu116 points away from the binding site into the interior wall of the protein. γSer161 faces into the binding pocket but is not in van der Waals contact with dTC. It seems feasible, however, that mutation to a cationic or large residue at this position could affect dTC binding, as observed, by virtue of its proximity.

It was also important to assess whether the model satisfied our observations on the affinities of the dTC analogs. The model agreed with our principal observation, the interaction between γTYr117 and the 2-N of dTC. We observed that 7'-modification increased affinity of dTC (Table I), consistent with proximity of this functional group to a cavity in the binding site model that may accommodate the added bulk of a substituent. The 12'-position appears in close contact with the protein, suggesting that added steric bulk should interfere with binding, consistent with the general decrease in affinity we observed for modification at this position. In contrast, we could not rationalize the effects of 13'-modification, a position that also appears in close contact with the protein, particularly with γyLeu109. Our preliminary experiments with mutations at γyLeu109 did not show interactions. Although the environment of the 13'-position is sterically crowded, it is not obvious why the affinities of the 13'-modified analogs tend to be sensitive to the γ117 side-chain structure nor why they induce increased desensitization of Torpedo AChR. We observe little change in affinity upon demethylation at the 6- and 6'-oxygen. The model predicts the 6'-hydroxymethyl of dTC to be in close contact with the protein, where loss of a methyl group might be expected to relieve steric strain and increase affinity. Although our model is consistent with many observations, it cannot rationalize all observations and therefore represents only a first step toward understanding dTC-AChR interactions.

We also sought to understand the nature of the interaction between γTYr117 and the dTC 2-N. The interaction between γTYr117 and the dTC analog metocurine had been proposed to occur through cation-π electron interactions (19). The proximity of the 2-N of dTC and γTYr117 in our model appears reasonably consistent with such a mechanism. However, we observe increased affinity of the mutation γY117F, which should yield cation-π interactions of similar strength (38). There is no obvious interaction of the tyrosine hydroxyl that explains this affinity change. Other factors clearly may influence the affinity as well; small side chains such as γY117A or γY117D yielded affinities as high as that of γY117F. It is possible that small side chains reduce steric hindrance and permit a lower energy conformation of dTC, thereby improving affinity.

A better understanding of dTC binding will appear with a direct structural determination and knowledge of the structural differences between the resting and desensitized conformations of the AChR. Our model clearly does not permit simple entry of dTC into the binding site without breathing or flexing in the binding pocket. Kinetic measurements of dTC association and dissociation rates may provide a test of our model and determine which protein conformation is better represented by the crystal structure of the AChBP. During energy minimization of various dTC conformations, we sometimes observed displacement of the α-subunit loop c. Such movement may be representative of the conformational changes that take place upon desensitization (or opening) of a native channel. Although proposing such a conformational movement is speculative, it may help explain some of the inconsistencies in our model. Structural changes in dTC may be accommodated by complementary structural changes in the binding site, affecting the overall conformational equilibrium, and yielding modest net changes in affinity.

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