Curariform Antagonists Bind in Different Orientations to the Nicotinic Receptor Ligand Binding Domain*

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Curariform alkaloids competitively inhibit muscle acetylcholine receptors (AChR) by bridging the α and non-α subunits that form the ligand-binding site. Here we delineate bound orientations of d-tubocurarine (d-TC) and its methylated derivative metocurine using mutagenesis, ligand binding measurements, and computational methods. When tested against a series of lysine mutations in the ε subunit, the two antagonists show marked differences in the consequences of the mutations on binding affinity. The mutations εL117K, εY111K, and εL109K decrease affinity of metocurine by up to 3 orders of magnitude but only slightly alter affinity of d-TC. At the α subunit face of the binding site, the mutation αY198T decreases affinity of both antagonists, but αY198F preferentially enhances affinity of d-TC. Computational and experimental docking orientations, based on our structural model of the α–ε subunit face of the human AChR, indicates distinct orientations of each antagonist; the flatter metocurine fits into a pocket formed principally by the ε subunit, whereas the more compact d-TC spans the narrower crevasse between α and ε subunits. The side chains of εTyr-111 and εThr-117 juxtapose one of two quaternary nitrogens in metocurine but are remote from the equivalent quaternary nitrogen in d-TC, which instead closely approaches αTyr-198. The different docked orientations arise through tilt of the curariform scaffold by −60° normal to the nitrogen-nitrogen axis, together with a 20° rotation about the axis. The overall mutagenesis and computational results show that despite their similar structures, d-TC and metocurine bind in distinctly different orientations to the adult human AChR.

The ligand binding domain of the nicotinic receptor has served as a prototype for understanding ligand recognition in members of the superfamily of pentameric ligand-gated ion channels. Contained within the synaptic protraction of these receptors, the ligand-binding sites are formed at interfaces between subunits. In the acetylcholine receptor (AChR) found at the motor end plate, the α subunit forms one face of each binding site, whereas a non-α subunit, γ, δ, or ε, forms the opposing face (1). Residues critical for ligand binding have been identified by using subunit chimeras and point mutations combined with measurements of binding of probes that interact with structures in both subunits at the interface. These probes helped establish the subunit interface structure of the ligand binding domain and include curare and its derivatives (2, 3), α-conotoxins (4, 5), α-neurotoxins (6), and Waglerins (7). The recent x-ray structure of acetylcholine-binding protein (AChBP) elegantly confirmed the subunit interface structure of the ligand binding domain (8). Curare and its derivatives competitively antagonize the AChR found at the motor end plate by occupying one of the two ligand-binding sites (9, 10). Curare binds more tightly to the α–ε than to the α–δ site, and thus its functional antagonism at tens of nanomolar concentrations results from occupancy of this high affinity binding site. Curariform antagonists contain a rigid, cyclic hydrocarbon scaffold bearing two tetrahydroisoquinoline rings and two electron-deficient nitrogen atoms separated by about 9 Å (11, 12). Thus two appropriately spaced, positively charged centers represent the minimal components required in a high affinity competitive antagonist, also known as the antagonist pharmacophore. At the core of the antagonist pharmacophore concept is the idea that structurally related antagonists block function by binding to the α–ε site in the same orientation.

We therefore sought to determine whether different methylated derivatives of curare bind in the same orientation to the human muscle AChR, and we addressed this question using mutagenesis, measurements of antagonist binding, and computational determinations of antagonist docking orientation. We examined binding of d-TC and its fully methylated derivative metocurine to AChRs containing mutations of key residues at the ligand-binding site. By using our previously described structural model of the AChR ligand binding domain, based on lysine scanning and homology modeling (13), we used molecular dynamics simulation to generate multiple snapshots of the binding site structure, and computed optimal docking orientations of antagonist for each snapshot. Unexpectedly, both our experimental and computational determinations of antagonist binding reveal that d-TC and metocurine bind in distinctly different orientations to the adult human muscle AChR. The results have important consequences for the design of neuromuscular blocking agents and of drugs targeted against other types of nicotinic receptors.

EXPERIMENTAL PROCEDURES

Materials—125I-α-Bungarotoxin was purchased from PerkinElmer Life Sciences and d-tubocurarine (d-TC) chloride from ICN Pharmaceuticals, Inc. The fully methylated analog of d-tubocurarine, metocurine iodide, was a gift from the Eli Lilly Co. Human embryonic kidney cells (HEK293) were from the American Type Culture Collection.

Plasmids and Mutagenesis—Sources of the adult human AChR subunit cDNAs subcloned into the cytomegalovirus-based expression vector pPRB4 are as described (14). Mutations were constructed using the QuickChange kit from Stratagene. The presence of each mutation and

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the absence of unwanted mutations were confirmed by automated dye terminator sequencing.

Expression of Mutant Receptors and Ligand Binding Measurements—HEK cells were transfected with mutant or wild type AChR subunit cDNAs using calcium phosphate precipitation as described (2). In brief, 3 days after transfection, intact HEK cells were harvested by gentle agitation in phosphate-buffered saline plus 5 mM EDTA. Ligand binding to intact cells was measured by competition against the initial rate of $^{125}$I-bungarotoxin ($\alpha$-bgt) binding (15). After harvesting, the cells were centrifuged, resuspended in high potassium Ringer’s solution, and divided into aliquots for ligand binding measurements. Potassium Ringer’s solution contains the following: 140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl$_2$, 1.7 mM MgCl$_2$, 25 mM HEPES, 50 mg/liter bovine serum albumin, adjusted to pH 7.4 with 10–11 mM NaOH. Specified concentrations of competing ligand were added prior to adding $^{125}$I-$\alpha$-bgt, which was allowed to occupy approximately half of the surface receptors. Binding was terminated by adding potassium Ringer’s solution containing 100 $\mu$M $d$-tubocurarine chloride. Cells were filtered through Whatman GF-B filters using a Brandel Cell Harvester and washed with potassium Ringer’s solution. Nonspecific binding was determined in the presence of 3 mM acetylcholine. The total number of $^{125}$I-$\alpha$-bgt-binding sites was determined by incubation with the toxin for 90 min. The initial rate of toxin binding was calculated as described (15) to yield fractional ligand occupancy. Competition measurements were analyzed according to the sum of two binding sites present in equal numbers as shown in Equation 1,

$$1 - Y = 0.5[f(1 + [ligand]/K_a) + 0.5f(1 + [ligand]/K_b)] \text{ (Eq. 1)}$$

where $Y$ is fractional ligand occupancy, and $K_a$ and $K_b$ are intrinsic dissociation constants.

Docking and Molecular Dynamics Simulation—To account for the contribution of protein dynamics to ligand docking, we carried out a 1-ns molecular dynamics (MD) simulation of our the structural model of human AChR (13) using the AMBER 7 program, which included the parm 99 force field (27). The protein was solvated in a water box using the TIP3P model for water molecules, extended at least 10 Å in each direction from the protein (28,165 water molecules included), and 66 Na$^+$ and 16 Cl$^-$ counter ions were added to neutralize the system by employing the LEAP module of AMBER 7. The entire system was first energy-minimized by employing the SANDER module of AMBER 7 and then gradually heated to 298 K using a 30-ns heating step; in these computations the Particle Mesh Ewald method was employed to calculate long range electrostatic interactions. Following the heating step, the system was maintained at 298 K, and the MD simulation was computed at 1-6 intervals with frames collected every 1 ps. Because the linker joining $\beta$-strands 8 and 9 in each subunit (8, 13) did not achieve a stable single structure in preliminary MD simulations, the protein main chain atoms were constrained in space, but side chain atoms were freely flexible. During the simulation, the SHAKE algorithm was turned on to constrain bonds involving hydrogen atoms, and the nonbonded interaction cut-off was set to 8.0 Å. Trajectories from the subsequent 1,000-ns MD simulation were collected for docking computations. From each of the 1,000 frames of the dynamic AChR structure, atomic coordinates of the $\alpha$ and $\epsilon$ subunits were collected for docking computations. Partial charges were then assigned to each atom of the $\alpha$ and $\epsilon$ subunits using the restrained electrostatic potential (RESP) charge model, which is used in the application of the AMBER force field.

Crystal structures of curcurine (12) and $d$-TC (11) were used to prepare the ligands for docking. Hydrogen atoms were added by employing QUANTA, followed by a 500-step energy minimization of all the hydrogen atoms using the CHARMm module in QUANTA. Partial charges were assigned to each atom of the ligand using the semiempirical charge model-1 (CM1) in the AMPSOL 6.7.2 program (16) with the following settings: the SM5.4PDA solvation mode, the AM1 semiempirical method, water as solvent, the “TRUSTE” optimizer, and “CHARGE” set to +2 based on the net charge of both ligands.

Docking computations were performed using the AUTODOCK 3.0.3 program. Nonpolar hydrogens were removed from each ligand, and their partial atomic charges were united with the bonded carbon atoms. For each frame of the $\alpha$-$\epsilon$ subunit pair, the ligand was arbitrarily positioned at the subunit interface using QUANTA, and then docking computations were performed using the Lamarckian genetic algorithm with grid spacing 40 x 40 x 40 (grid spacing 0.375 Å), yielding 10 docked conformations per frame. During the docking computation, free rotation was allowed about bonds to hydroxyl or methyl groups of each curare analog (Fig. 1), but the remaining atoms were maintained in the coordinates of the crystal structures. Parameters in AUTODOCK were assigned default values. The resulting 10 docking orientations for each ligand produced by AUTODOCK 3.0.3 were analyzed by grouping them into clusters with similar ligand orientations. We employed a cluster analysis that classified structures as similar in 1-Å intervals of the ligand root mean square deviation.

RESULTS

This study arose from the chance observation that a series of lysine mutations in the human $\epsilon$ subunit, previously used to assess surface or buried orientations of residue side chains (13), impaired binding of metocurine much more than its structural analog $d$-TC. The side chain of each mutated residue in this series was shown to project on the protein surface, rather than into the hydrophobic core, and to localize in loops $E$–$G$ at the $\epsilon$ subunit face of the ligand binding domain. We were also motivated by measurements of functional antagonism of mouse AChR showing different effects of mutations at $\epsilon 59$ on potency of several curare analogs (29).

We co-expressed each lysine mutation with complementary $\alpha$, $\beta$, and $\delta$ subunits in HEK293 cells, and we determined binding of $d$-TC and metocurine to receptors on intact cells by competition against the initial rate of $\alpha$-bungarotoxin binding (see “Experimental Procedures”). For each antagonist, occupancy of the adult human AChR is well described by the sum of two binding sites present in equal number, corresponding to the $\alpha$–$\epsilon$ and $\alpha$–$\delta$ subunit interfaces. Metocurine distinguishes between the two binding sites to the greatest degree, exhibiting a ratio of dissociation constants of 83, whereas for $d$-TC the ratio is 15 (Fig. 2 and Table I). For the lysine mutations in binding site loops $E$ and $F$ (loop $E$, $eW55K$, $eG57K$, and $eD59K$; loop $F$, $eL109K$, $eY111K$, and $eT117K$), large reductions in affinity are observed for metocurine, whereas much smaller reductions are observed for $d$-TC; notably, the mutation $eT117K$ exhibits almost absolute selectivity between the two antagonists. Exceptions to these metocurine-selective mutations include the loop F mutation $eL119K$ and the loop G mutations $eD173K$ and $eD175K$, which diminish affinity of both $d$-TC and metocurine. These findings show that the $\epsilon$ subunit contributes to binding of metocurine much more than binding of $d$-TC.

Each lysine mutation in this series increases the dissociation constant of one of the two ligand binding sites, presumably the $\alpha$–$\epsilon$ site, whereas the dissociation constant of the presumed $\alpha$–$\delta$ site is little changed (Table I). This site selectivity is evident from inspection of the log ratio of dissociation constants of mutant to wild type receptors computed for each of the two
Docking Modes of Curare Analogs to the AChR

Fig. 2. Mutations of key binding site residues selectively affect metocurine versus d-TC binding. a and b display measurements of binding of each antagonist to the indicated mutant receptors determined by competition against the initial rate of 125I-α-bungarotoxin binding (see “Experimental Procedures”). Individual competition determinations from single experiments are displayed; results from multiple experiments are summarized in Tables I and II. c and d exhibit the effects of the indicated mutations on the dissociation constant for the mutant relative to that of the wild type AChR-binding site ($K_i$ in Tables I and II). See Tables I and II for a summary of measurements for these and additional mutations.

binding sites; the ratio clearly deviates from zero for the affected site (dissociation constant $K_i$) but is close to zero for the other site (dissociation constant $K_o$; see Table I). Perturbation at only one of the two binding sites is expected because each mutation is located in the ε subunit, which contributes to the $\alpha-\epsilon$ site, whereas the remaining $\alpha-\delta$ site contains no mutation.

The results from lysine mutagenesis are combined with our computational determinations of antagonist docking orientation, described below, to illustrate the metocurine footprint at the ε subunit face of the binding site (Fig. 3). Highlighted in space-filling rendering are side chains of key residues in the ε subunit that, following mutation to lysine, markedly impair binding of metocurine. The mutagenesis-generated footprint reveals an elongated pocket in the ε subunit that closely complements the concave hydrophobic face of the metocurine scaffold.

The large size and positive charge of the lysine side chain make it a good probe of proximity to the positively charged antagonist. Lack of a mutational effect ensures that the ligand does not bind at the site of the mutation, but a large effect does not guarantee contact between the mutated residue and antagonist in the complex. We therefore examined the consequences of conservative mutations of several residues in the ε subunit, and we found the best evidence for direct interaction for residues in loops F and G. The loop F mutation εY111F markedly enhances affinity for metocurine but not that of d-TC, whereas the loop G mutation εD173A diminishes affinity of d-TC but not that of metocurine (Table II). The mutations εT117Y and εT117A show only minor effects on binding of either antagonist, indicating that polarity of the side chain at position e117 is not essential for binding. The overall results of conservative mutations in the ε subunit indicate εTyr-111 deep in the binding pocket contributes to binding of metocurine, whereas εAsp-173 at the periphery contributes to binding of d-TC; the intervening residues, while likely proximal to bound antagonist, may contribute hydrophobicity or van der Waals contacts rather than specific polar interactions.

Residues in the α subunit also contribute selectively to antagonist binding. Mutations targeting the three signature tyrosines found in all nicotinic receptors had to be structurally conservative to prevent loss of expression. The mutation αY198T markedly diminishes affinity of both antagonists, but the mutation αY198F selectively increases affinity of d-TC (Fig. 2 and Table II). Mutations of αTyr-93 and αTyr-190 have only minor effects on binding of both antagonists (Table II); the αY190T mutation eliminated α-bungarotoxin binding (not shown), either through misfolding or by preventing binding of the toxin, and could not be studied further. Thus αTyr-198 contributes to binding of both antagonists, but the selective enhancement of binding by αY198F makes it a good candidate for direct interaction with d-TC.

To determine the origin of the mutational specificity at the atomic structural level, we computed docking orientations of d-TC and metocurine to our structural model of the $\alpha-\epsilon$-binding site of the human AChR (13). Because proteins are intrinsically dynamic, we employed a computational strategy that accounts for this key property. We therefore used MD simulation to generate an ensemble of protein structures accessible to the antagonist and computed docking of antagonist to each snapshot of the dynamic structure. The computational analysis was patterned after the pioneering work of McCammon and co-workers (17) and our application of that approach to curariniform antagonist binding to AChBP (18). One difference, however, was the need to constrain the α-carbon backbone of the AChR ligand binding domain during MD simulation, while
Docking Modes of Curare Analogues to the AChR

Effects of lysine mutations on antagonist binding

Table I

| Metocurine | No. | $K_i$ | log$K_i/K_{1wt}$ | $K_o$ | log$K_o/K_{2wt}$ | d-Tubocurarine | No. | $K_i$ | log$K_i/K_{1wt}$ | $K_o$ | log$K_o/K_{2wt}$ |
|------------|-----|------|-----------------|------|-----------------|----------------|-----|------|-----------------|------|-----------------|
| WT         | 14  | 5.9e-8 | 4.9e-6          |      | 2               |                | 12  | 8.1e-8 | 1.2e-6          |      | 2               |                |
| z553K      | 2   | 5.6e-7 | 0.97            | 0.20 | 1               | 0.77           | 1   | 1.1e-6 | 1.9e-6          | 0.27 | 4.5e-7          |
| z553K      | 1.3e-7 | 0.25  | 6.1e-7          | 0.25 | 7.2e-7          | 1.3e-6         | 3   | 1.1e-6 | 9.6e-6          | 0.16 | 1.6e-6          |
| zW57K      | 2.8e-6 | 0.14  | 4.2e-8          | 0.11 | 8.1e-7          | 4.5e-7         | 3   | 4.8e-7 | 2.1e-6          | 0.20 | 1.6e-6          |
| zG57K      | 1.9e-6 | 0.10  | 1.7e-7          | 0.01 | 2.7e-7          | 3.4e-7         | 2   | 3.6e-7 | 2.2e-6          | 0.22 | 2.6e-6          |
| zD59K      | 2.4e-6 | 1.82  | 7.1e-6          | 0.10 | 2.8e-6          | 5.6e-7         | 3   | 3.0e-7 | 6.5e-7          | 0.22 | 7.6e-7          |
| zN107K     | 3.8e-8 | -0.02 | 4.9e-6          | 0.10 | 6.2e-8          | 1.5e-6         | 1   | 6.2e-8 | 1.5e-6          | 0.08 | 1.5e-6          |
| zL109K     | 2.6e-5 | 3.05  | 5.4e-6          | 0.11 | 1.7e-6          | 1.6e-6         | 1   | 6.5e-7 | 4.1e-6          | 0.52 | 3.1e-7          |
| zY111K     | 2.8e-6 | 1.68  | 4.0e-6          | 0.10 | 1.7e-6          | 1.2e-6         | 3   | 2.0e-7 | 1.1e-6          | -0.09 | 1.2e-6         |
| zS115K     | 5.3e-7 | 0.30  | 7.9e-7          | 0.07 | 8.2e-8          | 6.5e-7         | 3   | 8.2e-7 | 5.7e-7          | 0.21 | 6.5e-7          |
| zE117K     | 2.1e-7 | 0.55  | 3.7e-6          | 0.15 | 1.9e-7          | 1.6e-6         | 1   | 1.9e-7 | 6.5e-7          | 0.10 | 6.9e-7          |
| zE117K     | 7.2e-8 | 0.07  | 1.5e-6          | 0.34 | 4.2e-8          | 3.4e-7         | 3   | 2.6e-7 | 1.7e-6          | 0.16 | 3.4e-7          |
| zE117K     | 7.2e-8 | 0.07  | 1.5e-6          | 0.34 | 4.2e-8          | 3.4e-7         | 3   | 2.6e-7 | 1.7e-6          | 0.16 | 3.4e-7          |
| zL119K     | 2.5e-4 | 3.97  | 2.9e-6          | 0.18 | 2.6e-5          | 5.6e-7         | 2   | 2.6e-5 | 6.7e-7          | -0.46 | 5.9e-7         |
| zL119K     | 4.2e-4 | 0.26  | 7.3e-7          | 0.18 | 9.9e-6          | 1.3e-7         | 3   | 3.2e-6 | 1.8e-7          | 0.13 | 1.6e-6          |
| zD173K     | 3.8e-7 | 0.80  | 5.7e-6          | 0.03 | 1.3e-6          | 1.3e-6         | 3   | 3.2e-6 | 2.0e-7          | 0.06 | 3.4e-7          |
| zD175K     | 1.2e-7 | 0.15  | 1.2e-6          | 0.07 | 1.6e-6          | 3.4e-7         | 2   | 1.5e-6 | 1.2e-6          | 0.07 | 1.2e-6          |
| zD175K     | 2.6e-7 | 0.11  | 5.6e-7          | 0.11 | 4.9e-8          | 0.15           | 0   | 4.9e-8 | 0.15           | 0.10 | 0.10           |

Fig. 3. Mutagenic footprint of metocurine in the AChR ε subunit. Two views of the ε subunit are shown (green ribbons), with metocurine positioned in the optimal docked orientation determined from our computational results (see text). Residue side chains for which lysine mutations substantially affect metocurine affinity are displayed in space-filling representation, color-coded according to charge distribution, with red indicating negative and blue indicating positive. Metocurine is shown in ball and stick representation.

allowing free movement of all side chains (see “Experimental Procedures”). We therefore carried out a 1-ns MD simulation, with the α-carbon backbone constrained, and collected 1000 frames of the dynamic structure for computation of antagonist docking.

Application of AUTODOCK 3.0.3 generated 10 docked structures per frame to give a total of 10,000 docked structures for each antagonist. We then grouped these into clusters of similar structures by sorting them into 1-Å intervals of the ligand root mean square deviation (see “Experimental Procedures”). For both antagonists, our cluster analysis reveals a predominant ligand orientation within the binding pocket (cluster 1), along with several smaller clusters with different orientations (Fig. 4). For d-TC, comparison of orientations in clusters 1 and 2 reveals only a minor shift of the ligand within the binding pocket, whereas for metocurine, clusters 1 and 2 represent fundamentally different orientations (Fig. 4). The most frequent orientation produced by AUTODOCK has been shown for many protein-ligand complexes to agree well with the bound orientation observed in x-ray structures of the complexes (19). We nevertheless considered the orientation of metocurine in cluster 2 a formal possibility to be evaluated in light of our mutagenesis results.

Our computational determinations indicate that d-TC and metocurine, despite having identical molecular scaffolds and rigid cyclic structures, bind in distinctly different orientations to the ε-ε site of the adult human AChR (Fig. 5). The concave hydrophobic face of metocurine lies across β-strands 5, 6, and 2 of the ε subunit (8, 13), with one of the two quaternary nitrogens (N2) penetrating deep into the binding site to juxtapose εTyr-111 and εThr-117, and the other quaternary nitrogen (N1) extending peripherally to approach the β-β linker harboring εAsp-173 and εAsp-175. By contrast, the concave face of d-TC orients toward the ε subunit, with its quaternary nitrogen (N2) penetrating deep into the binding site to juxtapose εTyr-198, and its tertiary nitrogen (N1) projecting peripherally toward εAsp-173 in the β-β linker. The change in orientation from that of metocurine to that of d-TC results from tilt of the curariform scaffold by ~60° normal to the nitrogen-nitrogen axis, together with a 20° rotation about the axis.

Table III lists computationally determined distances between key atoms of each antagonist and determinants of binding at the ε-ε subunit interface. The key atoms of the antagonists include the most proximal nitrogen to the binding determinant in question, as well as the most proximal carbon atom in the curariform scaffold. These distances are mean values computed from all frames of the dynamic structure in cluster 1 (Fig. 4); for both antagonists mean values are also listed for cluster 2.

The computed separation distances for the predominant cluster 1 support our experimental observations that particular residues at the ε-ε interface are proximal to bound metocurine, whereas others are proximal to bound d-TC. The aromatic ring of εTyr-111 is 4.1 Å away from N2 of metocurine but is 10.4 Å from N2 of d-TC; these very different separation distances,
together with the selective effects of the mutations εY111K and εY111F on metocurine binding, strongly support a cation-π interaction at εTyr-111 stabilizing bound metocurine. Analogously, the aromatic ring of αTyr-198 is 3 Å from N2 of d-TC, whereas it is 6.6 Å from N2 of metocurine; these very different separation distances, and the selective enhancement of d-TC affinity by the mutation αY198F, strongly support a cation-π interaction at αTyr-198 stabilizing bound d-TC. The β-carbon atom of εThr-117 is 4.7 Å away from N2 of metocurine and 3.6 Å from the closest atom in the hydrocarbon scaffold, whereas the analogous distances are 9.8 and 6.2 Å for d-TC; these separation distances indicate close proximity of εThr-117 to bound metocurine but not to d-TC, in excellent agreement with the nearly absolute selectivity of the mutation εT117K in decreasing metocurine affinity. Our computationally determined separation distances indicate additional selective contributions to metocurine relative to d-TC affinity, including distances between metocurine and εLeu-109, εGly-57, and εAsp-59 (Table II), in agreement with the selective effects of the corresponding lysine mutations on metocurine affinity (Fig. 2 and Table I). Thus metocurine and d-TC encounter different residues at the α−ε site by binding in distinctly different orientations.

The computational results also disclose common contributions of binding site residues to both antagonists (Table III). The major common source of stabilization is εLeu-119, which achieves close contact of 3.5 Å with the hydrocarbon scaffold of metocurine and 3 Å with that of d-TC. Mutation of εLeu-119 to lysine markedly decreases binding affinity for both antagonists, in good agreement with the short separation distances, and suggesting that εLeu-119 stabilizes each bound antagonist through hydrophobic interactions.

Finally, the computational results confirm our observations that particular candidate residues do not affect antagonist binding following mutation. For example, εAsn-107, which is

| TABLE II | Effects of conservative mutations on antagonist binding |
|----------|----------------------------------|
| **No.** | **εMetocurine** | **εd-Tubocurarine** |
|         | K<sub>1</sub> | logK<sub>1</sub>/K<sub>1wt</sub> | K<sub>2</sub> | logK<sub>2</sub>/K<sub>2wt</sub> | No. | K<sub>1</sub> | logK<sub>1</sub>/K<sub>1wt</sub> | K<sub>2</sub> | logK<sub>2</sub>/K<sub>2wt</sub> |
| VT | 14 | 5.9e-8 | 4.9e-6 | 3.5e-7 | 12 | 8.1e-8 | 1.2e-6 |
| εD59A | 2 | 3.3e-8 | 3.1e-8 | 3.5e-8 | 3 | 4.0e-8 | 3.5e-7 |
| εY111T | 1 | 6.3e-9 | 3.5e-10 | 3.5e-10 | 3.5e-8 | 0.37 | 5.8e-7 | 0.25 |
| εY111S | 1 | 1.2e-8 | 3.1e-9 | 3.5e-8 | 1 | 3.5e-8 | 0.10 |
| εY111F | 2 | 5.3e-9 | 3.1e-8 | 3.5e-8 | 3 | 6.3e-8 | 0.10 |
| εT117 | 2 | 4.2e-9 | 3.1e-10 | 3.5e-10 | 3 | 6.3e-8 | 0.10 |
| εT117A | 1 | 4.2e-9 | 3.1e-10 | 3.5e-10 | 3 | 6.3e-8 | 0.10 |
| εY198T | 2 | 3.1e-9 | 3.1e-8 | 3.5e-8 | 3 | 6.3e-8 | 0.10 |
| εY198F | 1 | 4.2e-9 | 3.1e-8 | 3.5e-8 | 3 | 6.3e-8 | 0.10 |
| εY190F | 2 | 3.1e-9 | 3.1e-8 | 3.5e-8 | 3 | 6.3e-8 | 0.10 |
| εD195A | 1 | 4.2e-9 | 3.1e-8 | 3.5e-8 | 3 | 6.3e-8 | 0.10 |
| εD195N | 1 | 4.2e-9 | 3.1e-8 | 3.5e-8 | 3 | 6.3e-8 | 0.10 |
| εY198F | 3 | 9.5e-8 | 3.1e-9 | 3.5e-8 | 3 | 6.3e-8 | 0.10 |
| εY198T | 2 | 3.1e-9 | 3.1e-8 | 3.5e-8 | 3 | 6.3e-8 | 0.10 |

**FIG. 4.** Grouping of docked structures of d-TC and metocurine into clusters with similar structure (upper panel; see “Experimental Procedures”). Lower panel compares docked orientations of each antagonist in clusters 1 (heavy lines) and 2 (thin lines).
metocurine (d-TC) compared with that in cluster 2 (Fig. 4). In cluster 1, the
gether with our mutagenesis results, provide strong support for
weak effects of mutations of these conserved tyrosines.
Together with our mutagenesis results, provide strong support for
weak effects of mutations of these conserved tyrosines.

Separation distances for other key determinants of metocurine
binding also strongly favor the orientation in cluster 1 over that
span the subunit interface. The competitive antagonist phar-
macophore of the AChR together with the requirement of both
α and non-α subunits for antagonist binding suggest that an-
tagonists within a given structural family bridge the subunit
interface in the same orientation. Our observation to the con-
trary that d-TC and metocurine bind in distinctly different
orientations has profound implications for design of drugs tar-
ged against the family of nicotinic receptors.

The nicotinic receptor ligand binding domain is formed by
seven loops spaced far apart on the protein chains of the α and
non-α subunits (1, 21). Binding of curariform antagonists relies
on residues found within a subset of these seven loops and
include both conserved and non-conserved residues. Conserved
determinants include αTyr-198 in loop C, εTrp-55 in loop E,
εLeu-109 and εLeu-119 in loop F, and εAsp-173 in loop G.
Non-conserved determinants include εAsp-59 in loop E and
εTyr-111 and εThr-117 in loop F. The chemical nature of these
non-conserved residues varies widely among species and sub-
types of muscle AChR, with Asp (human ε) and Ala (frog ε)
found at the aligned position 59; Tyr (human ε), Ser (bovine ε),
and Arg (Torpedo ε) at the aligned position 111; and Thr
(human ε) and Tyr (frog ε) at the aligned position 117. The
presence of non-conserved determinants of ligand binding
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subtle structural differences at the binding site may affect the
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Promise for understanding the AChR ligand binding domain
at atomic resolution emerged with discovery and structural
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We recently reported that d-TC and metocurine bind in dis-

tinct orientations to AChBP using an analogous approach to
that used in this study (18). The origin of the different orien-
tations was traced to differences in the x-ray structures of the
two antagonists, likely to be present in solution (26), that allow
rearrangement of the positively charged ligands in the aromat-
ic-rich binding site. Distinct docking orientations have also
been observed for different steroids bound to sex hormone
binding globulin, as determined by x-ray crystallography and
confirmed by mutagenesis together with measurements of

Fig. 5. Stereo views of predicted docking orientations of
metocurine (upper panel) and d-tubocurarine (lower panel) to
the α–ε-binding site of the adult human AChR. Representative
frames from cluster 1 for each antagonist are displayed (see Fig. 4).
The α subunit face of the binding site is highlighted in red and the ε subunit
face in green. Bound antagonist, in space-filling representation, is color-
coded according to charge distribution, with red indicating negative and
blue indicating positive. Side chains of key binding site residues are
shown in stick representation. Note the different orientations of each
ligand in the binding pocket by referring to nitrogens N1 and N2.

Deep in the binding pocket, is 14.3 Å from N2 in metocurine and
12.1 Å from N2 in d-TC, in agreement with the lack of effect of
the lysine mutation at this position (Table 1). Similarly,
αTyr-93 and αTyr-190 are far from the nearest nitrogen atom of
either bound antagonist, with separation distances of 14.4 and
13.4 Å for metocurine and 13 and 12 Å for d-TC, in line with the
weak effects of mutations of these conserved tyrosines.

The computationally determined separation distances, to-
gether with our mutagenesis results, provide strong support for
the orientation of metocurine in the predominant cluster 1
compared with that in cluster 2 (Fig. 4). In cluster 1, the
β-carbon of εThr-117 is 4.7 Å away from N2 in metocurine and
3.4 Å from the nearest carbon atom in the metocurine scaffold;
for cluster 2 the analogous distances are greater, 5.9 and 7.6 Å
(Table III). Similarly, in cluster 1, the aromatic rings of εTyr-
111 and αTyr-198 are 4.1 and 6.6 Å from N2 in metocurine,
whereas in cluster 2 the separation distances are 6 and 10.2 Å.
Separation distances for other key determinants of metocurine
binding also strongly favor the orientation in cluster 1 over that
in cluster 2; these include distances between metocurine and
εLeu-109, εLeu-119, εAsp-173, and εAsp-59. For d-TC, on the
other hand, the key separation distances are similar for clus-

ters 1 and 2 (Table III), as expected from the similar orienta-
tions in these two clusters (Fig. 4).

DISCUSSION

Curare is the prototypical competitive antagonist of the
AChR found at the motor end plate, providing the structural
template that established the antagonist pharmacophore for
this receptor, as well as serving as a key probe for establishing
the subunit interface structure of the ligand binding domain.
Members of the curare family contain two positively charged
nitrogen atoms spaced 9–11 Å apart (11, 12), and these have
been shown to span the interface between α and non-α subunits
(20). Many structurally unrelated competitive antagonists of
the end plate AChR also contain two positively charged nitro-
gen, with the correct spacing, and like curare are thought to
span the subunit interface. The competitive antagonist phar-
macophore of the AChR together with the requirement of both
α and non-α subunits for antagonist binding suggest that an-
tagonts within a given structural family bridge the subunit
interface in the same orientation. Our observation to the con-
trary that d-TC and metocurine bind in distinctly different
orientations has profound implications for design of drugs tar-
ged against the family of nicotinic receptors.

The nicotinic receptor ligand binding domain is formed by
seven loops spaced far apart on the protein chains of the α and
non-α subunits (1, 21). Binding of curariform antagonists relies
on residues found within a subset of these seven loops and
include both conserved and non-conserved residues. Conserved
determinants include αTyr-198 in loop C, εTrp-55 in loop E,
εLeu-109 and εLeu-119 in loop F, and εAsp-173 in loop G.
Non-conserved determinants include εAsp-59 in loop E and
εTyr-111 and εThr-117 in loop F. The chemical nature of these
non-conserved residues varies widely among species and sub-
types of muscle AChR, with Asp (human ε) and Ala (frog ε)
found at the aligned position 59; Tyr (human ε), Ser (bovine ε),
and Arg (Torpedo ε) at the aligned position 111; and Thr
(human ε) and Tyr (frog ε) at the aligned position 117. The
presence of non-conserved determinants of ligand binding
means that among different species and subtypes of AChRs,
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Docking Modes of Curare Analogs to the AChR

TABLE III

| Atom pairs | Metocurine |   |   |   | Atom pairs |   |   |   |
|------------|------------|---|---|---|------------|---|---|---|
|            | Cluster 1  | Mean | ± S.D. | Mean | ± S.D. | Cluster 1  | Mean | ± S.D. | Mean | ± S.D. |
| Loop E     |            |      |        |      |        |            |      |        |      |        |
| εD57(Cα):C-E | 3.9        | 0.2  | 7.2    | 0.2  | 57(Cα):C-F | 4.5  | 0.2  | 3.9  | 0.2  |
| εW55(Cγ):N1 | 7.4        | 0.3  | 7.4    | 0.3  | 55(Cγ):N1 | 7.7  | 0.4  | 7.3  | 0.3  |
| εD59(Cγ):D-C | 4.2        | 0.3  | 8.2    | 0.4  | 59(Cγ):C-E | 6.9  | 0.3  | 7.5  | 0.2  |
| Loop F     |            |      |        |      |        |            |      |        |      |        |
| εN107(Oβ):N2 | 14.3       | 0.8  | 17.6   | 0.7  | 107(Oβ):N2 | 12.1 | 1.3  | 12.2 | 1.2  |
| εL109(Cγ):N2 | 5.7        | 0.4  | 9.3    | 0.4  | 109(Cγ):N2 | 7.8  | 0.6  | 6.1  | 0.3  |
| εL109(Cγ):C-AB | 3.8        | 0.3  | 11.2   | 0.3  | 109(Cγ):C-D | 5.6  | 0.3  | 5.6  | 0.4  |
| εY111(Cγ):N2 | 4.1        | 0.5  | 6.0    | 0.6  | 111(Cγ):N2 | 10.4 | 0.7  | 8.2  | 0.7  |
| εS115(Oγ):N1 | 15.9       | 0.3  | 15.9   | 0.3  | 115(Oγ):N1 | 13.6 | 0.5  | 15.9 | 0.4  |
| εT117(Cβ):N2 | 4.7        | 0.3  | 5.9    | 0.3  | 117(Cβ):N2 | 9.8  | 0.5  | 7.4  | 0.3  |
| εT117(Cβ):C-AB | 3.6        | 0.6  | 7.6    | 0.3  | 117(Cβ):C-D | 6.2  | 0.4  | 4.1  | 0.2  |
| εL119(Cγ):C-C | 3.5        | 0.2  | 5.3    | 0.1  | 119(Cγ):C-F | 3.0  | 0.2  | 3.4  | 0.3  |
| εL119(Cγ):C-D | 3.7        | 0.2  | 5.9    | 0.2  | 119(Cγ):C-F | 2.5  | 0.2  | 3.5  | 0.3  |
| Loop G     |            |      |        |      |        |            |      |        |      |        |
| εD173(Oβ):N1 | 4.2        | 0.4  | 9.4    | 0.3  | 173(Oβ):N1 | 5.1  | 0.4  | 2.8  | 0.5  |
| Loop A     |            |      |        |      |        |            |      |        |      |        |
| εY93(Cγ):N2 | 13.6       | 0.7  | 16.7   | 0.6  | Y93(Cγ):N2 | 6.1  | 0.6  | 8.2  | 0.5  |
| Loop B     |            |      |        |      |        |            |      |        |      |        |
| εW149(Oγ):N2 | 10.8       | 0.5  | 13.7   | 0.3  | W149(Oγ):N2 | 5.5  | 0.3  | 6.3  | 0.4  |
| Loop C     |            |      |        |      |        |            |      |        |      |        |
| εY190(Cγ):N1 | 13.4       | 0.3  | 7.9    | 0.30 | Y190(Cγ):N1 | 12.0 | 0.2  | 11.4 | 0.4  |
| εY198(Cγ):N2 | 6.6        | 0.6  | 10.2   | 0.3  | Y198(Cγ):N2 | 3.0  | 0.3  | 3.2  | 0.2  |

binding affinity (28). C19 androgens and estradiol were found to bind in opposite orientations where the A and D rings of the two steroids switch places within the binding pocket and the planes of the scaffolds flip by 180°. Thus distinct docking orientations of structurally similar ligands may be a widespread phenomenon among protein-binding sites.

Both AChBP and the AChR show considerable versatility in accommodating the flatter metocurine and the more compact d-TC. However the bound orientations of each antagonist differ between the AChR and AChBP. For metocurine bound to AChR, the concave hydrophobic face orients toward the α subunit, as observed here, but the nitrogen-nitrogen axis orients very differently between the two types of subunits, in agreement with a large body of work defining the subunit interface structure of the AChR ligand binding domain (1, 21). However, contrary to expectations, the two antagonists bind in mouse AChR, whereas it projects peripherally toward the β8-β9 linker in our complex with human AChR. The deduced complex between d-TC and the α-γ site of mouse AChR was based on the effects of mutations of γTyr-117 on binding of a series of structural analogs of curare (23). Our results show that bound orientations of d-TC and metocurine may not be interchangeable at a given AChR-binding site, posing difficulty interpreting results from a series of curare analogs to deduce a single bound orientation for the family of curariform antagonists.

Despite the excellent agreement between experimental and computational determinations of curariform antagonist docking observed here, there are limitations in the analysis and interpretation. The accuracy of our structural model, while apparently very good in the center of the binding pocket where the AChR and AChBP share many conserved residues, is uncertain in the peripheral linker joining the β-β strands 8 and 9 (13). Because the β8-β9 linker of the ε subunit contributes to d-TC binding, albeit weakly, the overall docked orientation could potentially change from that presented here once the structure of this non-conserved linker region is resolved. Also, the functional state of our structural model is uncertain, although recent electron microscopic detection of an ACh-induced conformational change in the α subunits suggest that the functional state is either active or desensitized (23). Curariform antagonists, however, show only small preferences for binding to active or desensitized states relative to the resting state (24, 25), suggesting that the docked orientations delineated here represent those that mediate functional antagonism of the adult human AChR.

Our overall results show that both d-TC and metocurine are stabilized by residues from both the α and non-α subunits, in agreement with a large body of work defining the subunit interface structure of the AChR ligand binding domain (1, 21). However, contrary to expectations, the two antagonists bind in
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different orientations at the α-ε subunit interface, where each antagonist contacts different residues. This fundamental result cautions against the broad application of the classical antagonist pharmacophore concept in interpreting drug structure-activity relationships, which tacitly assumes similar bound orientations of ligands within a given structural family. Structure-based drug design typically starts with a lead compound and adds or subtracts chemical groups in order to optimize binding affinity. However, our results show that subtle chemical modifications can substantially alter bound ligand orientations and, by extension, suggest that subtle differences between subtypes and species of the drug target could have the same effect. Clearly, rational drug design can be optimized by knowing the atomic coordinates of both the active site and the ligand, and by combining these with a docking analysis that incorporates the dynamics of the structure. Our overall findings have important implications for the design of drugs targeting nicotinic receptors as well as other members of the pentameric ligand-gated ion channel superfamily.

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