Interaction of d-Tubocurarine Analogs with the Mouse Nicotinic Acetylcholine Receptor

LIGAND ORIENTATION AT THE BINDING SITE

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The binding of d-tubocurarine and several of its analogs to the mouse nicotinic acetylcholine receptor (AChR) was measured by competition against the initial rate 125I-α-bungarotoxin binding to BC3H-1 cells. The changes in affinity due to methylation or halogenation at various functional groups on d-tubocurarine was measured to both the high affinity (αγ-site) and the low affinity site (αδ-site). We show that quaternization by methylation of the 2′-N ammonium group enhances the affinity for both the acetylcholine binding sites of mouse AChR, whereas this change does not affect affinity for the Torpedo AChR sites. The effect of N-methylation suggests the presence of interactions with the ammonium moiety that cannot be readily attributed to the known conserved residues thought to stabilize this functional group. Methylation of both the 7′- and 12′-phenols produced net affinity changes at both sites. The changes resulted from contributions at both the 7′- and the 12′-positions; however, these effects were dependent on whether the ammoniums were also methylated. Substitution of bromine or iodine at the 13′-position decreased the affinity considerably to the high affinity αγ-site of mouse AChR, whereas the affinity for the Torpedo αγ-site was slightly increased. Furthermore, binding to the mouse AChR was unaffected by the conformational state, whereas these ligands strongly preferred the desensitized conformation of the Torpedo AChR. Comparison of binding changes upon 13′-halogenation to the changes in amino acid residues at the ACh binding sites of the mouse and Torpedo AChR shows mouse residue Ile-γ116 as likely to be involved in interacting with the 13′-position of d-tubocurarine. It is predicted that this residue is involved in the conformational equilibrium between the resting and desensitized conformations.

The nicotinic acetylcholine receptor (AChR)1 of peripheral tissues such as Torpedo californica electric organ and mammalian muscle is a transmembrane protein that functions as a ligand gated cation channel. The receptor consists of homologous subunits with a stoichiometry of α2β2γδ. The five subunits traverse the lipid bilayer and form a pseudo symmetric rosette with the channel located at the central axis (1). Channel opening is regulated by the binding of two molecules of ACh to sites on the extracellular surface of the protein (see Ref. 2 for review). The two ACh binding sites are non-identical and can be distinguished by the differential binding of the plant alkaloid d-tubocurarine (3). A combination of one α-subunit and the γ-subunit of the AChR comprises the acetylcholine binding site with higher affinity for d-tubocurarine, the αγ-site, and the second α-subunit and the δ-subunit comprise the binding site with lower affinity, the αδ-site (4, 5).

Studies using affinity labels initially identified the α-subunit as the primary locus of ACh binding (6, 7). Subsequent affinity labeling experiments followed by mapping of the labeled sites have identified residues in the α-subunit involved in the ACh binding, specifically Cys-α192 and Cys-α193 (8) and the nearby residues Tyr-α190 (9, 10) and Tyr-α198 (11), as well as the residues more distant in the sequence, Trp- α149 (10) and Tyr- α93 (12). The region α186 to α211 itself binds α-bungarotoxin, albeit with lowered affinity, and the residues identified by affinity labeling contribute substantially to this binding (13). Residues Trp-γ55 and Trp-δ57 were identified by labeling and proteolytic mapping with [3H]d-tubocurarine (14). Mutagenesis of these residues indicated their importance in ACh binding affinity but had little effect on the d-tubocurarine binding affinity (15). Asp-β174 was identified by cross-linking experiments and has been confirmed to affect agonist affinity by site-directed mutagenesis experiments (16). Site-directed mutagenesis also suggested a role for Asp-α152 as stabilizing ACh binding through electrostatic interactions (17).

Site-selective binding of antagonists and agonists is due to the contribution of heterologous residues from the γ- and δ-subunits. Three homologous residues in the mouse γ- and δ-subunits, Met-γ116, Tyr-γ117, and Ser-γ161, were identified from chimeras of these subunits and demonstrated by site-directed mutagenesis to account for the differences in d-tubocurarine binding between the two agonist binding sites (18). A similar approach identified three residues, Lys-γ34, Ser-γ111, and Phe-γ172, and their δ-subunit homologues, as largely accounting for the strong site selectivity of conotoxin MI for the mouse AChR (19). Two of these residues, Lys-γ34 and Phe-γ172, were also shown to be important for the observed difference in agonist affinities for dimers expressed in cell culture (20). The extent that those two residues affect binding affinity was strongly dependent on the conformational state of the AChR, suggesting an indirect role in stabilizing binding but an important role in the transmission of conformational changes (20).

The exact roles of these various residues in stabilizing the binding of agonists and antagonists and in transmitting conformational changes to the ion channel are nonetheless poorly defined. Several of the conserved aromatic residues of the α- and γ-subunits, including Tyr-α93, Tyr-α190, Tyr-α198, Trp-
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1. Structures of d-tubocurarine analogs.

\[
\begin{array}{cccccccc}
R_1 & R_2 & R_3 & R_4 & \text{NAME} \\
G & H & H & H & S & R & d\text{-tubocurarine} \\
H & H & H & H & S & R & Tubocurarine \\
C & H & H & H & S & R & Chondcurarine \\
H & H & C & H & S & R & O,O\text{-dimethyl-tubocurarine} \\
C & H & C & H & S & R & O,O\text{-methyl-chondcurarine} \\
C & H & C & H & S & R & 12\text{-O-methyl-chondcurarine} \\
C & C & H & S & S & R & Metocurine \\
C & H & H & H & I & S & Iodo-d\text{-tubocurarine} \\
C & H & H & H & Br & R & Bromo-d\text{-tubocurarine} \\
H & H & H & H & R & R & I\text{-Beberine} \\
C & H & C & H & H & H & H & N,N\text{-dimethyl-I-beberine} \\
\end{array}
\]

**Stereo Isomer**

\(\alpha\)-149, and Tyr-\(\gamma\)-117, have been suggested to stabilize the amionium moiety that is critical for agonism. Effects on the binding of the minimal agonist, tetramethylammonium, were demonstrated by mutations of Tyr-\(\alpha\)-93, Tyr-\(\alpha\)-190, and Tyr-\(\alpha\)-198, suggesting a direct interaction with the ammonium (21). The effects of mutations on larger ligands, such as d-tubocurarine or its trimethylated derivative, metocurine, are harder to interpret as the mutations potentially affect interaction with different parts of the ligand surface.

To examine ligand-binding interactions sites in greater detail, we have developed a series of analogs of d-tubocurarine that can be used to analyze specific, pairwise interactions between particular functional groups on d-tubocurarine and particular binding site residues. We have previously demonstrated that the aromatic ring of d-tubocurarine that includes the 12\text{-} and 13\text{-}positions interacts with the Torpedo AChR binding sites in a site-selective manner (Ref. 22; see Fig. 1 for structures). In addition, we showed that the charged nitrogens in the d-tubocurarine need not be quaternary ammoniums for binding to the Torpedo AChR binding sites. In this article we present the data on binding properties of d-tubocurarine analogs to mouse AChR. There are several differences that suggest specific interactions between d-tubocurarine and ACh site residues and also suggest binding site interactions with, as yet, unidentified residues. In contrast to the Torpedo AChR, the affinity of curare compounds for the mouse AChR is enhanced by quaternization of the two ammonium groups (see Fig. 1). The substitution of bromine or iodine at the 13\text{-}position decreased the affinity considerably to the \(\alpha\)-\(\gamma\)-site of mouse AChR resulting in a loss of site selectivity. Based on the comparison of the previously mapped residues at the ACh binding sites between the mouse and Torpedo AChR, we have identified a likely site of interaction between the 13\text{-}position of d-tubocurarine and the AChR.

EXPERIMENTAL PROCEDURES

**Materials**—AChR-rich membranes were isolated from \(T.\) californica electric organ (Marinus Inc., Long Beach, CA) as described previously (23) with the addition of calpain inhibitors I and II (10 mg/kg organ).

Purified membranes typically contained 1–2 nmol of acetylcholine (ACH) binding sites/mg of protein measured by binding of \[^3H\]ACh. d-Tubocurarine was obtained from Sigma; metocurine was from Dionys Inc.; and HEPES was from Boehringer Mannheim. The tissue culture materials, chemicals, and media were purchased from Life Technologies, Inc. and Sigma. Tubocurine, chondocurarine, O,O-dimethyl tubocurine, 7\text{-}O-methylchondcurarine, 12\text{-}O-methylchondcurarine, iodo-d-tubocurarine, and bromo-d-tubocurarine were obtained as described previously (22). Other reagents were obtained from standard sources as described previously.

**Results**

125\text{-}I\text{-}BgTx Binding—Binding assays on Torpedo AChR-rich membranes were carried out in HEPES/Torpedo physiological saline solution that also included 0.1% bovine serum albumin (HTTPS: 250 mM NaCl, 5 mM KCl, 3 mM CaCl\(_2\), 2 mM MgCl\(_2\), 0.02% NaN\(_3\), 20 mM HEPES, pH 7.4). Binding of 125\text{-}I\text{-}BgTx was measured using the Debye filter binding method (25) as described previously (22). Surface binding of 125\text{-}I\text{-}BgTx to BC3H-1 cells was measured as follows: the cells were allowed to equilibrate at room temperature for 30 min and then changed to 350 \(\mu\)l of fresh differentiation media with competing ligand at the indicated concentrations. After incubation for another 30 min, 125\text{-}I\text{-}BgTx (~2 nM final concentration) was added to each well and incubated for 45 min. Each well was then washed twice with 0.7 ml of Dulbecco’s modified Eagle’s medium to remove unbound 125\text{-}I\text{-}BgTx. Bound 125\text{-}I\text{-}BgTx was determined in the presence of 250 nM o-BgTx.

Ligand Binding Data Analysis—The analysis of the inhibition data was done by nonlinear least squares fitting of the data to models for single site inhibition, \(B = A/(1 + K_I + Bcg)\), and for inhibition at two equimolar sites, \(B_i = A_i/(1 + K_{IC} + 1/(1 + K_{IC} + Bcg)) + Bcg\) (22). Where \(B_i\) is the concentration of bound ligand, \(A_i\) represents the maximum concentration of the bound ligand at each site, \(I\) the inhibitor concentration, \(Bcg\) the nonspecific binding, and \(K_{IC}\) the concentration of inhibitor required to produce 50% effect. For inhibition of binding to Torpedo AChR by halogenated curare analogs, the data were better fit to the following equation: \(B_j = \{A_j(1 + K_{IC}) + A_j(1 + K_{IC})\} + Bcg\). Where, \(A_1\) and \(A_2\) represent variable site stoichiometry (22). The data are plotted as the fraction of the maximum binding, as determined by the nonlinear regression analysis, after subtraction of the background, which was determined in the presence of excess o-hungarotoxin.

Ancillary Methods—Protein assays were performed using a bicinechonic assay (BCA microassay, Pierce) with bovine serum albumin as a standard. Absorbance measurements of 100 \(\mu\)l solutions of each curare analog in 10 mM HCl and in 1 mM NaOH were carried out on a DU-50 spectrophotometer to verify their concentrations.

RESULTS

The binding of metocurine to the Torpedo AChR differs from that of d-tubocurarine by a 10-fold decreased affinity for the \(\alpha\)-\(\gamma\)-site (22). In contrast, metocurine binds to mouse AChR with a higher affinity than d-tubocurarine (26, 27), suggesting a difference in interaction to the mouse AChR binding sites. To determine the structural basis for these differences, we examined the binding of d-tubocurarine analogs to the mouse AChR by inhibition of the initial rate of 125\text{-}I\text{-}BgTx binding to BC3H-1 cells. To facilitate direct comparison with binding to mouse AChR, we also re-examined the binding of d-tubocurarine analogs to Torpedo AChR by a similar assay and at a comparable pH (pH 7.4), whereas the previous work had been based primarily on inhibition of \[^3H\]ACh binding (22).
Torpedo AChR (panel B; see Fig. 1 for the structures of the various analogs). d-Tubocurarine shows biphasic inhibition of 125I-β-BGTx binding with 30% site selectivity for mouse AChR (the ratio of $K_{12}/K_{11}$, Table I), and 100-fold site selectivity for the Torpedo receptor. The decrease in affinity for the Torpedo αγ-site observed for metocurine, as compared with d-tubocurarine (Fig. 2, panel B, and Table I), was previously shown to be due largely to methylation of the 12′-phenol (22). The moderate change in affinity for the mouse AChR at the αγ-site was in the opposite direction: tri-methylation slightly increased the affinity (Fig. 2, panel A, and Table I). There was no significant change in affinity at the αδ-site. The affinities obtained with the binding of metocurine and d-tubocurarine to mouse AChR agreed well with results reported elsewhere (26, 27).

To determine the respective contributions of $N$-$N'$-dimethylation and $O$-$O$-dimethylation to affinity changes, the following analogs were examined for their binding affinities: the bis-

![Graph](image)

**Fig. 2. Effect of methylation of d-tubocurarine on binding to the mouse and Torpedo AChR.** The affinities of d-tubocurarine () and metocurine () for AChRs on BC3H-1 cells (panel A) and for Torpedo AChR-rich membranes (panel B) were determined by incubating with the indicated concentrations of the competing ligand in media and in HTPS, respectively. The initial rate of 125I-β-BGTx binding was then measured as described under “Experimental Procedures.” The solid lines indicate the best fits to a model for inhibition at two equimolar independent sites. Each data point is the average of duplicate determinations.

### Table I

| Compound                  | Mouse AChR | Torpedo AChR |
|---------------------------|------------|--------------|
|                           | $K_{12}$   | $K_{11}$     | Ratio | $n$ | $K_{12}$ | $K_{11}$     | Ratio | $n$ |
| d-Tubocurarine            | 0.39 ± 0.16 | 12 ± 2       | 30 8  | 0.056 ± 0.028 | 6 ± 2.6 | 108 11 |
| Tubocurine                | 1.3 ± 0.3  | 44 ± 11      | 34 5  | 0.12 ± 0.04  | 8 ± 2.4  | 64 5 |
| Chondocurarine            | 0.26 ± 0.08 | 8.4 ± 3      | 36 10 | 0.17 ± 0.04  | 4.3 ± 1.3 | 21 4 |
| $O,O$-DMTC                | 19 ± 3.5   | (1) 3        | 3.9 ± 2.5 | (1) 3 |
| 7′-O-Methyl-chondocurarine| 0.037 ± 0.011 | 1.3 ± 0.2      | 34 2  | 0.10 ± 0.03  | 10 ± 0.1  | 100 2 |
| 12′-O-Methyl-chondocurarine| 0.15 ± 0.03 | 31 ± 5       | 205 3  | 0.63 ± 0.3   | 31 ± 12  | 49 2 |
| Metocurine                | 0.147 ± 0.005 | 9.5 ± 0.7  | 64 4  | 1.5 ± 0.3    | 8 ± 5  | 6 3 |
| Iodo-d-tubocurarine       | 8 ± 1      | (1) 10       | 0.05 ± 0.03 | 7 ± 5 | 150 11 |
| Bromo-d-tubocurarine      | 3.8 ± 0.8  | 30 ± 10      | 11 3  | 0.045 ± 0.002 | 4.6 ± 0.5  | 184 2 |
| $l$-Bebeerine             | 57 ± 2     | (1) 3        | 6 ± 1.2 | (1) 3 |
| Dimethyl-$l$-bebeerine    | 6 ± 1.2    | (1) 3        | 6 ± 1.2 | (1) 3 |

*The selectivity ratios shown as (1) indicate that the data were equally well fit by a model for inhibition at a single binding site. Therefore, no value for $K_{12}$ is listed.
Effects of Individual O-Methylations—Although the net effect on the binding affinity of 7′,12′-O,O-dimethylation of the bis-quaternary chondocurarine to metocurine was small on the mouse AChR, the effects of individual 7′ and 12′ O-methylations were examined to determine whether this was due to cancellation of larger effects by the individual methylations or simply a lack of effect. The changes in binding upon methylation are shown in Fig. 5. 7′-O-Methylation increases affinity at both sites about 7-fold over the parent compound, chondocurarine, whereas 12′-O-methylation produces a small (less than 2-fold) increase in affinity at the 7′-site, the 7′- and 12′-O-methylations were opposite and negated each other. At the αγ-site, an increase in affinity for the mouse receptor would be predicted from the changes in affinities by the individually methylated compounds, but only a small change was observed.

7′-O-Methylation produces a larger change in affinity for the mouse AChR than the Torpedo AChR, and the effects are non-site-selective for both receptors; the affinity changes were similar in magnitude and direction at each site. 12′-O-Methylation, however, tended to be site-selective in its affinity changes, suggesting that this position interacts with a heterogeneous part of the binding site. For the mouse αγ-site, the effects of 7′-O-methylation were also dependent on the presence of the 12′-O-methyl; comparison of metocurine with 12′-
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O-methyl-chondocurarine does not show the 7-fold increase seen by comparing 7-O-methyl-chondocurarine with chondocurarine. The slightly higher affinity of metocurine for the mouse αγ-site, as compared with d-tubocurarine, was likely a result of O-methylation and, to a lesser extent, the effects of 2-N-methylation. In contrast, the strong decrease in Torpedo αγ-site affinity was due primarily to 12-O-methylation (22), an effect not seen on the mouse αγ-site. The effects of the desensitizing noncompetitive antagonist, proadifen, were examined to determine whether the conformational equilibrium between the resting desensitized states influenced this pattern. Proadifen changed the apparent affinities 3–5-fold without altering the pattern of affinity changes observed in its absence (data not shown).

Effect of 13'-Halogenation—The previous characterization of the binding of the 12'-O-methylated and the 13'-halogenated derivatives on Torpedo AChR suggested that these sites interact with the heterologous parts of the binding sites: the γ and δ-subunits (22). By analysis with 125I-α-BgTx inhibition, 13'-iodo-d-tubocurarine and 13'-bromo-d-tubocurarine had more modest changes in site selectivity than observed by inhibition of [3H]ACh binding (Fig. 6, panel B, and Table I). Analysis of binding to the mouse AChR showed a loss of site selectivity (Fig. 6, panel A, and Table I). This was due to a 20-fold decrease in affinity for the αγ-site as compared with d-tubocurarine. There was no significant change in binding to the αδ-site.

The binding of 13'-iodo-d-tubocurarine to the mouse αγ-site was ~100-fold weaker than to the Torpedo αγ-site, despite similar affinities for the αδ-site. Two possibilities were considered to account for this difference as follows: intrinsic differences at the binding site or differences in the conformational equilibrium between the resting and desensitized conformations of the AChR. In Torpedo, 13'-iodo-d-tubocurarine preferentially binds the desensitized conformation to a greater degree than the other curare analogs (22), and this preference was observed only for the αγ-site; the affinity for the αδ-site was unaffected by desensitization. The mouse and Torpedo AChR differ in their intrinsic propensity to desensitize. The equilibrium constant for desensitization, M, where M = [D]/[R] and D and R represent the desensitized and resting conformations, respectively, is 0.1–0.2 for the Torpedo AChR (29) and near 0.0001 for mouse AChR (28). The weaker affinity observed for 13'-iodo-d-tubocurarine binding to the mouse αγ-site could simply reflect a weaker binding to the resting state, which is more favored in the mouse AChR. To examine this question, the binding of 13'-iodo-d-tubocurarine was determined in the presence of the noncompetitive antagonists phenocyclidine and tetracaine. These compounds shift the equilibrium toward the desensitized and resting states, respectively (29, 30, and as discussed above).

Binding of 13'-iodo-d-tubocurarine to mouse AChR was completely unaffected by the presence of either phenocyclidine or tetracaine (Fig. 7, panel A, and Table III). Nor did the noncompetitive antagonist proadifen affect binding to the mouse AChR; the Kf values do not differ significantly (Table III). For the Torpedo αγ-site, phenocyclidine enhanced binding affinity and tetracaine decreased binding affinity of 13'-iodo-d-tubocurarine (Fig. 7, panel B), as expected for its preference in binding the desensitized conformation. There were only small effects at the αδ-site for either mouse or Torpedo AChR. These results suggest that poorer binding to the resting conformation than to the desensitized conformation does not contribute for the weaker affinity of 13'-iodo-d-tubocurarine for the mouse receptor, but rather, intrinsic differences in the binding site structure are likely to cause this difference.

Effect of Stereosimerization on Binding—The binding of the l-stereoisomers of tubocurarine and chondocurarine, l-bebeberine and dimethyl-l-beberine, to the mouse AChR displays no site
selectivity for either compound (Fig. 8 and Table I). l-Bebeerine binds with 10-fold weaker affinity than dimethyl-l-bebeerine, consistent with the similar changes in affinity observed for tubocurine and chondocurarine. Thus, N,N'-dimethylation enhances affinity equally at both the αγ-site and the αδ-site of the mouse AChR for both the l- and d-isomers. The affinity of the two l-isomers for the Torpedo AChR did not differ dramatically for the αγ-site, as shown previously (22). This was consistent with the lack of effect of N,N'-dimethylation on the affinities for the Torpedo AChR.

DISCUSSION

The data presented here describe the interaction of d-tubocurarine analogs with the mouse AChR. The conclusions from these data that address the orientation of the various functional groups within the binding sites are similar to those derived originally by analysis of [3H]ACh binding to the Torpedo AChR (22); the methylation of the 2-N', 2'-N, and 7'-O-positions results in changes that are similar for the two binding sites and likely reflects interactions with residues that are conserved among the two binding sites. Methylation of the 12'-O-position or halogenation of the 13'-position results in affinity changes that differ between the sites and suggests that these positions interact with heterologous residues contributed by the γ- or δ-subunit at each of the two binding sites.

Several aspects of the binding of the d-tubocurarine analogs to the mouse AChR differed markedly from that observed for the Torpedo AChR. The binding to the mouse AChR was sensitive to methylation of the 2-N and 2'-N ammoniums, whereas such methylation did not affect binding to the Torpedo AChR. This quaternization of the ammoniums also affected the extent to which 7'- and 12'-O-methylation modulated affinity. The effect of 7'-O-methylation was more pronounced in the mouse AChR (7-fold changes) than for the Torpedo AChR (2-fold changes). The effect of 13'-halogenation on binding the mouse AChR was opposite from that in Torpedo: halogenation weakened affinity for the mouse αγ-site. From these distinctions in binding we infer a role for particular amino acids in stabilizing the binding of d-tubocurarine.

Effects of N,N'-Methylation—The enhanced binding affinity for the mouse AChR upon quaternization of the ammoniums appeared primarily as an effect of the 2'-N-methyl group, suggesting that this position is more important than the 2-N position. The binding of the l-stereoisomers, l-bebeerine and dimethyl-l-bebeerine, to the mouse AChR followed the same pattern as the corresponding d-isomers, tubocurine and chondocurarine. The loss in affinity due to inversion at the 1-carbon

Table III

| Antagonist | Mouse AChR, K<sub>m</sub> | Torpedo AChR, K<sub>n</sub> |
|-----------|--------------------------|---------------------------|
| None<sup>a</sup> | 6.8 ± 0.7 | 54 ± 6 |
| Phencyclidine<sup>a</sup> | 9.2 ± 1.0 | 19 ± 6 |
| Tetracaine<sup>a</sup> | 11 ± 2 | 170 ± 30 |
| None<sup>b</sup> | 7.9 ± 0.4 | ND<sup>c</sup> |
| Proadifen<sup>c</sup> | 6.5 ± 0.1 | ND<sup>c</sup> |

<sup>a</sup> The errors are the standard error as determined by the nonlinear regression of the data shown in Fig. 7.
<sup>b</sup> The errors are the range of determinations of the K<sub>n</sub> values from two independent experiments that each directly compared binding of 13'-iodo-d-tubocurarine in the absence and presence of 26 μM proadifen.
<sup>c</sup> ND, not determined.

(see Fig. 1) did not affect the enhancement in affinity by N,N'-dimethylation. Because the inversion at this position strongly affects the conformation of curare compounds in the vicinity of the 2-N-ammonium, the data suggest that this position is not involved in the affinity changes caused by N-methylation. The result supports the conclusion from the affinity changes of the various d-isomers that the 2-N ammonium interacts with the receptor locus that stabilizes the ammonium associated with agonists.

The affinity changes caused by quaternization are not readily explained by differences in the repertoire of amino acids thought to stabilize cholinergic binding. Attention has focused on amino acids that potentially stabilize the ammonium by aromatic π-electron interactions, such as Tyr-α190, Tyr-α198, Tyr-α93, and Tyr-γy117, or by electrostatic stabilization via residues such as Asp-γ174 or Asp-α152. The effects of site-directed mutagenesis of Tyr-α190, Tyr-α198, and Tyr-α93 on the binding of tetramethylammonium, a small symmetric agonist, provides evidence for direct interactions (21). However, comparison of the homologous residues from Torpedo and mouse AChR (Table IV) reveals no differences in any of the α-subunit residues; nor do any of the identified γ- and δ-subunit residues remain conserved between the αγ- and αδ-sites and differ from mouse to Torpedo. The difference in the affinity shift upon quaternization, therefore, suggests the presence of interactions of the ammonium with unidentified residues that are not accounted for by the present set of amino acids. Because of the importance of the ammonium to agonism, such an interaction is likely to be critical for receptor function.

Effects of N,N'-Dimethylation and O,O-Dimethylation Are Linked—The affinity changes for the mouse AChR caused by 7'- and 12'-O-dimethylation were dependent on whether the ammoniums were quaternized; the affinity differences between chondocurarine and metocurine were small, whereas the differences between tubocurine and O,O-dimethyl tubocurine were larger (Table I). This suggests a linked effect. The linkage becomes apparent by analyzing the changes in the free energy of binding (ΔΔG<sub>x</sub>) for pairs of the four compounds, tubocurine, chondocurarine, metocurine, and O,O-dimethyl tubocurine in a thermodynamic cycle (Fig. 9). The free energies of binding are listed in the upper part of Table V. The differences in free energies for each pair of ligands (ΔΔG<sub>x</sub> ), as indicated in Fig. 9, are calculated in the lower part of Table V. If the effects of N,N'-dimethylation were independent from the effects of O,O-dimethylation, then the changes in free energy should be independent of the other modification: ΔΔG<sub>1</sub> = ΔΔG<sub>2</sub> and ΔΔG<sub>5</sub> = ΔΔG<sub>6</sub>. The data in Table IV clearly show show a 1.9 kcal/mol difference between ΔΔG<sub>1</sub> and ΔΔG<sub>2</sub> and between ΔΔG<sub>5</sub> and
The lack of changes at the ad position. The affinity changes caused by halogenation differed between the contrast, it had low affinity for the mouse Torpedo mouse and shown to affect the site selectivity of dimethylation was independent of the O,O of the ligand in the binding site, or the ligand itself can change other methylated sites, the methylation can alter the position causes as follows: the methylations can induce a conformational change in the receptor that affects the interaction at the other methylated sites, the methylation can alter the position of the ligand in the binding site, or the ligand itself can change its conformation. The latter possibility seems unlikely as similar linked effects were not observed for binding the Torpedo AChR. Analysis of the binding of these four ligands in the presence of noncompetitive antagonists that stabilize either the resting conformation or the desensitized conformation of the AChR showed that this conformational change did not affect the pattern of affinity changes. Thus, if a receptor conformational change mediates the linkage, it is unrelated to desensitization. Most likely is that one type of methylation affects the positioning of the ligand in the binding site such that the effects of methylating other sites are altered.

13' Halogens Interact with Residue 116 of the γ- and δ-Subunit—Iodination or bromination of the 13'-position of d-tubocurarine affected the binding and the conformation of the AChR differently in mouse than in Torpedo: 13'-iodo- d-tubocurarine had high affinity for the Torpedo γ-site; in contrast, it had low affinity for the mouse γ-site. Because the affinity changes caused by halogenation differed between the γ-site and αβ-site, this functional group likely interacts with a residue that differed between the sites. The magnitude of the change was also dramatically different between mouse and Torpedo receptor. Thus, residues that differ between the mouse and Torpedo γ-sites are most likely to interact with the 13'-position. The lack of changes at the αβ-site further suggested that the residue be conserved at that site.

Of the residues identified as potentially involved in ligand binding, the following γ-subunit residues differ between the mouse and Torpedo AChR: Ser-γ111, Ile-γ116, and Phe-γ172 (Table IV). The homologous residue of mouse Ser-γ111, Torpedo Tyr-γ111, was labeled by [3H]d-tubocurarine (31) and was shown to affect the site selectivity of α-conotoxin MI (19). However, the mouse αβ-site also has a Tyr at this position, which differs from the Torpedo αβ-site residue (Arg-δ113, Table IV). Thus, the changes in this position do not correlate completely with the observed affinity changes. The homologous residues of mouse Ile-γ116 are Torpedo Met-γ116 and Thr-δ119 (mouse and Torpedo). The amino acid changes at this position correlate well with the affinity changes upon iodination, and this residue is one of three identified by Sine (18) that accounted for the metocurine affinity difference between the mouse AChR γ-site and αβ-site. The homologous residues of mouse Phe-γ172 are Torpedo His-γ172 and Ile-δ178 (mouse and Torpedo). The amino acid changes at this position also correlate well with the affinity changes. This residue was identified as important for determining the site selectivity of conotoxin MI and was implicated as important for the site-selective binding of carbamylcholine to the resting state of the AChR (20). However, it does not appear to affect d-tubocurarine site selectivity.

The closest correlation of the affinity changes induced by iodination at the 13'-site with a binding site residue and with importance for interaction with d-tubocurarine appears to be with Ile-γ116. Site-directed mutation of Ile-γ116 by Sine (18) showed enhanced metocurine affinity when the residue was changed to Val, the δ-subunit homologue, although the magnitude depended on the subunit context and on the other two residues involved in site selectivity. The effect was opposite to the direction of the net decrease in affinity from the γ-subunit. Ile is one methyl group larger than Val. The decrease in affinity for the mouse γ-site due to halogenation may simply reflect increased steric repulsion between the Ile and 13'-iodine. The smaller Val may not show this effect. How-
The second remarkable effect of iodination is the discrepancy in the stabilization of the two predominant conformations. In *Torpedo*, 13'-iodo-d-tubocurarine stabilizes the desensitized conformation better than *d*-tubocurarine. In the mouse AChR, 13'-iodo-d-tubocurarine had no conformational preference although *d*-tubocurarine and the variously methylated analogs preferentially bound the desensitized state. Addition of the halogen clearly affects the ligand's conformational preference, and this preference correlates with the changes in affinity caused by halogenation: higher affinity is associated with the desensitized conformation. Nonetheless, the complete lack of sensitivity of 13'-iodo-*d*-tubocurarine to the conformational state is surprising since all the other analogs are consistent in their modest preference for the desensitized state. It may be that in the mouse AChR, the presence of the halogen disrupts normal interactions of the ligand that induce desensitization (e.g. of the quaternary ammonium). Alternatively, the halogen may interact with residues involved in the conformational change to an extent such that the net propensity to desensitize is reduced. Clear identification of residues that interact with this moiety will be important for understanding how ligands induce conformational transitions in the AChR.

**Orientation of d-Tubocurarine in Its Binding Site**—The data presented here with the conclusions from the previous characterization of curare analogs (22) suggest a crude positioning of d-tubocurarine within its binding site. The interactions of the 2'-N- and 7'-O-positions appear non-site-selective, suggesting interaction with conserved parts of the binding site, most likely constituted of α-subunit residues. Moreover, the 2'-N-ammonium appears likely to interact with the locus that stabilizes the ammonium group of agonists. The heterologous changes in affinity associated with modification of the 12'- and 13'-positions suggest that they interact with nonconserved residues contributed by the γ- or δ-subunits and particularly that the 13'-position interacts with residue 116. The effects of stereo-isomerization about the 1-carbon were also site-selective, suggesting interaction of that end of the molecule with the γ- or δ-subunit.

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