Interaction of d-Tubocurarine Analogues with the Torpedo Nicotinic Acetylcholine Receptor

METHYLATION AND STEREOISOMERIZATION AFFECT SITE-SELECTIVE COMPETITIVE BINDING AND BINDING TO THE NONCOMPETITIVE SITE*

(Received for publication, May 3, 1995, and in revised form, September 11, 1995)

Steen E. Pedersen and Rao V. L. Papineni
From the Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030

Analogues of d-tubocurarine were used to determine the individual effects of methylation, stereoisomerization, and halogenation of d-tubocurarine on the affinity for each of the two acetylcholine (ACh) binding sites of the Torpedo nicotinic acetylcholine receptor (AChR) and for the noncompetitive antagonist site. Eight analogues were synthesized, including three new compounds: 7-O-methylchondocurarine, 12'-O-methylchondocurarine, and 13'-bromo-d-tubocurarine. The two ACh sites differ in their affinities for d-tubocurarine, whereas their affinity ratio for metocurine, the trimethylated derivative of d-tubocurarine, is reduced to 30 due to a decreased affinity for high affinity sites. Binding analysis of five d-tubocurarine analogues demonstrates that methylation of the phenols alone is responsible for the observed changes in affinity. Substitution with bromine or iodine at the 13'-position affected affinity at both sites with a net increase in site selectivity. Stereoisomers of d-tubocurare had decreased affinity for only the high affinity ACh site. Thus, the ring systems, including the 12'- and 13'-positions and the 1-position stereocenter, appear to be important in discriminating between the two ACh binding sites. Desensitization of the AChR was measured by increased affinity for [3H]phenylcyclidine. Binding to the single, high affinity acetylcholine binding site, comprised by the αγ-subunits, was required for partial desensitization of the AChR by d-tubocurarine and its analogs. Stronger desensitization, to the same extent observed in the presence of the agonist carbamylcholine, occurred upon binding by iodinated or brominated d-tubocurarine. Interaction of the analogs at the noncompetitive antagonist site of the AChR was also measured by [3H]phenylcyclidine binding. The bis-tertiary ammonium analogs of either the d- or l-stereoisomers bound to the noncompetitive antagonist site with 100-fold higher affinity than the corresponding quaternary ammonium analogs.

The nicotinic acetylcholine receptor (AChR) from Torpedo californica electric organ is a ligand gated cation channel composed of homologous subunits with a stoichiometry of αβγδ (Raftery et al., 1980; Noda et al., 1983). The five subunits each traverse the lipid bilayer and form a pseudo-symmetric pentameric rosette with the channel located at the central axis (Unwin, 1993). Channel opening is regulated by the binding of two molecules of acetylcholine (ACh) to sites on the extracellular surface of the protein (see Devillers-Thiery et al. (1993) for review). The ACh binding sites are nonidentical and can be distinguished by the differential binding of the plant alkaloid d-tubocurarine, a competitive antagonist (Neubig and Cohen, 1979). One α-subunit and the γ-subunit of the AChR comprise the acetylcholine binding site with higher affinity for d-tubocurarine, while the second α-subunit and δ-subunit comprise the site with lower affinity (Pedersen and Cohen, 1990; Blount and Merlie, 1989). Because the two α-subunits are identical, the distinct affinities of the two sites are likely to arise from interactions with the sites formed by differing aspects of the γ- and δ-subunits.

Affinity labeling of ACh binding sites using sulfhydryl reactive compounds initially identified the α-subunit as the site of acetylcholine binding (Damle and Karlin, 1978; Wolosin et al., 1980). The snake venom toxin α-bungarotoxin could also be shown to bind with moderate affinity to α-subunit, even when denatured and proteolysed (Wilson et al., 1984). Further studies using affinity labeling followed by mapping of the labeled sites to the amino acid sequence has identified residues in the α-subunit involved in acetylcholine binding: Cys-192 and Cys-193 (Kao et al., 1984) and the nearby residues, Tyr-190 (Abramson et al., 1989; Dennis et al., 1988), Tyr-198 (Middleton and Cohen, 1991), as well as residues more distant in the sequence, Trp-149 (Dennis et al., 1988), and Tyr-193 (Cohen et al., 1991). The region α186 to α211 also binds α-bungarotoxin with low affinity and the residues identified by affinity labeling contribute substantially to this binding as judged by mutagenesis of these residues (Chaturvedi et al., 1993).

Several residues of the AChR that interact particularly with d-tubocurarine have been identified by affinity labeling or by site directed mutagenesis. The homologous residues Trp-56 and Trp-157 were identified by labeling and proteolytic mapping with [3H]d-tubocurarine (Chiara and Cohen, 1992). Site-directed mutagenesis of three residues of the mouse muscle AChR γ-subunit (Ile-116, Tyr-117, and Ser-161) to the corresponding residues of the δ-subunit could completely change the affinity to that characteristic of the αδ-site (Sine, 1993). The contribution of the subunits to the binding sites by particular amino acids is well characterized, but form an incomplete picture. Some of the amino acids are predicted to stabilize the quaternary ammonium of acetylcholine (e.g. Tyr-190, Tyr-
Interaction of d-Tubocurarine Analogs with the AChR

α93, Tyr-α198, and Tyr-γ117: Cohen et al., 1991; Sine et al., 1994; Fu and Sine, 1994).

An alternative approach to examining the structure of the acetylcholine binding sites is to compare ligand structural analogs and determine quantitatively the influence of various substitutions upon binding energy (see Gund and Spivak (1991) for review). Many analogs of d-tubocurarine have been analyzed in search of a better muscle relaxant, but such studies utilized in vivo assays of potency that cannot be readily converted to binding affinity and do not take into account pharmacokinetics and other mechanisms such as open channel blockade. Further complications of d-tubocurarine structure-function analysis was the correction of the structure by Everett et al. (1970) from a bis-quaternary ammonium to a mono-quaternary, mono-tertiary ammonium as well as the appreciation by Naghaway and Soine (1978a, 1978b) that some previous synthetic procedures did not yield the expected derivatives (e.g. Marshall et al., 1967). Interpreted of such experiments often emphasized the importance of bis-onium structure for antagonism (Sobell et al., 1972).

Experiments that examined the binding of d-tubocurarine and metocurine, its trimethylated derivative, revealed a difference in their site selectivity for binding to the two distinct acetylcholine binding sites on the AChR (Neubig and Cohen, 1979). Because the difference in structure lay in the methylation of the tertiary ammonium to a quaternary ammonium and methylation of the two phenols (see Fig. 1), we examined whether the difference could be ascribed to a particular site of methylation, or resulted from smaller, additive effects on binding. In this report we describe the analysis of the binding properties of d-tubocurarine and 10 analogs. Binding is characterized for each of the acetylcholine binding sites and the noncompetitive antagonist site of the AChR. To determine how methylation at individual sites affects affinity, five analogs of d-tubocurarine were prepared, including two new compounds: 7'-O-methylchondocurarine and 12'-O-methylchondocurarine. In addition, two halogenated derivatives were prepared: 13'-iodo-d-tubocurarine (Menex et al., 1973) and 13'-bromo-d-tubocurarine. The results demonstrate that the charged nitrogens in d-tubocurarine need not be quaternary ammoniums for binding to the acetylcholine binding sites and that the changes in affinity are accounted for solely by methylation of the phenols. The effect of 13'-halogenation and stereoisomerization are also characterized. To further understand the binding of cholinergic ligands at the acetylcholine binding sites, it is desirable to determine a complete set of amino acids that interact with the ligands and to correlate the receptor sites that are important for binding with their points of contact on the ligand. The series of analogs presented here should facilitate such a characterization.

**EXPERIMENTAL PROCEDURES**

**Materials—**AChR-rich membranes were isolated from Torpedo californica electric organ (Marinus Inc., Long Beach, CA) as described in Pedersen et al. (1986) 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 as described below. Membranes were stored in 37% sucrose, 0.02% NaN3 at −80 °C under argon. Lower specific activity fractions (0.2–0.5 nmol of acetylcholine binding sites/mg of protein) were used for [3H]ACh binding assays. Lauryl sulfate, α-bungarotoxin (α-BgtX), carbamylcholine, I-beberine and Tris were from Sigma. Dissopropyl fluorophosphate and thiophenol were from Aldrich. D-tubocurarine was obtained from either Sigma or Aldrich. Metocurine was from Diosynth Inc. HEPES was from Boehringer Mannheim. [3H]ACh (74 Ci/mol) and [3H]-a-BgTx (200 Ci/mmol) were obtained from Amersham Corp., [3H]-glycine (12 Ci/mmol) from DuPont NEN, and [3H]-d-tubocurarine (11 Ci/mmol) was kindly donated by David C. Chiara and Jonathan B. Cohen. [3H]-d-Tubocurarine was isotopically diluted to 0.22 Ci/mmol for binding assays and was shown to be 80% radiochemically pure by assay for ability to bind the AChR. I-Beberine was crystallized from 4:1 methanol:methylene chloride prior to using in assays. Mepradin was synthesized according to Krödel et al. (1979) as described by Pedersen (1995).

**Synthesis of d-Tubocurarine Analogs—**The structures of the d-tubocurarine analogs used in this study are shown in Fig. 1. Three compounds were obtained commercially: d-tubocurarine (7,12-dihydroxy-6,6'-dimethoxy-2,2'-trimethylcurcurianum chloride), metocurine (6,6',7,12'-tetramethoxy-2,2'-tetramethylcurcurianum diiodide), and I-beberine (12,12'-dihydroxy-6,6'-dimethoxy-2,2'-dimethylcurcurianum). The remaining compounds were synthesized as described below. The synthesis and purification of each compound was conveniently monitored using reversed phase high pressure liquid chromatography (HPLC). The elution of the compounds is shown in Fig. 2. HPLC was also used to establish purity of newly synthesized compounds, particularly the lack of contamination by related compounds. This was particularly important for proper interpretation of differences in binding affinity for the various compounds. Electrospray mass spectrometry was routinely used to confirm the structure and assess possible purity of the synthesized compounds.
contamination by other curare compounds. The (M + nLi)z peaks observed in mass spectra of I by Naghaway and Soine (1979) were not seen. Electrospray mass spectroscopy is likely a gentler method that is less prone to breakdown of the specimen; nonetheless we frequently observed a minor (M – 58)z peak, even with commercial compounds after recrystallization.

(-)-Tubocurarine (II) was obtained by demethylation of d-tubocurarine using the sodium thiophenoxide method of Shamma et al. (1966). This procedure was also used by Naghaway and Soine (1978b) to obtain II in good yield. Sodium thiophenoxide was prepared by treatment of thiophenol with NaOH and extracted with CHCl3. This yielded 0.18 g (0.29 mmol, 54%) another 9% of the product was recovered by conversion to the chloride by methylation with methyl iodide as described by Naghaway and Soine (1978). This material was purified as the chloride and was dissolved by rotary vacuum evaporation to remove solvent. The product was redissolved in 100 ml NH4HCO3 pH 9.0, and applied to a 2.5 x 5-cm CM-Sephadex C25 cation exchange column. The column was eluted with a 1-liter exponential gradient from 100 to 300 mM NH4HCO3 pH 9.0. Fractions were assayed by absorbance at 280 nm and by HPLC. The starting material (II) eluted first and was completely separated from V and VI. Metocurine (VII) was eluted last with a subsequent 2 x NH4HCO3 pH 9.0 wash.

Fractions containing pure VI were neutralized with acetic acid and lyophilized yielding a yellow powder that contained significant salt contamination. This was rechromatographed over the same column and eluted with a step change to 0.4 mM NH4HCO3 pH 9.0. The product was dried by rotary vacuum evaporation, redissolved in water, and brought to pH 2.0 with trifluoroacetic acid. This material was applied to a preparative C18 reversed phase HPLC column (22 x 150 mm, Beckman Ultrasphere) and eluted with a step change to 30% acetonitrile. The eluate was dried to a yellow powder that was essentially salt free (0.127 g, 0.18 mmol, 15%).

Fractions from the first cation exchange column that contained V also contained significant quantities of VI. To completely purify V, the fractions were neutralized with acetic acid, lyophilized, and redissolved in 10 ml of 100 mM NH4HCO3 pH 9.0. This material was reapplied to the same cation exchange column and eluted with a gradient from 100 to 400 mM NH4HCO3 pH 9.0. This achieved sufficient separation of VI from V. The fractions containing pure V were pooled and concentrated to dryness by rotary vacuum evaporation (41 mg, 54% of theoretical yield). Compounds V and VI were deemed pure by HPLC. HPLC traces of V contained no visible contaminants, while VI contains a small impurity visible as a shoulder on peak VI in Fig. 2. This minor contaminant was not a product of the reaction but appeared during purification. Absorption spectra of V and VI in 10 mM HCl (Fig. 3) revealed extinction coefficients at 280 nm similar to those of chondocurarine (I) and metocurine (VI). Mass spectra of the two compounds were similar yielding the predicted double ion mass peak (m/z = 284.2).

Since the two products are similar and have identical molecular weights, the location of the methoxy groups were assigned by 13CNMR. The data for the region, including the methoxy groups, are as follows:

88.5, 72.3, and 57.6 ppm is characteristic of the middle aryl methoxy substituent (Breitmaier and Voelter, 1987) and thus identifies compound V as 12-O-methylchondocurarine. The NMR data corresponded well to those obtained by Koke et al. (1981) for the corresponding analogs of I-beberine.

Iodo-d-tubocurarine (7′, 12′-Dihydroxy-6′, 6′-dimethoxy-2, 2′-dibromo-tubocuraranium) and 12′-O-Methylchondocurarine (7′, 12′-Dihydroxy-6′, 6′-dimethoxy-2, 2′-dibromo-tubocuraranium) was synthesized and purified essentially as described by Menez et al. (1973). The product was pure as judged by HPLC and by mass spectroscopy (m/z = 735.2 and 368.2).
344.2 for the protonated double ion).

**π,π’-Dimethyl-1-beberine (I)** was treated by preparation of X (0.1 g, 0.18 mmol) with 0.36 ml of methyliodide in 1.8 ml of methanol plus 1 ml of CHCl3. The product precipitated from solution slowly after overnight reaction. This material was crystallized from water to yield a white powder (55 mg, 62 μmol, 35%) that was pure by HPLC and mass spectroscopy (m/z = 312.2).

Ligand Binding Assays—Binding assays were carried out in HEPES-Torpedo physiological saline solution (HTPS: 250 mM NaCl, 5 mM KCl, 3 mM CaCl2, 2 mM MgCl2, 0.02% NaN3, 20 mM HEPES, pH 7.0). Ligand binding experiments with [3H]ACh binding assays, the AChR-rich membranes, or a less spure side fraction of membranes from the discontinuous sucrose gradient fractionation, were first incubated with diisopropylfluorophosphonate to inactivate acetylcholinesterase. Membranes (100 μg) were then incubated in HEPES at room temperature with the indicated concentrations of ligand for 30 min and then centrifuged at 19,000 × g for 30 min in a TOMY MTX-150 microcentrifuge to separate bound from free ligand. The free ligand concentration was determined by counting an aliquot of the supernatant. Bound ligand was determined by counting the pellet after dissolving in 10% SDS. Nonspecific binding was determined by including a competitive inhibitor in high concentration.

**125I-a-BgTx Binding Assay**—Binding of 125I-a-BgTx was measured using the DE-81 filter binding method of Smith and Raftley (1973). AChR-rich membranes were incubated in 60 μl of HTx containing 0.1% BSA with 125I-a-BgTx. After incubation for 45 min or for 1 h in the reaction was diluted 5-fold into 10 ml Tris, pH 7.4, 0.1% Triton X-100, 0.1% BSA with 300 nM a-BgTx to stop any further binding. Aliquots (60 μl) of the samples were then spotted onto DE-81 filters. The filters were then washed twice with 100 ml of 10 ml Tris, pH 7.4, 50 mM NaCl, 0.1% Triton X-100, blotted, and then counted for bound 125I-a-BgTx. In HTx, the incubation time with 125I-a-BgTx is within the linear portion of the binding reaction and this condition measures the initial rate of binding. When competing ligands were included they were preincubated with the AChR for 30 min to addition of 125I-a-BgTx. Extended incubation in HTx reveals binding to approximately half of the sites measured by [3H]ACh binding, whereas incubation in 10 mM Tris, pH 7.4, 0.1% Triton X-100, 0.1% BSA shows stoichiometric binding. These results are consistent with those of Conti-Tronconi et al. (1990) that indicate negative cooperativity of a-BgTx binding to the AChR in physiological buffers.

Ligand Binding Data Analysis—Inhibition data were analyzed by nonlinear least squares fitting of the data to models for single site inhibition. Bt = A(1 + I/Kapp) < Bcg, and for inhibition at two equimolar sites, Bt = A0D(1+I/K1app)+1/(1+I/K2app)+Bcg, where Bt is the concentration of bound ligand, A the maximum concentration of ligand at each site, I the inhibitor concentration, Bcg the nonspecific or background level of binding, and Kapp the concentration of inhibitor required to produce a 50% effect. Inhibition of 125I-a-BgTx binding by halogenated analogs was not well fit by this equation but could be better described using the following equation: Bt = A0(1 + I/K1app) + A2(I + I/K3app) + Bcg, A1 and A2 represent variable site stoichiometry. Although such an assumption is inconsistent with the expected 1:1 ratio of ACh binding sites on the AChR, this equation nonetheless consistently fit this particular data set better than other models. Nonlinear least squares fitting was performed using the program SigmaPlot (Jandel Scientific version 4.1 or Windows version 2.0). Equilibrium dissociation constants for inhibitors (Ki) were determined from the Kapp values: Ki = KappK1/1L + Kapp + L, where L is the free radioactive ligand concentration, and Kapp is the equilibrium dissociation constant for the radioactive ligand. The Ki for [3H]ACh was determined in independent experiments to be 17 nM. For inhibition of the initial rate of 125I-a-BgTx binding, Kapp = Kapp.

For [3H]PCP binding experiments performed in the absence of carbamylcholine, binding was sometimes increased due to the allosteric effect of binding to the ACh sites. Such data were fit to a model describing simple binding: Bt = A(I + I + K) + Bcg. For some ligands, this effect was followed by direct competitive inhibition at higher concentrations of ligand. These data were modeled using an equation to describe binding modulated by a direct inhibition function.

\[
B_t = \left( B_0 + \frac{A}{1 + K_1} \right) \frac{K_2}{K_2 + Bcg} \quad \text{(Eq. 1)}
\]

In this equation, B0 represents the binding of [3H]PCP in the absence of other ligands, A is the amplitude of binding induced by ligand I, K1 is the corresponding dissociation constant, and K2 is the binding constant for inhibition. Data from experiments performed in the presence of carbamylcholine were fit to the equation described above for single site competitive inhibition.

**RESULTS**

Analogues of d-tubocurarine (I) were synthesized to analyze the effects of specific modifications on the binding affinity for the AChR. The procedures for the isolation and synthesis of many analogs and derivatives of d-tubocurarine have been described previously (Dutcher, 1946, 1952). Compounds II, III, IV, VIII, IX, and XI were, therefore, prepared essentially according to published procedures with the modifications indicated under "Experimental Procedures." In each case they were the predominant expected product, and the structure was corroborated by mass spectroscopy.

To examine the particular effects of O-methylation on binding to the AChR, two new compounds were synthesized: 7′-O-methylchondocurarine (V) and 12′-O-methylchondocurarine (VI). The UV absorption spectra of the starting material, compound II, and compounds V, VI, and VII in acid and in base are shown in Fig. 3. The absorbance peak of II increases and shifts from 280 to 290 nm with increased pH. The change in absorbance is most pronounced in the region from 295 to 310 nm and is similar to the changes seen with d-tubocurarine (data not shown; Kalow, 1954). The change in the spectra are ascribed to titration of the 7′- and 12′-phenols to phenolate ions. Because the corresponding methoxy groups are untitrat-
The binding of ammonium and one phenol) in each of these analogs. The changes in the pK values expected from the assignments of Kalow (1954). Titration of compounds [3H]ACh binding. The data for the various binding sites was analyzed by competitive inhibition of Kapp values determined for inhibition at each site were used to fitted curves is shown by the corresponding solid lines. The Kapp values determined for inhibition at each site were used to calculate the corresponding dissociation constants, K1 values, as described under “Experimental Procedures.” Inhibition of [3H]ACh binding by d-tubocurarine (I) shows biphasic inhibition (Fig. 4A) with a 450-fold site selectivity (the ratio of K1/K12, Table II). The dissociation constants (K1 values, Table II) agree well with previously published binding constants determined by direct binding of [3H]d-tubocurarine and by competitive binding assays (Neubig and Cohen, 1979; Pedersen and Cohen, 1990). The higher affinity site is comprised by the α- and γ-subunits of the AChR and will be referred to as the αγ-site in the following discussion; the low affinity site will be referred to as the αδ-site.

The site selectivity of metocurine, the trimethylated analog of d-tubocurarine, is reduced compared with that of d-tubocurarine. This is a result of a 10-fold decrease in affinity for the αγ-site with no significant change in the affinity for the αδ-site (Fig. 4A; Table II). To determine the effect of methylation at the amines, the binding of the bis-tertiary ammonium analog, tubocurarine (II), and the bis-quaternary ammonium analog, chondocurarine (III), were examined (Fig. 4B). The affinities at each site were comparable with those of d-tubocurarine, exhibiting less than 2-fold changes in the K1 values, and demonstrate that tertiary and quaternary ammoniums interact equally well at the binding site (Table II). This result further suggested that methylation of the two phenols was responsible for the affinity change displayed by metocurine at the αγ-site. This was confirmed by the binding of the bis-tertiary ammonium analog, O,O-dimethyl tubocurarine (IV), which had binding affinities indistinguishable from metocurine (Fig. 4B and Table II).

To examine whether the affinity change due to methylation of the phenols could be accounted for by a single modification or resulted from effects exerted by both modifications, the binding of 7′-O-methylchondocurarine (V) and 12′-O-methylchondocurarine (VI) was determined (Fig. 4C). Comparison of the K1 values calculated from the titration curves by nonlinear fitting to one or two titratable sites.

### Table I

| Compound            | No. | pK1 | pK2 |
|---------------------|-----|-----|-----|
| d-Tubocurarine      | I   | 8.24| 9.21|
| Chondocurarine      | III | 8.30| 8.85|
| 7′-O-Methylchondocurarine | V | 8.68|     |
| 12′-O-Methylchondocurarine | VI | 8.39|     |
| Metocurine          | VII |     |     |

### Table II

| Compound            | No. | K1  | K12 | Selectivity |
|---------------------|-----|-----|-----|-------------|
| d-Tubocurarine      | I   | 30± 13 | 2 | 450 |
| Tubocurine          | II  | 25± 22 | 5 | 870 |
| Chondocurarine      | III | 45± 11 | 2 | 240 |
| O,O-Dimethyl tubocurarine | IV | 380± 14 | 3 | 37 |
| 7′-O-Methylchondocurarine | V | 39±5 | 5 | 130 |
| 12′-O-Methylchondocurarine | VI | 315±34 | 6 | 110 |
| Metocurine          | VII | 340±10 | 2 | 28 |
| Iodo-d-tubocurarine | VIII| 16± 47 | 10 | 2900 |
| Bromo-d-tubocurarine| IX  | 18± 44 | 10 | 2400 |
| 1-Bebeerine         | X   | 300±80 | 20 | 57 |
| N,N′-Dimethyl-1-bebeerine | XI | 220± 400 | 15 | 7 |

Fig. 4. Effect of methylation of d-tubocurarine analogs on binding to the AChR as determined by inhibition of [3H]ACh binding. AChR-rich membranes (100 μg; 36 pmol of ACh binding sites) were incubated with 100 nM [3H]ACh and the indicated concentrations of competing ligand in 1 ml of HTPS. Bound [3H]ACh was then determined after removal of free [3H]ACh by centrifugation as described under “Experimental Procedures.” Each panel shows a separate experiment that included controls with no added ligand (●) and with 100 μM carbamylcholine (○). A, d-tubocurarine (I, ●), metocurine (VI, ○), B, chondocurarine (III, ●); tubocurarine (II, ○); O,O-dimethyl tubocurarine (IV, △), C, 12′-O-methyl chondocurarine (VI, ○), 7′-O-methyl chondocurarine (V, ○). Each set of data was fitted to a model for inhibition at two equimolar independent sites (190). Each data point is the average of duplicate determinations that generally varied less than 5%.
values for V and VI with the parent compound III (Table II) indicates that 7'-O-methylation has no effect on the binding to the αγ-site and increased affinity 2-fold for the αδ-site. Methylation at the 12'-position decreases affinity at the αγ-site 7-fold and at the αδ-site 3-fold. Thus, 7' and 12' methylation both affect binding affinities, but the effect exerted by 12' modification applies to both sites, whereas the effect of 7' modification is weaker and applies only at the αδ-site.

The effect of methylation on binding affinity to the αγ- and αδ-site were also examined by inhibition of the initial rate of binding of 125I-<i>v</i>-BgTx. The net site selectivity was lower using this assay (typical selectivity for d-tubocurarine was 50–150-fold). Nonetheless, the changes in site selectivity were similar to those observed using inhibition of [3H]ACh binding. The conclusions on the effects of methylation on the affinity and site-selectivity are similar (data not shown).

Effect of 13'-Halogenation on Binding Affinity—The more pronounced effect of 12'-O-methylation on binding affinity suggested that this portion of d-tubocurarine interacted directly at the binding site. The structure of d-tubocurarine shows this portion of the molecule pointing into the surrounding solution. Iodination at the adjacent 13'-position had been demonstrated by Menez et al. (1973), and this compound was shown to behave biologically active, but its binding properties have not been characterized in detail. Therefore, we examined the binding properties of the iodo- and bromo-derivatives of d-tubocurarine, compounds VIII and IX. Inhibition of [3H]ACh binding to the ACh binding sites is shown in Fig. 5A, and the corresponding values are listed in Table II. Both VIII and IX displayed 2-fold increased affinity for the αγ-site and 4-fold decreased affinity for the αδ-site. This resulted in a significant increase in site selectivity to more than 2000-fold.

Examination of the affinities of d-tubocurarine and iodo-d-tubocurarine by inhibition of the initial rate of binding of 125I-<i>v</i>-BgTx yielded a similar 5-fold increase in site selectivity for iodo-d-tubocurarine (Fig. 5B; for I, K<sub>11</sub> = 45 nM, K<sub>12</sub> = 2.5 μM, and selectivity is 57-fold; for VIII, K<sub>11</sub> = 50 nM, K<sub>12</sub> = 15 μM, and selectivity is 300-fold). Bromo-d-tubocurarine (IX) displayed a similar inhibition pattern. However, the inhibition by the halogenated derivatives could not be fit using the equation for inhibition at two equimolar sites. These data were consistently better fit to an equation with variable site stoichiometry. A ratio of 2 to 1 of high affinity to low affinity sites was typically observed. The inhibition curves for all other derivatives tested could be fit well using the model with two equimolar sites. This difference may be related to the ability of the halogenated analogs to desensitize the AChR more strongly as shown below in Fig. 8, thereby causing noncompetitive effects on the binding of 125I-<i>v</i>-BgTx at the low affinity site. Similar noncompetitive effects on α-toxin binding have also been observed by a desensitizing noncompetitive antagonist (Krodel et al., 1979).

The High Affinity Site Is the αγ-Site for the d-Tubocurarine Derivatives—To ensure that the effects of methylation and halogenation on site selectivity were not the result of more radical affinity changes that resulted in inversion of site selectivity between the two sites, [3H]d-tubocurarine was used as the radioligand at a concentration such that binding was primarily to the αγ, high affinity site. The ability of the methylated and halogenated analogs to compete for this binding at concentrations consistent with binding to the αγ-site demonstrated that no inversion of site selectivity had occurred (data not shown).

Effect of Altered Stereochemistry on Binding—Various stereoisomers related to d-tubocurarine have been isolated from natural products. Most have been characterized as noncholinergic, suggesting that they bind the AChR poorly despite the structural homology to d-tubocurarine. I-Bebeerine (X) is a stereoisomer of tubocurarine (II) with an inverted configuration at carbon 1 (Fig. 1). Comparison of the binding properties should reveal the importance of the correct stereoisomer. The dimethylidiole of I-bebeerine, a stereoisomer of chondocurarine, was also synthesized (compare XI versus III in Fig. 1).

Inhibition of [3H]ACh binding to the ACh binding sites by X and XI is shown in Fig. 6A and the corresponding values in Table II. Inhibition by X was incomplete at 300 μM and K<sub>12</sub> is therefore poorly determined. Higher concentrations of X could not be used as they disrupted membrane pelleting in the assay. Therefore, binding of X was also examined by inhibition of the initial rate 125I-<i>v</i>-BgTx binding as shown in Fig. 6B. These data are well fit by a single inhibition constant of 5.6 μM, suggesting no site selectivity. This value is reasonably consistent with the K<sub>11</sub> determined by inhibition of [3H]ACh binding, but differs substantially from K<sub>12</sub>. Inhibition of 125I-<i>v</i>-BgTx binding by d-tubocurarine is also shown (Fig. 6B, K<sub>11</sub> = 25 nM and K<sub>12</sub> = 4 μM). Inhibition of [3H]ACh binding by XI was also described by a two-site fit (Fig. 6A). The value for K<sub>12</sub> was similar to those of the d-isomers but K<sub>11</sub> was substantially higher. The K<sub>11</sub> value for X and XI was similar (Table II). Thus, the primary effect of stereoisomerization appears to be a 50-fold reduction in affinity at the αγ site with only a small effect at the αδ-site.
Interaction of Curare Analogs with the Noncompetitive Antagonist Binding Site—The data for inhibition of $[^3H]ACh$ binding and $[^{125}I]$-BgTx binding by $l$-bebeerine and iodo-$d$-tubocurarine suggested the presence of allosteric effects in addition to strictly competitive binding at the $ACh$ sites. One potential source of allosteric modulation is through the noncompetitive antagonist (NCA) site of the $AChR$. Therefore, the binding of the curare analogs to the NCA site was examined by inhibition of $[^{3H}]PCP$ binding. $PCP$ binds at the NCA site with $-5$-fold higher affinity to the desensitized conformation than the resting conformation. The following experiments were carried out using a low concentration of $[^{3H}]PCP$ ($-1$ nm). Because this concentration is substantially lower than the dissociation constant (near $1 \mu M$), only a small fraction of the $AChR$ are occupied. The amount bound, therefore, may reflect changes in affinity due to allosteric modulation (e.g. by agonist binding to the $ACh$ binding sites) in addition to being inhibitable by direct competitive binding at the NCA site. The allosteric regulation is illustrated by the data performed in the presence of carbachol, which induces desensitization, and therefore results in more observed binding than in its absence (Fig. 7, compare filled with open squares). The inclusion of $1 \mu M$ carbachol also serves to block binding to the $ACh$ sites by the competing ligand. 

Tubocurarine inhibited $[^{3H}]PCP$ binding in the presence of carbachol, whereas chondocurarine had little effect (Fig. 7A, filled symbols). In the absence of carbachol, both compounds increased $[^{3H}]PCP$ binding to a level near $60\%$ of the binding observed in the presence of carbachol (Fig. 7A, open symbols). For tubocurarine, this was followed by inhibition of binding at higher concentrations (Fig. 7A, open triangles) yielding a bell-shaped curve. The enhanced binding induced at the lower concentrations was presumably due to desensitization of the $AChR$ induced upon binding of the ligand at the high affinity $ACh$ binding site ($\alpha_\gamma$-site). The increase in binding was well fit to curves describing binding at a single site (Fig. 7A, solid lines), and the $K_{app}$ values ($230 \, \mu M$ for chondocurarine) were consistent with titration of the high affinity binding sites. The $K_{app}$ was substantially lower than the $K_{1/2}$ for binding to the low affinity $\alpha_\delta$-site.

The $l$-isomers of chondocurarine and tubocurarine, dimethyl-$l$-bebeerine and $l$-bebeerine, were likewise examined for their effects on $[^{3H}]PCP$ binding (Fig. 7B). In the presence of carbachol, $l$-bebeerine could fully inhibit binding, whereas dimethyl-$l$-bebeerine required $100\%$-fold higher concentrations for inhibition (Fig. 7B, filled symbols). The $l$-isomers also increased binding in the absence of carbachol, as did the $d$-isomers, but the effect was only $2$-fold (Fig. 7B, open symbols), and only $l$-bebeerine inhibited binding at higher concentrations (open triangles).

Thus, $[^{3H}]PCP$ binding to the NCA site was inhibited $100\%$ more potently by the $biss$-tertiary analogs, tubocurarine, and $l$-bebeerine, than their $biss$-quaternary counterparts, chondocurarine and dimethyl-$l$-bebeerine. The $K_{app}$ values for inhibition are given in Table IIII. The $K_{app}$ values were decreased in the presence of carbachol, suggesting that tubocurarine and $l$-bebeerine bind with higher affinity to the NCA site in the desensitized conformation. Likewise, metocurine increased $[^{3H}]PCP$ binding in a manner similar to that of chondocurarine whereas $O,O$-dimethyl tubocurarine displayed a pattern similar to tubocurarine (data not shown). Thus, only the $biss$-tertiary compounds bind the NCA site with appreciable affinity. 

Iodo-$d$-tubocurarine increased $[^{3H}]PCP$ binding to the same extent as carbachol whereas $d$-tubocurarine increased binding to only $60\%$ of that level (Fig. 8). In each case, the $K_{app}$...
for the binding increase was consistent with titration of only the high affinity, \( \alpha_2 \)-site. Bromo-d-tubocurarine increased binding to the same extent as ido-d-tubocurarine (data not shown). Only the halogenated analogs increased binding to the level observed in the presence of carbamylcholine.

**DISCUSSION**

The work presented in this article correlates the structure of d-tubocurarine with its site selectivity for the nicotinic acetylcholine binding sites. The initial observation that metocurine has 10-fold lower affinity for the \( \alpha_2 \)-site and unchanged affinity for the \( \alpha \)-site, as compared with d-tubocurarine, provided a starting point for determination of structural features that affect site selectivity. The data demonstrate that methylation of the phenolic groups alone was responsible for the affinity changes observed with metocurine. It was further shown that halogenation at the 13'-position and stereoisomerization at the 1-position also affect site selectivity. Together the data support the notion that the phenyl ring bearing the 12' and 13' substituents and the fused rings that bear the carbon 1 stereocenter interact with residues important for site selectivity.

Independent Effects of Methylation upon Binding Affinity—The insignificant changes in affinity observed upon changes in N-methylation from tertiary to quaternary ammoniums demonstrated that there is no requirement for a quaternary ammonium in d-tubocurarine for binding to the AChR sites. While the importance of the positive charge is undisputed, this observation is consistent with the general lack of correlation between the potency of agonists and the successive state of methylation of the positive center associated with agonists (see Gund and Spivak (1991) for review). Recent studies of mutant AChR have indicated the importance of Tyr-\( \alpha_9 \), Tyr-\( \alpha_190 \), and Tyr-\( \gamma_117 \) (Sine et al., 1994; Fu and Sine, 1994) to the binding of metocurine by stabilization of the quaternary ammonium. The results presented here show that a quaternary ammonium interaction is not required. It may be that tertiary ammonium ligands are equally well accommodated by these particular residues or that other portions of metocurine actually interact with these residues.

As \( N \)-methylation did not account for the observed affinity changes in metocurine, the unmethylated II was compared with its O,O-dimethyl analog, IV, to reveal differences due to methylation at only the phenolic groups. Those differences fully account for the change in affinity between d-tubocurarine and metocurine. The individual contributions of each \( O \)-methyl modification were established using 7'-O-methylchondocurarine and 12'-O-methylchondocurarine and demonstrated that each methylation contributed to the total affinity change. The free energy of binding (\( \Delta G \)) to each of the sites was calculated from the binding constants for compounds III, V, VI, and VII (Table IV). The change in free energy of binding (\( \Delta G \)) relative to III was then calculated for each compound at each site. From the values it can be seen that the free energy changes due to methylation at the individual sites when summed are nearly equal to the free energy change when metocurine is compared with chondocurarine. This demonstrates that the effects of individual methylation accounts for the observed affinity changes of metocurine compared with chondocurarine. The individual effects of methylation are, therefore, additive and unlikely to involve allosteric or synergistic effects on binding and the changes in binding due to each methylation can be interpreted separately.

The effect of 7'-O-methylation is weaker and increases binding affinity only to the \( \alpha \)-site. Methylation at the 12'-phenol decreases binding affinity at both sites but to different extents, having a nearly 10-fold effect on binding to the \( \alpha \)-site. The compensatory effects at the \( \alpha \)-site result in unchanged affinity when both 7'- and 12'-O-methylations are present. Halogenation of 13'-position produced smaller affinity changes at each site, but in opposite directions and resulting in a substantial change (5-fold) in site selectivity. The adjacent 12' and 13'-positions have the strongest effects observed with the modifications tested here and suggests that this part of the structure interacts directly with the binding site. It is tempting to speculate that this portion of d-tubocurarine interacts particularly with a portion of the site that affects site selectivity, particularly residues of the \( \gamma \) and \( \delta \) subunits, but such a conclusion must be tempered by the observation that mutagenesis of an \( \alpha \)-subunit residue can also affect site selectivity (Tyr-\( \alpha_190 \); Sine et al., 1994).

**Allosteric Interactions**—To determine the binding constants for the d-tubocurarine analogs, we relied upon inhibition of [\( ^3H \)]ACh binding with saturating concentrations of [\( ^3H \)]ACh and calculated the \( K_v \) values based on the affinity of [\( ^3H \)]ACh for the AChR. However inhibition at the low affinity and high affinity sites reflect different situations. Competition for binding at the high affinity site occurs with [\( ^3H \)]ACh presumably present on the low affinity site, whereas competition for the low affinity site presumably has the competing ligand present at the high affinity site. In the case of the low affinity site, it is possible that the equilibrium dissociation constant for [\( ^3H \)]ACh differs from the experimentally determined value because of the presence of the competing ligand at the high affinity site. This may compromise the calculation used to determine the \( K_v \).
at the low affinity site (see "Experimental Procedures"). The dominant conformational change is the equilibrium between the resting and desensitized conformations. The Torpedo AChR is 10-20% desensitized in the absence of ligand (Cohen and Stnrd, 1987 and references therein), defining an allosteric equilibrium constant of $M = 0.1-0.2$ for this conformational change. d-Tubocurarine and its variously methylated analogs will desensitize the AChR to 60%, effectively changing $M$ to near 1. This could potentially result in a 5-fold change in affinity for $[3H]$ACh. More importantly, the change will be similar for each of the methylated analogs as they desensitize to the same extent (Figs. 7 and 8 and associated text). Thus, while the accuracy of the binding constant to the low affinity site with moderate affinity (Table III) and with a slight, 2-fold change in binding affinity to the $\alpha\gamma$-site, $\Delta G_1$ refers to the $\alpha\gamma$-site, $\Delta G_2$ refers to the $\alpha\delta$-site.

Energy minimization of l-bebeerine using the MM2 algorithm (Hyperchem version 2.0) was compared with the energy minimized structure of d-tubocurarine (data not shown). An exact alignment of the molecules in the vicinity of the 2'-ammonium and the carbon 1' stereocenter was achieved with the major deviations in the fused ring structure associated with the carbon 1. While it is tempting to speculate that inversion at the carbon 1 decreases binding affinity due to local changes in that vicinity, and therefore that portion of tubocurarine interacts most closely with the ACh sites, it is equally possible that perturbation of the structure as a whole affects the binding affinity. Nonetheless, the ring system that includes the 1-position must interact uniquely with the $\alpha\gamma$-site and suggests that site-specific interactions are closely associated with this portion of the molecule. Testing the other stereoisomer of d-tubocuraine, d-bebeerine (chondodendrine), which has inverted stereochemistry at the carbon 1', should elucidate this issue.

Interaction with the NCA Site—l-Bebeerine could potently inhibit binding of $[3H]$PCP to the NCA site of the AChR in the presence of high concentrations of agonist. N,N'-dimethylation reduced the affinity 100-fold. A similar inhibition pattern was seen for the d-isomers. Although d-tubocurarine has been characterized as a voltage-dependent open channel blocker on the frog neuromuscular junction at $\mu M$ concentrations (Colquhoun et al., 1979), the affinity for the NCA site in Torpedo AChR has been shown to be low ($K_a < 9\, \text{mM}$; Cohen et al., 1985). For both l-bebeerine and the d-isomer, tubocurarine, binding at the NCA site is preferentially to the desensitized conformation as seen by the ability to inhibit binding in the presence and absence of agonist (Table III). The large loss of affinity upon quaternization of these compounds suggests that the ammonium binds in a sterically constrained portion of the NCA site, which is within the ion channel, or disrupts a particular interaction with the tertiary ammonium (such as a hydrogen bond).

**Desensitization by Binding to the $\alpha\gamma$-Site—Occupancy of the $\alpha\gamma$-site by the tubocurarine analogs increased the observed binding of $[3H]$PCP. This increase in binding is interpreted as higher affinity for $[3H]$PCP due to desensitization of the AChR. The l-isomers induced significantly less $[3H]$PCP binding than the d-isomers. This likely reflects the inability of the altered structure to induce the desensitized conformation. d-Tubocurarine and its methylated analogs produced partial desensitization (60%), whereas the iodo- and bromo-d-tubocurarine analogs desensitized the AChR to the same extent as the agonist carbamylcholine. The $K_{app}$ for increased $[3H]$PCP binding (Figs. 7 and 8) is correlated with binding to the high affinity $\alpha\gamma$-site. There was no evidence that binding to the $\alpha\delta$-site...
further altered [3H]PCP binding. Thus, binding to only the single αγ-site is required for desensitization. The extent of desensitization by d-tubocurarine was consistent with that observed by Cohen and Strnad (1987) using a [3H]ACh titration assay (Boyd and Cohen, 1984) and with the effects of d-tubocurarine on the binding affinity of [3H]histrionicotoxin (Cohen et al., 1986).

Desensitization by ACh appears to require binding to only a single site, as suggested by a Hill coefficient of 1 for the slow transition to the state that has high affinity for ACh (Neubig et al., 1982). Desensitization by proadifen of mouse muscle type AChRs when expressed in limited subunit combinations (αγβγ2 and αγβγ) suggests that desensitization more strongly affects agonist affinity at the αγ-site (Sine and Claudio, 1991). That result and the data presented here suggest that the binding energy that drives desensitization is derived predominantly, but not exclusively, from binding to the αγ-site. Nonetheless, the dramatic difference in the K_{app} for channel opening (near 100 μM; Neubig et al., 1982), which reflects binding to the resting conformation, and the equilibrium affinity (~20 nM) dictates significant conformational changes at both binding sites upon desensitization. The complete absence of effect on [3H]PCP affinity by the d-tubocurarine analogs upon binding the αδ-site is unlikely to be due only to a lesser contribution of this site to desensitization but probably also reflects the weaker preference of d-tubocurarine for the desensitized conformation, compared with the strong preference of agonists.

By determining features of the structure that affect site-selectivity, due to the orientation of d-tubocurarine in the binding site relative to the surrounding αγ and αδ subunits are obtained. This initial set of data begins a description of the structural basis of binding affinity of tubocurarine compounds. The series of analogs described should serve as a starting point for a point-to-point mapping of contact sites between amino acids on the AChR and specific functional groups on d-tubocurarine. This can be accomplished by studying changes in affinity of the analogs upon mutation of specific binding site residues. Such studies should lead to full description of the ACh binding sites and be able to elucidate the relative location of amino acids within the site.

Acknowledgments—We thank Hyunah Choi for technical assistance and David Chiara and Jonathan B. Cohen of Harvard Medical School, Department of Neurobiology for their gift of [3H]d-tubocurarine.

REFERENCES
Abrahamson, S. N., Li, Y., Culver, P., and Taylor, P. (1989) J. Biol. Chem. 264, 12666–12672
Bick, I. R. C., and McLeod, L. J. (1974) J. Pharm. Pharmacol. 26, 985–987
Blount, P., and Merlie, J. P. (1989) Neuron 3, 349–357
Boyd, N. D., and Cohen, J. B. (1984) Biochemistry 23, 4023–4033
Bretmaier, E., and Voelter, W. (1987) Carbon-13 NMR Spectroscopy, VCH Verlagsgesellschaft, Weinheim, Germany
Chaturvedi, V., Donnelly-Roberts, D. L., and Lentz, T. L. (1993) Biochemistry 32, 9570–9576
Chiarra, D. C., and Cohen, J. B. (1992) Biophys. J. 62, A106
Cohen, J. B., and Strnad, N. P. (1987) in Molecular Mechanisms of Desensitization to Signal Molecules (Konijn, T. M., ed) Springer-Verlag, Berlin
Cohen, J. B., Medynski, D. C., and Strnad, N. P. (1985) in Effects of Anesthesia (Fozard, H. A., ed) pp. 53–64, American Physiological Society, Bethesda, MD
Cohen, J. B., Correll, L. A., Dreyer, E. B., Kuisk, I. R., Medynski, D. C., and Strnad, N. P. (1986) in Molecular and Cerebral Cell Mechanisms of Anesthetics (Roth, S. H., and Miller, K. W., eds) Plenum Publishing Corp., New York
Cohen, J. B., Sharp, S. D., and Liu, W. S. (1991) J. Biol. Chem. 266, 23354–23364
Conti-Tronconi, B. M., Tang, S., Walgrave, S., and Gallagher, W. (1990) Biochemistry 29, 1046–1054
Cox, D., Dreyer, F., and Sheridan, R. E. (1979) J. Physiol. (London) 293, 247–284
Dame, V. N., and Karlin, A. (1979) Biochemistry 18, 2039–2045
Dennis, M., Giraudet, J., Kitzyba-Hilbert, F., Goedner, M., Hirth, C., Chang, J.-Y., Lazure, C., Chretien, M., and Changeux, J.-P. (1988) Biochemistry 27, 2346–2357
Devillers-Thiry, A., Galzi, J. L., Eisele, J. L., Bertrand, S., and Changeux, J.-P. (1993) Membr. Biol. 136, 97–112
Dutcher, J. D. (1946) J. Am. Chem. Soc. 68, 419–424
Dutcher, J. D. (1952) J. Am. Chem. Soc. 74, 2221–2225
Evertt, A. J., Lowe, L. A., and Wilkinson, S. (1970) J. Am. Chem. Soc. 92, 13735–13743
Kalow, W. (1954) J. Pharm. Pharmacol. 110, 433–442
Kao, P. N., Dwork, A. J., Kaldany, R. J., Silver, M. L., Wideman, J., Stein, S., and Karlin, A. (1984) J. Biol. Chem. 259, 11652–11656
King, H. J. (1935) Chem. Soc. 1381–1389
Kolke, L., Marsaidi, A. J., and Reis, P. de A. M. (1981) J. Org. Chem. 46, 2385–2389
Kroedel, E. K., Bedkman, R. A., and Cohen, J. I. (1979) Mol. Pharmacol. 15, 296–312
Marshall, I. G., Murray, J. B., Smail, G. A., and Stenlake, J. (1967) J. Pharm. Pharmacol. 19, 535–570
Menez, A., Bouet, F., Morel, J.-L., Ronseray, A.-M., Bouquet, P., Changeux, J.-P., and Fromageot, P. (1973) Biochimie (Paris) 55, 919–924
Middleton, R. E., and Cohen, J. B. (1991) Biochemistry 30, 6987–6997
Naghashy, J. A., and Soine, T. O. (1978a) J. Pharm. Sci. 67, 473–477
Naghashy, J. A., and Soine, T. O. (1978b) J. Pharm. Sci. 67, 1204–1207
Naghashy, J. A., and Soine, T. O. (1979) J. Pharm. Sci. 68, 655–656
Neubig, R. R., and Cohen, J. B. (1979) Biochemistry 18, 5464–5475
Neubig, R. R., Boyd, N. D., and Cohen, J. B. (1982) Biochemistry 21, 3460–3467
Noua, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., and Numa, S. (1983) Nature 302, 528–532
Petersen, S. E. (1995) Mol. Pharmacol. 47, 1–9
Petersen, S. E., and Cohen, J. B. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2785–2789
Petersen, S. E., Dreyer, E. B., and Cohen, J. B. (1986) J. Biol. Chem. 261, 13735–13743
Raftery, M. A., Hunkapiller, M. W., Strader, C. D., and Hood, L. E. (1980) Science 208, 1454–1457
Schmidt, J., and Raftery, M. A. (1973) Anal. Biochem. 52, 349–354
Shamma, M., Deno, N. C., and Remar, J. F. (1966) Tetrahedron Lett. 13, 1375–1379
Sine, S. M. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 9436–9440
Sine, S. M., and Claudio, T. (1991) J. Biol. Chem. 266, 13969–13977
Sine, S. M., Quiriam, P., Papanikolaou, F., Kreinikamp, H.-J., and Taylor, P. (1994) J. Biol. Chem. 269, 8808–8816
Soell, H. M., Sakore, T. D., Tavare, S. S., Canepa, F. G., Pauling, P., and Petcher, T. J. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 2212–2215
Unwin, N. (1993) J. Mol. Biol. 229, 1101–1124
Wilson, P. T., Gershoni, J. M., Hawrot, E., and Lento, T. L. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 2533–2537
Wolsin, J., Lyddiatt, A., Dolly, J. O., and Barnard, E. A. (1980) Eur. J. Biochem. 109, 495–505
Interaction of d-Tubocurarine Analogs with the Torpedo Nicotinic Acetylcholine Receptor: METHYLATION AND StereoISOMerIZATION AFFECT SITE-SELECTIVE COMPETITIVE BINDING AND BINDING TO THE NONCOMPETITIVE SITE
Steen E. Pedersen and Rao V. L. Papineni

J. Biol. Chem. 1995, 270:31141-31150.
doi: 10.1074/jbc.270.52.31141

Access the most updated version of this article at http://www.jbc.org/content/270/52/31141

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 14 of which can be accessed free at http://www.jbc.org/content/270/52/31141.full.html#ref-list-1