Functional Effects on the Acetylcholine Receptor of Multiple Mutations of γAsp174 and δAsp180†

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ABSTRACT: Residues γAsp174 and δAsp180, previously implicated in the binding of acetylcholine (ACh) by the mouse muscle ACh receptor, were each mutated to nine other residues, Asn, Glu, Thr, Ala, Cys, His, Val, Tyr, and Lys. The effects of the mutations on ACh-induced current was determined on surface receptors containing wild-type α and β subunits and mutant γ and δ subunits. The mutations increased the concentration of ACh eliciting half-maximal current (EC_{50}) by factors from 22 for the Glu mutant to 660 for the Lys mutant. Analysis of the effects in terms of the difference in the accessible surface areas of the mutant and wild-type side chains and the difference in side-chain charges indicated that, per binding site, $\Delta\Delta G^0$ for activation was a sum of 10 cal mol^{-1} Å^{-2} of change in side-chain accessible surface area and of 0.95 kcal mol^{-1} positive step^{-1} in side-chain charge, equivalent to 1 mol of charge falling through 42 mV. The effects on the concentration of ACh ($IC_{50,ACh}$) and of d-tubocurarine ($IC_{50,dTC}$) causing half-maximal retardation of α-bungarotoxin binding were determined on complexes containing wild-type α and β subunits and either mutant γ or mutant δ subunit. The effects on $IC_{50,ACh}$ correlated well with the effects on EC_{50}, with a similar magnitude for the influence of side-chain charge on the free energy of binding (in this case to the desensitized state) and on the electrostatic potential at the binding site. The effects on $IC_{50,dTC}$ were in all cases less than the effects on $IC_{50,ACh}$, and the two sets of effects were poorly correlated. In line with the higher ACh affinity and lower d-tubocurarine affinity of the α-δ binding site compared to the α-γ binding site, mutations of δAsp180 had a greater effect on $IC_{50,ACh}$ than did the same mutations of γAsp174, and vice versa for effects on $IC_{50,dTC}$. Consequently, all mutations decreased the asymmetry in the binding properties of the two types of sites.

The binding of acetylcholine (ACh)[1] by nicotinic receptors promotes the transitions of the receptor from the resting state to the open and the desensitized states (*1*). An initial step in these transitions was suggested by the functional effects of quaternary ammonium affinity labels of different lengths: longer labels acted like tethered antagonists and shorter labels acted like tethered agonists (*2*). One explanation was that these labels acted as bridges between two parts of the binding site, a subsite around the Cys residues and a negative subsite that bound the quaternary ammonium group, and further, that the shorter labels stabilized a contracted, active state of the site. Reversibly binding agonists, but not bulkier competitive antagonists, could also activate the receptor by inducing a contraction of the binding site (*2, 3*). The affinity labels were attached specifically to one of a pair of adjacent cysteine residues, normally cross-linked by a disulfide bond, which was reduced prior to labeling. These residues are αCys192 and αCys193 (*4, 5*). Since the distance from the end of the affinity labels that reacted with the Cys residue to the positively charged, quaternary ammonium end was 9−12 Å, a reasonable assumption was that the binding site contains a negatively charged group at this distance from the disulfide (*2*). Such a group, the carboxylate of δAsp180, was cross-linked to αCys192/193 with a 9-Å cross-link (*6, 7*). The question is whether or not δAsp180 and the aligned γAsp174 interact directly with bound ACh.

Muscle-type ACh receptors contain two nonidentical ACh binding sites (*8*−*10*). Normally, these receptors are composed of four types of subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ (*11*−*13*). One of the ACh binding sites is formed in the interface between the first α subunit and the γ subunit, and the other site is formed between the second α subunit and the δ subunit (*6, 14*−*18*).

The mutation to Asn of δAsp180 (*19*) or of γAsp174 (*20*) increased the apparent dissociation constant for ACh and other agonists by 2 orders of magnitude. Mutations to Asn or Gln of nine other neighboring Asp and Glu residues in the γ subunit and 11 in the δ subunit had much less effect on binding. The mutations to Asn of δAsp180 and of γAsp174 also increased the apparent dissociation constants for competitive antagonists, but only by 1 order of magnitude (*20*). The effects of the mutations are consistent with a close, electrostatic interaction of the Asp carboxylates with agonists and a less significant interaction with antagonists. If the quaternary ammonium groups of the agonists and antagonists bind in the same location, then the greater effect of the mutations on agonist binding implies that γAsp174 and δAsp180 are also involved in the conformational changes

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[1] Abbreviations: ACh, acetylcholine; dTC, d-tubocurarine; DTT, dithiothreitol; MBTA, 4-(*N*-maleimido)benzyltrimethylammonium; NEM, *N*-ethylmaleimide.

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induced by agonists but not by antagonists. Mutations of other binding-site residues, αTyr93 and αTyr190 (*21*−*24*), also had a much greater effect on agonist binding than on antagonist binding (*25*), and some of these differences were ascribed to changes in gating kinetics (*26*, *27*). In contrast, mutation of αAsp152 to Asn caused similar decreases in the apparent affinities for different agonists and for the antagonist d-tubocurarine (*28*).

In order to test further that the effects of the mutations of γAsp174 and δAsp180 to Asn were primarily due to the loss of the negative charges, we made nine additional mutations of each and tested their effects on both ACh-induced current and on ACh and d-tubocurarine (dTC) retardation of α-bungarotoxin binding. The results are consistent with a change in the electrostatic potential affecting agonist binding at each ACh binding site of 42 mV for each positive step in charge at these positions.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression. Mouse muscle acetylcholine receptor subunit cDNAs (provided by T. Claudio, Yale University) were subcloned into pSP64T at the *Bgl*II site. Mutations of the γ subunit were made in a cassette defined by the restriction enzymes *Bst*X1 and *Msc*I, by the Altered Sites in vitro mutagenesis system (Promega). All mutations were confirmed by sequencing the cassette in both directions. The cDNA for each subunit in the SP64T plasmid was transcribed with SP6 polymerase under standard conditions (Promega) to generate capped mRNA. Mutations and the mutant subunits are designated by subunit, wild-type residue, position, and mutant residue, with residues indicated in single-letter code, e.g., γ(D174N). Oocytes from *Xenopus laevis* were prepared as described by Czajkowski *et al*. (*19*). The oocytes were injected with 50 nL of total mRNA (200 pg/nL), consisting of mRNA for each subunit in the ratio of 2α: 1β: 1γ: 1δ or 2α: 1β: 2γ, or 2α: 1β: 2δ. Oocytes were used 1−6 days after injection for electrophysiological experiments and for preparing membrane fractions.

Since the expressed subunits can form various complexes, we indicate the combination injected as the subunits separated by plus signs. Mutations, if any, are indicated within parentheses following the appropriate subunit.

Electrophysiology. ACh-induced currents were recorded by two-electrode voltage clamp, at a holding potential of −40 mV, as described by Akabas *et al*. (*29*). The currents elicited by various concentrations of ACh were fit by the Hill equation, $I = I_{max}/\{1 + (EC_{50}/[ACh])^n\}$.

Toxin Binding Measurements. The binding of ^{125}I-α-bungarotoxin at different concentrations was determined as described by Martin *et al*. (*20*). The concentration of toxin giving half-maximal binding, K'_{tox}, was estimated by a nonlinear least-squares fit of the specifically bound toxin, *Y*, to the equation $Y = Y_{max}/[1 + (K'_{tox}/X)^n]$, where X is the ^{125}I-α-bungarotoxin concentration. Given the rate constants for the association and dissociation of α-bungarotoxin (*17*), the binding would not be expected to reach equilibrium in 24 h, especially at low concentrations of α-bungarotoxin, and therefore K'_{tox} is not an equilibrium constant.

Ligand Binding Measurements. The IC_{50}s of ACh and d-tubocurarine were determined from their slowing of the binding of ^{125}I-α-bungarotoxin. A total membrane fraction was prepared (*19*) from oocytes expressing wild-type or mutant receptor. Membrane (50 μL) in NP50 buffer (50 mM NaCl, 10 mM $NaPO_4$, and 1 mM EDTA, pH 7.0) was pretreated for 20 min with 200 μM diisopropyl phosphofluoridate to inhibit cholinesterases and combined with 0.75 nM ^{125}I-α-bungarotoxin (50 μL) and various concentrations of agonist or antagonist (50 μL), all in NP50 buffer, in a final volume of 150 μL. The final ^{125}I-α-bungarotoxin concentration was 0.25 nM. After 3 h at room temperature, the suspension was filtered through Reeves−Angel glass-fiber filters presoaked in 2% poly(ethylenimine) and washed two times with 5 mL of ice-cold buffer as described by Martin *et al*. (*20*). The amount of ^{125}I-α-bungarotoxin on the filters was determined by liquid scintillation counting.

The inhibition constant, $IC_{50,ligand}$, for ligand was obtained by a nonlinear least-squares fit of the binding data to

$$Y = (Y_0 - U)/[1 + (A/IC_{50,ligand})^n] + U \qquad (1)$$

where *Y* is the specifically bound ^{125}I-α-bungarotoxin, defined as that blocked by 1 μM α-bungarotoxin, Y_0 is the specifically bound ^{125}I-α-bungarotoxin in the absence of ligand, and *A* is the concentration of ligand. *U* is the specifically bound ^{125}I-α-bungarotoxin not blocked by saturating concentrations of the ligand; *U* was estimated by the fit.

Titration of γ(D174H). To determine the IC_{50} for ACh of αβγ(D174H) receptor at different pH values, membrane was sedimented in a Beckman Airfuge at 100000*g* for 5 min, and the pellet was suspended in a citric acid−phosphate buffer, providing a buffering range from pH 5.0 to 8.0 (100 mM NaCl, 1 mM EDTA, 10 mM Na_2PO_4, and 3−50 mM citric acid). The remainder of the experiment followed the protocol described above. The acid dissociation constant, K_a, for the substituted histidine was estimated by a nonlinear least-squares fit to

$$IC_{50,ACh} = ({}^{+}IC_{50,ACh})([H^+]/K_a)/[1 + ([H^+]/K_a)] + {}^{0}IC_{50,ACh}/[1 + ([H^+]/K_a)] \qquad (2)$$

where $^{+}IC_{50,ACh}$ is the inhibition constant of ACh for the receptor when the histidine is charged, $^{0}IC_{50,ACh}$ is the inhibition constant of ACh when the histidine is uncharged, and $[H^+]$ is the hydrogen ion concentration.

Determining Rate of MBTA Labeling. Membrane in NP50 buffer was incubated with an equal volume of 0.4 mM DTT (100 mM NaCl, 20 mM Tris, and 1 mM EDTA, pH 8.3) for 30 min at room temperature. Membrane was sedimented in a Beckman Airfuge at 100000*g* for 3 min. The pellet was suspended in NP100 buffer (100 mM NaCl, 20 mM $NaPO_4$, and 0.4 mM EDTA, pH 7.0) with 10 μM DTT, added to either water or various concentrations of MBTA, and incubated for 3 min at room temperature. The reaction with MBTA was stopped by reacting the membrane with 100 mM NEM for 10 min at room temperature. Membrane was sedimented again in the Airfuge. The pellet was suspended in TNP50 buffer (50 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, and 0.2% Triton X-100, pH 7.0) and held for 15 min at room temperature.

To determine the rate of MBTA labeling of ^{125}I-α-bungarotoxin binding sites, membrane was incubated for 3 h with 2.5 nM ^{125}I-α-bungarotoxin and either TNP50 buffer for total binding or 10 mM ACh or 1 μM unlabeled

α-bungarotoxin to determine nonspecific binding. Membrane was then filtered, washed, and counted as described previously. The rate of MBTA labeling of receptor was determined by the fit to

$$Y = (Y_0 - U)\exp(-ktm) + U \quad (3)$$

where Y is specific ^{125}I-α-bungarotoxin binding, Y_0 is the specific binding in the absence of MBTA, U is the binding that cannot be blocked by MBTA, k is the rate constant for MBTA reaction, t is a fixed time, and m is the concentration of MBTA. Since m was varied at constant t, the fit yielded k.

Sucrose Density Gradients. Oocyte membrane was harvested in NP50 buffer as described previously (*20*). The level of receptor expression was determined for each membrane sample with both ^{125}I-α-bungarotoxin binding and protein assays, and approximately 10 fmol of receptor was loaded on each gradient. To label receptors, we incubated membrane with 10 nM ^{125}I-α-bungarotoxin at 4 °C, for 3−18 h. Membrane was washed with NP50 buffer and pelleted to remove unbound radioactivity. Membrane pellets were solubilized at 4 °C in 100 μL of TNP50 buffer with the Triton X-100 concentration raised to 1.1%. After 1 h, the membrane suspension was layered on a 4 mL 5−20% sucrose gradient in TNP50. Gradients were centrifuged in a Beckman SW60 rotor for 5 h at 60 000 rpm at 4 °C. Fractions of 135 μL were collected; 60 μL of each fraction was removed for enzyme assays, and the remaining volume was counted in a scintillation counter. Sedimentation coefficients were estimated by comparison with carbonic anhydrase (3.3S) (*30*), alkaline phosphatase (6.5S) (*31*), and catalase (11S) (*32*) cosedimented with the receptor.

RESULTS

Effects of Mutations of γD174 and δD180 on ACh-Induced Current. Previously, α + β + δ(D180N) and α + β + γ + δ(D180N) (*19*) and α + β + γ(D174N) + δ and α + β + γ(D174E) (*20*) formed functional, presumably pentameric complexes on the oocyte surface membrane. All other mutants of γD174 or δD180, when injected with wild-type α and β, failed to form functional complexes at the surface. Injection of mutant γ *and* mutant δ with wild-type α and β did yield functional complexes at the oocyte surface in all cases except one, the mutants γD174Y and δD180Y. In this last case, there was also no ^{125}I-α-bungarotoxin binding activity on the oocyte surface.

For each of the mutations expressed as a functional complex at the cell surface, the effect of the mutation on the $EC_{50,ACh}$ for ACh-induced current was large (Figure 1; Table 1). Even the conservative mutation of D to E increased the $EC_{50,ACh}$ by a factor of 22; the largest effect was a 656-fold increase in $EC_{50,ACh}$ due to the mutation of D to K. The current extrapolated to infinite ACh concentration, I_{max}, was smallest for the mutants with the largest increases in $EC_{50,ACh}$, but whether the lower maximum currents were due to a decrease in the number of surface receptors or to a decrease in the open probability of the channels is not known. There was little effect of these mutations on the Hill coefficients characterizing the dose−response relationships.

Effects of Mutations on ACh Binding Assayed by Competition with α-Bungarotoxin Binding. The mutant γ or mutant δ were injected as α + β + γ(D174X) or as α + β + δ(D180X). We measured the binding of ^{125}I-α-bungarotoxin to a total membrane fraction of the oocytes. The specific binding varied from 0.1 to 5 fmol/oocyte (Table 2). There was little effect of tested mutations on the apparent binding constant for ^{125}I-α-bungarotoxin: this was 130 ± 30 pM for α + β + γ, 160 ± 30 pM for α + β + γ(D174N), 111 ± 11 pM for α + β + γ(D174K), 98 ± 1 pM for α + β + γ(D174V), and 108 ± 13 pM for α + β + γ(D174T).

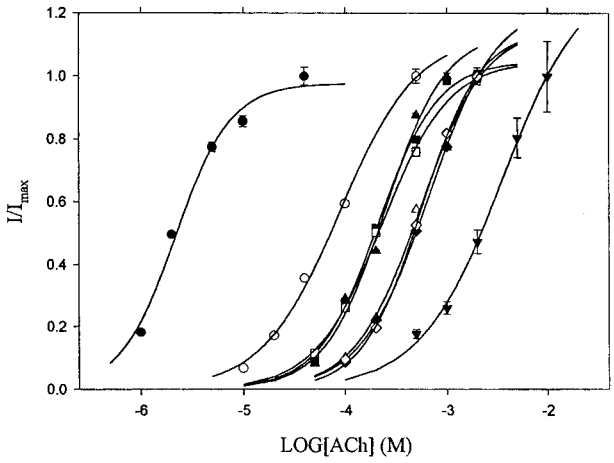

FIGURE 1: Current as a function of ACh concentration for wild-type and mutant receptor. The receptor complexes are wild-type α + β + γ + δ, or mutant α + β + γ(D174X) + δ(D180X). Oocytes were superfused with five or six concentrations of ACh, each for 10 s, with Ca^{2+}-free frog Ringers solution for 5 min between ACh applications, and the series of applications was repeated. The ACh-induced currents were measured with a two-electrode voltage clamp at −40 mV (see Experimental Procedures). The peak currents obtained during the two 10-s applications of each ACh concentration were averaged (I), and the averages were fit by the Hill equation (Table 1). The relative currents (I/I_{max}) and their errors in representative experiments are plotted against the ACh concentration, and the least-squares fits are shown. Data shown are for complexes of wild-type α + β + γ + δ (●), α + β + γ(D174E) + δ(D180E) (○), α + β + γ(D174T) + δ(D180T) (■), α + β + γ(D174A) + δ(D180A) (□), α + β + γ(D174C) + δ(D180C) (▲), α + β + γ(D174N) + δ(D180N) (△), α + β + γ(D174H) + δ(D180H) (◆), α + β + γ(D174V) + δ(D180V) (◇), and α + β + γ(D174K) + δ(D180K) (▼).

The fraction of total specific ^{125}I-α-bungarotoxin binding blockable by ACh varied from 61% to 92% (Table 2). In the cases of complexes obtained from α + β + γ, α + β + γ(D174N), α + β + δ, and α + β + δ(D180N), the fractions of ^{125}I-α-bungarotoxin binding blockable by ACh were the same as the fraction of ^{125}I-α-bungarotoxin-binding complexes that sedimented faster than α monomer in a sucrose density gradient (data not shown); i.e., only multisubunit complexes bound ACh.

The inhibition of ^{125}I-α-bungarotoxin binding by ACh was fit well in all cases by one $IC_{50,ACh}$ and a Hill coefficient (Figure 2; Table 2). In all cases, this fit was at least as good as a fit with two independent IC_{50}s. Nevertheless, it appears that in all wild-type α + β + γ + δ and α + β + δ, there were two classes of ACh binding sites that differed in affinity by 12 and 22 times, respectively, whereas there was only one class of sites in α + β + γ.

For α + β + γ, the $IC_{50,ACh}$ was 6.0 μM, and for the nine mutants of γ tested, $IC_{50,ACh,mut}/IC_{50,ACh,wt}$ varied from 0.9 to 616 (Table 2). For α + β + δ, the $IC_{50,ACh}$ was 0.3 μM ACh. For the seven mutants tested, $IC_{50,ACh,mut}/IC_{50,ACh,wt}$ varied from 248 to 3712.

Table 1: Effects of Mutations in the γ and δ Subunits on the ACh-Induced Current[a]

subunit	γ substitution	δ substitution	EC_{50ACh} (μM)	EC_{50mut}/EC_{50wt}	Hill coefficient	I_{max} (−nA)	*n*
$\alpha\beta\gamma\delta$	wt	wt	3.8 ± 0.5	1	1.4 ± 0.04	3597 ± 184	9
$\alpha\beta\gamma\delta$	D174E	D180E	84 ± 9	22 ± 4	1.1 ± 0.03	1618 ± 447	3
$\alpha\beta\gamma\delta$	D174T	D180T	232 ± 18	61 ± 10	1.5 ± 0.03	2029 ± 354	3
$\alpha\beta\gamma\delta$	D174A	D180A	245 ± 45	64 ± 15	1.3 ± 0.03	2975 ± 348	3
$\alpha\beta\gamma\delta$	D174C	D180C	275 ± 36	72 ± 14	1.4 ± 0.12	1429 ± 125	5
$\alpha\beta\gamma\delta$	D174N	D180N	521 ± 44	137 ± 22	1.5 ± 0.1	680 ± 150	3
$\alpha\beta\gamma\delta$	D174H	D180H	527 ± 60	140 ± 25	1.4 ± 0.06	2371 ± 263	5
$\alpha\beta\gamma\delta$	D174V	D180V	553 ± 19	146 ± 21	1.6 ± 0.03	264 ± 32	4
$\alpha\beta\gamma\delta$	D174K	D180K	2495 ± 326	656 ± 126	1.3 ± 0.08	288 ± 43	4
$\alpha\beta\gamma$	wt		8.8 ± 1.1	1	1.5 ± 0.1	2500 ± 230	5
$\alpha\beta\gamma$	D174E		6 ± 0.8	0.7 ± 0.5	1.3 ± 0.1	600 ± 250	3
$\alpha\beta\gamma$	D174N	wt	24 ± 2	6.4 ± 3.3	1.4 ± 0.1	3500 ± 410	10
$\alpha\beta\delta$		wt	2.7 ± 0.3	1	1.1 ± 0.2	1808 ± 276	7
$\alpha\beta\delta$		D180N	227 ± 26	84 ± 13	1.5 ± 0.1	693 ± 109	9
$\alpha\beta\gamma\delta$	wt	D180N	5.1 ± 0.8	1.9 ± 0.4	1.6 ± 0.03	3775 ± 743	2

[a] Subunit mRNA was injected into *Xenopus* oocytes, and after 1−3 days ACh-induced currents were recorded, as described under Experimental Procedures. The mutant complex $\alpha + \beta + \gamma$(D174Y) + δ(D180Y) produced no detectable ACh-induced current. Peak current as a function of ACh concentration was fitted by the Hill equation. The means, standard errors of the means, and number of independent experiments are given.

Table 2: Effects of Mutations on the Binding of Acetylcholine[a]

subunits	residue substitution	toxin sites/oocyte (fmol/oocyte)	blockable by ACh (%)	IC_{50} (μM)	IC_{50mut}/IC_{50wt}	Hill coefficient	IC50′ (μM)	IC50″ (μM)	*n*
$\alpha\beta\gamma\delta$	none	6.5 ± 1.7	87 ± 3	0.7 ± 0.1		0.74 ± 0.11	0.21 ± 0.07	2.6 ± 0.2	5
$\alpha\beta\gamma$	none	2.7 ± 0.5	83 ± 2	6.0 ± 0.6	1	0.91 ± 0.10	4.0 ± 1.4	4.2 ± 1.3	6
$\alpha\beta\gamma$	D174E	1.0 ± 0.2	85 ± 2	5.1 ± 0.8	0.9 ± 0.2	0.80 ± 0.11			4
$\alpha\beta\gamma$	D174H	4.9 ± 0.2	61 ± 4	35 ± 7.4	5.9 ± 1.4	0.87 ± 0.10			6
$\alpha\beta\gamma$	D174T	2.2 ± 0.1	87 ± 2	181 ± 18	30 ± 4.8	0.73 ± 0.06			4
$\alpha\beta\gamma$	D174C	0.83 ± 0.02	73 ± 2	250 ± 62	42 ± 12	0.70 ± 0.06			4
$\alpha\beta\gamma$	D174V	1.8 ± 0.7	84 ± 2	284 ± 37	48 ± 8	0.91 ± 0.23			4
$\alpha\beta\gamma$	D174A	0.35 ± 0.02	82 ± 5	533 ± 235	89 ± 41	0.83 ± 0.11			3
$\alpha\beta\gamma$	D174N	5.0 ± 1.4	87 ± 4	701 ± 217	117 ± 39	0.69 ± 0.06			5
$\alpha\beta\gamma$	D174K	3.2 ± 0.2	87 ± 1	1176 ± 198	197 ± 41	0.69 ± 0.05			4
$\alpha\beta\gamma$	D174Y	0.08 ± 0.02	92 ± 2	3682 ± 810	616 ± 155	0.50 ± 0.04			2
$\alpha\beta\delta$	none	1.5 ± 0.2	72 ± 6	0.3 ± 0.1	1	0.73 ± 0.06	0.08 ± 0.01	1.8 ± 0.4	6
$\alpha\beta\delta$	D180N	0.35 ± 0.05	68 ± 2	84 ± 22	248 ± 78	0.62 ± 0.08			2
$\alpha\beta\delta$	D180C	0.6 ± 0.02	58 ± 4	100 ± 27	294 ± 98	0.51 ± 0.02			3
$\alpha\beta\delta$	D180A	1.5 ± 0.2	84 ± 6	138 ± 34	402 ± 124	0.92 ± 0.14			3
$\alpha\beta\delta$	D180H	2.5 ± 0.3	64 ± 3	164 ± 19	484 ± 107	0.65 ± 0.07			4
$\alpha\beta\delta$	D180V	1.3 ± 0.1	81 ± 3	282 ± 97	832 ± 325	0.56 ± 0.05			4
$\alpha\beta\delta$	D180T	0.9 ± 0.4	72 ± 14	346 ± 26	1016 ± 206	0.68 ± 0.26			2
$\alpha\beta\delta$	D180K	0.9 ± 0.3	91 ± 1	1262 ± 497	3712 ± 1617	0.54 ± 0.08			3

[a] The inhibition constants (IC_{50}) for ACh were determined from the retardation of the binding of ^{125}I-α-bungarotoxin, as described under Experimental Procedures. Expression of mutant complexes $\alpha + \beta + \delta$(D180E) and $\alpha + \beta + \delta$(D180Y) did not result in specific toxin binding to either membrane homogenates or intact oocytes. For wild-type subunit complexes, both the one-site and two-site fits are shown. Means, standard errors of the means, and number of experiments are given.

Effects of pH on $IC_{50,ACh}$ of $\alpha\beta\gamma$(D174H). For this mutant, the $IC_{50,ACh}$ for the inhibition of ^{125}I-α-bungarotoxin binding increased markedly with decreasing pH (Figure 3). Assuming that $IC_{50,ACh}$ varied as the sum of the fraction of complexes with uncharged His, multiplied by $^{0}IC_{50,ACh}$ characteristic of the site with an uncharged His, plus the fraction with charged $HisH^+$, multiplied by $^{+}IC_{50,ACh}$ characteristic of the site with a charged $HisH^+$ (see Experimental Procedures), we estimate that $^{0}IC_{50,ACh} = 9.2 \pm 6.5$ μM, $^{+}IC_{50,ACh} = 185 \pm 29$ μM, and $pK_a = 5.4$ for the substituted His. Thus, the complex with the positively charged His appears to have an $IC_{50,ACh}$ 20 times greater than the complex with the uncharged His. The apparent dissociation constant for ^{125}I-α-bungarotoxin varied little over the range of pH tested: 67 ± 21 pM at pH 5, 107 ± 33 at pH 7, and 57 ± 4 at pH 8, $n = 3$ for each.

Effects of Mutations on dTC Binding Assayed by Competition with α-Bungarotoxin Binding. For comparison of the mutants, we fit the inhibition curves with a single $IC_{50,dTC}$ and a Hill coefficient, which was as good as a two-site fit (Table 3). As with ACh binding, the two-site fit was consistent with two classes of dTC binding sites in all wild-type $\alpha + \beta + \gamma + \delta$, differing in affinity by about 12 times. In contrast to ACh binding, however, $\alpha + \beta + \gamma$ also showed two classes of dTC binding sites that differed in affinity by about 12 times, whereas if there were two classes of dTC binding sites in $\alpha + \beta + \delta$, their affinities differ by less than 3-fold.

For the nine mutants of γ tested, the ratio of the $IC_{50,dTC,mut}/IC_{50,dTC,wt}$ varied from 1.8 to 29, and for the seven mutants of δ tested, the $IC_{50,dTC,mut}/IC_{50,dTC,wt}$ varied from 0.8 to 16 (Table 3).

Effect of D to N Mutation on the Rate of Labeling by MBTA. After the reduction of the disulfide bond between αCys192 and αCys193, 4-(*N*-maleimido)benzyltrimethylammonium (MBTA) affinity-labels the ACh binding site, alkylating one of these cysteine residues (*2, 4*). This reaction is much faster with one of the two ACh binding sites than

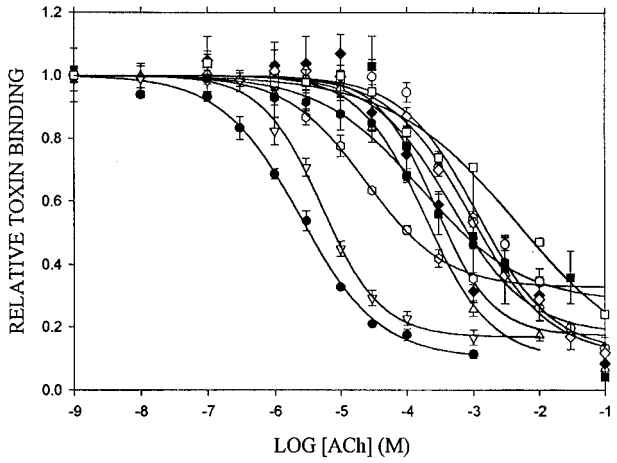

FIGURE 2: Inhibition by ACh of ^{125}I-α-bungarotoxin binding to membranes for wild-type and mutant receptor. Relative toxin binding is the ^{125}I-α-bungarotoxin specifically bound at each ACh concentration divided by the ^{125}I-α-bungarotoxin specifically bound in the absence of ACh, determined as described under Experimental Procedures. Representative single experiments, in which each point is the mean of triplicates ± standard error of the means, are shown. The data were fit as described under Experimental Procedures. Data shown are for complexes of wild type α + β + γ (●), α + β + γ(D174A) (■), α + β + γ(D174T) (△), α + β + γ(D174K) (○), α + β + γ(D174V) (◆), α + β + γ(D174N) (◇), α + β + γ(D174E) (▽), α + β + γ(D174C) (⬢), α + β + γ(D174H) (⬡), and α + β + γ(D174Y) (□).

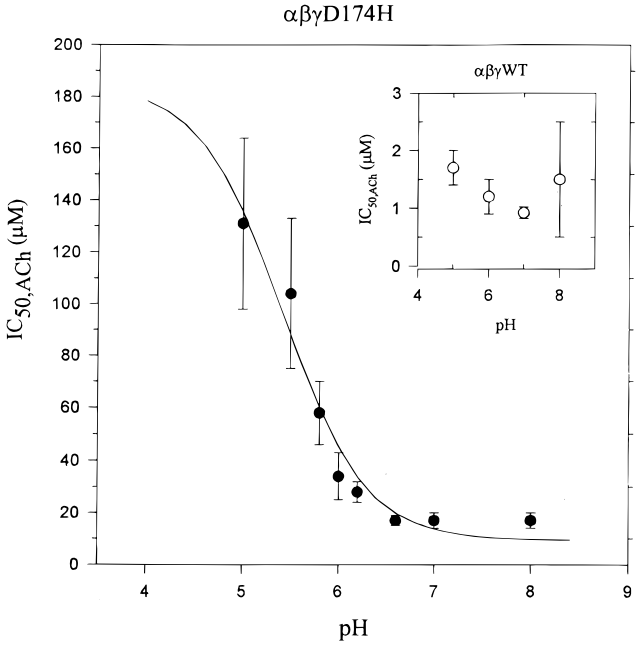

FIGURE 3: Dependence of the IC_{50} for α + β + γ(D174H) on pH. IC_{50} was determined as described in Experimental Procedures. The mean and SEM of 3−5 determinations are plotted (●). The dependence of IC_{50} for all wild-type α + β + γ is shown in the inset (○). The data for the mutant were fit by eq 2 (Experimental Procedures). The least-squares fit yielded $^{0}IC_{50} = 9.2 \pm 6.5$ μM, $^{+}IC_{50} = 185 \pm 29$ μM, and $K_a = 3.9 \pm 1.6$ μM ($pK_a = 5.4$).

with the other (*8*, *33*). We found that the rate constant for the reaction of MBTA with reduced mouse α + β + γ + δ expressed in oocyte membranes was, within a factor of 2, the same as that obtained previously with ACh receptor from *Electrophorus electricus* and *Torpedo californica* electric tissues [(6−8) × 10^5 M^{-1} s^{-1}] (*34*). Also, as with *Torpedo* ACh receptor, half of the ACh-blockable α-bungarotoxin binding sites were blocked by MBTA (Table 4). We determined the rate constants for the reaction of MBTA with α + β + γ and with α + β + δ. These two rate constants were the same and were also close to the rate constant we obtained with α + β + γ + δ. Furthermore, as with α + β + γ + δ, with both α + β + γ and α + β + δ, MBTA blocked half of the ACh-blockable α-bungarotoxin binding sites. The β subunit was required for the reaction of MBTA with half of the sites, since with α + γ and α + δ, 100% of ACh-blockable toxin sites were blocked by MBTA.

We tested the roles of γD174 and δD180 in the affinity reactions with α + β + γ and α + β + δ by determining the effect of mutating these Asp to Asn (Table 4). With α + β + γ(D174N), the rate constant was 5 times smaller than with wild-type α + β + γ, and 100% of the ACh-blockable α-bungarotoxin binding sites were blocked. With α + β + δ(D180N), the rate constant was the same as with wild-type α + β + δ, and 100% of the sites were blocked. Only γD174 is relevant to the rate of the affinity labeling reaction; both γD174 and δD180 are relevant to the reaction of MBTA with just one of the two sites.

Analysis of the complexes by sedimentation in a sucrose density gradient showed no difference in the distributions of complexes between wild-type α + β + γ and α + β + γ(D174N) or between wild-type α + β + δ and α + β + δ(D180N).

DISCUSSION

Influence of Side-Chain Charge on the Binding of ACh. Previous observations are consistent with γD174 and δD180 contributing electrostatically to the binding of ACh. δD180, and, by homology γD174, are located within 1 nm of αC192 and αC193 (*6*, *7*), known to be at the ACh binding sites (*4*, *5*). Thus, these negatively charged Asp residues are at the inferred distance from the pair of Cys to the negative subsite (*2*). Furthermore, neutralizing mutations of Asp to Asn of γD174 and δD180 decreased the EC_{50} and IC_{50} for ACh by 2 orders of magnitude (*19*, *20*). Also, the ACh binding site was shown to be at a negative electrostatic potential relative to bulk solution (*35*). The current studies were undertaken to test further the idea that these residues do contribute electrostatically to the binding of ACh.

We made nine mutations of γD174 and δD180. Of these, eight gave functional receptors on the surface of oocytes when expressed as the combination α + β + γ(D174X) + δ(D180X). We determined the $EC_{50,ACh}$ for each of the α + β + γ(D174X) + δ(D180X) mutants. The functional complex formed from α + β + γ(D174X) + δ(D180X) at the cell surface was a pentamer incorporating all the subunits since, with two exceptions, neither α + β + γ(D174X) nor α + β + δ(D180X) expressed at the cell surface. In the exceptional cases of γ(D174E) and δ(D180N), the EC50s of α + β + γ(D174E) and α + β + δ(D180N) are different than those of α + β + γ(D174X) + δ(D180X) (Table 1).

We estimated the separate influences of mutant side-chain charge and side-chain geometry on the $EC_{50,ACh}$ by fitting the data (Table 1) to

$$\log(EC_{50,mut}/EC_{50,wt}) = a|\Delta A| + b\Delta q \qquad (4)$$

in which *a* and *b* are coefficients, $|\Delta A|$ is the absolute value of the difference between the accessible surface areas of the fully exposed mutant side chain and the wild-type Asp side chain, and Δq is the difference in the algebraic charges of

Table 3: Effects of Mutations on the Binding of d-Tubocurarine[a]

subunits	residue substitution	blockable by ACh (%)	IC_{50} (μM)	IC_{50mut}/IC_{50wt}	Hill coefficient	IC50′ (μM)	IC50″ (μM)	*n*
$\alpha\beta\gamma\delta$	none	80 ± 3	50 ± 9		0.73 ± 0.03	15 ± 4	179 ± 26	8
$\alpha\beta\gamma$	none	78 ± 5	46 ± 17	1	0.84 ± 0.10	20 ± 7	234 ± 156	3
$\alpha\beta\gamma$	D174E	77 ± 2	82 ± 17	1.8 ± 0.78	0.78 ± 0.17			3
$\alpha\beta\gamma$	D174T	80 ± 2	99 ± 14	2.1 ± 0.84	0.80 ± 0.04			4
$\alpha\beta\gamma$	D174Y	65 ± 3	455 ± 86	9.8 ± 4.1	1.14 ± 0.18			4
$\alpha\beta\gamma$	D174N	77 ± 2	466 ± 66	10 ± 4	0.98 ± 0.08			4
$\alpha\beta\gamma$	D174C	72 ± 8	758 ± 198	16.4 ± 7.5	0.94 ± 0.32			4
$\alpha\beta\gamma$	D174V	78 ± 1	835 ± 366	18 ± 10	0.77 ± 0.10			4
$\alpha\beta\gamma$	D174A	50 ± 3	845 ± 220	18 ± 8	0.98 ± 0.12			4
$\alpha\beta\gamma$	D174K	73 ± 2	1338 ± 261	29 ± 12	0.93 ± 0.09			4
$\alpha\beta\gamma$	D174H	55 ± 3	1633 ± 33	35 ± 13	1.10 ± 0.09			3
$\alpha\beta\delta$	none	74 ± 4	337 ± 39	1	0.91 ± 0.06	267 ± 132	726 ± 187	4
$\alpha\beta\delta$	D180H	64 ± 4	260 ± 62	0.8 ± 0.2	0.72 ± 0.09			3
$\alpha\beta\delta$	D180N	77 ± 4	738 ± 76	2.2 ± 0.34	1.04 ± 0.14			2
$\alpha\beta\delta$	D180C	79 ± 7	940 ± 560	2.8 ± 1.6	0.73 ± 0.06			3
$\alpha\beta\delta$	D180V	73 ± 6	1220 ± 329	3.6 ± 1.0	0.85 ± 0.28			3
$\alpha\beta\delta$	D180T	62 ± 10	1700 ± 100	5.0 ± 0.64	0.86 ± 0.21			4
$\alpha\beta\delta$	D180A	68 ± 1	2000 ± 615	5.9 ± 1.9	1.22 ± 0.37			3
$\alpha\beta\delta$	D180K	69 ± 12	5533 ± 649	16.4 ± 2.7	1.44 ± 0.53			

[a] The inhibition constants (IC_{50}) for dTC were determined from the retardation of the binding of ^{125}I-α-bungarotoxin, as described under Experimental Procedures. Expression of mutant complexes $\alpha + \beta + \delta$(D180E) and $\alpha + \beta + \delta$(D180Y) did not result in specific toxin binding to either membrane homogenates or intact oocytes. For wild-type subunit complexes, both the one-site and two-site fits are shown. Means, standard errors of the means, and number of experiments are given.

Table 4: Effects of Mutations on the Rate of Irreversible MBTA Block of ^{125}I-α-Bungarotoxin Binding to Wild-Type and Mutant Receptors[a]

subunits	residue substitution	$(10^{-5})kR$ (M^{-1} s^{-1})	fractional block of toxin binding (%)	*n*
$\alpha\beta\gamma\delta$		3.7 ± 1.0	47 ± 6	7
$\alpha\beta\gamma$		2.8 ± 1.0	55 ± 3	5
$\alpha\beta\gamma$	D174N	0.49 ± 0.13	96 ± 3	5
$\alpha\beta\delta$		3.0 ± 0.5	54 ± 4	6
$\alpha\beta\delta$	D180N	2.9 ± 1.1	99 ± 1	9

[a] Rate constants for the reaction of MBTA with reduced receptor were determined as described under Experimental Procedures. Percent toxin block represents the fraction of ACh-blockable ^{125}I-α-bungarotoxin binding sites blocked by MBTA. Means, standard errors of the means, and number of experiments are given.

the mutant and native side chains (Figure 4). The least-squares fit gives $a = 0.015 \pm 0.01$ and $b = 1.43 \pm 0.03$ ($R^2 = 0.8$). (This equation fits the data better than one in which the difference in the side-chain volumes is used instead of ΔA; the fit is also better with the absolute value of ΔA.) If we take $\Delta\Delta G^0 = 2.303RT \log (EC_{50,mut}/EC_{50,wt})$ to be the difference, mutant *minus* wild type, in the standard free energy for ACh-induced activation of the receptor,[2] then at $T = 298$ K, $2.303RTa = 85.5$ J/mol^{-1} Å^{-2} = 20 cal mol^{-1} Å^{-2}. There are, however, two ACh binding sites, and channel opening is mainly from the doubly occupied receptor. Thus, the free energy difference per ACh binding site is 10 cal mol^{-1} Å^{-2}. This value is about one-third of the magnitude of the hydrophobic effect, which is characterized by a free energy for the transfer of nonpolar groups from nonpolar solvent to water of about 25 cal mol^{-1} Å^{-2} (for discussion see ref 36). The use of the absolute value of ΔA can be rationalized as expressing the free energy penalty of departures from the optimal nonelectrostatic interactions of the native Asp side chain, in the unoccupied state of the receptor and/or in the bound, active state of the receptor. In neither state would we expect the native Asp or mutant side chain to be sequestered from water completely.

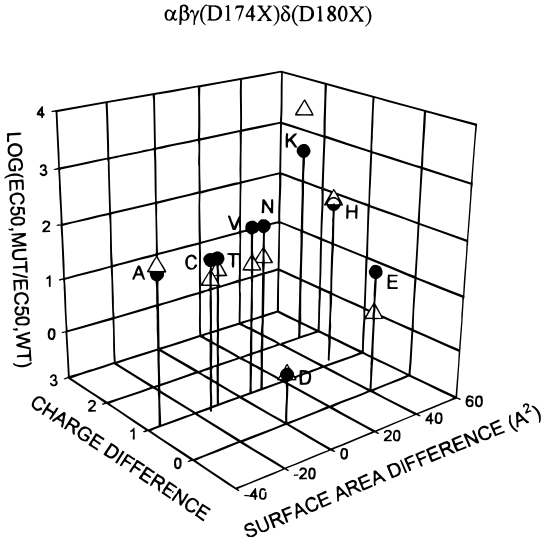

FIGURE 4: $EC_{50,ACh}$ for the ACh-induced current in intact oocytes expressing wild-type α and β subunits and both mutant γ and mutant δ subunits. The mutations were γ(D174X) and δ(D180X), where X is given next to the data point. The wild-type receptor is indicated by D. The $EC_{50,ACh}$ was calculated by fitting the Hill equation to the data (Table 1). The log of the ratio of the means of the observed $EC_{50,ACh}$ for the mutant and wild-type receptors are plotted as a function both of the charge difference and of the accessible surface-area difference between the side chain of the substituted residue and the side chain of the wild-type aspartate. The accessible surface areas of the fully exposed side chains were taken from Chothia *et al.* (*40*). The data (●) were fitted by eq 4 (Discussion), and the fitted values are plotted (△).

[2] The EC_{50} is the product of equilibrium constants for binding of agonist and for isomerization to the open state for the simple kinetic scheme $A + R = AR_{closed} = AR_{open}$, where A is agonist, R is receptor, and the isomerization is strongly toward AR_{open}. This is too simple a scheme, however, to describe accurately the activation of the ACh receptor, which has two nonidentical agonist binding sites and which undergoes desensitization as well as activation. In more realistic schemes, the EC_{50} is a complicated function of the different binding constants and isomerization constants. Hence, the log of the ratios of the EC_{50}s is only an approximation for $\Delta\Delta G^0$.

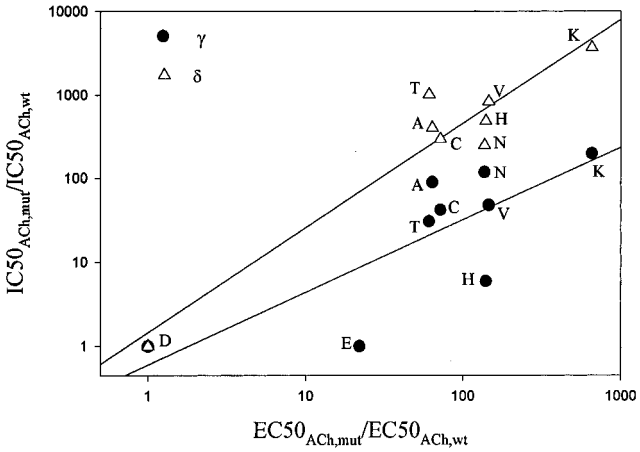

FIGURE 5: Correlation of the binding of ACh and the ACh-induced current in mutants of γ and δ. The binding of ACh was characterized by the $IC_{50,ACh}$ for the retardation of α-bungarotoxin binding (Experimental Procedures). The logarithms of the IC_{50}s for $\alpha + \beta + \gamma$(D174X) divided by the IC_{50} of all wild-type $\alpha + \beta + \gamma$ (●) and the logarithms of the IC_{50}s of $\alpha + \beta + \delta$(D180X) (△) divided by the IC_{50} of all wild-type $\alpha + \beta + \delta$ were plotted against the logarithms of the ratios of the EC_{50}s for $\alpha + \beta + \gamma$(D174X) + δ(D180X) to the EC_{50} of all wild-type $\alpha + \beta + \gamma + \delta$. The regression lines were characterized by r^2 and P values of 0.61 and 0.005 for the γ mutants and 0.92 and 0.0002 for the δ mutants, and hence were statistically significant.

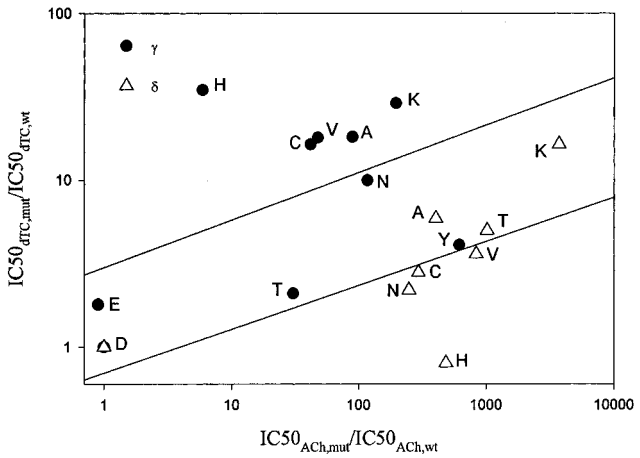

FIGURE 6: Correlation of the binding of ACh and of d-tubocurarine in mutants of γ and δ. The logarithm of relative $IC_{50,dTC}$ is plotted as a function of the logarithm of relative $IC_{50,ACh}$ for $\alpha + \beta + \gamma$(D174X) (●) and $\alpha + \beta + \delta$(D180X) (△) receptor complexes. The r^2 and P values for the γ mutants are 0.24 and 0.16, and for the δ mutants, 0.43 and 0.08. These correlations are not statistically significant.

The second term on the right of the equation is the electrostatic term and can be interpreted as $2.303RTb = z_{ACh}F(\Delta\psi/\Delta q)$, where z_{ACh} is the algebraic charge of two ACh molecules, namely 2, one at each ACh binding site, dropping through a potential difference from bulk solution to the binding site of $\Delta\psi$, and Δq is the difference between the charge of the mutant side chain and the Asp side chain. Given that $b = 1.43$, $\Delta\psi/\Delta q = 42$ mV; i.e., for each positive step in the side-chain charge, the electrostatic potential at each binding site increases by 42 mV. Previously, we estimated the electrostatic potential at reduced αCys192/193 (excluding any contribution of the thiols) as −30 mV at an ionic strength of 135 mM (*35*). It is reasonable to assume that 1 nm away from the Cys residues, close to the Asp residues, the electrostatic potential is even more negative than −30 mV. We also inferred previously that this electrostatic potential arose from two or more negative charges at each site. Therefore, since the contribution of each of γD174 and δD180 alone is −42 mV, it is likely that in the native receptor the electrostatic potentials in the vicinity of γD174 and δD180 are more negative than −42 mV.

The above estimate of the contribution of γD174 and δD180 to the electrostatic potential at the binding sites presupposes that the effects of mutation on $EC_{50,ACh}$ were due to the direct electrostatic interaction of the Asp residues with ACh. This need not have been the case. The principal effects of the mutations could have been on the kinetics of gating or of desensitization, consistent with the greater effect of the mutations on the IC_{50} for agonists than competitive antagonists (see below) (*20*). Characterizing the ACh binding to $\alpha + \beta + \gamma$(D174X) and $\alpha + \beta + \delta$(D180X) as ACh retardation of α-bungarotoxin, we obtained $IC_{50,ACh}$ values that correlate well with the $EC_{50,ACh}$ values (Figure 5). It is apparent that the effect of each mutation in $\alpha + \beta + \gamma + \delta$ on $EC_{50,ACh}$ was close to the geometric mean of the effects of the mutation on $IC_{50,ACh}$ in $\alpha + \beta + \gamma$ and $\alpha + \beta + \delta$ (Figure 5). This correlation between the effects on $EC_{50,ACh}$ and $IC_{50,ACh}$ suggests that the mutations affected steps that are common to activation and to desensitization, which include binding itself and local conformational changes attendant on the binding of agonists. The correlation of the effects on EC_{50}, which only pertains to pentameric complexes of all of the injected subunits functionally expressed at the cell surface, and the effects on IC_{50}, which pertains to both pentameric and possibly smaller complexes expressed both at the cell surface and in intracellular membranes, argues against these effects arising from alterations in assembly. Even though there are certainly intermediate assembly forms inside the cell (*37*), we did not observe any difference between the distribution after sedimentation in sucrose density gradients of ^{125}I-α-bungarotoxin binding in complexes formed from wild-type $\alpha + \beta + \gamma$ and $\alpha + \beta + \gamma$D174N; also, there was no difference between wild-type $\alpha + \beta + \delta$ and $\alpha + \beta + \delta$D180N. Nevertheless, some of the effects of the mutations could be indirect, acting through local or global distortions in the structure of the receptor.

The fitting of eq 4 to log ($IC_{50,ACh,mut}/IC_{50,ACh,wt}$) for $\alpha + \beta + \gamma$(D174X) gave $a = 0.008 \pm 0.008$ and $b = 1.2 \pm 0.3$ ($R^2 = 0.66$), and for $\alpha + \beta + \delta$(D180X), $a = -0.2 \pm 0.2$ and $b = 2.7 \pm 0.4$ ($R^2 = 0.85$). Thus, the dependence of the effects of the mutations on the change in side-chain charge are not less for binding measured by IC_{50} than for activation measured by EC_{50}.

The magnitude of the effect of side-chain charge is also apparent in the dependence of $IC_{50,ACh}$ on pH in $\alpha + \beta + \gamma$D174H (Figure 3). We calculated that the $IC_{50,ACh}$ for a complex with a positively charged His residue is 20 times the $IC_{50,ACh}$ for a complex with an uncharged His residue. If we apply eq 4 to this ratio, where $|\Delta A| = 0$ and $\Delta q = 1$, then $b = \log 20 = 1.3$, which is not significantly different than 1.2 obtained by fitting $IC_{50,ACh}$ for all mutants. This implies $\Delta\psi/\Delta q = 38$ mV/positive step in algebraic charge.

Comparison of the Effects on ACh Binding and dTC Binding. Except for the mutations to E and H, all mutations had a greater effect on ACh binding than on dTC binding (Figure 6; note the different scales). We previously observed the much greater effect of the Asp to Asn mutation on three

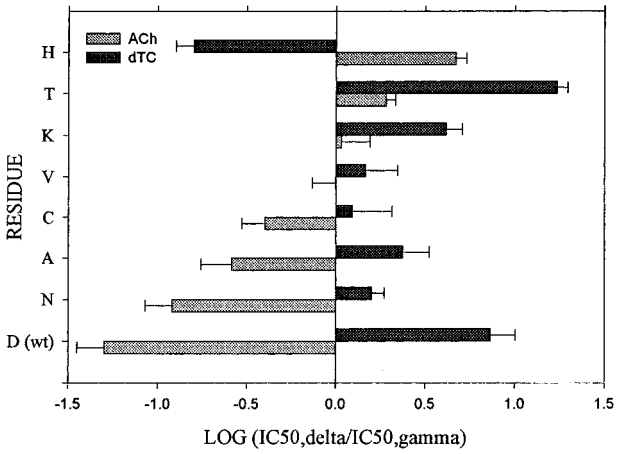

FIGURE 7: Differences in the α-γ and the α-δ binding sites in wild-type and mutant α + β + γ and α + β + δ. For ACh (light bars) and d-tubocurarine (dark bars), the log of the mean $IC_{50,\delta(D180X)}/IC_{50,\gamma(D174X)}$ (±SEM) is plotted for wild type and for each mutation.

agonists compared to three antagonists (*20*). Furthermore, the correlation of the effects of mutations on dTC binding with the effects on ACh binding was not statistically significant. Fitting log ($IC_{50,dTC,mut}/IC_{50,dTC,wt}$) to eq 4 gave $a = 0.004 \pm 0.005$ and $b = 0.84 \pm 0.18$ ($R^2 = 0.69$) for α + β + γ(D174X) and $a = -0.008 \pm 0.008$ and $b = 0.69 \pm 0.21$ ($R^2 = 0.5$) for α + β + δ(D180X). Thus, the effect on $IC_{50,dTC}$ of the change in side-chain charge was roughly half the effect on either $EC_{50,ACh}$ or $IC_{50,ACh}$. Agonists and competitive antagonists may bind within the same binding pockets; however, the interactions within these pockets may be quite different. Another explanation, which does not exclude the first, is that a large part of the effects of the mutations are on isomerization steps following agonist but not antagonist binding.

Comparison of the Effects of the γ and δ Mutations. All mutations of δ, expressed as α + β + δ, had larger effects on ACh binding than did all mutations of γ, expressed as α + β + γ (Figure 5). Conversely, almost all mutations of γ had larger effects on dTC binding than did mutations in δ. These differences correlated with the higher apparent affinities of α + β + δ for ACh and α + β + γ for dTC (Tables 2 and 3) (*15*, *17*). In addition, for all α + β + δ(D180X) and of α + β + γ(D174X), $\delta IC_{50,ACh}/\gamma IC_{50,ACh}$ is closer to 1 than it is for wild-type γ and δ, and except for the D to T mutation, this is also true for $\delta IC_{50,dTC}/\gamma IC_{50,dTC}$ (Figure 7). These decreases in the functional asymmetry of the α-γ and α-δ binding sites are consequences of the greater effects of the mutations on the site that has the higher affinity.

Effects of the Mutations on MBTA Labeling. The initial observation of the asymmetry of the two ACh binding sites was that MBTA labeled only half of the toxin binding sites (*8*). We believed that this asymmetry arose from the different neighbors of the two α subunits in the pentameric complex $\alpha_2\beta\gamma\delta$ (*38*). With the evidence that the binding sites are in the interface between α and γ and between α and δ (*6*, *14*, *15*, *17*), we anticipated that the presence of γ or δ would determine whether or not a reduced binding site would react with MBTA. Thus, we were surprised that MBTA reacted with only half of the sites of both α + β + γ and α + β + δ. This implies that β, which contacts only one of the two α subunits and on the side of α opposite the location of the binding site (*19*), determines the susceptibility of the binding site to MBTA. This effect is abolished with the mutation of γD174 to N or δD180 to N. It is also remarkable that, of the two mutations, only the mutation γ(D174N) decreased the rate of reaction of MBTA with the reduced binding site. Thus, it seems likely to us that in $\alpha_2\beta\gamma\delta$ the α-γ binding site in the appropriate location in the ring of subunits (*39*) is susceptible to labeling by MBTA, and the α-δ site is not. In $\alpha_2\beta\gamma_2$, the α-γ site, in its native position, is susceptible, and the α-γ site formed at the native position of the α-δ site is not susceptible. In $\alpha_2\beta\delta_2$, it is the α-δ site in the native position of α-γ that is susceptible and not the α-δ site in its native position. Also, as was evident above (Figure 7), the Asp residues contribute to the differences between the two ACh binding sites and may be differently disposed in the two sites. Other residues in γ and δ also contribute to the differences between the α-γ and the α-δ binding sites (*18*).

One possibility is that the α-δ site preexists in a conformation close to the active conformation induced by agonist binding and that the α-γ site preexists in a resting conformation that is stabilized by competitive antagonist binding. Thus, the binding of ACh to the α-γ site would bear a larger free energy burden of altering its conformation than would ACh binding to the α-δ site; hence the higher affinity for the α-δ site. The opposite would be true for dTC binding. MBTA acts like a competitive antagonist of the unreduced receptor (*2*) and hence would be expected to bind more tightly to the native α-γ site.

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