The Contributions of Aspartyl Residues in the Acetylcholine Receptor $\gamma$ and $\delta$ Subunits to the Binding of Agonists and Competitive Antagonists*

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The acetylcholine (ACh) receptors in muscle have the composition $\alpha_2\beta_2\delta_2$ and contain two ACh binding sites. One is formed between an $\alpha$ subunit and the $\gamma$ subunit, and the other is formed between an $\alpha$ subunit and the $\delta$ subunit. Among the residues in the ACh binding sites are Cys-192 and Cys-193. The negatively charged Asp-180 is at an appropriate distance from Cys-192/193 also to be in the ACh binding site and to interact electrostatically with the positively charged ammonium group common to agonists and competitive antagonists. Mutation to Asn of either Asp-180 or the aligned residue in the subunit, Asp-174, decreased the affinities of three agonists, acetylcholine, tetramethylammonium, and succinylcholine 170–560-fold. By contrast, these mutations decreased the affinities of three competitive antagonists, (+)-tubocurarine, hexamethonium, and dihydro-β-erythroidine, only 2–15-fold. Agonists, but not antagonists, promote the transitions of the receptor from the resting state to the higher affinity active and desensitized states, and the greater effects of the mutations of $\gamma$Asp-174 and $\delta$Asp-180 on the apparent affinities of agonists could reflect the involvement of these residues in the conformational changes of the receptor corresponding to its transitions to higher affinity states. In these transitions, one possibility is that $\gamma$Asp-174 and $\delta$Asp-180 move closer to bound agonist.

The binding of acetylcholine (ACh) by nicotinic receptors promotes the transitions of the receptor from the resting state to the open and the desensitized states (Katz and Thesleff, 1957). Muscle-type ACh receptors contain two nonidentical ACh binding sites (Damle and Karlin, 1978; Neubig and Cohen, 1979; Dowding and Hall, 1987). Normally, these receptors are pentamers composed of four types of subunits in the stoichiometry $\alpha_2\beta_2\delta_2$ (Reynolds and Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980). One of the ACh binding sites is formed in the interface between the first $\alpha$ subunit and the $\gamma$ subunit, and the other site is formed between the second $\alpha$ subunit and the $\delta$ subunit (Kurosaki et al., 1987; Blount and Merlie, 1989; Pedersen and Cohen, 1990; Sine and Claudio, 1991; Czajkowski and Karlin, 1991).

Several residues have been identified as in or close to the ACh binding sites. In the $\alpha$ subunit, disulfide-linked Cys-192 and Cys-193, and four aromatic residues, Tyr-93, Tyr-189, and a Tyr-198 were labeled by binding site-directed reagents (Kao et al., 1984; Kao and Karlin, 1986; Dennis et al., 1988; Abramson et al., 1989; Galzi et al., 1990; Cohen et al., 1991; Middleton and Cohen, 1991). Furthermore, the involvement of these six residues in ACh binding was supported by the functional consequences of site-directed mutagenesis (Mishina et al., 1985; Tomasselli et al., 1991; Galzi et al., 1991a; O'Leary and White, 1992). The adjacent cysteines and the four aromatic residues are highly conserved among all $\alpha$-subunit sequences.

An early assumption was that the ammonium group common to all potent agonists and antagonists of the ACh receptor is bound to a negative subsite of the binding site. From the rates of reaction and functional effects of affinity labels of different lengths, it was inferred that this negative subsite is about 12 Å from the binding site disulfide in the resting state of the receptor and about 9 Å from the binding site disulfide in the open state (Karlin, 1969). Using a radiolabeled cross-linker that reacts with a sulfhydryl at one end and a carbonyl group at the other end, we showed that three negatively charged residues in the $\delta$ subunit of Torpedo ACh receptor, Asp-165, Asp-180, and Glu-182 (see Fig. 1) are within approximately 9 Å of Cys-192/193 (Czajkowski and Karlin, 1991, 1995). Furthermore, in the complex of mouse ACh receptor $\alpha$, $\beta$, and $\delta$ subunits, the mutation of mouse AAsp-180 to Asn decreased the apparent affinity of the receptor for ACh by 2 orders of magnitude (Czajkowski et al., 1993). In addition, mutation of Glu-189 to Gln decreased the apparent affinity for ACh by 1 order of magnitude. Therefore, both Asp-180 and Glu-189 could contribute to the negative subsite of the ACh binding site formed between the $\alpha$ and $\delta$ subunits. By contrast, mutations of Asp-165, Glu-182, or eight other negatively charged residues in $\delta$ had only small effects on the apparent affinity for ACh.

The cross-linking of Cys-192/193 to Asp-180 supports the location of an ACh binding site in the interface of an $\alpha$ subunit and the $\delta$ subunit. The photolabeling of the aligned residues $\gamma$Trp-55 and $\delta$Trp-57 by the competitive antagonist (+)-tubocurarine also supported the location of ACh binding sites in the interfaces between $\alpha$ and $\gamma$ and between $\alpha$ and $\delta$ (Pedersen and Cohen, 1990; Chiara and Cohen, 1992). Mutation of these Trp residues had modest effects on the binding of agonists and antagonists (O'Leary et al., 1994; Corringer et al., 1995). These Trp residues and some of the aromatic residues identified in the

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1 The abbreviation used is: ACh, acetylcholine.

2 Because the cross-linker limits the distance but not the direction from Cys-192/193, the side chain carboxylates need not be adjacent.
α subunit could also contribute to the negative subsite, i.e., to the binding of the ammonium group (Dougherty and Stauffer, 1990; Galz et al., 1990).

The γ subunit is the most similar in sequence to the δ subunit, and mouse-muscle γ and δ subunits for each other in forming fully functional receptor complexes with just three types of subunits, presumably with the stoichiometries α2γ2β2 or α2δβ2 (Kullberg et al., 1990; Sine and Claudio, 1991). The residues in γ that align with δα32-180 and δGlu-189 are γα32-174 and γGlu-183 (see Fig. 1). These two residues are identically conserved among all aligned sequences of the γ, δ, and ε subunits (Czajkowski et al., 1993). The ε subunit substitutes for γ in adult muscle ACh receptor (Mishina et al., 1986). We mutated to Asn or Gln, γα32-174, γGlu-183, and each of the other eight Asp and Glu residues in a 60-residue segment preceding the first membrane-spanning segment (see Fig. 1) and expressed each mutant γ subunit, together with wild-type α and β subunits, in Xenopus oocytes. We also expressed δα32-180 mutated to Asn and δGlu-189 mutated to Gln together with wild-type α and β subunits. The salient findings were that the mutation of γα32-174 to Asn, just like the mutation of δα32-180 to Asn, decreased the apparent affinity for ACh by 2 orders of magnitude and that both of these mutations decreased the apparent affinities for agonists much more than the affinities for competitive antagonists.

**EXPERIMENTAL PROCEDURES**

Acetylcholine bromide, tetramethylammonium, hexamethonium, and (+)-tubocurarine were obtained from Sigma, and succinyldicholine and dihydro-β-erythroidine were obtained from Research Biochemicals International. Mouse muscle acetylcholine receptor subunit cDNAs (provided by T. Claudia, Yale University) were subcloned into pSP64T at the BglI site. Mutations of the γ subunit were made in a cassette defined by the restriction enzymes BstXI and MsdI either by polymerase chain reaction mutagenesis (Nelson et al., 1989; Kupers et al., 1991) or by the Altered Sites in vitro Mutagenesis system (Promega). All mutations were confirmed by sequencing in both directions. Capped mRNA for each subunit was transcribed with SP6 polymerase under standard conditions (Promega) from the cDNA in the pSP64T plasmid. Mutations and the mutant subunits are designated as “subunit,” “wild-type residue position,” and “mutant residues” with residues indicated in single-letter code, e.g., γD174N. Oocytes from Xenopus laevis were prepared as described by Czajkowski et al. (1993). The oocytes were injected with 50 nl of mRNA (200 pg/ml) containing subunit mRNA in the ratios 2α:1β:1γ, 2α:1β:2γ (wild type), 2α:1β:γ (mutant), and 2α:2β:γ (mutant), and 2α:1γ:Oocytes were used 1–6 days after injection for electrophysiological studies and for preparing membrane fractions.

ACh-induced currents were recorded with a two-electrode voltage clamp at a holding potential of −40 mV as described (Akabas et al., 1992). The currents induced by various concentrations of ACh were fit by the Hill equation, \[ I = I_{max}/(1 + (EC_{50}/[ACh])^n) \] (Eq. 1)

Oocyte membranes were prepared as described (Czajkowski et al., 1993). The membrane suspension contained about 1.5 μg of protein/μl. The yield was approximately 5 fmol of 125I-α-bungarotoxin binding sites and about 15 μg of protein/oocyte.

The binding of 125I-labeled α-bungarotoxin at different toxin concentrations was determined by diluting 20 μl of membrane suspension to 400 μl with NP50 (50 mM NaCl/10 mM sodium phosphate/1 mM EDTA, pH 7.0) containing 0.2% Triton X-100, mixing with 125I-α-bungarotoxin (at five concentrations from 0.05 to 5 nM), and incubating for about 24 h at 18°C. The samples were diluted with 5 ml of ice-cold wash buffer (0.2% Triton X-100/10 mM NaCl/10 mM sodium phosphate, pH 7.4), filtered through Reeves-Angel glass-fiber filters pre-soaked in 2% polyethyleneimine, and washed two times with 5 ml of wash buffer. The amount of 125I-α-bungarotoxin on the filters was determined by liquid scintillation counting. Specific binding was defined as that binding of 125I-α-bungarotoxin blocked by 1 μM nonradioactive α-bungarotoxin.

The concentration of toxin giving half-maximal binding, \( K_{\text{app}} \), was estimated by a nonlinear least squares fit of the specifically bound toxin, \( Y = Y_{\text{max}}/(1 + (K_{\text{app}}/X)) \), to the equation, \( Y = Y_{\text{max}}/(1 + (K_{\text{app}}/X)) \), where \( X \) is the 125I-α-bungarotoxin concentration. Given the rate constants for the association and dissociation of α-bungarotoxin (Sine and Claudia, 1991), the binding would not be expected to reach equilibrium in 24 h, especially at low concentrations of α-bungarotoxin, and therefore \( K_{\text{app}} \) is not an equilibrium dissociation constant.

The binding of 125I-α-bungarotoxin to intact oocytes was determined by placing 5–10 oocytes in a final volume of 400 μl of 0.5 mM 125I-α-bungarotoxin in MOR2 (82 mM NaCl/2.5 mM KCl/1 mM Na2HPO4/5 mM MgCl2/0.2 mM CaCl2/5 mM Hepes, pH 7.4), containing 0.1% bovine serum albumin, and incubating at 18°C overnight. The oocytes were placed on a single DE81 filter and washed four times with 5 ml of ice-cold MOR2 (without bovine serum albumin). The amount of 125I-α-bungarotoxin on the filter was determined by liquid scintillation counting. In the case of intact oocytes, specific binding was defined as that blocked by 100 nM nonradioactive toxin.

The binding of agonists and antagonists was determined by their inhibition of the binding of 125I-α-bungarotoxin to wild-type and mutant receptors to a crude membrane fraction of oocytes. Membranes (50 μl), pretreated for 20 min with 200 μM disopropyl phosphofluoridate, 0.75 mM 125I-α-bungarotoxin (50 μl), and various concentrations of agonist or antagonists (50 μl), all in NP50, were combined in a final volume of 150 μl. The final 125I-α-bungarotoxin concentration was 0.25 nM. After 5 h at room temperature, the suspension was filtered, and the bound 125I-α-bungarotoxin was determined as above.

The dissociation constant, \( K_{\text{diss}} \), for ligand was obtained by the nonlinear least squares fit of the following equation to the binding data:

\[ Y = (Y_0 - U)(1 + (A/K_{\text{diss}})) + U \] (Eq. 1)

where \( Y \) is the specifically bound 125I-α-bungarotoxin (that blocked by 1 μM α-bungarotoxin), \( Y_0 \) is the specifically bound 125I-α-bungarotoxin in the absence of ligand, and \( A \) is the concentration of ligand. \( U \) is the specifically bound 125I-α-bungarotoxin not blocked by saturating concentrations of the ligand; \( U \) was estimated by the fit. Over all the ligands tested, \( U \) ranged from 13 to 42% of specifically bound 125I-α-bungarotoxin.

The inhibition data was also analyzed in terms of two binding sites by the following equation:

\[ Y = (Y_0 - U)(1 + (A/K_{\text{diss}})) + (1 - g)(1 + (A/K_{\text{diss}})) + U \] (Eq. 2)

where \( g \) is the fraction of blockable binding to site 1, and \( K_{\text{diss}} \) is the dissociation constant of the ligand for site 1, and \( K_{\text{diss}} \) is the dissociation constant for site 2, and the other parameters are defined above.

**RESULTS**

The ten acidic residues between and including γGlu-163 and γGlu-203 (Fig. 1) were mutated one at a time, Asp to Asn and Gln to Gln. In addition, γAsp-174 was mutated to Glu, and γGlu-183 to Asp. These mutant γ subunits were expressed in Xenopus oocytes together with wild-type α and β subunits, and
in some cases, together with α, β, and δ subunits. We determined the effects of each of these mutations on the whole cell currents elicited by ACh and on the inhibition by ACh of the binding of 125I-α-bungarotoxin. For three mutations of γ, γD174N, γD174E, and γE183Q, and for two mutations of δ, δD180N and δE189Q, we also determined the binding of two additional agonists and of three antagonists (Fig. 2).

The complex of wild-type α, β, and γ (α2β2γ2 receptor), similar to the complex of α, β, and δ (α2β2δ2 receptor; Czajkowski et al., 1993), yielded an EC50 that was close to that of the complex of wild-type α, β, γ, and δ (α2β2γδ receptor; Table I). No ACh-induced current was obtained with just α and β (Table I). Also, Liu and Brehm (1993) found that complexes of α and γ or of α and δ yielded ACh-induced currents that were 200 or 40 times smaller than the currents yielded by complexes of α, β, or α, δ, and β, respectively. Initially, we tested the γ mutants in complexes just with α and β.

For 10 of the 12 γ mutants, the complexes with α and β gave maximal ACh-induced currents that were 2-50% of wild-type currents (Table I). The largest change in the EC50 for ACh was a 4.6-fold increase shown by γE176Q; γE183Q gave a 4-fold increase; the other EC50 values were within a factor of 3 of the EC50 of wild-type α2β2γ2 receptor (Table I). The Hill coefficients of all mutants were very close to the Hill coefficient of wild-type receptor. Also, for these 10 mutants the Kd,αCh, determined by the inhibition of ACh of 125I-α-bungarotoxin binding, was slightly less than that of wild-type α2β2γ2 (Table I).

Two mutants, γD174N and γE202Q, co-expressed with α and β, gave no detectable ACh-induced current at ACh concentrations up to 2 mM. Furthermore, there was no specific binding of 125I-α-bungarotoxin to the surface of the intact oocytes expressing α+β+γD174N or α+β+γE202Q. Therefore, we conclude that the complexes formed by these subunits were not transported to the cell surface. These subunits did form agonist-binding complexes in cytoplasmic membranes, the predominant constituents of the crude membrane fraction used in the binding experiments. Because the dissociation constant for ACh of the complex containing γE202Q was slightly less than that of all wild-type α+β+γ, this mutant was not further characterized (Table I).

Unlike the complex of α, β, and γD174N, the complex of α, β, δ, and γD174N was expressed on the cell surface. The ACh-induced current was characterized by an EC50 of 2.7 μM, six times the EC50 of all wild-type αβγδ (Fig. 3; Table I). Because the complex of all wild-type αβδ2 receptor had an EC50 of 2.7 μM (Czajkowski et al., 1993) and the complexes of αβ+γD174N were not expressed on the cell surface, the increase in the EC50 must have been due to the expression of the pentameric complex of αβδγD174N. Given that wild-type αβδ2 complex may also have been present, the observed EC50 of 24 μM ACh is a lower limit to the EC50 of the complex of αβδγD174N.

The binding of ACh by complexes of α, β, and γD174N in subcellular membrane fraction of oocytes was characterized as Kd,αCh, derived from the inhibition by ACh of 125I-α-bungarotoxin binding. Kd,αCh for αβ+γD174N was 170 times the Kd,αCh for wild-type α+β+γ (Fig. 4A and Table I). All of the other 11 mutants tested had Kd,αCh slightly less than that of wild-type α+β+γ.

The mutation γD174N also affected the binding of ACh in the
TABLE I
Effects of mutations in the gamma subunit on ACh-induced current and on ACh binding

Subunit mRNA was injected into Xenopus oocytes, and after 1–3 days ACh-induced currents were recorded, as described under “Experimental Procedures.” Peak current as a function of ACh concentration was fitted by the Hill equation. The dissociation constants of ACh were determined from its retardation of the binding of toxin (see “Experimental Procedures”). Where two values are given, the data were better fitted by a two-site than a one-site fit. Means, S.E.s, and number of independent experiments are given.

| Wild-type subunits | Mutant subunit | Mutation | EC_{50} | EC_{50mut}/EC_{50wt} | -1_{max} | Hill coefficient | n | K_{ACH} | K_{mut/Kwt} | n |
|--------------------|----------------|----------|---------|----------------------|----------|------------------|---|----------|-------------|---|
| aβγδ               | aβγD174N       | γ        | 3.8 ± 0.4 | 1                    | 3600 ± 180 | 1.4 ± 0.0 | 9 | 0.21 ± 0.04 | 5.7 ± 0.67 | 1 | 5 |
| aβγ                | aβγE163Q       | γ        | 20 ± 3   | 2.3 ± 0.5           | 400 ± 50  | 1.3 ± 0.0 | 4 | 1.4 ± 0.1  | 0.4 ± 0.1  | 3 |
| aβγδ               | aβγD164N       | γ        | 15 ± 2   | 1.7 ± 0.8           | 1200 ± 120| 1.2 ± 0.0 | 3 | 1.4 ± 0.1  | 0.4 ± 0.1  | 3 |
| aβγδ               | aβγE169Q       | γ        | 24 ± 1   | 2.7 ± 1.3           | 370 ± 180 | 1.4 ± 0.0 | 4 | 2.9 ± 0.4  | 0.8 ± 0.3  | 7 |
| aβγδ               | aβγD174N       | γ        | 0.7 ± 0.5| 0.7 ± 0.5           | 600 ± 250 | 1.3 ± 0.1 | 3 | 4.6 ± 0.4  | 1.3 ± 0.3  | 4 |
| aβγδ               | aβγE167Q       | γ        | 41 ± 4   | 4.6 ± 2.5           | 630 ± 60 | 1.2 ± 0.1 | 2 | 2.2 ± 0.2  | 0.6 ± 0.2  | 4 |
| aβγδ               | aβγE180Q       | γ        | 28 ± 2   | 3.2 ± 1.6           | 240 ± 80 | 1.5 ± 0.2 | 4 | 2.9 ± 0.2  | 0.8 ± 0.3  | 3 |
| aβγ                | aβγE183Q       | γ        | 35 ± 6   | 4.0 ± 2.0           | 60 ± 10 | 1.4 ± 0.0 | 2 | 2.8 ± 0.3  | 0.4 ± 0.3  | 4 |
| aβγδ               | aβγE183D       | γ        | 6.0 ± 0.2| 0.7 ± 0.4           | 330 ± 40 | 1.2 ± 0.1 | 6 | 1.7 ± 0.3  | 0.5 ± 0.2  | 3 |
| aβγδ               | aβγD190N       | γ        | 12 ± 2   | 1.5 ± 0.8           | 340 ± 60 | 1.2 ± 0.2 | 3 | 2.2 ± 0.1  | 0.6 ± 0.1  | 5 |
| aβγ                | aβγE202Q       | γ        | 0.0 ± 4  | 0.1 ± 0.3           | 11 ± 4   | 0.2 ± 0.1 | 3 | 1.9 ± 0.2  | 0.3 ± 0.1  | 4 |
| aβγδ               | aβγE203Q       | γ        | 22 ± 2   | 2.5 ± 1.2           | 460 ± 80 | 1.2 ± 0.1 | 4 | 1.9 ± 0.2  | 0.5 ± 0.2  | 3 |

Fig. 3. Current as a function of ACh concentration. The receptor complexes are all wild-type αβγδ (circles), αβγD174N (squares), and αβγE183Q (triangles). Oocytes expressing these complexes were superfused with five or six concentrations of ACh for 10 s each with a 5-min wash between, and the peak currents were recorded under two-electrode voltage clamp at –40 mV (see “Experimental Procedures”). Each concentration was added twice. The peak current (I) is the average of the duplicates and is plotted as a fraction of the peak current (I_{max}) at infinite ACh concentration, calculated by fitting the Hill equation to the data (Table I). Average errors are shown. Representative data from individual oocytes are shown.

Fig. 4. Inhibition by ACh of [125I]-bungarotoxin binding to membranes. Relative toxin binding is the [125I]-bungarotoxin bound in each ACh concentration divided by the [125I]-bungarotoxin bound in the absence of ACh, determined as described under “Experimental Procedures.” Representative single experiments in which each point is the average of triplicates are shown. The errors are S.E. The data were fit as described under “Experimental Procedures.” A shows data for complexes of αβγδ, αβγD174N, αβγE183Q, and αβγD174E. B shows data for complexes of αβγδ, αβγD174N, αβγD174N + δD180N, and αβγD174E.
The dissociation constants for ligands were determined from their retardation of the binding of toxin, as described under "Experimental Procedures." Where two values are given, the data were better fitted by a two-site fit than a one-site fit. S.E.s and the number if independent experiments are described.

### Table II

**The effects of mutations on the binding of agonists**

| Wild-type subunits Mutant subunit Mutation | Acetylcholine $K_D$ (μM) | Tetracaine $K_D$ (μM) | Sucinylcholine $K_D$ (μM) |
|------------------------------------------|--------------------------|------------------------|--------------------------|
| $\alpha\beta\gamma$                      | $0.21 \pm 0.04, 5.7 \pm 0.7$ (5) | $45 \pm 4 (6)$ | $15 \pm 2 (4)$ |
| $\alpha\beta\gamma$ D174N                | $3.6 \pm 0.2 (5)$ | $225 \pm 20 (5)$ | $8.2 \pm 0.9 (4)$ |
| $\alpha\beta\gamma$ D174E                | $600 \pm 60 (7)$ | $40,500 \pm 6,000 (4)$ | $2445 \pm 390 (5)$ |
| $\alpha\beta\gamma$ E183Q                | $6.5 \pm 0.2 (4)$ | $165 \pm 30 (4)$ | $180 \pm 50$ |
| $\alpha\beta\delta$                      | $0.13 \pm 0.03 (3)$$^*$ | $345 \pm 30 (4)$ | $2445 \pm 390 (5)$ |
| $\alpha\beta\delta$ D180N                | $1.1 \pm 1 (4)$ | $11.4 \pm 1.1 (4)$ | $12 \pm 0.11 (3)$ |
| $\alpha\beta\delta$ E189Q                | $26 \pm 7 (3)$$^*$ | $2850 \pm 760 (4)$ | $675 \pm 100 (4)$ |

$^*$ Data from Czajkowski et al. (1993).

**Discussion**

We previously identified two residues in the $\delta$ subunit, $\delta$Asp-180 and $\delta$Glu-189, that could contribute to the negative subsite of the ACh binding site formed between $\alpha$ and $\delta$ (Czajkowski and Karlin, 1991; Czajkowski et al., 1993; Czajkowski and Karlin, 1995). One of these, $\delta$Asp-180, was cross-linked via a 9-A cross-link to one of the adjacent Cys residues, $\alpha$Cys-192 or $\alpha$Cys-193, that form the binding site disulfide, and the mutation of $\delta$Asp-180 to Asn caused a 2 orders of magnitude decrease in the apparent affinity of the $\alpha+\beta+\delta$ receptor complex for ACh, measured both by activation and by binding. Although $\delta$Glu-189 was not cross-linked to $\alpha$Cys-192/193, its mutation caused a 1 order of magnitude decrease in the apparent affinity for ACh, also measured by both methods. ACh binding was uniquely sensitive to the mutation of these two residues among the 11 negatively charged residues in a stretch of 60 residues just preceding the first membrane-spanning segment of the $\delta$ subunit. Because $\gamma$ and $\delta$ each forms an ACh binding site with an $\alpha$ subunit (Kuroskai et al., 1987; Blount and Merlie, 1989; Pedersen and Cohen, 1990; Sine and Claudio, 1991; Czajkowski and Karlin, 1991), we determined the effects on agonist and competitive antagonist binding of mutating to Asn or Glu each Asp or Glu residue in the aligned stretch of residues in the $\gamma$ subunit. Among the 10 acidic residues in this stretch of $\gamma$, only the mutation of $\gamma$Asp-174, which aligns with $\delta$Asp-180, caused...
a large decrease in the apparent affinity for ACh (Table I). The dissociation constant for ACh, determined by competition with 
$^{125}$I-bungarotoxin binding to the $\alpha+\beta+\gamma$ complex, was 170 times greater for $\gamma\Delta D174N$ than for wild-type $\gamma$.

The effects of the mutations of $\gamma\Delta D174N$ and $\delta\Delta D180N$ were most obvious in complexes of just three of the four types of receptor subunits. In mouse receptor, $\gamma$ and $\delta$ substitute for each other to form a functional complex of three types of subunits with properties very similar to those of the complex with all four types of subunits (Kullberg et al., 1990; Sine and Claudio, 1991). On the other hand, $\alpha$ and $\beta$ alone, gave no current and $\gamma$ or $\delta$. $\gamma$ and $\delta$ complexes (Liu and Brehm, 1993). Therefore, to the extent that we were able to characterize the mutations by their effect on agonist-induced currents, we could be certain that we were characterizing only complexes containing the mutant subunit when either the mutant $\gamma$ or the mutant $\delta$ was expressed with wild-type $\alpha$ and $\beta$ subunits. This approach was successful with most of the mutants tested.

$\gamma\Delta D174N$, however, was not expressed on the oocyte surface with $\alpha$ and $\beta$ alone. Nevertheless, $\gamma\Delta D174N$ was incorporated into a functional complex on the oocyte surface when it was expressed together with wild-type $\alpha$, $\beta$ and $\delta$. The EC$_{SO}$ characterizing the ACh-induced current was six times the EC$_{SO}$ for this analysis, we can ignore $\alpha$ and $\delta$. In this case, the binding of ACh was characterized by two dissociation constants. The value for the low affinity binding site, presumably the $\alpha\gamma$ site (Blount and Merlie, 1989; Sine and Claudio, 1991), was 27 times higher than that for all wild-type $\alpha+\beta+\gamma+\delta$. When both $\gamma\Delta D174N$ and $\delta\Delta D180N$ were co-expressed with wild-type $\alpha$ and $\beta$, a single dissociation constant was obtained that was greater than both dissociation constants of wild-type $\alpha+\beta+\gamma+\delta$. Therefore, both $\gamma\Delta D174N$ and $\delta\Delta D180N$ affect binding, even in complexes of all four types of subunits.

The largest effects of the mutation $\gamma\Delta D174N$ were obtained after coexpression just with wild-type $\alpha$ and $\beta$. In this case, the binding of agonists and competitive antagonists were entirely to complexes in intracellular membranes. These complexes could include $\alpha\gamma$ dimers, $\alpha\gamma\beta$ trimers, ($\alpha\gamma$)$_2$ tetramers, and ($\alpha\gamma$)$_2\beta$ pentamers (Kreibik et al., 1995). We obtained little $\alpha$-bungarotoxin binding and negligible toxin binding blocked by ACh when we coexpressed just $\alpha$ and $\gamma$ (data not shown); thus, for this analysis, we can ignore $\alpha$-dimers and ($\alpha\gamma$)$_2$ tetramers. The mutation $\gamma\Delta D174N$ could, however, have shifted the distribution between $\alpha\beta\gamma$ trimers and $\alpha\beta\gamma$ pentamers. If these complexes had different binding properties, the effect of $\gamma\Delta D174N$ on binding could have been due to the shift in the distribution of complexes. $\gamma$Asp-174, however, is not in a region found to effect receptor assembly (Kreibik et al., 1995).

Furthermore, the mutation of the aligned residue, $\delta$Asp-180N, had a comparable effect on ACh binding in functional $\alpha_2\beta_2$ pentamers expressed on the oocyte surface (Czajkowski et al., 1993).

It is remarkable that the mutation to Asn of either $\gamma$Asp-174 or $\delta$Asp-180 had a 10–100 times greater effect on the binding of agonists than on the binding of competitive antagonists (Tables II and III). Agonists and competitive antagonists bind to overlapping sites; some of the same residues are labeled by agonist and antagonist affinity labels (Kao et al., 1984; Galzi et al., 1990; Cohen et al., 1991; Middleton and Cohen, 1991), and the mutations of these residues affect the binding of both agonists and antagonists, albeit not equally. Furthermore, ACh receptors altered by chemical modification (Karlin and Winnik, 1968) or by mutations (Bertrand et al., 1992) can be activated by ligands that normally are competitive antagonists, consistent with the overlap of agonist and competitive antagonist sites. Thus, the difference in the effects of mutations on agonist and competitive antagonist binding was not likely due to completely separate sites for the two types of ligands. Nevertheless, the differences could have resulted from nonidentical contacts of agonists and antagonists within overlapping binding sites. In the results presented here, it is clear that the difference was not dependent on the number of ammonium groups on the ligands; the binding of all three agonists was affected much more than the binding of all three antagonists (Tables II and III).

The binding of agonists promotes the transitions of the receptor from the resting state to the higher affinity active and desensitized states. Therefore, alteration of the kinetics of these transitions could affect both the EC$_{SO}$ and K$_{ligand}$. The binding of competitive antagonists does not normally promote activation, and the observed K$_{ligand}$ for a competitive antagonist is likely to be simply an equilibrium dissociation constant.

The uniformly greater effects of the mutations on agonist binding than on antagonist binding is therefore likely due to effects on the kinetics of the agonist-induced transitions. Consistent with this interpretation, coexpression of $\gamma\Delta D175N$ ($\epsilon$Asp-175 aligns with $\gamma$Asp-174 and $\delta$Asp-180), with wild-type $\alpha$, $\beta$, and $\delta$, caused an 8-fold increase in EC$_{SO}$, most of which could be accounted for by a decrease in the channel opening rate (Zhang et al., 1995).

Mutations of other residues in or close to the ACh binding site also affected the kinetics of state transitions. Based on photoaffinity labeling, $\epsilon$Tyr-93 and $\epsilon$Tyr-190 are in or close to the ACh binding site (Dennis et al., 1988; Abramson et al., 1989; Galzi et al., 1990; Cohen et al., 1991). Mutations of these residues also had a much greater effect on agonist binding than on competitive antagonist binding (Sine et al., 1994). An analysis of $\epsilon$Y190F showed that the 2 orders of magnitude increases
in the EC_{50} were attributable to changes both in binding and in gating kinetics (O'Leary and White, 1992; Chen et al., 1995).

The structure of the binding site changes on the binding of agonists (Karlin, 1969; Damle and Karlin, 1980) or on the transition to the desensitized state (Galzi et al., 1991b). The involvement of residues in or close to the ACh binding site in the agonist-induced transitions between states is consistent with these residues moving during the transitions. We have determined by cross-linking that δA8p-180 is close to the α-δ ACh binding site (Czajkowski and Karlin, 1995), and by symmetry, γA8p-174 and εA8p-175 are also likely to be close to the α-γ and α-ε ACh binding sites. Nevertheless, we do not know whether or not these residues participate directly in the binding of agonists. One possible mechanism, however, that places these residues in the binding sites and incorporates their movement as an integral part of activation is that on the binding of agonist to the α subunit, the side chain of the Asp on the neighboring subunit, γA8p-174, εA8p-175, or δA8p-180, moves closer to the agonist ammonium group, increasing the electrostatic interaction between these oppositely charged groups and bringing other side chains into more favorable interactions with the agonist. We previously postulated a negative subsite that interacted with the ammonium group of agonists and inferred that on the binding of agonist this negative subsite moved a few Ångstroms closer to the binding site disulfide (Karlin, 1969). We now suggest that γA8p-174 contributes to the negative subsite of the ACh binding site formed between α and γ, and similarly δA8p-180 contributes to the negative subsite of the ACh binding site formed between α and δ (Czajkowski and Karlin, 1991, 1995; Czajkowski et al., 1993). The postulated contraction of the binding site crosses the subunit interface and could trigger the sliding of neighboring subunits. This relative movement of the subunits could be a mechanism for the propagation of structural changes across the membrane, from the ACh binding sites, in the extracellular domain, to the gates, close to the intracellular end of the channel (Czajkowski et al., 1993; Akabas et al., 1994; Unwin, 1995).

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