Two anionic residues in the nicotinic acetylcholine receptor, Asp-152 in the α-subunit and Asp-174 in the γ-subunit or the corresponding Asp-180 in the δ-subunit, are presumed to reside near the two agonist binding sites at the αγ and α δ subunit interfaces of the receptor and have been implicated in electrostatic attraction of cationic ligands. Through site-directed mutagenesis and analysis of state changes in the receptor elicited by agonists, we have distinguished the roles of anionic residues in conferring ligand specificity and ligand-induced state changes. αAsp-152 affects agonist and antagonist affinity similarly, whereas γAsp-174 and δAsp-180 primarily affect agonist affinity. Combining charge neutralization on the α subunit with that on the γ and δ subunits shows an additivity in free energy changes for carbamylcholine and d-tubocurarine, suggesting independent contributions of these residues to stabilizing the bound ligands. Since both aromatic and anionic residues stabilize cationic ligands, we substituted tyrosines (Y) for the aspartyl residues. While the substitution, αD152Y, reduced the affinities for agonists and antagonists, the γD174Y/δD180Y mutations reduced the affinity for agonist binding, but surprisingly enhanced the affinity for d-tubocurarine. To ascertain whether selective changes in agonist binding stem from the capacity of agonists to form the desensitized state of the receptor, carbamylcholine binding was measured in the presence of an allosteric inhibitor, proadifen. Mutant nAChRs carrying αD152Q or γD174N/δD180N show similar reductions in dissociation constants for the desensitized compared with activable receptor state and a similar proadifen concentration dependence. Hence, these mutations influence ligand recognition rather than the capacity of the receptor to desensitize. By contrast, the αD200Q mutation diminishes the ratio of dissociation constants for two states and requires higher proadifen concentrations to induce desensitization. Thus, the contributions of αAsp-152, γδAsp-174/180, and αAsp-200 in stabilizing ligand binding can be distinguished by the interactions between agonists and allosteric inhibitors.

The nicotinic acetylcholine receptor (nAChR) in muscle is a pentamer composed of four homologous subunits present in a stoichiometry α2β2γδ and arranged to surround a central channel (1–3). Simultaneous occupation of agonist at the two binding sites that reside at the interfaces of the αγ and αδ subunits activates the nAChR by increasing the probability of channel opening. Continued exposure to agonist shifts the receptor into the desensitized state characterized by a greater than two orders of magnitude increase in agonist affinity and by a closed channel.

The extracellular domain in each subunit is formed principally from the amino-terminal 210 amino acids; this domain is followed by four transmembrane spanning regions. Since only a small segment between membrane spans 2 and 3, and the very carboxyl terminus of ~8 residues reside at the extracellular face, residues within the amino-terminal 210 amino acids should largely contribute to the agonist binding site. Three segments of the α-subunit, residues surrounding Tyr-93, the regions between residues 149 and 153 and between residues 180 to 200, have been shown by chemical labeling and site-directed mutagenesis to contribute to agonist binding (2, 4). Four segments on the opposing subunit interface of the γ and δ subunits (identified by residues 34, 55–59, 111–119, and 172–174 on the γ subunit and corresponding residues on the δ subunit) present surfaces which also appear to contribute to ligand binding (5–12). A model of the extracellular portion of the nAChR based on sequence identity with proteins of known structure and on identification of interacting residues details the known information on the extracellular domain of the receptor (13).

Long range electrostatic attractive forces and stabilization of paired charges through Coulombic interactions have long been known to control binding of charged ligands to proteins. More recently, the importance of interaction between quaternary ammonium moieties and aromatic side chains became evident through studies of model binding sites (14) and crystallographic studies of proteins that associate with quaternary ammonium ligands (15, 16). Charged residues involved in ligand binding on the γ and δ subunits of the nAChR have been identified by chemical cross linking (17, 18) and site-directed mutagenesis (7, 19). Residue proximity revealed from cross-linking and the reduction in agonist affinity associated with charge removal point to γAsp-174 and δAsp-180 as critical residues. Our search for anionic residues in α-subunit identified αAsp-152 to be at the αγ and αδ subunit interfaces, where it affects the binding of agonists and competitive alkaloid antagonists as well as subunit assembly (20). Since αAsp-152 and γAsp-174/δAsp-180 are critical to ligand binding, yet differentially affect agonist and antagonist binding, herein we examine the role of these residues in conferring specificity and affecting state changes in the receptor.

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The abbreviation used is: nAChR, nicotinic acetylcholine receptor.
These residues are compared with other anionic residues critically positioned in the extracellular region of the receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carbamylcholine, suberylcholine, decamethonium, and d-tubocurarine were purchased from Sigma. Dimethyl-d-tubocurarine was obtained from Lilly. 123I-β-Bungarotoxin (specific activity ~16 μCi/μg) was a product of NEN Life Science Products.

**Site-directed Mutagenesis**—cDNAs encoding mouse nAChR subunits were subcloned into a cytoplasmal-galilose-based expression vector, pBRG4. Single-stranded cDNAs were prepared as described previously (20). All mutations were introduced using single-stranded templates. After the mutagenesis reaction, restriction fragments containing the mutated site were subcloned into the original pBRG4 plasmid. Confirmation of the mutations and the absence of spontaneous mutations in the polymerase generated segment were established by dideoxy sequencing.

**Cell Transfection**—cDNAs encoding the wild type and mutant subunits were transfected into human embryonic kidney (HEK-293) cells using Ca3(PO4)2 in the ratios of (20). All mutations were introduced using single-stranded templates. After the mutagenesis reaction, restriction fragments containing the mutated site were subcloned into a cytomegalovirus-based expression vector, (7.5 μg/β (7.5 μg/β (7.5 μg/β or δ (15 μg/β)).

**Ligand Binding Measurements**—Cells were harvested in phosphate-buffered saline, pH 7.4, containing 5 mM EDTA, 2–3 days after transfection. They were briefly centrifuged, resuspended in potassium-Ringer's buffer, and divided into aliquots for binding assays. Assayed concentrations of agonists, antagonists, and proadifen were added to the samples 20 min prior to initiating the rate of association assay with 123I-β-bungarotoxin. Dissociation constants of the ligands were determined from their competition with the initial rate of 125I-

**RESULTS**

**Influence of nAChR α Subunit Mutations at Positions 99, 152, 180, and 200 on Ligand Binding**—Candidate anionic residues were selected in three segments of the extracellular domain of the α subunit known to affect ligand binding (Table IA). In segment A (amino acids 88–99), aspartate 99 was mutated to asparagine. In segment B (amino acids 144–154), aspartate 152 was mutated to glutamine. Glutamine substitution precludes insertion of a glycosylation signal. In segment C (amino acids 180–200), two anionic residues glutamate 180 and aspartate 200 were mutated into asparagine and glutamine, respectively.

Fig. 1A shows the effect of charge neutralization of these anionic side chains. Both the αD152Q and αD200Q mutations shift the binding curves to more than 10-fold higher agonist concentrations, whereas αD99N and αE180N mutations produce only slight shifts in concentration dependence and a loss of positive cooperativity for the agonists (Table II). For the antagonist d-tubocurarine, the αD152Q mutation produces a reduction in affinity similar to that seen for the agonist (Fig. 1B) (20). In contrast, the αD200Q mutation showed only a small influence on d-tubocurarine binding (Fig. 1B, Table II). This was also true for the αD99N and αE180N mutations.

**Influence of γD174N/δD180N Mutations on Ligand Binding**—γD174N and δD180N are situated in the most carboxyl-terminal position of the four segments in the γ and δ subunits known to contribute to ligand binding (Table IB). After cotransfection of α, β, γ, and δ subunits carrying the γD174N/δD180N mutations, the binding curve for the agonist carbamylcholine shifts over two orders of magnitude to higher concentration (Fig. 1C), whereas less than a 10-fold shift is seen for the antagonist d-tubocurarine (Fig. 1D). To distinguish between the two binding sites, subunits containing γD174N or δD180N were expressed as pentamers of either α2β2γ or α2β2δ subunit composition on the cell surface by transfection of the requisite three cDNAs; these subunit arrangements possess apparently equivalent subunit interfaces for binding (α2β2γ with two α–γ sites, α2β2δ with two α–δ sites). A reduction in agonist affinity that is greater than those for antagonists is again evident with these unnatural subunit compositions (Fig. 2). The loss of co-
opativity on agonist binding for αβδ2 pentameric receptor was also evident in previous studies (23), and likely reflects the diminished capacity of this subunit combination to elicit state transitions in response to agonists. Binding of d-tubocurarine is largely unaltered by these mutations with the αβγ2 or αβδ2 subunit combinations. As expected for a pentamer containing two equivalent sites, the respective Hill coefficients also approach 1.0.

**Ligand Specificity of αD152Q and γD174N/δD180N Receptor Mutations**—The bis-quaternary agonist, suberyldicholine, shows a reduction in affinity similar to carbamylcholine for the charge neutralization mutations in the alpha and γδ subunits. Thus, mono- and bis-quaternary agonists are not differentially affected by these mutations. For bis-quaternary antagonist, dimethyl d-tubocurarine, the αD152Q mutation showed over a 10-fold reduction in affinity, whereas the γD174N or the δD180N mutation showed only a 3–4-fold reduction; values similar to d-tubocurarine, which contains single cationic tertiary and quaternary moieties. For the bis-quaternary partial agonist, decamethonium, the loss of affinity with the mutations falls between d-tubocurarine and suberyldicholine, but somewhat closer to the antagonist (Table III).

**Combining the Mutations of αD152Q and γD174N/δD180N**—Upon expression of receptor containing charge neutralization mutations on both the α and γδ subunits, the change of free energy (ΔΔG) between the double subunit mutant combining αD152Q with γD174N/δD180N and wild-type receptor for carbamylcholine is in close accord with the sum of ΔΔG calculated from separate mutations on the individual subunits contained in the pentameric receptor (Fig. 1C; Table II). For d-tubocurarine, ΔΔG between the wild-type receptor and the receptor with combined α and γδ substitutions is also

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**Table II**

| Composition | Carbamylcholine | d-Tubocurarine |
|-------------|----------------|---------------|
|             | K<sub>eq</sub> | Hill slope | K<sub>eq</sub>mt/K<sub>eq</sub>wt | ΔΔG | K<sub>1m</sub> | K<sub>1m</sub>/K<sub>1w</sub> | ΔΔG1 | K<sub>2</sub> | K<sub>2</sub>/K<sub>2</sub>wt | ΔΔG2 |
| αβγδ         | 16 | 1.3 | 41 | 1000 |
| αD99N        | 22 | 1.1 | 16 | 0.39 | 0.56 | 690 | 0.69 | 0.22 |
| αD152Q       | 1.3 | 16 | 1.17 | 1900 | 37 | 2.1 | 2200 | 22 | 1.8 |
| αD152Y       | 690 | 1.1 | 44 | 2800 | 85 | 2.5 | 41000 | 41 | 2.2 |
| αE180N       | 14 | 1.0 | 0.90 | 0.063 | 46 | 1.1 | 0.057 | 1100 | 1.1 | 0.057 |
| αD200Q       | 470 | 0.91 | 30 | 530 | 13.0 | 1.5 | 530 | 0.53 | 0.38 |
| γD174N/δD180N| 1900 | 0.89 | 120 | 210 | 5.2 | 0.98 | 2700 | 2.7 | 0.59 |
| αD152Q/γD174N/δD180N | 34000 | 0.88 | 2100 | 4.6 | 7500 | 190 | 3.1 | 80000 | 80 | 2.6 |
| γD174N/δD180Y | 1300 | 0.76 | 81 | 19 | 0.41 | 0.46 | 330 | 0.33 | 0.66 |
| αβγ2         | 3.3 | 1.0 | 50 |
| αD152Q       | 280 | 0.75 | 85 | 340 | 6.7 | 1.1 |
| αβδ2         | 0.74 | 0.51 | 780 |
| δD180N       | 55 | 0.54 | 74 | 1100 | 1.3 | 0.15 |
in close accord with the sum of ΔΔG values calculated for two respective receptors with individual substitutions in the two subunits (Fig. 1D; Table II). These results suggest that the charged residues, α1D152Q and γD174N/δD180N, confer independent, noninteracting electrostatic contributions to the binding site.

**Influence of the α1D152Q and γD174N/δD180N on Receptor Desensitization**—The selectivity of the γD174N/δD180N mutations for agonist, but not antagonist, affinity might be a consequence of these residue positions affecting the characteristic state transitions associated with the binding of agonists, but not antagonists. When agonists are allowed to equilibrate the receptor, the overall equilibrium constant (K_equil) reflects binding to at least three discrete receptor states: activable, active (open channel), and desensitized. The last state exhibits the highest affinity for agonists, and allosteric ligands such as local anesthetics, such as proadifen (27), the apparent dissociation constant for carbamylcholine is equivalent to that for the desensitized state (K_desens).

We examined the possibility that a diminished capacity for desensitization was responsible for the apparent reduction of agonist affinity associated with the charge neutralization on the receptor. Fig. 3 shows binding profiles for the agonist carbamylcholine at various concentrations of a local anesthetic, proadifen. Upon increasing the concentration of proadifen, the binding curves shift to toward lower concentrations of carbamylcholine. The degree of enhancement of affinity by proadifen can be monitored from the shift in the family of carbamylcholine binding curves. In both wild type and two mutant receptors (α1D152Q and γD174N/δD180N), 10 μM proadifen is nearly sufficient for saturation (Fig. 3, panels A–C).

Similar degrees of shift of carbamylcholine affinity in the presence and absence of proadifen are observed for the wild type (K_equil/K_R = 120), α1D152Q (K_equil/K_R = 88) and γD174N/δD180N (K_equil/K_R = 130) mutant receptors (Fig. 4). Therefore, the α1D152Q and the γD174N/δD180N mutants retain their capacity for desensitization or conversion to a high affinity state, and the large influence of the charge neutralization mutation on agonist binding does not arise from a compromised capacity of the mutant receptor to desensitize. Furthermore, the concentration dependence for proadifen in effecting the increase in carbamylcholine affinity for the α1D152Q and the γD174N/δD180N mutant receptors are superimposable with that found for wild-type receptor (Fig. 4, panels A–C).

**Influence of α2D200Q Mutation on Receptor Desensitization**—A previous study of the permeability response of the mouse nAChR expressed from mRNAs encoding the individual subunits when injected into *Xenopus* oocytes showed a only minor influence of α2D200Q on the concentration dependence for activation and functional antagonism (8). Our study shows a larger influence of this mutation on carbamylcholine binding at equilibrium, over one order of magnitude reduction in affinity.
ity (Fig. 1). d-Tubocurarine affinity is less affected by this mutation (Fig. 1). Exposure of the ligand to the receptor prior to assay allows all of the receptor states to equilibrate in the binding measurement, whereas the concentration dependence of the permeability response does not reflect the fraction of receptor in the desensitized state. Therefore, transitions to the desensitized states may explain the difference between the two studies.

For the αD200Q mutant, the ratio of dissociation constants ($K_{eq}$/Kr = 22) (Figs. 3 and 4, panel D) is significantly reduced compared with wild-type and the other receptor mutations with charge neutralization in the α or γδ subunits. The proadifen concentration dependence also increases where half-maximal conversion occurs at 61 μM, a value more than 10-fold higher than for wild type (Figs. 3D and 4D). These data indicate that the capacity for desensitization and for conversion to the high affinity state contributes to the overall dissociation constant measured at equilibrium and may well account for the interesting differences seen for this mutant between the previous measurements of permeability (8) and our binding studies.

Substitutions of Tyrosine for the Anionic Residues in the α and γδ Subunits—Since both anionic and aromatic residues are known to stabilize complexes of quaternary ligands and their binding sites, we determined whether substitutions of tyrosine for the charged residues would still result in a binding site that accommodates the bound agonist and antagonist. Although capacity for surface expression of receptor with the αD152Y mutation is about 30% of that of its αD152Q mutation, ligand binding affinities for carbamylcholine and d-tubocurarine are similar to those seen with αD152Q (Fig. 5). The γD174Y/δD180Y mutation also results in a loss of affinity for carbamylcholine, but leads to an enhanced affinity for d-tubocurarine (Fig. 5; Table II). This increase in affinity is also seen for structurally related bisquaternary antagonist, dimethyl d-tubocurarine (Table III).

The Influence of Proadifen on the γD174Y/δD180Y Mutant Receptor—The profile of binding curves for carbamylcholine in the presence of proadifen differs between the γD174Y/δD180Y and γD174N/δD180N (Fig. 3, panels E and C). Proadifen at 1 μM does not affect carbamylcholine binding and only at 10 μM...
gives a shift in the binding profile comparable to that found for wild type and the δD174/D180N mutant receptors at 1 μM proadifen. The proadifen concentration which gives a half-maximal shift in carbamylcholine affinities is nearly an order of magnitude greater than the wild-type receptor (Fig. 3, A and E). Moreover, the magnitude of proadifen elicited shift in carbamylcholine binding to higher affinity for the δD174Y/δD180Y mutant is approximately 4 times smaller than that found for wild-type receptor.

**DISCUSSION**

**Hydrophobic and Electrostatic Forces Affecting Ligand-nACHR Complexes**—The forces involved in stabilization of quaternary ammonium complexes with macromolecules have been studied for nearly a half-century and reveal both hydrophobic and electrostatic contributions to their stabilization (31). Electrostatic forces have been quantified by ionic strength masking of rates of association enhanced by electrostatic attraction or slowed by repulsion. Apart from long range forces generated from an electrostatic dipole in the binding site, charge may serve to stabilize particular orientations of the bound ligand or to entrap the ligand in the target site (16, 32). Recent studies with model compounds and crystallographic studies of complexes with quaternary ligands also show the importance of aromatic residues in interacting with cations through an ion quadrupole interaction (14–16). Perspectives on the importance of charged versus aromatic amino acid side chains depend in large part on the measurement technique. Photolytic labeling of the nACHR reveals the involvement of proximal aromatic residues in binding since those very residues are susceptible to photolysis (33). The crystal structures of the phosphorylcholine antibody (15) and acetylcholinesterase (16) show their binding sites for quaternary ligands to be heavily surrounded with aromatic residues. Nevertheless strategically placed charges, where longer range electrostatic forces (1/r in coulombic interactions versus 1/r^6 for hydrophobic dispersion forces and ion-quadrupole interactions), appear important for stabilization of these complexes.

Formation of site-specific mutants of the cholinesterase have clearly delineated the importance of both aromatic and charged residues on the enzyme in stabilizing quaternary ions (34, 35). With the nAChR, chemical cross-linking (17, 18) and subsequently mutagenesis studies have pointed to the cationic residue, on the γ subunit Asp-174 (7), and the corresponding residue Asp-180 (19), on the δ subunit, to lie within 10 Å of cysteine 192 or 193 of the α subunit and to be critical for the binding of agonists.

Martin and Karlin (23) have introduced several mutations at the γAsp-174 and δAsp-180 positions and have analyzed the electrostatic and steric influences of these residues on ligand binding and activation. Their studies, conducted by co-injection of mouse subunit receptors into *Xenopus* oocytes, also revealed that these mutations primarily decreased agonist, rather than antagonist affinity (19, 23), and that the loss of affinity was observed equivalently in receptor states involving activation and desensitization (23). Other studies analyzing the electrostatic potential by introduction of charged methanethiosulfonates at cysteines are consistent with an agonist binding site containing 2–3 negative charges (36). Elimination of the charge on αAsp-152 substantially reduces both agonist and antagonist affinities (20). Thus, distinctions between the charged residues at α152, β-174/180, and δ200 are evident, for the charge at α152 influences the binding of both agonists and antagonists, whereas the charges on γ174, δ180, and α200 selectively influence agonist binding.

**Distinguishing the Role of Anionic Charges in Ligand Binding to the nACHR**—Charge neutralization in the vicinity of a ligand recognition site and subunit interface can be expected to exert an influence on ligand selectivity and subunit assembly by multiple mechanisms. Indeed the mRNA encoding the γD174N mutation, when co-injected with mRNAs encoding the α and β subunits, did not result in expressed receptor on the *Xenopus* oocyte surface (19); yet we find upon cDNA transfection into mammalian cells, the combination of αβγ(δD174N)g yields an expression level similar to the wild-type αβγg. The αD152N mutation inserts an additional glycosylation signal in the sequence and increased glycosylation of the α-subunit receptor expressed in mammalian cells (20). This mutation yields diminished receptor expression which appears to be a consequence of compromising the initial dimer formation between the α and δ subunits, and particularly the α and γ subunits. Since αD152Q and especially αD152Y also yield diminished receptor expression when cotransfected with the complement of other subunits, this perturbation of subunit assembly cannot be attributed solely to steric hindrance by added oligosaccharide. Assembly of the pentamer can be monitored by sedimentation analysis, but even when assembly occurs, slight alterations in residue apposition at the subunit interface may influence the capacity of the receptor to undergo state changes. Diminished cooperativity seen with receptors of αβγz, and αβδz compositions with Hill slopes less than unity (28) are indicators of either a compromised state transition or a lack of identity of the two binding sites in these receptors assembled from three distinct subunits.

To underscore the differences in agonist and antagonist selectivity seen between charge neutralization at α152 and γD174/180, we examined these mutations in combination and by substitution of tyrosine for the charges. The influence of the
two charges on distinct subunits for both agonist and antagonist binding shows a summation of free energy contributions, presumably reflecting the independent influence of the charges of the receptor on the ligand and, for agonists, the preservation of cooperative state transitions in these particular mutants.

Agonist binding is compromised by substitution of tyrosine for the diacidic amino acid at α152 and γ174/δ180, but antagonists show an enhanced affinity with the substitutions of tyrosine at γ174/δ180. This is of particular interest since d-tubocurarine is a rigid molecule with a fixed distance of 10.5 Å between the two cationic nitrogen centers and distinct hydrophilic and hydrophobic surfaces (37). Hence, the d-tubocurarine, with its multiple aromatic moieties, may actually bind in a different orientation on the receptor where tyrosine has replaced aspartate in the γ and δ subunits. For agonists, it appears on basis of proadifen sensitivity that at least part of the affinity reduction seen for carbamylcholine when tyrosine is substituted at γ174 and δ180 arises from the compromised state transition elicited by the agonist.

Mutations in Relation to Multiple States of the Receptor—Our studies also reveal that elimination of charge of the various anionic residues can affect agonist binding by influencing the degree of desensitization. A multistate scheme where two agonists, L, and one heterotropic ligand, H, bind to the receptor is shown in Scheme 1.

In equilibrium binding for agonists, the observed dissociation constant at equilibrium ($K_{\text{eq}}$), not only reflects the intrinsic dissociation constants for the activable and desensitized states, $K_a$ and $K_{\text{des}}$, respectively, but is influenced by the allosteric constant, $M$ (R'R'/R), for the state transitions between the low affinity, activable state and desensitized state (24–30). For simplicity, the channel opening constant, $\beta/a$, for the doubly liganded species, $LRLR$, has been omitted. A heterotropic ligand, such as proadifen, will bind to a distinct site. Its relative affinities for the R'R', and RR receptor states will influence the ratio of high affinity, desensitized to low affinity, activable states of the receptor.

Fractional occupation of the agonist ($L$), carbamylcholine in our case, can be described in the absence of $H$ by

$$\bar{Y} = \frac{[L]}{K_{\text{eq}}(1 + [L]/K_a)} + M \frac{[L]}{K_{\text{eq}}(1 + [L]/K_a)}\left(\frac{1 + [L]/K_a}{1 + [L]/K_{\text{eq}}} + M \frac{1 + [L]/K_a}{1 + [L]/K_{\text{eq}}}\right)^{-1}$$

(Eq. 1)

A heterotropic ligand $H$, such as proadifen, may be described in terms of altering the allosteric constant $M$, to yield an apparent constant, $M'$

$$M' = M\left(\frac{1 + [H]/K_{\text{eq}}}{1 + [H]/K_{\text{eq}} + M}\right)^{\alpha}$$

(Eq. 2)

A previous study estimated $K_a$ for carbamylcholine and the wild type receptor to be $6 \times 10^{-5}$ (21). Using this value, $M$ for wild type receptor is calculated to be $6.6 \times 10^{-5}$, a value similar to $1.0 \times 10^{-4}$ derived from mouse receptors in BC3H-1 cells (25). Although we have no data on $K_{\text{eq}}$ for γD174N/δD180N mutant, a dose-response analysis for acetylcholine with this mutant receptor showed activation to occur at a 140-fold higher concentration (23). Since the difference in concentrations for activation and binding is similar for acetylcholine and carbamylcholine (38), $K_a$ for γD174N/δD180N can be approximated to $8.2 \times 10^{-3}$ μM. Then $M$ may be calculated to be $5.6 \times 10^{-5}$, a value very close to the constant for wild type receptor. Furthermore, the proadifen concentration dependence of the γD174N/δD180N mutant receptor is superimposable with that of wild type (Fig. 4, panels A and C), suggesting the allosteric constant $M$ in Scheme 1 is similar for this mutant and wild type receptor.

The αD200Q mutation shifts the proadifen concentration dependence to higher concentration as well as diminishes the total capacity for desensitization. Hence, αAsp-200 appears crucial for not only for receptor channel opening (39), but also for achieving full desensitization. The lack of influence of γD174N/δD180N mutations on the capacity to desensitize shown previously (23) and in these studies suggests that these charges are not required for the state transition associated

### Table IV

| Subunit mutation | Carbamylcholine | Proadifen |
|------------------|-----------------|-----------|
|                  | $M$             | $K_{\text{eq}}$, wt | $|H_{\text{eq}}|$ |
|                  | μM              | μM        | μM |
| Wild type        | 6.6 × 10^{-5}   | 120       | 3.7 |
| αD152Q           | 6.6 × 10^{-5}   | 120       | 3.7 |
| γD174N/δD180N    | 5.6 × 10^{-5}   | 120       | 3.7 |
| γD174Y/δD180Y    | 5.6 × 10^{-5}   | 120       | 3.7 |
| αD200Q           | 22              | 21        | 61  |

*mt, mutant; wt, wild type.
with desensitization. Hence, the overall dissociation constant measured for agonists at equilibrium is a composite of various contributory equilibria between receptor states. For agonists, at least three states (activable, open channel, and desensitized) are involved and the charge mutations differentially affect the various equilibria.

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