A Distinct Contribution of the δ Subunit to Acetylcholine Receptor Channel Activation Revealed by Mutations of the M2 Segment

Jian Chen and Anthony Auerbach
Department of Biophysical Sciences, State University of New York at Buffalo, Buffalo, New York 14214 USA

ABSTRACT Acetylcholine receptor (AChR) channels with proline (P) mutations in the putative pore-forming domain (at the 12′ position of the M2 segment) were examined at the single-channel level. For all subunits (α, β, ε, and δ), a 12′P mutation increased the open channel lifetime >5-fold. To facilitate the estimation of binding and gating rate constants, subunits with 12′P mutations were co-expressed with α subunits having a binding site mutation that slows channel opening (αD200N). In these AChRs, a 12′P mutation in ε or β slowed the closing rate constant ~6-fold but had no effect on either the channel opening rate constant or the equilibrium dissociation constant for ACh (K_d). In contrast, a 12′P mutation in δ slowed the channel closing rate constant only ~2-fold and significantly increased both the channel opening rate constant and the K_d. Pairwise expression of 12′P subunits indicates that mutations in ε and β act nearly independently, but one in δ reduces the effect of a homologous mutation in ε or β. The results suggest that a 12′P mutation in ε and β has mainly local effects, whereas one in δ has both local and distributed effects that influence both agonist binding and channel gating.

INTRODUCTION

Acetylcholine receptor (AChR) channels of adult muscle are composed of five subunits: two α, and one each of β, δ, and ε (Changeux et al., 1992; Karlin and Akabas, 1995). Each subunit has four hydrophobic segments, the second of which (M2) forms the narrow region of the ion conduction pathway (Lester, 1992; Unwin, 1993; Akabas et al., 1994). The M2 segment consists of ~19 hydrophobic residues (Fig. 1) and is thought to span the membrane with its amino terminus on the cytoplasmic side. All subunits have a highly conserved leucine located nine residues from the amino terminus of M2, i.e., at the 9′ position. It has been suggested that these leucines project into the channel lumen to form a hydrophobic barrier, or gate, that prevents ion conduction when the channel is closed (Unwin, 1993, 1995; Labarca et al., 1995; Filatov and White, 1995; Kearney et al., 1996). Here we focus on the function of residues that are three removed from these leucines, at the 12′ position of each subunit.

Besides being located near the central leucines, there are some suggestions that the 12′ and 13′ residues play an interesting role in channel activation. In humans, a spontaneous mutation of the 12′ residue in the ε subunit (threonine to proline; Ohno et al., 1995) or of the 13′ residue of the β subunit (valine to methionine; Engel et al., 1996) prolongs the open channel lifetime and causes a slow-channel congenital myasthenic syndrome. In *Torpedo*, the 12′ residue of the δ subunit is photolabeled by a snake α-neurotoxin (Machold et al., 1995). Finally, as shown in Fig. 1, the side chains at the 12′ and 13′ positions are highly conserved. The 12′ residue is a T in all subunits except δ where it is an S, and the 13′ residue is a V in all subunits.

We have examined the effects of proline substitutions to the 12′ position of α, β, δ, and ε subunits. The rate constants for channel closing and dose-response profiles were measured both for single and multiple 12′P mutants. The results show that the 12′P mutation has a different effect in the δ subunit than it does in the others. Specifically, the mutation in β or ε appears to have mainly local effects, whereas the mutation in δ has a more nonlocal character that influences transmitter binding, channel gating, and the consequences of pore mutations in other subunits. We speculate that the δ12′ residue is an important link between binding and gating processes.

MATERIALS AND METHODS

Mutagenesis and expression

Recombinant mouse cDNAs were generously provided by Drs. J. Merlie and N. Davidson. The α subunit had a background mutation in M4 (V433A; Zhou et al., 1998). Mutations αT254P, βT265P, and δS268P were introduced into corresponding sequences by ligating the DNA fragments produced by polymerase chain reaction at common restriction sites. All constructs were confirmed by restriction mapping and dideoxy sequencing of the insert. The mouse mutant εT264P (Ohno et al., 1995) and αD200N (Akk et al., 1996) cDNAs in vector pRBG4 were generously provided by Dr. Steven Sine (Mayo Foundation, Rochester, MN).

Human kidney embryonic (HEK) 293 cells were transiently transfected by calcium phosphate precipitation (Ausubel et al., 1992). A total of 3.6 μg of cDNA per 35-mm culture dish in the ratio 2:1:1:1 (α:β:δ:ε) was used. Approximately 12 h after transfection the medium was changed, and 48 h later electrophysiological recordings were made.

Electrophysiology

Electrophysiology was performed using the patch-clamp technique in the cell-attached configuration (Hamill et al., 1981). The bath solution was Dulbecco's phosphate-buffered saline (mM): 137 NaCl, 0.9 $CaCl_2$, 2.7 KCl, 1.5 KH_2PO_4, 0.5 $MgCl_2$, and 6.6 Na_2HPO_4, pH 7.3. The pipette solution was (mM): 142 KCl, 5.4 NaCl, 1.8 $CaCl_2$, 1.7 $MgCl_2$, 10 HEPES/

Received for publication 19 September 1997 and in final form 16 April 1998.

Address reprint requests to Dr. Anthony Auerbach, Department of Biophysical Sciences, SUNY-Buffalo, 118 Cary Hall, Buffalo, NY 14214. Tel.: 716-829-2435; Fax: 716-829-2415; E-mail: auerbach@xenopus.med.buffalo.edu.

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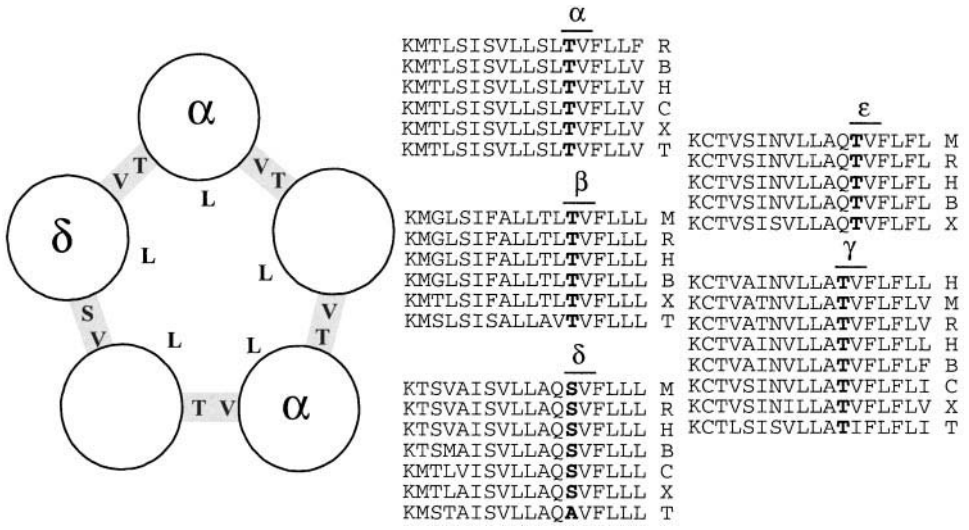

FIGURE 1 Schematic representation of an AChR pentamer with sequence alignments of M2 segments of muscle-type subunits. The 12′ residue is bold; single-letter species abbreviations are M, mouse; R, rat; H, human; B, bovine; C, chick; X, *Xenopus*; T, *Torpedo*. The 9′–13′ residues are LxxTV for α, β, ϵ, and γ subunits (γ13′ is I in *Torpedo*) and S for the δ subunit (A in *Torpedo*). Not shown: the 9′–13′ residues are also LxxTV in all neuronal AChR subunits of all vertebrate species in the database (26 genes) with the exceptions of α_5 and β_3 (LVxxTV; R, C, H) and β_4 (LxxTF; H, R). Left, a helical wheel representation of the closed AChR pore. The subunit between the two α subunits is either β or ϵ. The 9′ leucines project toward the pore axis. In this model, the 12′ and 13′ residues project into the intersubunit space at a level ~4.5 Å above the leucines and would be separated from each other in the vertical dimension by ~1.5 Å. If the M2 segment is a right-handed helix, then the 12′ residue would project in the counterclockwise direction (i.e., $\delta \rightarrow \epsilon/\beta$, as shown). If it were left-handed, it would project in the clockwise direction (i.e., $\delta \rightarrow \alpha$). The TV channel motif is present at all intersubunit spaces except in δ where it is SV.

KOH, pH 7.4. Unless stated otherwise, the potential of the patch pipette was +70 mV, and the estimated membrane potential was −100 mV. Currents were recorded with an Axopatch 200A amplifier, low-pass filtered at 20 kHz, and stored on videotape in digital format (Instrutech VR-10).

Data analysis

The current record was transferred to a computer via a digital interface (Instrutech VR111) at a sampling frequency of 94 kHz. Opening events were detected after digital filtering (Gaussian low-pass filter, f_c = 3 kHz) using a half-amplitude threshold-crossing criterion. At high agonist concentrations, AChR currents occur as clusters of openings that reflect the entrance and exit of a single protein molecule in long-lived desensitized states (Sakmann et al., 1980). Typically, the analysis focused on closed and open intervals within clusters, the durations of which reflect agonist binding and channel gating processes. A cluster was defined as a series of openings separated by closed intervals shorter than a critical duration, τ_{crit} (Auerbach, 1993). τ_{crit} was >4 times longer than the slowest closed interval component within clusters and ranged from 10–2000 ms depending on the concentration of ACh in the pipette. Clusters having >10 openings, and lasting >200 ms, were selected for further analysis. Closed-open transitions within these clusters were again idealized using a higher filter cutoff (5 kHz; dead time = 35 μs) and a correction to compensate for the effects of filtering (Colquhoun and Sigworth, 1995). In some experiments, for example, at low ACh concentrations (<2 μM for wild type), the currents were not clustered and all open intervals in the record were measured.

Open and closed interval durations within clusters were compiled into histograms that were fitted by sums of exponentials. Usually, multiple components were apparent in these histograms. We have limited the analysis to the open channel lifetime, defined as the time constant of the slowest component of the open interval duration histogram. The effective opening rate (β') was defined as the inverse of the time constant of the slowest component of the intracluster closed interval duration histogram. Under the experimental conditions, the slowest component of the open and closed duration histograms was also the predominant component.

RESULTS

Effects of single mutations

We first examined single-channel currents from AChR having a threonine-to-proline (T-to-P) mutation at the 12′ residue of only one subunit (αT254P, βT265P, ϵT264P, or δS268P). The results of these experiments are shown in Fig. 2 and Table 1.

With wild-type AChR activated by 2 μM ACh (Fig. 2 *A*), subconductance openings were not observed and open interval duration histograms were described by a single exponential with a time constant of ~1 ms. The 12′P mutations all increased the open channel lifetime (Fig. 2, *B–E*). For each 12′P mutant, subconductance events and short-lived open interval components were apparent. The 12′P mutation lengthened the open channel lifetime to a different extent in each subunit (Table 1). A 12′P mutation in the α or ϵ subunit had a larger effect (~20-fold) than one in the β or δ subunits (~7-fold).

Co-expression of 12′ mutants with αD200N

The following model served as the basis for quantitative analysis:

$$C \rightleftarrows AC \underset{k_{-2}}{\rightleftarrows} A_2C \overset{\beta}{\underset{\alpha}{\rightleftarrows}} A_2O,$$

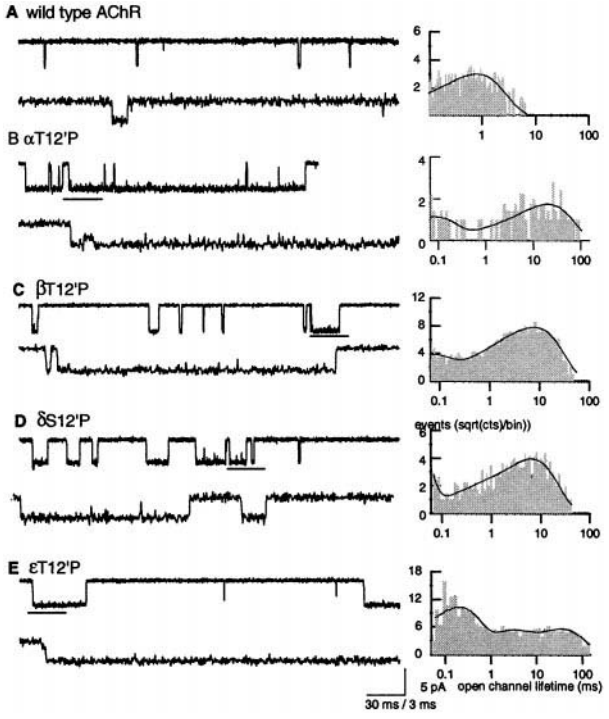

FIGURE 2 AChR with 12′P mutations. (*Left*) Single-channel currents elicited by 2 μM acetylcholine, shown at low (*top trace*) and high (*bottom trace*) time resolutions. (*Right*) Histograms of open channel lifetimes were fitted by one or more exponentials. (*A*) In wild-type AChR, lifetimes are described by a single exponential (τ_o = 0.98 ms) and subconductance states are rare. (*B*) With 12′P mutations in the two α subunits, the lifetime is prolonged (τ_o = 16 ms). Additional open interval components and subconductance states are apparent. (*C*) With a 12′P mutation in the β subunit, the lifetime is prolonged (τ_o = 7.8 ms). (*D*) With a 12′P mutation in the δ subunit, the lifetime is prolonged (τ_o = 5.6 ms). (*E*) With a 12′P mutation in the ϵ subunit, the lifetime is prolonged (τ_o = 23 ms) and multiple open interval duration components and subconductance states are apparent. All 12′ mutations significantly prolong the open channel lifetime, but a δ mutation has the smallest effect.

where C is a receptor with a closed channel, O is a receptor with an open channel, A is a molecule of ACh, β is the opening rate constant of a doubly liganded AChR, α is the closing rate constant of a doubly liganded AChR, and k_{-2} is the rate constant for one ACh molecule to dissociate from a doubly liganded, closed AChR. According to this scheme the open channel lifetime (τ_o) is

$$\tau_o = \alpha^{-1}\left(1 + \frac{\beta}{k_{-2}}\right) + \left(\frac{\beta}{k_{-2}}\right)\left(\frac{1}{\beta + k_{-2}}\right) \quad (1)$$

Because the A_2C state is brief (i.e., $(\beta + k_{-2}) \gg \alpha$), Eq. 1 reduces to (2)

$$\tau_o = \alpha^{-1}\left(1 + \frac{\beta}{k_{-2}}\right) \quad (2)$$

In wild-type, adult mouse AChR, both the opening rate constant (~50,000 s^{-1}; Sine et al., 1995) and the dissociation rate constant (18,000 s^{-1}; Akk et al., 1996) are large, and discrete sojourns in the doubly liganded closed and open conformations are not usually resolved. Therefore, the observed increase in the open channel lifetime after a 12′ mutation of wild-type AChR could arise from a combination of effects on the closing, opening, and agonist dissociation rate constants.

TABLE 1 Open channel lifetimes of 12′P mutant AChR

12′P mutation	Open channel lifetime (ms)	
	Wild-type binding site	αD200N
None	0.98 ± 0.23 (9)	0.25 ± 0.03 (4)
ϵ	23.2 ± 8.1 (12)	1.32 ± 0.18 (5)
β	7.8 ± 1.2 (5)	1.72 ± 0.10 (3)
δ	5.6 ± 0.95 (5)	0.59 ± 0.95 (5)
α	17.8 ± 1.7 (5)	2.36 ± 0.89 (3)

The open channel lifetime is the time constant of the slowest component of the open interval duration histogram. In AChR with wild-type binding sites this duration reflects opening, closing, and dissociation rate constants. In αD200N AChR, this duration is equal to the inverse of the channel closing rate constant. The values are mean ± SD (*n* patches).

To further simplify the interpretation of the open channel lifetime, the 12′ mutants were co-expressed with an α subunit having a binding site mutation, αD200N. Residue αD200 is located near the transmitter binding site, and mutation of this amino acid reduces the channel opening rate constant while only modestly affecting receptor affinity (Akk et al., 1996). Because this mutant has such a slow opening rate constant (~600 s^{-1}), the inverse of the open channel lifetime is essentially equal to the true channel closing rate constant. That is, in αD200N receptors, where $\beta \ll k_{-2}$, Eq. 2 reduces to

$$\tau_o = \alpha^{-1}$$

Fig. 3 shows example currents and open channel lifetime distributions for αD200N AChR, with and without additional 12′P mutations. These results are summarized in Table 1. The αD200N mutation alone shortens the open channel lifetime to 250 μs, which is ~4 times shorter than in wild-type AChR. As was the case with αD200 AChR, inserting a 12′P mutation in αD200N AChR prolongs the openings, but to a different extent for each subunit. Mutations in the α, β, or ϵ subunit slow down the channel closing rate constant ~5-fold, whereas a 12′P mutation in the δ subunit slows down the channel closing rate constant only ~2-fold.

We next determined the ligand binding and channel gating properties of AChR having both a 12′P and a αD200N mutation. Fig. 4 shows dose-response curves for αD200N AChR having a proline mutation at the ϵ, β, or δ subunit. The P_{open} profile gives the probability of being open within a cluster versus [ACh]. The intervals between clusters, which reflect sojourns in desensitized states, are not factored into the P_{open} value. Moreover, because P_{open} is calculated from idealized intervals, channel block (which for ACh is mainly manifest as reduction in the single-channel amplitude) has little influence on the P_{open} profile. The effective opening rate curve plots the time required for a

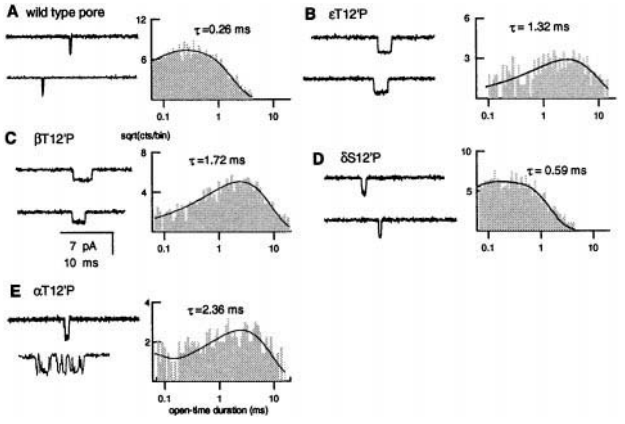

FIGURE 3 Open intervals of αD200N AChR with 12′P mutations activated by 50 μM ACh. Each panel shows example openings and an open interval duration histogram of AChR activated by 50 μM ACh. The αD200N mutation reduces the channel opening rate constant ~100-fold, thereby allowing the channel closing rate constant to be calculated from the inverse of the open mean duration (Table 2). A single 12′P mutation increased the open channel lifetime ~5-fold in the α, β, and ε subunits, but only ~2-fold in the δ subunit. (*E*) In AChR with two mutations (D200N and T12′P), each in both α subunits, the openings often occurred in bursts.

vacant receptor to bind ACh and open and is thus independent of the channel closing rate constant.

The 12′P mutation in ε and β did not significantly alter the activation characteristics of αD200N AChR. For both mutant and wild-type AChR, both dose-response curves superimpose with those of αD200N AChR having no 12′ mutations. In these receptors, the channel opening rate constant (determined from the saturation in the effective opening rate profile) was ~600 s^{-1}, and the K_d for ACh was ~35 μM. We conclude that in αD200N receptors a proline mutation at the 12′ position of ε or β does not alter the equilibrium dissociation constant for ACh or the channel opening rate constant.

In contrast, a 12′P mutation in δ caused a marked rightward shift in the dose-response curves. In addition, the effective opening rate curve did not saturate (up to 2 mM ACh), indicating that the channel opening rate constant for this double mutant was at least 2000 s^{-1}. Thus, a 12′P mutation in the δ subunit significantly increased both the equilibrium dissociation constant for ACh and the channel opening rate constant of αD200N AChR. This pattern was particular to the δ subunit, as 12′P mutations to ε or β had neither effect. The δ12′P pore mutation reverses, in part, the major effect of the αD200N binding site mutation.

Subunit interactions in the pore

In the pore region, near the position of the 12′ side chains, all five subunits come into close proximity. We therefore tested whether 12′P residues of different subunits interact. In the experiments shown in Fig. 5, *A–C*, 12′P mutants were expressed in pairs, along with wild-type α subunits (i.e., having neither a 12′ or a D200 mutation). When both ε and β had the 12′P mutation (Fig. 5 *A*), the open channel lifetime was 28 ± 7.4 ms (n = 4). This is somewhat longer than with either a single ε or β 12′P mutation (Table 1); i.e., the combined effect of the two mutations on the open channel lifetime is greater than the effect of either single mutation. Surprisingly, when one of the 12′P mutations was in the δ subunit, the combined effect of the two mutations was less than the effects of either single mutation. The open channel lifetime of (ε + δ)12′P was 12 ± 2.1 (n = 5) ms and that of (β + δ)12′P was 4.0 ± 0.78 ms (n = 4), both of which are significantly shorter than with single β or ε subunit 12′P mutations. The presence of a δ12′P mutation partially reversed the effects of the single ε or β mutations.

The above experiments used wild-type α subunits, so the increased open channel lifetime could not be unambiguously attributed to a change in the channel closing rate constant. To directly estimate the effect of double 12′P mutations on channel closure they were co-expressed with αD200N subunits. Example currents and histograms are shown in Fig. 5, *D–F*. As was the case with wild-type α, the open lifetime of (ε + β)12′P was 5.13 ± 0.12 ms (n = 3), longer than either single mutation (1.3 ms for ε and 1.7 ms for β; Table 1). Again, when one of the mutation subunits was δ, the open lifetime was shorter than in the case of the single mutants. The lifetime of (ε + δ)12′P was 0.97 ± 0.23 ms (n = 5), which is shorter than ε alone, and the open lifetime of (β + δ)12′P was 0.96 ± 0.12 ms (n = 4), which is shorter than β alone.

These results are summarized in Table 2 in terms of closing rate constants and activation free energies. In αD200N AChR, a single 12′P mutation in the β or ε subunit reduces the channel closing rate ~6-fold, which translates to an increase in the activation free energy for closing of ~1 kcal/mol (1.8 k_BT). A single 12′P mutation in the δ subunit has approximately one-half this effect and reduces the closing rate constant only ~2-fold (or increases the activation energy by ~0.5 kcal/mol). In the case of the (ε + β)12′P AChR, the net effect of the two mutations is only somewhat less than would be expected if each 12′P residue contributed independently to the activation free energy. In terms of free energies, the effects of the two mutations are nearly additive. However, when one of the 12′P mutations was in δ, the net effect was significantly less than would be expected from independent action. If we assume that the effect of the δ subunit 12′P mutation in these double M2 mutants is the same as in δ alone (0.5 kcal/mol) then the effect of the second ε or β mutation is ~3 times smaller (0.3 kcal/mol) than it is without the a δ mutation (1 kcal/mol).

DISCUSSION

The results indicate that the 12′ position of the δ subunit plays a role in AChR activation that is distinct from that played by the homologous 12′ residues of β and ε. In retrospect, there were some clues that pointed to an unusual function for the δ12′ amino acid. In all other subunits, and

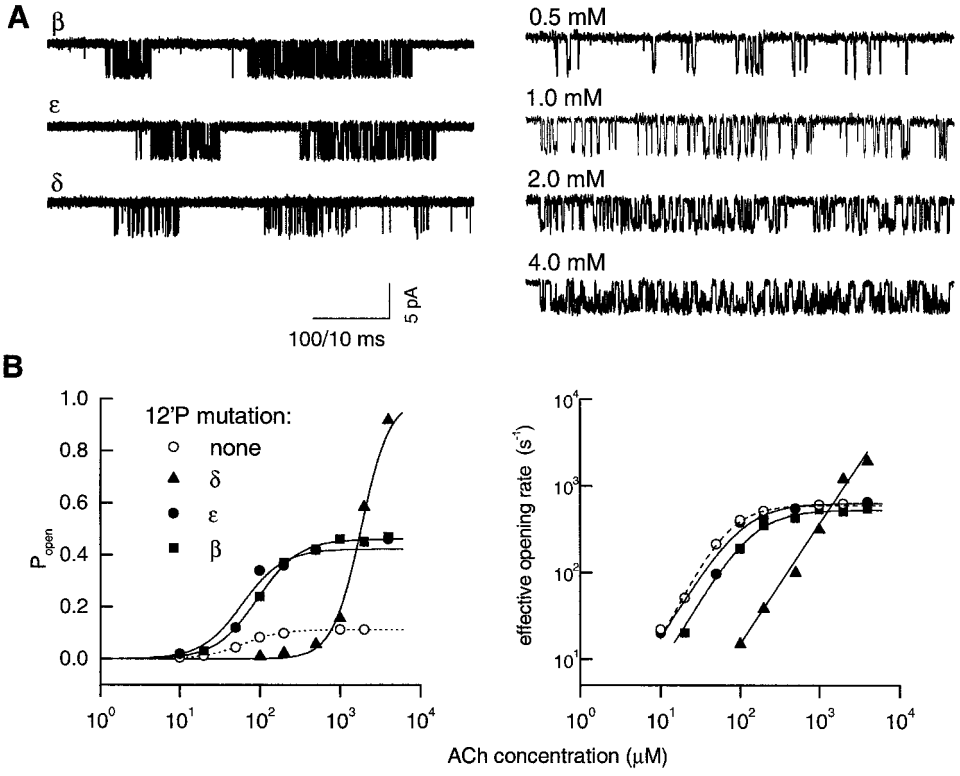

FIGURE 4 Properties of αD200N AChR with 12′P mutations. (*A*) Example clusters. (*Left*) Clusters activated by 500 μM ACh. At this concentration, the δ12′P mutant has a lower open probability than the ϵ- and β12′P mutants. (*Right*) δ12′P (αD200N) clusters at different ACh concentrations. The δ12′P mutant is capable of achieving a high open probability at very high ACh concentrations. (*B*) Dose-response curves. (*Left*) Open probability (P_{open}) of clusters. In αD200N AChR without a 12′P mutation, (- - -), the maximal P_{open} is ~0.1 and is half-maximal at ~80 μM. The maximal P_{open} is increased by all 12′P mutations; the ACh concentration required to produce a half-maximal response is not altered by 12′P mutations in β or ϵ but is increased >10-fold by a 12′P mutation in δ. (*Right*) Effective opening rate versus [ACh]. The durations of the slowest component of closed intervals are similar for AChR with no 12′ mutation (- - -) and AChR with a 12′P mutation in β or ϵ, indicating that these 12′P mutations do not alter the channel opening rate constant or the receptor's affinity for ACh. With a δ12′P mutation, both the maximal effective opening rate and the ACh concentration required to produce a half-maximal response are increased, indicating that this 12′P mutation increases the opening rate constant and decreases the receptor's affinity for ACh.

for all species, the 12′ residue is a T, but in δ it is an S (or an A in *Torpedo*; Fig. 1). In addition, cysteine mutagenesis experiments show that in the α subunit the 12′ position is inaccessible from the extracellular compartment (Akabas et al., 1994), although affinity labeling experiments show that externally applied α-neurotoxin from *Naja naja oxiana* binds to the 12′ residue of the δ subunit (*Torpedo)*, indicating that this side chain is accessible from the extracellular side (Machold et al., 1995).

In our experiments, two sets of observations point to a special role for δ12′ in AChR activation. First, in αD200N receptors the consequences of mutating the 12′ position in δ are quantitatively different than in the other subunits. Although for all subunits a proline mutation at 12′ increases the free energy required for channel closing, the effect of this substitution in δ is only approximately one-half that in the other subunits. Second, although 12′P residues of β and ϵ act essentially independently of each other, the 12′P residue in δ interacts with homologous residues in ϵ or β. The extent of this interaction is significant, as the β or ϵ mutations have approximately one-third the effect when coupled with a δ12′P as when they are expressed alone.

The special character of the δ12′ position is also apparent when the interactions of this pore residue with the transmitter binding site are considered. In αD200N AChR, the δ12′P mutation increases the equilibrium dissociation constant for ACh ~10-fold, whereas a similar mutation in β or ϵ has virtually no effect on agonist affinity. In addition, the δ12′P mutation, but not one in β or ϵ, restores, in part, fast opening to receptors with a binding site mutation that would otherwise have a very slow channel opening rate constant.

We consider whether this restoration indicates an interaction between that δ12′ and αD200 residues, or whether it might be a consequence of independent effects of the two mutations. An αD200N mutation alone decreases the channel opening rate constant ~10-fold, which is equivalent to a 1.4 kcal/mol increase in the activation free energy for opening (Table 2; Akk et al., 1996). The effect of a lone δ12′P mutation can be estimated by assuming that this mutation changes only the opening rate constant. The apparent lifetime of δ12′P AChR is 5.7 times longer than the wild type. From Eq. 2 we calculate that the opening rate constant of this mutant (Fig. 3 *D*) would have to be ~300,000 s^{-1}, which is equivalent to a decrease in the

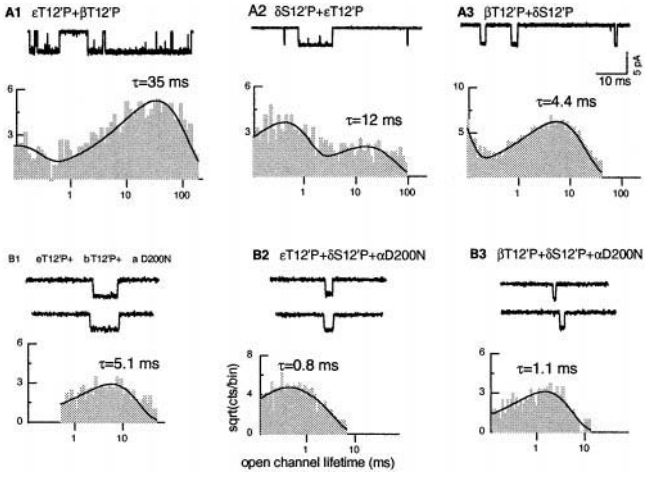

FIGURE 5 Effect of multiple 12′P mutations. (*A*) Pairwise expression of 12′P mutations in AChR with wild-type binding sites. The double $\epsilon + \beta$ mutant has a lifetime that is longer than that of either the β or ϵ single 12′P mutant AChR, but the double mutants with a δ12′P mutation have a lifetime that is shorter than the β or ϵ single mutants. (*B*) Pairwise expression of 12′P mutations in AChR with αD200N binding sites. The double $\epsilon + \beta$ mutant has a lifetime that is longer than that of either the β or ϵ single 12′P mutant AChR (see Table 1), but the double mutants with a δ12′P mutation have a lifetime that is shorter than the β or ϵ single mutants (see Table 2).

activation energy for opening of ~1 kcal/mol. If the two mutations were independent, the net activation energy for opening would be 0.4 kcal/mol higher than the wild type, so that the opening rate constant would be approximately one-half of the wild type, or ~30,000 s^{-1}. The measured opening rate constant for the double mutant is >2000 s^{-1} (Fig. 4), which is consistent with the hypothesis of independent action of the αD200N and δ12′P mutations. If this were so, the true channel closing rate constant (Eq. 2) for δ12′P + αD200N AChR would be approximately twice the apparent rate constant (Table 2) and would be nearly equivalent to that of αD200N alone.

However, an increased opening rate constant is not the sole manifestation of the δ12′P mutation. The shift in the equilibrium dissociation constant by this mutation indicates that there is some interaction between this pore position and the transmitter binding site. In addition, short-lived gaps were not observed in δ12′P + αD200N AChR single-channel currents, suggesting that the increase in the open channel lifetime of αD200N + δ12′P AChR is a direct effect on the channel closing rate constant rather than a consequence of faster opening. Nonetheless, in the absence of a direct measurement of the opening rate constant of αD200N + δ12′P AChR we cannot reject the hypothesis that the lifetime of the double mutant receptor is due, perhaps in part, to additive energetic consequences of the two mutations.

TABLE 2 Channel closing kinetics of 12′P+αD200N AChR

12′P mutation	α (s^{-1})	$\Delta\Delta G^{\ddagger}$ (kcal/mol)
None	4025 ± 332 (4)	
ϵ	741 ± 49 (5)	1.00
β	607 ± 30 (3)	1.12
δ	1693 ± 216 (5)	0.51
$\epsilon + \beta$	195 ± 15 (3)	1.79
$\epsilon + \delta$	1024 ± 390 (5)	0.81
$\beta + \delta$	1041 ± 143 (4)	0.80

All values pertain to αD200N AChR, where the channel closing rate constant (α) is the inverse of the open channel lifetime (Eq. 1–3; see Table 1). The activation energy for channel closing $\Delta\Delta G^{\ddagger} = 0.59 \ln(\alpha_{wt}/\alpha_{12'P})$; values are mean ± SD (*n* patches). The effects of the $\beta + \epsilon$ 12′P mutations are close to independent because the double M2 mutant $\Delta\Delta G^{\ddagger}$ value is only 0.3 kcal/mol less than the sum of the single mutant values. The effects of the $\delta + \epsilon$ and $\delta + \beta$ 12′P mutations are not independent because the double M2 mutant $\Delta\Delta G^{\ddagger}$ values are less than each single mutant value.

Regardless of the mechanism, the effects of the 12′P mutation in δ are quite distinct from the homologous mutations in β or ϵ. Although mutations to all three residues slow closing, only the δ mutation increases the opening rate constant and alters the equilibrium dissociation constant. Also, the effect of δ12′P with regard to the closing rate constant is much less than in β or ϵ. The consequences of α12′P and γ12′P mutations in αD200N AChR were not examined, so we cannot be certain that the δ subunit is unique in this regard.

We can attempt to provide a structural basis for interpreting the effects of 12′ mutations on channel gating by assuming that the M2 segment is a helix and that for each subunit the 9′ leucine side chain projects into the pore (Unwin, 1993). Because the rotation angle for each amino acid in a helix is ~100°, the 12′ and 13′ side chains should each protrude at ~±60° from the pore radius. Given the 5-fold symmetry of the channel, this arrangement would place the 12′ residue at a subunit interface ~0.45 nm above the 9′ leucines and projecting toward the 13′ residue of the adjacent subunit. In all non-δ subunits, the 12′ residue is a threonine and the 13′ residue is a valine; thus, this configuration would result in ring of T-V side chains at a level just above the central leucine (a TV channel motif; Fig. 1). Molecular models of the AChR pore (Smith and Sansom, 1997), however, suggest that the M2 domains of adjacent subunits may be too widely separated to allow these side chains to make direct contact.

A proline mutation would be expected to change the rotation angle of the peptide bond between the 11′ and 12′ residue from −180° *trans* to something <−180°, i.e., to produce a kink in the M2 segment, although not introducing a bulky or charged side chain into the intersubunit space. Cryo-electron microscopic studies of the closed and open state of *Torpedo* ($\alpha_2\beta\delta\gamma$) AChR suggest that the upper region of the M2 helix both rotates and moves with respect to the pore axis during gating (Unwin, 1995); thus, it is expected that the presence of a kink near the 12′ position would alter the energetics of this transition.

A slower channel closing rate constant is consistent with either a more stable open channel configuration and/or an increased height of the closed-open transition state barrier. The observation that in αD200N AChR a 12′P mutation in ϵ or β does not change the opening rate constant suggests

that the transition state energy has not been altered by the pore mutations. We therefore hypothesize that the 12′P mutations in these subunits introduce a kink in M2 that serves to stabilize the open conformation of the pore.

The five subunits of the receptor come into a close proximity in the pore region; thus, it is not surprising that a conformational change in one subunit can influence other subunits. Interactions between the subunits have also been reported for unnatural amino substitution at the 9′ positions (Kearney et al., 1996). Our results indicate that there are negative interactions between the δ12′P position and the 12′P positions of β and ε, i.e., that the δ mutation reduces the effect of a mutation at these other subunits.

The effect of a mutation can be subdivided into local and nonlocal effects. The main local effect of a 12′P mutation is to increase the stability of the open channel conformation. Nonlocal effects include a reduction in the local effects of other 12′P mutations and a change in the affinity of the transmitter binding site. The overall effect of multiple mutations on channel activation is determined by a balance of local and nonlocal effects. The observations suggest that the net effect of putting a kink at the 12′ position has a similar consequence in all subunits but that in αD200N AChR the effects in β and ε are mainly local (~1 kcal/mol per mutation), whereas the effect in δ is both local (~0.5 kcal/mol) and nonlocal (~0.7 kcal/mol).

The strong interactions of δ with β and ε apparently do not arise from direct subunit contacts. The α subunits are not adjacent; thus, regardless of whether the ε or the β subunit (Kubalek et al., 1988) lies between the two α subunits, δ cannot contact both. Moreover, if the M2 helices are left-handed, the δ12′ residue would lie near the α-δ interface and contact neither β nor ε. We hypothesize that the transfer of energy from δ12′P to the ε- and β12′ residues reflects a more distributed structural change in the protein.

The location of the binding site relative to the δ12′ position is not known with certainly, but affinity labeling, ultrastructural, and fluorescence studies (Machold et al., 1995; Unwin, 1993; Valenzuela et al., 1994) suggest that these regions are separated by >20 Å. The observation that only in δ does the proline substitution alter the affinity of the αD200N AChR transmitter binding site supports the idea of a more widespread change in structure consequent to the δ12′P mutation. By the same logic, the near independence of the β and ε 12′P mutations, and the unaltered affinity of the transmitter binding sites following these mutations, is consistent with the notion that structural changes induced by these prolines are local.

The gating of the AChR pore is a concerted process: the step-like nature of the conductance change indicates that in the channel domain all five subunits undergo an effectively synchronous change in structure (within ~1 μs; Maconochie et al., 1995). Our results suggest that the conformation of the δ subunit backbone at the 12′ position plays a different role in gating than the homologous residues of the other subunits. Whether this distinction extends to other regions of the δ subunit is unknown.

We thank Dr. Steven Sine for critical comments and for providing αD200N and εT264P and Karen Lau for technical contributions.
This work was supported by National Institutes of Health grant NS23513 to A. Auerbach.

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