

We here trace the cause of a CMS to two pathogenic mutations in the AChR δ subunit: δ L42P in the β_1 strand which causes abnormally brief channel openings, and δ I58K in the β_2 strand which hinders AChR assembly. The principle effect of δ L42P is to attenuate channel gating by reducing both its speed and efficiency. Thermodynamic mutant cycle analysis reveals that the functional consequences of δ L42P result from structural perturbation of an inter-subunit link between α - and δ -subunits essential for channel gating.

Results

Characteristics of CMS patient. A 20-year-old woman had moderately severe to severe myasthenic symptoms since birth, no anti-AChR antibodies, and a 34-71% decremental response of the compound muscle action potential on repetitive stimulation of motor nerves at 2 Hz. She responded poorly to pyridostigmine alone but improved markedly after the addition of 3,4-diaminopyridine. A similarly affected sibling died at age 11 months.

Endplate studies. The configuration of the endplates (EPs), evaluated from the cytochemical reaction for acetylcholinesterase on longitudinally oriented teased single muscle fibers, was abnormal, with numerous small EP regions distributed over a 2- to 4-fold increased span of the muscle fiber surface. The reaction for AChR, detected in cryostat sections with rhodamine-labeled α -bungarotoxin (α -bgt) was greatly attenuated.

On EM, the structural integrity of the junctional folds was preserved. Morphometric analysis of individual EP regions revealed that nerve terminal size was normal but the postsynaptic area of junctional folds and cleft was reduced to 58% of normal. Some postsynaptic regions were simplified and the postsynaptic membrane length per unit postsynaptic area was 70% of normal. The reaction for AChR, detected with peroxidase-labeled α -bgt, was patchy as well as attenuated (Figure 1) and the AChR-reactive postsynaptic membrane length was 33% of normal (Table 1). The number of ^{125}I α -bgt sites per EP was decreased to 16% of normal (Table 2).

In vitro microelectrode studies of intercostal muscle EPs showed that quantal release by nerve impulse was normal. The miniature EP potential (MEPP) and current (MEPC) amplitudes were reduced to 6% and 10% of normal, respectively (Table 2).

Mutation analysis. Direct sequencing of each AChR subunit gene revealed three heterozygous mutations in CHRND: 125T>C, predicting Leu>Pro at codon 42 (δ L42P) in the β_1 strand; 173T>A, predicting an Ile>Lys at codon 58 (δ I58K) in the β_2 strand, and 277G>C predicting Val>Leu at codon 93 (δ V93L) in the β_4 strand (Figure 2A). δ L42 and δ V93L are conserved across AChR δ subunits of different species; I58K is conserved in δ subunits of mammals, and in human ϵ and γ subunits (Fig 2A). None of the mutations is present in 200 normal alleles of 100 unrelated subjects. Family analysis indicates that each mutation is recessive with δ I58K and δ V93L in the paternal and δ L42P in the maternal allele (Figure 2B).

Expression studies of mutant AChRs expressed in HEK cells. To determine whether the mutations hinder the surface expression of AChR, we engineered the observed mutations into the human δ subunit and coexpressed them with complimentary wild-type α , β , and ϵ subunits in HEK cells. As controls, we coexpressed wild-type α , β , δ , and ϵ subunits, and also wild-type α , β , and ϵ subunits without the δ subunit. Compared to wild-type, surface [125 I] α -bgt binding was enhanced by δ V93L, reduced to 37% by δ L42P, and decreased to 5% by δ I58K and by the δ -omitted $\alpha_2\beta\epsilon_2$ construct (Figure 3A). Surface expression of the double mutant δ I58K+V93L-AChR was similar to that of δ I58K-AChR, indicating that δ V93L did not mitigate the effects of δ I58K (Figure 3A). We also compared expression of the wild α/δ dimer with that harboring δ I58K, and with expression of only the α subunit. Toxin binding by HEK cells expressing the mutant dimer was only 15% of that of the wild-type dimer and not different from toxin binding by HEK cells expressing only the α subunit (Figure 3B), indicating that δ I58K hinders δ/α subunit association, an early step in AChR assembly (14). We also compared the apparent affinity for ACh of the δ L42P receptor relative to wild-type by measuring ACh binding at steady state by competition against the initial rate of [125 I] α -bgt binding to intact cells (15). The apparent dissociation constant of δ L42P-AChR was reduced 5-

fold compared to that of wild-type AChR, indicating an increased affinity of ACh for one or more of the closed, open or desensitized states of the mutant receptor (data not shown).

Activation kinetics of the δ L42P mutant. To determine whether the mutations affect the kinetics of AChR activation, we recorded single channel currents elicited by a low concentration of ACh from HEK cells expressing wild-type, δ L42P, and δ V93L AChRs. At a concentration of 50 nM ACh, channel openings of di-liganded AChRs appear in bursts of several openings in quick succession and provide an indirect measure of the decay rate of post-synaptic currents. Burst duration histograms of δ L42P AChR displayed two components whereas those of wild-type and the other mutant AChRs displayed three components (Figure 4). The mean duration of the longest component of bursts for the δ L42P receptor was 0.76 ms, much briefer than the corresponding value of 3.3 ms for the wild type AChR, while that of the δ V93L receptor was mildly but significantly prolonged (Table 3). Because δ I58K is essentially a null mutation and δ V93L is not pathogenic, δ L42P determines the phenotype.

To determine the mechanism by which the δ L42P mutant shortens bursts of channel openings, we examined the activation kinetics of the δ L42P mutant AChR by recording single channel currents over a range of ACh concentrations (see Experimental Methods). For both wild type and δ L42P AChRs, the distributions of closed durations exhibited several exponential components that shifted from long to brief durations with increasing ACh concentrations (Fig. 5A, B). However for the δ L42P mutant AChR, the shift toward brief durations was significantly less, and at a saturating concentration of ACh, closed durations remained prolonged. Open durations for the δ L42P mutant changed little across the range of ACh concentrations and were uniformly briefer than observed for the wild type AChR.

To quantify changes in elementary rate constants underlying activation of the mutant AChR, we fitted Scheme 1 (Fig. 6) to the single channel closed and open intervals elicited by the entire range of ACh concentrations displayed in Fig. 5. The scheme depicts AChR activation as the reversible binding of two agonists (A) to AChRs in the closed state (R) followed by reversible formation of the open state (R*). At high concentrations, the agonist blocks the open channel to form the blocked state (R_B).

For both wild type and δ L42P mutant AChRs, Scheme 1 describes the dwell time distributions over the entire range of ACh concentrations, as shown by the smooth curves overlying the histograms. The analysis gives estimates of rate constants for ACh association and dissociation and opening and closing of the channel (Table 4). The most significant effect of δ L42P is a 12.5-fold decrease of the di-liganded channel gating equilibrium constant θ_2 , which results from a decrease of the channel opening rate constant β_2 and an increase of the channel closing rate constant α_2 . Elementary steps for ACh binding were modestly affected by the mutation, with rate constants for ACh dissociation, k_{-1} and k_{-2} , slowed; although the estimate of k_{-2} was well-defined, the estimate of k_{-1} showed a larger error and thus greater uncertainty.

The fitted rate constants account quantitatively for the dependence of channel open probability (P_{open}) on ACh concentration, showing that δ L42P shifts P_{open} to lower ACh concentrations and decreases its maximum (Fig. 5C). Furthermore the burst duration, approximated by $(1 + \beta_2/k_{-2})/\alpha_2$, predicts a value of 0.7 ms which agrees closely with the independently determined burst duration of 0.76 ms recorded with 50 nM ACh (Table 3); both values predict abnormally fast decay of synaptic currents typical of the fast-channel CMS. The probability a di-liganded channel will open following a pulse of nerve-released ACh, given by

$\beta_2/(\beta_2 + k_{-2})$, decreases from 0.84 for the wild type AChR to 0.53 for the δ L42P mutant AChR, predicting a reduced peak synaptic response.

Other substitutions of δ L42. δ L42P introduces a proline residue in the lower third of the β_1 strand (Figure 7A) which could destabilize the anti-parallel inner β -sheet by breaking inter-strand hydrogen bonds. To determine whether the mutation effects are proline-specific, we introduced into δ codon 42 the small and flexible residue Gly as well as the charged residues Lys and Asp. Each of these mutations generated abnormally brief channel bursts (Table 3) and reduced the channel gating equilibrium constant 12- to 25-fold, mainly by slowing β_2 (Table 4). Thus the attenuated gating by δ L42P is not proline-specific and not due to disruption of hydrogen bonds; the effects may instead result from a local structural disturbance of the subunit interface or the hydrophobic core of the δ subunit.

Proline substitution in other subunits of residues aligning with δ L42. To determine whether the effects of δ L42P are subunit specific, we mutated the corresponding Leu in the ϵ , β , and α subunits to Pro. Compared to wild-type AChR, ϵ L40P decreases the burst duration by ~50% (Figure 4 and Table 3), reduces P_{open} to a lesser extent than δ L42P (Fig. 5C), and attenuates the channel gating equilibrium constant 4-fold (Table 4). β L40P decreases the burst duration by 30% (Table 3), but affects neither P_{open} (Fig. 5C) nor the gating equilibrium constant (Table 4). In striking contrast to mutations in the β -, δ - and ϵ -subunits, mutation of the equivalent residue in the α -subunit α L40P prolongs the longest component of opening bursts ~4 fold (Figure 4 and Table 3). The overall results indicate that the lower β_1 strands of the different subunits contribute to AChR activation in distinct and in some cases opposing ways.

Proline mutations of residues adjacent to δ L42. To determine whether the effects of the mutation are specific to the location of δ L42P within the β_1 strand, we performed proline

scanning mutagenesis two residues upstream and downstream of δ L42. We found that δ N41P, δ I43P, and δ S44P decreased the major long component of bursts to 0.6-0.71 ms, like δ L42P, and δ S40P decreased it to 0.35 ms (Table 3). Thus, over a span of four consecutive residues within the β_1 strand of the δ -subunit, substitution of proline attenuates receptor activation.

Mutant cycle analysis. δ L42 and the equivalent ϵ L40 are located at subunit interfaces, suggesting they contribute to channel gating affecting interaction between the subunits. Moreover, previous mutant cycle analyses revealed that the adjacent residues, δ N41 and ϵ N39, couple energetically with the proximal α Y127 from the juxtaposed α -subunit (13). We therefore used mutant cycle analyses (16) to determine whether δ L42 and ϵ L40 also couple energetically with α Y127. Using $-RT\ln\theta_2$ the free energy of channel gating of each mutant AChR, we generated a 2-dimensional mutant cycle composed of gating equilibria for wild type, δ L42P/ ϵ L40P, α Y127T and α Y127T/ δ L42P/ ϵ L40P AChRs (Fig. 7). The resulting inter-residue coupling free energy of 3.9 kcal/mol is large and approaches the value of 5.8 kcal/mol obtained for the previously described mutant cycle generated using the mutations α Y127T, δ N41A and ϵ N39A.

To determine the origin of the coupling energy, we cast three more 2-dimensional mutant cycles using subsets of the three residue substitutions. The mutant cycles α Y127/ δ L42P and α Y127/ ϵ L40P exhibit approximately equal coupling free energies approaching 2 kcal/mol, showing that at the two subunit interfaces, the residue pairs contribute equally to the overall coupling energy. The mutant cycle δ L42P/ ϵ L40P reveals a low coupling energy of 0.7 kcal/mol, indicating δ L42P and ϵ L40P, which are far apart in the 3D structure, are essentially independent in contributing to gating. Thus although δ L42P and ϵ L40P are independent of each other, their contributions to channel gating depend on the juxtaposed α Y127 of the neighboring α -subunit.

Discussion

We identify three mutations in β strands of the AChR δ subunit in a patient with moderately severe to severe myasthenic symptoms since birth. One mutation, $\delta V93L$ has no appreciable kinetic effects and allows for robust AChR expression in HEK cells. The second mutation on the same allele, $\delta I58K$, hinders δ/α subunit association and is essentially a null mutation. Hence the third mutation, $\delta L42P$, determines the phenotype. Although this mutation modestly enhances affinity of the di-liganded closed receptor, it decreases the gating efficiency of the diliganded receptor 13-fold, and reduces AChR expression in HEK cells to 37% of wild-type. The activation rate constants of the $\delta L42P$ -receptor in Table 4 predict a mean burst duration of 0.7 ms, close to that measured for bursts elicited by a low ACh concentration (Table 3). This value predicts a 5-fold faster decay of synaptic currents at patient than at normal EPs. The activation rate constants also predict that channel opening probability during synaptic transmission is decreased from 0.84 to 0.53; thus only ~60% of the synaptic AChRs would be opened by an instantaneous pulse of ACh. The amplitude of the synaptic response is additionally curtailed by reduced expression of the $\delta L42P$ -AChR, and by the simplified junctional folds which decrease (17). Thus, the safety margin of neuromuscular transmission is compromised by the combined effects of EP AChR deficiency, reduced opening probability of the available receptors, and abnormally fast decay of the synaptic current.

We also find that the consequences $\delta L42P$ are not Pro specific, because substitution with a charged Asp or Lys, or with a small and flexible Gly, has similar kinetic effects. Pro mutants at $\delta N41$, $\delta I43$, and $\delta S44$ mimic the effects of $\delta L42P$, indicating that a span of 4 residues in the $\beta 1$ strand of the δ subunit is critical for maintaining gating efficiency. Mutation of residues that align with $\delta L42$ in the ϵ , β , and α -subunit impede, fail to alter, or enhance gating; therefore the

effects of the mutation are subunit specific. Finally, using mutant cycle analysis we show that δ L42 and ϵ L40 contribute to an inter-subunit linkage required for efficient channel gating. The patient mutation likely causes a local structural perturbation that alters the primary inter-subunit linkage between δ N41, adjacent to the patient mutation, and the juxtaposed α Y127.

Although the identified Leu in the β 1 strand is conserved at equivalent positions of the α , β , ϵ and δ -subunits, the contributions of the Leu to AChR activation depend on the subunit. Differences in residues proximal to the identified Leu may therefore contribute to the different functional consequences of the mutations. The Leu in the δ and ϵ subunits is adjacent to the key Asn residue that spans the subunit interface and mediates inter-subunit interactions with α Y127 required for efficient channel gating. None of the other subunit interfaces contain the Tyr/Asn linkage, suggesting inter-subunit interactions at the α - δ and α - ϵ interfaces contribute uniquely to channel gating. Before the current study, it was not known whether residues at equivalent positions of the other three subunit interfaces mediate inter-subunit interactions that contribute to channel gating. However by examining the equivalent Leu in the α -subunit, we find that two interfaces, ϵ - α and β - α , contribute to channel gating but in a novel manner. Structural perturbation of these interfaces by the mutation α L40P enhances rather than attenuates channel gating. Thus residue differences at these interfaces, whether proximal or distal to α L40, emerge as candidates for inter-subunit linkages mediating global communication required for channel gating.

Methods

Muscle specimens. Intercostal muscle specimens were obtained intact from origin to insertion from patients and control subjects without muscle disease undergoing thoracic surgery. All human studies were in accord with the guidelines of the Institutional Review Board of the Mayo Clinic.

AChR and acetylcholinesterase were detected in cryostat sections by two-color fluorescence (18). Endplates (EPs) were localized for electron microscopy and analyzed by the established methods (19). Peroxidase-labeled α -bgt was used for the ultrastructural localization of AChR (20). The number of AChRs per EP was measured with [125 I] α -bungarotoxin (α -bgt) (21).

Electrophysiology of muscle specimens. Recordings of EP potentials and currents (EPCs) and estimates of the number of transmitter quanta released by nerve impulse were performed as described elsewhere (21, 22), except that the amplitude of the miniature EP potentials (MEPPs) and currents (MEPCs) were estimated from the quantal components of the EPP and EPC, respectively (23).

Mutation analysis. We directly sequenced AChR α , β , δ , and ϵ subunit genes using genomic DNA. The mutations were traced with allele-specific PCR in family members and in 200 normal alleles of 100 unrelated controls.

Construction and expression of wild-type and mutant AChRs. Sources of human α , β , ϵ and δ subunit cDNAs were as previously described (24). All four cDNAs were subcloned into the CMV-based expression vector pRBG4 for expression in human embryonic kidney fibroblast (293 HEK) cells (24). The artificial mutations were engineered into wild-type AChR subunit cDNAs in pRBG4 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The presence of each mutation and absence of unwanted mutations was confirmed by sequencing the entire inserts. HEK

cells were transfected with plasmids comprising of pRBG4- α , - β , - δ , - ϵ , and pEGFP-N1 in a ratio of 2:1:1:1:1 using FuGene6 transfection reagent (Roche).

α -Bungarotoxin binding measurements. The total number of [125 I] α -bgt sites on the surface of transfected human embryonic kidney (HEK) cells and ACh competition against the initial rate of [125 I] α -bgt binding were determined as described elsewhere (24). ACh competition measurements were analyzed using Hill equation: $1 - Y = 1/(1 + ([ACh]/K_{ov})^n)$, where Y is fractional occupancy by ACh, n is the Hill coefficient, K_{ov} is an overall dissociation constant.

Patch-clamp recordings and single-channel kinetic analysis. Recordings were obtained in the cell-attached configuration at a membrane potential of -80 mV, at 22°C, and with bath and pipette solutions containing (mM): KCl 142, NaCl 5.4, CaCl₂ 1.8, MgCl₂ 1.7, HEPES 10, pH 7.4 (24). Single-channel currents were recorded using an Axopatch 200A amplifier (Axon Instruments) at a bandwidth of 50 kHz, digitized at 5 μ s intervals using a Digidata 1322A (Axon Instruments), and recorded to hard disk using the program Clampex 8 (Axon Instruments). Records were analyzed at a uniform bandwidth of 11.7 kHz for recordings at ACh concentration of 50 nM and 10 kHz for recordings at 3 μ M or above with TACx4.0.9 software (Bruyton). Dwell-time histograms were plotted on a logarithmic abscissa and fitted to the sum of exponentials by maximum likelihood (25).

To estimate rate constants underlying AChR activation, we employed desensitizing concentrations of ACh that cause events from a single channel to cluster into identifiable activation episodes (26). Clusters were identified as a series of closely spaced openings preceded and followed by closed intervals greater than a defined critical time. The critical time was determined by a method that misclassifies an equal number of events between two adjacent closed-time components (27). For each receptor, the critical time that provided the best fit for the

closed time histogram was chosen for the final analysis. Clusters with fewer than five openings were excluded from analysis. Individual clusters were examined for homogeneity by determining the mean open probability and open duration for each cluster, and clusters within two standard deviations of the means were accepted for further analysis (28, 29). The resulting global set of open and closed dwell times of wild-type and mutant AChRs were analyzed using the program MIL which uses an interval-based maximum likelihood method that also corrects for missed events (28). The kinetic analysis of wild-type and mutant receptors yielded a set of rate constants that were fitted to a scheme of receptor activation (see Figure 6 under Results).

For each type of AChR, single channel dwell times obtained over a range of ACh concentrations were fitted simultaneously. Data were obtained over a range of ACh concentrations from 10-300 μM for wild-type and $\alpha\text{Y127T}/\epsilon\text{L40P}$ - AChR, from 100-1000 μM for αY127T - AChR, and from 3-1000 μM for other AChRs; 1,562-11,250 (mean 4,800) events were analyzed for each ACh concentration. The final set of rate constants were checked by superimposing probability density functions calculated from the rate constants on the experimental dwell time histograms, and by their ability to predict burst length at low ACh concentrations (30, 31).

Acknowledgments

This work was supported by NIH grants to A.G. Engel (NS-6277) and to S.M. Sine (NS-31744) and by a Muscular Dystrophy Association Grant to A.G. Engel. We thank Dr. Isabella Illa for patient referral.

Table 1. Morphometric analysis of endplate regions

Parameter	Patient	Controls
Nerve terminal area (μm^2)	3.28 ± 0.40 (27)	3.88 ± 0.39 (63)
Postsynaptic area (μm^2)	$6.13 \pm 0.42^{\text{C}}$ (27)	10.60 ± 0.79 (39)
Normalized postsynaptic membrane length ($\mu\text{m}/\mu\text{m}^2$) ^A	$4.07 \pm 0.20^{\text{C}}$ (27)	5.83 ± 0.25 (47)
Normalized AChR-reactive membrane length ^B	$1.00 \pm 0.13^{\text{C}}$ (16)	3.01 ± 0.11 (85)

Values indicate mean \pm SE; numbers in parentheses represent number of endplate regions. More than one region can occur at an endplate.

^APostsynaptic membrane length divided by postsynaptic area.

^BAChR reactive postsynaptic membrane length divided by length of primary synaptic cleft.

^C $P < 0.001$

Table 2. In vitro microelectrode and α -bgt binding studies

	Patient	Controls
m^A	32 ± 5 (17)	31 ± 1 (190)
MEPP amplitude (mV) ^B	0.067 ± 0.011 (17)	1.00 ± 0.025 (165)
MEPC amplitude (nA) ^C	0.44 ± 0.064 (11)	3.95 ± 0.10 (79)
[¹²⁵ I] α -bgt binding sites/EP	2.06×10^6	$12.82 \pm 0.79 \times 10^6$ (13)

Values indicate mean \pm SEM. Measurements at $29^\circ\text{C} \pm 0.5^\circ\text{C}$ for MEPPs and EPPs recordings, and at $22^\circ\text{C} \pm 0.5^\circ\text{C}$ for MEPCs recordings. Numbers in parenthesis indicate number of subjects for [¹²⁵I] α -bgt binding sites/EP and number of EPs for other measurements.

^AQuantal content of EPP at 1 Hz stimulation corrected for a resting membrane potential of -80 mV, nonlinear summation, and non-Poisson release.

^BEstimated by dividing the corrected EPP amplitude by m , and corrected for a fiber diameter of 50 μm .

^CEstimated by dividing the EP current amplitude by m .

Table 3. Burst durations of wild-type and mutant AChRs in HEK cells

AChR	$\tau_1(a_1)$	Burst (ms)	
		$\tau_2(a_2)$	$\tau_3(a_3)$
Wild-type	0.036 ± 0.002^A (0.24 ± 0.02)	0.47 ± 0.06 (0.21 ± 0.03)	3.31 ± 0.12 (0.58 ± 0.04)
δ L42P	0.18 ± 0.040 (0.69 ± 0.02)	0.76 ± 0.23 (0.31 ± 0.016)	
δ V93L	0.12 ± 0.038^B (0.14 ± 0.02)	1.41 ± 0.30 (0.4 ± 0.05)	4.83 ± 0.44 (0.52 ± 0.07)
δ L42G	0.068 ± 0.011 (0.55 ± 0.06)	0.38 ± 0.02 (0.42 ± 0.08)	
δ L42D	0.16 ± 0.024 (0.73 ± 0.04)	0.63 ± 0.31 (0.27 ± 0.04)	
δ L42K	0.080 ± 0.009 (0.56 ± 0.03)	0.43 ± 0.02 (0.44 ± 0.03)	
ϵ L40P	0.062 ± 0.01 (0.31 ± 0.03)	0.23 ± 0.04 (0.59 ± 0.03)	1.71 ± 0.14 (0.1 ± 0.008)
β L40P	0.48 ± 0.10 (0.50 ± 0.10)	2.27 ± 0.08 (0.50 ± 0.10)	
α L40P	0.11 ± 0.032 (0.22 ± 0.07)	1.67 ± 0.58 (0.41 ± 0.05)	12.98 ± 0.90 (0.36 ± 0.06)
δ S40P	0.17 (0.23)	0.72 (0.53)	2.98 (0.24)
δ N41P	0.074 ± 0.007 (0.37 ± 0.02)	0.61 ± 0.03 (0.63 ± 0.02)	
δ I43P	0.030 ± 0.02 (0.24 ± 0.07)	0.69 ± 0.07 (0.76 ± 0.1)	
δ S44P	0.024 ± 0.0007 (0.21 ± 0.04)	0.35 ± 0.03 (0.64 ± 0.04)	2.05 ± 0.54^C (0.15 ± 0.03)

Twenty-one patches for wild-type, 5 for δ V93L, 4 for β L40P and α L40P, multiple combined patches for δ S40P, and 3 patches for all other AChRs were analyzed. Values indicate means \pm SE. τ_n and a_n indicate time constants and fractional histogram areas. ACh = 50 nM; membrane potential = -80 mV; bandwidth = 11.7 kHz.

^{A,B,C} not detected at 3, 2, and 1 patches, respectively.

Table 4. Activation kinetics of wild-type and mutant AChRs expressed in HEK cells

AChR	k_{-1}	k_1	K_1 / μ M	k_{+2}	k_2	K_2 / μ M	β_1	α_1	θ_1	β_2	α_2	θ_2	k_{+b}	k_b	K_B / μ M
Wild-Type	98 ± 7	1,817 ± 185	19	86 ± 2	10,449 ± 186	121	191 ± 16	3,052 ± 276	0.063	56,291 $\pm 1,495$	2,223 ± 75	25	90 ± 6	144,500 $\pm 2,515$	1.61
δ L42P	193 ± 51	139 ± 66	0.7	96 ± 6	6,072 ± 362	63	438 ± 37	6,919 ± 334	0.063	6,772 ± 146	3015 ± 37	2	44 ± 6	143,409 $\pm 46,824$	3.26
δ L42G	101 ± 29	115 ± 48	1	153 ± 7	10,525 ± 502	69	111 ± 12	11,149 ± 760	0.010	4,741 ± 85	3,978 ± 39	1	7 ± 1	58,093 $\pm 4,344$	8.30
δ L42D	80 ± 16	20 ± 8	0.3	135 ± 4	9,992 ± 336	74	97 ± 6	8,522 ± 538	0.011	5,816 ± 118	3,136 ± 30	2	54 ± 3	143,436 $\pm 2,520$	2.66
δ L42K	671 ± 63	4540 ± 641	7	293 ± 18	13,386 ± 670	46	1489 ± 168	18,085 ± 727	0.082	13,076 ± 312	6,065 ± 113	2	7 ± 1	71,284 $\pm 8,194$	10.18
ϵ L40P	199 ± 32	595 ± 162	3	79 ± 5	8,523 ± 434	108	200 ± 24	3,510 ± 182	0.057	6,815 ± 235	1,221 ± 21	6	39 ± 3	111,670 $\pm 2,846$	2.86
β L40P	229 ± 33	1,313 ± 207	6	159 ± 7	21,706 ± 754	137	ND	ND	ND	28239 $\pm 1,197$	1,106 ± 30	26	13 ± 2	103,260 $\pm 7,671$	7.94
α Y127T	ND	ND	ND	ND	ND	ND	ND	ND	ND	182 ± 2	3,568 ± 46	0.051	14 ± 1	66,021 $\pm 3,060$	4.72
α Y127T+ δ L42P	150 ± 39	1,182 ± 393	8	195 ± 22	12,159 $\pm 1,330$	62	44 ± 11	13,674 ± 779	0.003	1,208 ± 23	7,541 ± 74	0.16	11 ± 1	67,221 $\pm 4,129$	6.11
α Y127T+ ϵ L40P	ND	ND	ND	50 ± 9	4,900 ± 927	98	102 ± 4	10,051 ± 348	0.01	707 ± 20	3,735 ± 51	0.19	14 ± 2	71,756 $\pm 6,135$	5.13
δ L42P+ ϵ L40P	ND	ND	ND	368 ± 38	1,225 ± 141	3	ND	ND	ND	885 ± 11	5,965 ± 53	0.15	9 ± 1	61,364 $\pm 5,848$	6.82
α Y127T+ δ L42P+ ϵ L40P	82 ± 8	189 ± 56	2	368 ± 55	12,056 $\pm 1,706$	33	ND	ND	ND	1,823 ± 45	8,462 ± 104	0.22	6 ± 1	45,991 $\pm 8,050$	7.67

Rate constants are in units per μM per sec for association rate constants, and per sec for all others. The dissociation constants, K_1 , K_2 are the ratio of k_-/k_+ . The channel gating equilibrium constants, θ_1 , θ_2 are the ratios of the opening to closing rate constants, β_1/α_1 , β_2/α_2 , respectively.

Figure legends

Figure 1. EM localization of AChR with peroxidase labeled α -bgt at patient (A) and control (B) endplates. Bars = 1 μ m.

Figure 2. Mutation analysis. (A) Multiple alignment of the β_1 , β_2 , and β_4 strands of AChR subunits. Leu 42 is conserved in all human ACR subunits and in δ subunits of all species. Note the δ L42P, δ I58K and δ V93L in the β_1 , β_2 , and β_4 strand, respectively. (B) Family analysis.

Figure 3. α -Bungarotoxin binding studies. (A) [125 I] α -bgt binding to surface receptors on intact HEK cells transfected with the indicated AChR subunits. The results are normalized for α -bgt binding to wild-type AChR ($\alpha_2\beta\delta\epsilon$) and represent mean and SD of three to six experiments. (B) Total α -bgt binding to saponin-permeabilized cells transfected with the indicated constructs. Amounts of bound [125 I] α -bgt are normalized to that measured for the wild-type dimer ($\alpha\delta$).

Figure 4. Single-channel events elicited by 50 nM ACh from HEK cells expressing wild-type and mutant AChRs. Left column: representative channel openings, shown as upward deflections. Right column: logarithmically binned burst duration histograms fitted to the sum of exponentials.

Figure 5. Activation kinetics of wild-type- and δ L42P-AChR (A and B) and open channel probabilities (C). In (A) and (B), left column shows representative single-channel currents at indicated AChR concentrations recorded from HEK cells. Currents are shown as upward deflections; bandwidth 10 kHz. Center and right columns show corresponding histograms of closed and open durations with superimposed global fits for Scheme 1 for the entire range of

ACh concentrations. Fitted rate constants are shown in Table 4. (C) Open probability (P_{open}) as function of ACh concentration. Symbols and vertical lines indicate mean and standard deviation. Smooth curves represent functions predicted by the rate constants in Table 4.

Figure 6. Scheme of AChR activation.

Figure 7. Structural models of AChR (A and B) and mutant cycle analyses (C-F). Location of interacting residues on α and δ subunits in A with magnified view in B are shown on a structural model AChR generated with Torpedo AChR as template (Protein Data Bank code 2BG9). (C-F). In each mutant cycle, the coupling free energy ($\Delta\Delta G_{\text{int}}$) between the residues at upper right and low left corner was computed from $-RT\ln[(\theta_{\text{ww}}\theta_{\text{mm}})/(\theta_{\text{wm}}\theta_{\text{mw}})]$ (kcal/mol), where θ (β/α) is the gating equilibrium constant for diliganded receptors generated by kinetic fitting for wild-type (ww), single-mutant (wm, mw) or double-mutant (mm) receptors. Table 4 shows θ for each moiety of AChR. Free energy changes for channel gating are shown for each limb of the cycle. Traces above or below the indicated AChR constructs in panel C are representative single-channel currents at 100 μM ACh recorded from HEK cells. Currents are shown as upward deflections; bandwidth 10 kHz. Horizontal bar = 20 ms for wild-type and 100 ms for other moieties; vertical bar = 5 pA.