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The Role of Conserved Leucines in the M2 Domain of the Acetylcholine Receptor in Channel Gating

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SUMMARY

A highly conserved leucine is found in the middle of the pore-lining (M2) domain of the members of the ligand-gated ion channel family. Two very different roles have been proposed for this leucine. In one model, this residue swings into the lumen of the channel during desensitization to form the nonconducting desensitized state, whereas in the other model, the leucines from each subunit interact with each other to form a constriction in the channel that constitutes the actual gate of the channel. We examined the role of this leucine in the muscle-type acetylcholine receptor by replacing it with the polar amino acid threonine. Replacement of the leucine in any one subunit slows desensitization and shifts the dose-response relationship toward lower concentrations. Replacement of leucines in additional subunits leads to progressively larger shifts in the dose-response curves. The shift depends only on the number of leucines replaced, not on which particular subunits contain the mutation; in other words, the mutations act independently. At the single-channel level, the mutation greatly increases the channel mean open time. We conclude that the role of the conserved leucine is to set the mean open time of the channel through interactions with other regions of the receptor rather than to serve as the gate per se of the ion channel.

AChR is a member of the ligand-gated ion channel gene superfamily, which includes neuronal nicotinic AChRs, $GABA_A$Rs, GlyRs, and 5-HT_3Rs. All members of the family exhibit sufficient similarity to be considered homologous, and the muscle-type receptor is considered the "prototype" of all the other members of the family (1–3). The structure of the *Torpedo* electroplax AChR has been determined by Unwin (4, 5) to a resolution of 9 Å using electron image reconstruction in both the closed and open channel configurations. The five subunits of the receptor are arranged in a ring around the ion permeation pathway that spans the membrane, and the walls of the ion channel are believed to be formed by an α-helical segment (the so-called M2 domain) from each subunit. A number of site-directed mutagenesis studies have suggested that the ion channel is organized into a series of rings of amino acids consisting of the five amino acids contributed by each subunit and that these rings govern the pharmacological and ion transport properties of the pore (6–8).

The members of the ligand-gated channel family show many structural similarities to those of the AChR and can be considered variations on a common structural theme. A leucine residue (αL251 in the α subunit of the muscle-type AChR) is found in the middle of the M2 domain of the members of the family cloned to date. Using the terminology of Miller (9), which assigns position 1′ to the amino terminus of the M2 domain (the cytoplasmic end), this leucine is found at position 9′. Two roles for this leucine residue (L9′) have been proposed. Based on examination of the structure of the *Torpedo* AChR in both the closed- and open-channel conformation, Unwin has suggested that these leucines serve as the gate of the channel through an association that forms a constriction in the closed state of the channel (4). Agonist binding to the receptor induces conformational changes that disfavor ring formation, and the disruption of the ring creates the open-channel configuration of the pore. On the other hand, Changeux *et al.* (10, 11) proposed that the leucines from each subunit move into the lumen of the channel during desensitization to occlude the pore. This conclusion was based on results from experiments on the homomeric α7 neuronal AChR in which the leucine was changed to threonine. This substitution markedly reduced the rate of receptor desensitization and shifted the macroscopic dose-response relationship toward lower concentrations, and an additional conductance state of the channel was observed for mutant but not wild-type receptors. These alterations in gating were ascribed to the notion that the polar threonine converted the normally nonconducting desensitized state to a conducting

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ABBREVIATIONS: AChR, acetylcholine receptor (muscle-type); ACh, acetylcholine; $GABA_A$R, γ-aminobutyric type A receptor; GlyR, glycine receptor; 5-HT_3R, serotonin type 3 receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

one. Substitution of threonine for the analogous leucine in the 5-HT_3R has a similar effect on macroscopic gating and desensitization (12).

These two models call for very different roles for the conserved leucines. We used the combination of site-directed mutagenesis, electrophysiology, and ligand-binding techniques to address the role of these leucines in ligand-gated ion channel gating. We used the muscle-type AChR for two main reasons. First, the muscle-type receptor consists of four different subunits in a fixed stoichiometry ($\alpha_2\beta\gamma\delta$), so one can control the number of leucine substitutions per receptor complex in a predefined manner, something that cannot be done with the homomeric α7 neuronal AChRs. Second, the muscle-type AChR has a fixed arrangement of subunits around the pore (13), so one can examine any effects of the spatial arrangement of substituted residues on receptor function. Our data show that threonine-for-leucine substitutions in the muscle-type AChR have effects on the macroscopic gating similar to those observed for the α7 homomers and 5-HT_3Rs but that the effects at the single-channel level are quite different for those reported for the homomeric α7 AChRs. Our results can be explained by an alteration in the equilibrium between the closed and open forms of the channel, not an effect on the permeability properties of the desensitized state.

Materials and Methods

Plasmids and site-directed mutagenesis. Mouse muscle AChR subunit cDNAs were obtained from Dr. J. P. Merlie (Washington University; α subunit), N. Davidson (California Institute of Technology; β and δ subunits), and S. Heinemann (Salk Institute; γ subunit) and subcloned into the pALTER-1 vector (Promega, Madison, WI). For mammalian cell transfection, mouse AChR subunits subcloned into pSM (14) were obtained from Dr. Z. W. Hall (University of California, San Francisco). Mutagenesis was carried out using the commercially available Altered Sites system (Promega, Madison, WI). The mutagenic primers were 17–21 nucleotides long and were synthesized using an Applied Biosystems Model 391 oligonucleotide synthesizer. Mutations were confirmed by sequence analysis using the Sanger dideoxy termination method (Sequenase, US Biochemicals, Cleveland, OH) to verify that only the desired nucleotide changes were present.

***In vitro* transcription and expression in *Xenopus* oocytes.** Plasmid DNAs were linearized with the appropriate restriction enzymes and transcribed *in vitro* using SP6 RNA polymerase, and oocytes were harvested from mature female *Xenopus laevis* and prepared for injection as described previously (15). AChR subunit RNAs were mixed in a molar stoichiometry of 2:1:1:1 ($\alpha/\beta/\gamma/\delta$), and 8–15 ng was injected into the oocyte cytoplasm. Experiments were performed 18–72 hr after RNA injection.

Electrophysiology. Currents elicited by bath application of various concentrations of ACh to oocytes expressing either wild-type or mutant AChRs were measured with a standard two-microelectrode voltage clamp at a holding potential of −70 mV. Electrodes were filled with 3 M KCl and had resistances of 0.5–3 MΩ. The recording chamber was continuously perfused with a low-Ca^{2+} saline (100 mM NaCl, 2 mM KCl, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.6) containing 0.3 μM atropine to block any residual muscarinic ACh receptors that remained after removal of the follicle cells.

Dose-response curves from individual oocytes were normalized to the maximum current and were fit to eq. 1 using a Levenberg-Marquardt algorithm in a commercially available software package (Igor, WaveMetrics, Oswego, OR) as described previously (16):

$$\theta = [1 + (EC_{50}/[A])^n]^{-1} \quad (1)$$

where θ is the normalized current, EC_{50} is the concentration of ACh required to obtain half-maximal current, and n is the apparent Hill coefficient.

Single-channel currents were recorded in the cell-attached mode (17) at a holding potential of +100 mV. Pipettes were filled with 145 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, and 25 mM HEPES, pH 7.4, supplemented with 2 μM ACh and had resistances between 7 and 20 MΩ. Single-channel currents were filtered at 5 kHz (−3-dB frequency, eight-pole Bessel filter) and digitized at 47 kHz (VR-10B digitizer, Instrutech, Elmont, NY) before storage on videotape. Single-channel data were transferred from tape to an 80486-based computer system running pClamp (Axon Instruments, Foster City, CA). Single-channel transitions were detected with the use of a half-amplitude detection algorithm, and open-time histograms were binned and displayed using a square-root ordinate and a logarithmic time axis (18). Mean open times were determined by fitting the open-time histograms to a single-exponential relation using a maximum likelihood method. Only stretches of data without overlapping events were used in the analysis.

Ligand binding measurements of transfected mammalian cells. tsA201 cells, a derivative of the human embryonic kidney 293 cell line, were obtained from Dr. R. Horn (Thomas Jefferson University) and maintained at 37° in Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% fetal bovine serum. Cells were transfected with the calcium phosphate precipitation technique using a total of 15 μg of AChR cDNAs in a subunit stoichiometry of 2:1:1:1 ($\alpha/\beta/\gamma/\delta$) per 100-mm plate. Cells were incubated with the DNA-calcium phosphate precipitate for 10 hr, and then fresh medium was added. Twelve hours later, the cells were lightly trypsinized and replated into 24-well culture dishes (one per 100-mm plate of cells), and ligand-binding assays were carried out the next day. The affinity of curare (*d*-tubocurarine) for the AChR was estimated by measuring the inhibition of the initial rate of ^{125}I-α-bungarotoxin binding (19). Cells in 24-well dishes were preincubated at 37° for 30 min with medium containing the desired concentration of curare. The medium was removed and replaced with medium containing the same concentration of curare and 5 nM ^{125}I-α-bungarotoxin (14 μCi/μg; New England Nuclear), and the cells were incubated for an additional 30 min. The medium was removed, and the cells were washed twice with phosphate-buffered saline and then solubilized in 0.1 M NaOH for counting with a γ-counter. Nonspecific binding was determined with 2 μM unlabeled α-bungarotoxin.

Data were analyzed in terms of a two-site model for curare (20), consisting of one high and one low affinity site per receptor complex (eq. 2):

$$\phi = 0.5\{[1 + ([dTC]/K_1)^{-1}] + [1 + ([dTC]/K_2)^{-1}]\} \quad (2)$$

where ϕ is the fractional amount of labeled bungarotoxin bound in the presence of curare at concentration [dTC] relative to that in the absence of curare, and K_1 and K_2 are the dissociation constants for the high and low affinity binding sites, respectively.

Results

We investigated the role of L9′ in the gating of the muscle-type AChR. Our strategy relies on the examination of the gating behavior of mutant AChRs containing one or more subunits in which L9′ is replaced by a polar threonine residue (L9′T mutants). The basic effect of a threonine for leucine substitution is shown in Fig. 1. Receptors containing a mutant γ subunit (γL9′T) expressed in *Xenopus* oocytes exhibit a decreased rate of desensitization and a shift of the dose-response relationship toward lower concentrations relative to wild-type AChRs. The fact that a single substitution is sufficient to alter both channel opening and desensitization suggests that L9′ does not swing into the lumen during

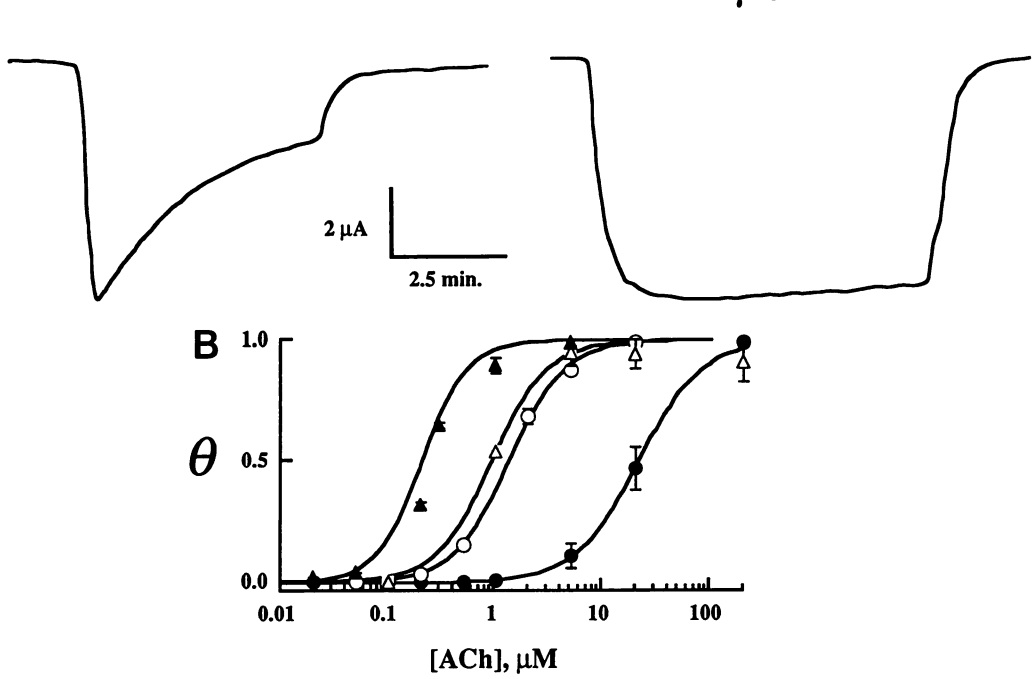

Fig. 1. Replacement of a single L9′ alters AChR gating. A, Currents elicited by bath application of 20 μM ACh to voltage-clamped oocytes expressing either *wild-type* or *γL9′T* AChRs. Note the marked lack of desensitization of γL9′T AChRs. B, Macroscopic ACh dose-response curves for wild-type (○), γL9′T (●), δL9′T (△), and γL9′TδL9′T (▲) AChRs. *Solid curves* are described by eq. 1, with EC_{50} values of 21 μM (wild-type), 1.3 μM (γL9′T), 0.9 μM (δL9′T), and 0.2 μM (γL9′TδL9′T).

desensitization because there still are four leucines that could swing into the pore to form a nonconducting channel.

On the other hand, if L9′ forms part of the gate of the channel, then all single-subunit substitutions should have essentially the same effect on gating. Fig. 1B shows that receptors containing either a mutant γ (γL9′T) or δ (δL9′T) subunit give essentially identical shifts in the dose-response curve and that when both mutant γ and δ subunits are present, the dose-response curve is shifted further to the left. Fig. 2 shows the effect of all possible permutations of subunits containing threonine substitutions on the dose-response relationship, ranging from none (i.e., wild-type receptors) to five substitutions (all subunits contain threonine). It can be seen that the EC_{50} depends on the number of substitutions and not on which particular subunits contain the substitution(s). Furthermore, the effect of each substitution is additive on the effective free energy of channel opening, with each threonine-for-leucine substitution decreasing the effective ΔG by ~0.7 kcal/mol.

The midpoint of the dose-response relationship, EC_{50}, is an empirical parameter that depends on the rate constants for ligand binding and unbinding, as well as those for channel opening and closing (21). Although one might expect a mutation at a point in the channel that is approximately 45 Å from the ligand binding site (5) to have no effect on ligand binding, there is no *a priori* reason that this should be true. The competitive antagonist curare binds to the two ligand-binding domains of the AChR. In general, alterations in the EC_{50} for ACh activation due to mutations in the ligand-binding domain are paralleled by alterations in the affinity of the competitive antagonist curare (22), so we used curare in ligand-binding assays as a probe of the structure of the ligand-binding domain of the AChR. γL9′T receptors transiently expressed in tsA201 cells show the same slowing of desensitization and a decrease in the EC_{50} for ACh activation as they do in oocytes (data not shown). Table 1 shows that the

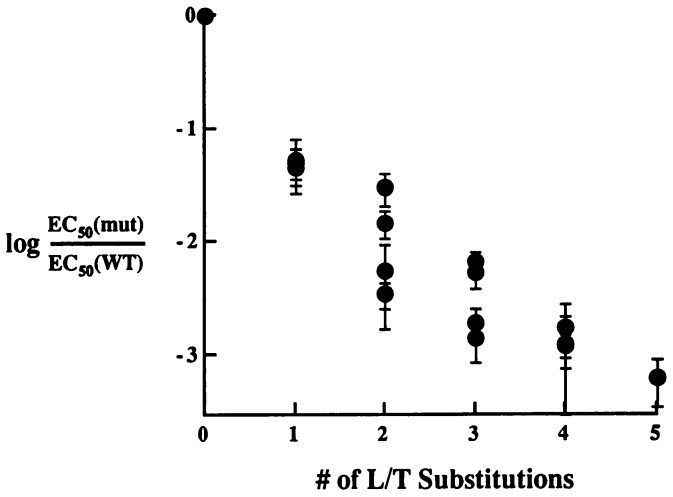

Fig. 2. The effects of threonine (*T*) substitutions are position independent and additive. Ratios of the EC_{50} values for each L9′T mutant (*mut*)/wild-type (*WT*) subunit combination (total of 16) relative to that of the wild-type AChRs are plotted as a function of the number of threonine substitutions ranging from none (all five subunits contain leucine (*L*) at this position; i.e., wild-type receptors) to five (all five subunits contain threonine at this position). Ratios are plotted on a log scale, which presents the data on a linear free-energy scale ($\Delta G = -RT\ln[EC_{50}]$). It can be seen that each leucine substitution makes a similar energetic contribution to the gating process (on the order of 0.7 kcal/mol per leucine) and that the EC_{50} depends on the absolute number of substitutions rather than on any particular geometry of substitutions.

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TABLE 1

Threonine substitution does not affect the ligand-binding site

The decrease in the initial rate of ^{125}I-α-bungarotoxin binding to transfected cells expressing either wild-type or γL9'T AChRs by curare was used to determine the affinities of the high and low affinity ligand-binding sites as described in Materials and Methods. Each value is the mean ± standard error of three determinations.

Receptor	K_1	K_2
	nM	μM
Wild-type	153.4 ± 53.3	5.5 ± 1.5
γL9'T	180.1 ± 46.0	4.8 ± 0.9

0.9 μM for mutant). Therefore, as expected, the mutation in the channel domain has no major effect on ligand affinity, and the alterations in the EC_{50} can be associated with changes in opening and closing of the channel.

If the L9s from each subunit come together to form the "gate" that holds the channel closed, then replacement by threonine would remove the stabilizing effect of a given leucine, making it easier to open the channel. If this view is correct, then this effect should manifest itself at the single-channel level as an increase in the channel-opening rate without a concomitant change in the closing rate constant. Fig. 3 shows single-channel currents and open-time histograms recorded in the presence of 2 μM ACh from oocytes that express either wild-type or γL9'T AChRs. Wild-type and γL9'T channels have identical single-channel conductances, and at this concentration the open-time histograms could be described by a single-exponential component. We see no indication of an additional conductance state for the mutant receptors, as has been reported for homomeric α7 neuronal AChRs (10). It is clear that one effect of the mutation is to greatly prolong the mean open time of the receptor. Wild-type receptors have a mean open time at +100 mV of 2.4 ± 0.3 msec (mean ± standard error of 5 patches), whereas that of γL9'T receptors was 174 ± 44 msec (8 patches). These results strongly suggest that a major effect of the threonine substitution is to stabilize the open state of the receptor by decreasing the channel-closing rate constant, which in turn would lead to a decrease in the EC_{50} value of the mutant receptor by biasing the equilibrium between the closed- and open-channel states of the biliganded receptor toward the open-channel form.

Discussion

Our results clearly demonstrate that the conserved leucines in the M2 domain play an important role in muscle-type AChR gating. Substitution of the more polar amino acid threonine for leucine affects the rate of desensitization and the EC_{50} for ACh activation of the receptor. The macroscopic data are consistent with either model proposed for the role of L9′ in AChR function. However, there are several features of the effects of the substitutions on the gating of the mouse muscle receptor that distinguish them from those reported for the homomeric α7 neuronal AChR. First, we do not see any evidence of an additional conductance state of the receptor, as has been reported for the α7 receptor and ascribed to the conducting desensitized state (10). Second, mutant muscle AChRs do not show activation by curare or other antagonists (data not shown), as has been reported for the mutant α7 receptors (11). Third, the fact that we observe progressively larger shifts of the dose-response relationship toward lower concentrations as we increase the number of threonine substitutions is inconsistent with the "desensitization" model (10) because that should predict only a single unique position for the dose-response curve of the mutant AChRs, that of the high affinity desensitized state. Whether these differences are due to an intrinsic difference between muscle AChRs and neuronal AChRs in general or are a reflection of the fact that homomeric α7 AChRs may not exist *in vivo* (24) and thus may prove to be a poor model for muscle-type AChRs and other ligand-gated ion channels is not clear. Nonetheless, we do not need to invoke a conducting desensitized state to explain our data.

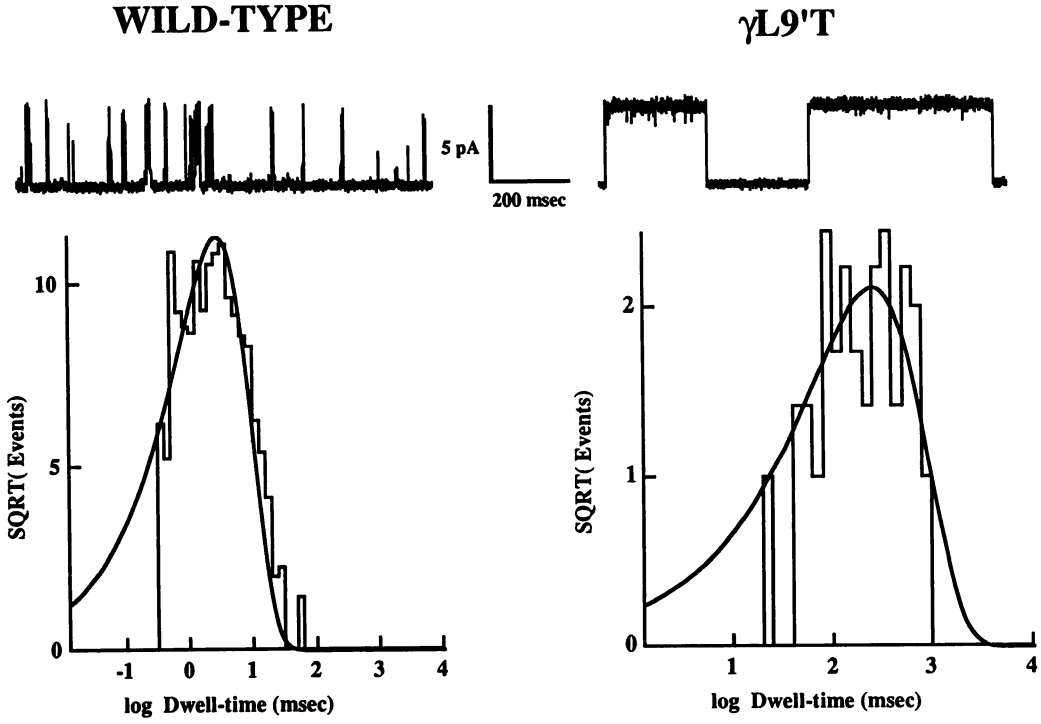

Fig. 3. Threonine substitution stabilizes the open state of the channel. Single channels were recorded at +100 mV in the presence of 2 μM ACh from cell-attached patches containing either *wild-type* or *γL9'T* receptors. *Upward deflections* represent channel openings. The *bottom* of each plot shows the channel open-time distribution for channels recorded from the same patches shown in the *top*. Mean open time was 2.68 msec for the wild-type receptors and 248 msec for the γL9'T receptors.

The ligand binding data presented in Table 1 show that the γL9'T mutation has no effect on the affinity of the competitive antagonist curare. It is possible, of course, that the L9'T mutation can affect agonist, but not antagonist, affinity, but we consider this unlikely. With one explainable exception (25), mutations that affect ligand binding give identical alterations in the affinity of curare and the EC_{50} for ACh activation, so this result suggests that, as expected, the change in EC_{50} value is not due to alterations in agonist affinity.

A major effect, therefore, of the threonine substitution is to stabilize the open state of the AChR channel by decreasing the channel-closing rate. This stabilization would shift the dose-response curve toward lower concentrations by biasing the open/closed equilibrium toward the open state. The observed decrease in the rate of desensitization may also be a result of the open-state stabilization. A stabilization of the open state is somewhat different than one might expect from Unwin's model (4) of AChR gating. In that model, the leucines hold the channel shut through leucine-leucine interactions. Replacement of leucine by threonine might be expected to weaken this interaction, thus destabilizing the closed state and making it easier to open the channel. This view of the role of the leucines does not predict that mutations that might weaken the leucine-leucine interactions would stabilize the open channel, which is what we observed. One way in which this might happen is that when the channel opens, the leucines move into a somewhat polar environment, and the residence time in this position, and thus the channel open time, depends on the polarity of the amino acid. Substitution of the more polar threonine stabilizes this type of interaction, leading to a longer mean open time.

The prolonged channel open times result in a great deal of overlap between single-channel events and, when coupled with the reduction in desensitization for the mutants, do not allow us to determine the opening rate constant. The lack of interruptions in the mutant single-channel records suggest that they are long openings rather than bursts of activity. The number of interruptions per opening is directly proportional to the rate constant for channel opening (26), and the lack of interruptions suggests that the opening rate is not greatly increased by the mutation, but detailed kinetic analysis must be done to determine the effect of the mutation on the opening rate. However, using the equations developed by Colquhoun and Ogden (21), it can be shown that a 50-fold decrease in the channel-closing rate is in itself sufficient to give the observed >10-fold decrease in the EC_{50} value. In any case, stabilization of the open state is not predicted by Unwin's model.

This particular mechanism also is consistent with the finding that the mutations act independently. When the channel opens, the leucines are isolated from each other and can interact with another region of the receptor in a truly independent fashion. In the leucine-leucine interaction model, one can imagine a situation where the spatial arrangement of the substituted residues might make a difference. For example, substitution of two adjacent residues might have a different effect than substitution of two residues that are opposite each other. In the former case, three leucines are adjacent, allowing two leucine-leucine interactions to take place, whereas in the latter, only one such interaction can take place. One would imagine that these two cases might have different stabilizing effects on the closed channel if leucine-leucine interactions were important for keeping the channel closed. On the other hand, if these residues are involved instead in holding the channel open, then this should not matter if the leucines no longer need to contact each other.

The energies involved in this sort of stabilizing interaction are relatively weak, on the order of 0.7 kcal/mol per leucine residue. This is in the low end of the range of hydrogen bonds involving uncharged groups (0.5–1.5 kcal/mol; 27) and other weakly polar interactions in proteins (28). If we assume that the role of these residues is not to hold the channel closed but rather to control the mean open time of the channel, then one would expect the forces involved to be rather weak, given the fact that the channels need be open for only a very brief time during synaptic transmission.

Mutations of other residues far removed from the ligand-binding site have been shown to alter AChR gating. The series of cysteine-substitution mutants in the M2 domain of the α subunit of the mouse AChR studied by Akabas *et al.* (29, 30) show a wide range of alterations in the midpoint of the ACh dose-response curve, with $EC_{50_{mut}}/EC_{50_{wt}}$ values ranging from 0.045 to 50. These authors postulated that these effects were due to alterations in the free energy of the closed state relative to that of the open state, similar to our conclusion concerning L9'T mutations. A congenital myasthenic syndrome caused by prolonged channel openings has been shown to be due to a proline-for-threonine substitution in the M2 domain of the ε subunit (εT12'P) of the AChR (31). Substitution of tryptophan for conserved cysteine residues in the M4 domain of the α and β subunits of the *Torpedo* AChR leads to prolonged channel open times and an increase in channel open probability (32). In conjunction with our data, it is clear that channel gating involves interactions between many different amino acid residues, and there may not be a particular structure that forms the "gate" of the channel.

During the process of AChR channel opening, kinked α helices from each subunit rotate so that the constriction observed in the closed form of the receptor is opened and the channel can now conduct ions. Unwin (4) has postulated that the M2 domain is the kinked helix and that conserved leucines occur at the kink, forming the actual gate of the channel. Support for this notion is provided by photoincorporation studies with the noncompetitive antagonist 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine that suggest that the nserved leucines form a barrier to ion flow in the resting state of the receptor (33). On the other hand, Akabas *et al.* (30) argued that their results obtained with a series of cysteine substitutions throughout the M2 domain of the α subunit of the mouse muscle AChR demonstrate that the gate of the channel is much farther down the channel than L9'. Although our data demonstrate that the L9s from each subunit do play a role in channel gating, they do not address the question as to whether the leucines form the gate. Nevertheless, they provide some insight into some of the interactions that take place during the gating process. Given the highly conserved nature of this leucine, it is likely that similar interactions take place in the other members of the ligand-gated ion channel family.

Acknowledgments

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Note Added in Proof

Similar data have been obtained for L9'S mutations (Labarca, C., M. Nowak, H. Zhang, X. Tang, P. Deshpande, and H. Lesler. *Nature (Lond.),* in press.

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