Transmitter molecules bind to synaptic acetylcholine receptor channels (AChRs) to promote a global channel-opening conformational change. Although the detailed mechanism that links ligand binding and channel gating is uncertain, the energy changes caused by mutations appear to be more symmetrical between subunits in the transmembrane domain compared with the extracellular domain. The only covalent connection between these domains is the pre-M1 linker, a stretch of five amino acids that joins strand β10 with the M1 helix. In each subunit, this linker has a central Arg (Arg\(^{3}\)), which only in the non-α-subunits is flanked by positively charged residues. Previous studies showed that mutations of Arg\(^{3}\) in the α-subunit alter the gating equilibrium constant and reduce channel expression. We recorded single-channel currents and estimated the gating rate and equilibrium constants of adult mouse AChRs with mutations at the pre-M1 linker and the nearby residue Glu\(^{45}\) in non-α-subunits. In all subunits, mutations of Arg\(^{3}\) had similar effects as in the α-subunit. In the ε-subunit, mutations of the flanking residues and Glu\(^{45}\) had only small effects, and there was no energy coupling between εGlu\(^{45}\) and εArg\(^{3}\). The non-α-subunit Arg\(^{3}\) residues had Φ-values that were similar to those for the α-subunit. The results suggest that there is a general symmetry between the AChR subunits during gating isomerization in this linker and that the central Arg is involved in expression more so than gating. The energy transfer through the AChR during gating appears to mainly involve Glu\(^{45}\), but only in the α-subunits.

Acetylcholine receptors (AChRs)\(^{2}\) are ligand-gated ion channels that mediate fast chemical synaptic transmission (1). The binding of two acetylcholine molecules to the extracellular domain (ECD) triggers a rapid, global, and reversible conformational change that increases the affinity of the two agonist-binding sites and opens a cation conduction pathway through the transmembrane domain (TMD). Defects in AChRs originating from inherited mutations that alter gating kinetic properties or expression cause myasthenic disorders (2). Electrophysiology studies have identified many mutations that produce abnormal AChR gating, but a detailed understanding of the mechanism by which the agonist affinity change and channel opening/closing are linked is not yet available.

The adult neuromuscular AChR consists of five homologous subunits (two α-subunits and one each of the β\(_{1}\), δ\(_{1}\), and ε-subunits) folded symmetrically around the central axis of the pore (3). Its structure is modular, as the extracellular N-terminal half of each subunit is a β-barrel and the transmembrane C-terminal half is a four-α-helix bundle (M1–M4). The five sets of β-barrels form the ECD, and the five sets of α-helices form the TMD. Several structures have revealed important features that are relevant to understanding the mechanism of energy transfer through the protein in the gating isomerization, including the Torpedo AChR (4), two prokaryotic pentameric ligand-gated ion channels crystallized in either a non-conducting (ELIC) (5) or a presumably conducting conformation (GLIC) (6, 7), an ECD fragment of the mouse AChR α-subunit (8), and the ECD homolog, the acetylcholine-binding protein (9). A comparison of the ELIC and GLIC x-ray structures suggests that the pore-lining M2 helices tilt tangentially and radially as part of the channel-opening process (6, 7). Despite this structural information, we are still unsure of the molecular events that constitute the affinity change at the binding sites and the conductance change at the pore or the intermediate events that couple structural changes in these two widely separated domains (10).

The interface between the ECD and TMD is a complex region that has been studied in several members of the Cys loop receptor channel family (11–18). This region has many charged residues and hence the potential for many non-bonded interactions. The only covalent link between the ECD and TMD is a stretch of five residues that join strand β10 with the M1 helix, known as the “pre-M1” linker (Fig. 1). This linker has a central positively charged Arg that is conserved among all pentameric ligand-gated receptor channels (12). Here, we will call this position Arg\(^{3}\) to mark it as the third position in this linker. Lee and Sine (12) proposed that the perturbation of a salt bridge between αArg\(^{3}\) and loop 2 residue εGlu\(^{45}\) is the principal event that links the ECD and the TMD in gating, but other results suggest that εArg\(^{3}\) plays a smaller role in gating but is important for receptor expression (14, 17).

The affinity change at the binding sites involves mainly α-subunit residues, but the opening and closing of the gate near the M2 equator involve the rearrangements of atoms in all five subunits. One goal of our experiments was to assay the symmetry of the gating energy changes at the pre-M1 linker, a location that is about halfway between the binding sites and the gate. In general, previous results indicate that large gating energy changes in the ECD are predominantly in the α-subunit but become more evenly spread among all subunits in the TMD (12, 18).
19–25). Although the mechanism of this spreading of the gating energy changes is not clear, an intersubunit energy transfer has been found between αTyr^{127} and εAsn^{39} or δAsn^{41} (19).

So far, only one mutation at one pre-M1 linker position has been studied in all subunits using single-channel kinetic analysis. A Gln substitution at the fourth linker residue modestly increases diliganded gating (by ∼3-fold) in the α-subunit but is without effect in the non-α-subunits (26). Here, we report the effects of mutations of multiple pre-M1 residues in non-α subunits, estimated from the single-channel rate and equilibrium constants of the adult mouse AChR gating isomerization.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis and Expression*—A detailed description of our methods is described by Jha et al. (27). Mutant AChR cDNAs were made by QuikChange™ site-directed mutagenesis (Stratagene) and confirmed by sequencing. We made 26 mutants of the ε-subunit, three double-mutant combinations of the ε-subunit, two mutants of the β-subunit, and two mutants of the δ-subunit. HEK293 cells were transiently transfected by the calcium phosphate precipitation method. HEK cells were incubated with 2.5–5 μg of mouse WT or mutant cDNAs in a 35-mm culture dish at a subunit ratio of 2:1:1:1 (α/β/δ/ε). After ∼16 h of incubation at 37 °C, the transfected cells were washed with HEK culture medium. Electrophysiology recordings were performed 20–40 h post-transfection.

*Single-channel Recordings and Kinetic Analysis*—Recordings were carried out in the cell-attached patch configuration at room temperature (23 °C). The pipette and bath solutions were both Dulbecco’s PBS (137 mM NaCl, 0.9 mM CaCl_2, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM MgCl_2, and 8.1 mM Na_2HPO_4 at pH 7.2). Pipettes were pulled from borosilicate capillaries to a resistance of ∼10 meqohms and coated with SYLGARD (Dow Corning Corp., Midland, MI). The pipette solution contained 0.5 mM acetylcholine, 20 mM choline, or 5 mM carbamylcholine. These agonist concentrations are approximately five times the corresponding equilibrium dissociation constants (K_d); thus, almost all currents arose from diliganded AChRs. Because the mutations were far from the binding site, we assumed that they did not change K_p. The high concentration of agonist caused partial channel block, which decreases both the apparent single-channel current amplitude and the apparent closing rate constant. Although none of the mutations changed the degree of channel block, nonetheless we measured the closing rate constant using low concentrations of agonist (30 μM acetylcholine, 200 μM choline, and 200 μM carbamylcholine), at which channel block is insignificant. The diliganded gating equilibrium constant (E_d) was calculated as the ratio of the opening/closing rate constant. Choline was used to measure the diliganded opening rate constant for AChR mutants where E_d was larger than or equal to the WT; acetylcholine was used for mutants where E_d was less than the WT; and carbamylcholine was used to measure mutants where E_d was approximately equal to the WT. It has been shown for many non-binding site mutations that different agonists support the same Φ-values and fold-changes in E_d (21).

Cells were held at a pipette potential of +70 mV, which corresponds to a membrane potential of approximately −100 mV. Errors in the rate constants associated with the errors in the membrane voltage are small (in WT AChRs, an ∼70-mV depolarization is necessary to decrease E_d by e-fold) (28). Currents were filtered at 20 kHz and digitized at a sampling frequency of 50 kHz. Kinetic analyses were done using QUB software. Currents were idealized using the SKM (segmental K-Means) algorithm filtered at 12 kHz with a C ↔ O (closed ↔ open) model
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with starting rate constants of 100 s⁻¹. The diliganded opening ($f_2$) and closing ($b_2$) rate constants were estimated from idealized interval durations using a maximum interval likelihood algorithm after incorporating a dead time of 25 μs (29).

The diliganded rate constants were measured multiple times ($n = 2$ to 5 patches) and then averaged. Φ was estimated as the slope of the linear fit to the log-log rate-equilibrium free energy relationship ($R/E$ analysis). The range energy (kcal/mol) = −0.59 ln($E_{\text{max}}$/E$_{\text{min}}$), where the superscripts are the $E_2$ values for the side chains generating the largest and smallest $E_2$ values, respectively. The coupling free energy was calculated as ΔΔG (kcal/mol) = −0.59 ln($E_{\text{double mutant}}$/E$_{\text{mutant 1}^\phi$E$_{\text{mutant 2}^\phi$}).

RESULTS

$\varepsilon$-Subunit Linker—Table 1 shows a sequence alignment of the pre-M1 linker and that Arg$^3$ is completely conserved. In the $\alpha$-subunit linker, this is the only positively charged residue, but in the non-$\alpha$-subunits, it is flanked by two additional basic amino acids.

We estimated residue range energy (see “Experimental Procedures”) and Φ-values for AChR with a mutation of one of the three positively charged amino acids in the $\varepsilon$-linker (εArg$^2$, εArg$^3$, εLys$^3$). None of the side chain substitutions at εArg$^2$ (Ala, Cys, Asp, Asn, Trp, or Val) changed the diliganded gating equilibrium constant ($E_2$) by >2-fold (Fig. 2 and Table 2). This set of substitutions was selected to include residues of different size, charge, and hydrophobicity. All mutations caused a slight reduction in $E_2$, with the largest being for Asn (1.8-fold), which corresponds to a range energy of 0.3 kcal/mol. We conclude that like its homolog in the $\alpha$-subunit, the $\varepsilon^2$-side chain is nearly isoenergetic between the ground state conformations, which suggests that this amino acid does not move with respect to its local environment during the gating isomerization.

Nine substitutions of the central position εArg$^3$ were examined, but only Ala, His, Lys, Asn, and Glu expressed functional channels. We were unable to observe single-channel currents from the Cys, Asp, Glu, and Val mutants (3–10 patches per mutant, recording for 15–25 min/patch). In this respect, position ε$^3$ behaves similarly to its homolog in the $\alpha$-subunit (14) and to $\alpha_1$Arg$^{220}$ in the GABA receptor channel (17), where mutations also reduce the expression of functional channels. Mutations of εArg$^3$ had substantial effects on $E_2$ (Table 2). The Ala, His, Lys, Asn, and Glu substitutions all decreased $E_2$ compared with the

### TABLE 2
Kinetic parameters for $\varepsilon$-subunit pre-M1 mutants

| Construct | Agonist | $f_2$ | $b_2$ | $E_2$ | Normalized $f_2$ (mutant/WT) | Normalized $E_2$ (mutant/WT) | n |
|-----------|---------|------|------|------|---------------------------|----------------------------|---|
| WT$^a$    | ACh     | 48,000 | 1750 | 28.00 | 1.00                      | 1.00                       | 3 |
| WT        | Cho     | 120    | 2583 | 0.05  | 1.00                      | 1.00                       | 3 |
| εR2'A     | Cho     | 104 ± 10 | 783 ± 88 | 2934 ± 329 | 0.04 ± 0.02 | 0.86                       | 0.76 | 3 |
| εR2'C     | Cho     | 139 ± 24 | 933 ± 202 | 3232 ± 700 | 0.05 ± 0.02 | 1.16                       | 0.97 | 3 |
| εR2'D     | Cho     | 95 ± 15 | 869 ± 60 | 3372 ± 235 | 0.03 ± 0.01 | 0.79                       | 0.61 | 3 |
| εR2'N     | Cho     | 90 ± 17 | 849 ± 217 | 3586 ± 918 | 0.03 ± 0.01 | 0.75                       | 0.56 | 3 |
| εR2'W     | Cho     | 131 ± 24 | 1247 ± 209 | 3891 ± 652 | 0.04 ± 0.01 | 1.09                       | 0.76 | 3 |
| εR2'V     | Cho     | 129 ± 10 | 896 ± 109 | 3719 ± 454 | 0.04 ± 0.01 | 1.08                       | 0.76 | 3 |
| εR3'A     | Cho     | 991 ± 171 | 4397 ± 826 | 6572 ± 1234 | 0.15 ± 0.02 | 0.02                       | 0.005 | 3 |
| εR3'K     | Cho     | 9019 ± 1907 | 2623 ± 648 | 6194 ± 1530 | 1.56 ± 0.63 | 0.19                       | 0.055 | 3 |
| εR3'N     | Cho     | 6363 ± 856 | 2579 ± 261 | 4627 ± 468 | 1.40 ± 0.29 | 0.13                       | 0.050 | 4 |
| εR3'V     | Cho     | 1559 ± 159 | 6813 ± 980 | 10,311 ± 1484 | 0.15 ± 0.04 | 0.03                       | 0.006 | 3 |
| εR3'Q     | Cho     | 1446 ± 35 | 3180 ± 840 | 5726 ± 1513 | 0.27 ± 0.07 | 0.03                       | 0.009 | 4 |
| εR4'A     | Cho     | 325 ± 56 | 431 ± 76 | 1634 ± 286 | 0.20 ± 0.03 | 2.71                       | 4.31 | 3 |
| εR4'K     | Cho     | 300 ± 60 | 712 ± 251 | 2327 ± 820 | 0.15 ± 0.08 | 2.50                       | 3.14 | 3 |
| εR4'D     | Cho     | 221 ± 20 | 593 ± 46 | 2260 ± 174 | 0.10 ± 0.01 | 1.84                       | 2.11 | 3 |
| εR4'N     | Cho     | 111 ± 8 | 525 ± 141 | 2213 ± 596 | 0.05 ± 0.01 | 0.93                       | 1.12 | 3 |
| εR4'W     | Cho     | 351 ± 36 | 351 ± 109 | 1059 ± 79 | 0.32 ± 0.03 | 2.92                       | 6.87 | 3 |
| εR4'V     | Cho     | 135 ± 40 | 706 ± 43 | 2600 ± 158 | 0.05 ± 0.01 | 1.12                       | 1.12 | 3 |

$^a$ Agonist concentration was ~5-fold the equilibrium dissociation constant (0.5 mM acetylcholine or 20 mM choline).

$^b$ Wild-type constants for acetylcholine and choline were taken from Chakrapani and Auerbach (32) and Mitra et al. (24), respectively.
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FIGURE 3. Mutations of εArg$_3$ (εArg$_{3^{18}}$) decrease the gating equilibrium constant. A, example clusters elicited by 0.5 mM acetylcholine (ACh). Single-channel currents were not detected for the Cys, Asp, Glu, Val, and Trp mutants. B, R/E analysis. Each point represents the average of three to four patches with its S.D. (Table 2). The WT is boxed. The Φ-value (lower right) was estimated as the linear slope of log $f_2$ versus log $E_2$ and gives the relative timing of the residue’s gating energy change (1 to 0, start to end). The εArg$_3$ side chain changes its energy relatively early in the channel-opening process.

WT (Fig. 3). The Ala and Asn substitutions had the largest effect and decreased $E_2$ by ~180-fold. The range energy for εArg$_3$ was ~3.1 kcal/mol, which is about the median value for two α-subunit mutations (10, 30). The R/E plot for εArg$_3$ had a slope (Φ-value) of 0.73 ± 0.06 (Fig. 3B), which indicates that the change in $E_2$ was caused mainly by a reduction in the forward channel-opening rate constant ($f_2$). This Φ-value is the same as for αArg$_3$ (14), which suggests that the central Arg residues experience a change in energy at about the same time in the gating reaction in the α- and ε-subunits.

All substitutions tested at εLys$_4$ expressed functional AChRs. Ala, Cys, Asp, and Trp mutations increased $E_2$, but only modestly (Fig. 4 and Table 2). The Trp mutant showed the largest energy change (~1.1 kcal/mol). AChRs with an Asn or Val side chain here had WT gating properties, as did a Glu substitution (26). The Φ-value of the εLys$_4$ series was 0.63 ± 0.09, similar to that of its neighbor εArg$_3$. However, in the α-subunit, the Φ-value of αLeu$_4$ (Φ = 0.35) was distinctly lower than that of the adjacent residue αArg$_3$ (Φ = 0.72) (14).

Energetic Coupling between εArg$_3$ and εGlu$_{45}$—In the cryo-EM structure of the Torpedo AChR (4), the positively charged side chain of αArg$_3$ faces the negatively charged side chain of αGlu$_{45}$, and swapping charges here restores functional gating (12). We investigated the effects of five side chain substitutions at εGlu$_{45}$ and three εGlu$_{45}$/εArg$_3$ double-mutant combinations to explore the interactions between these ε-positions during gating.

Fig. 5 and Table 3 show the effects of mutating εGlu$_{45}$. The εGlu$_{45}$ substitutions Cys, Arg, Trp, and Val all decreased $E_2$, but only slightly (<1 kcal/mol). The Φ-value measured for the εGlu$_{45}$ mutation series was 0.50 ± 0.15. Because of the near-WT gating properties of the εE45A mutant, we conclude that a negatively charged side chain at this position in the ε-subunit is not critical for efficient gating. The small range energy makes the εGlu$_{45}$ Φ-value estimate imprecise (31), but it may be that this residue changes its energy somewhat after its α-subunit homolog (Φ = 0.80) (14).
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**TABLE 3**

Kinetic parameters for eGlu<sup>45</sup> mutants

See the legend to Table 2. No single-channel currents were observed with the eR3′E/eE45R double mutant. Cho, choline; ACh, acetylcholine.

| Construct | Agonist | $f_2$ | $b_2$ | $b_2$′ | $E_2$ | $E_2$′ | Normalized $E_2$ | $\Delta \Delta G$ | $n$ |
|-----------|---------|------|------|-------|------|-------|---------------|-------------|-----|
| eE45A     | Cho     | 164 ± 27 | 1238 ± 112 | 3519 ± 319 | 0.05 ± 0.004 | 1.01 | Observed | Predicted | kcal/mol |
| eE45C     | Cho     | 61 ± 13 | 1041 ± 26 | 3365 ± 83 | 0.02 ± 0.004 | 0.40 | 3 |
| eE45R     | Cho     | 56 ± 4 | 1551 ± 74 | 6321 ± 301 | 0.01 ± 0.001 | 0.19 | 3 |
| eE45W     | Cho     | 102 ± 7 | 1156 ± 141 | 6032 ± 733 | 0.02 ± 0.003 | 0.37 | 2 |
| eE45V     | Cho     | 76 ± 8 | 2443 ± 1 | 7404 ± 1 | 0.01 ± 0.001 | 0.22 | 2 |
| eR3′Q/eE45A | ACh     | 2258 ± 382 | 3219 ± 521 | 5631 ± 911 | 0.41 ± 0.10 | 0.015 | 0.009 | −0.30 | 5 |
| eR3′K/eE45A | Cho     | 38 ± 12 | 1076 ± 181 | 3986 ± 669 | 0.01 ± 0.01 | 0.216 | 0.050 | −0.86 | 4 |

**FIGURE 6.** Energy coupling between eArg<sup>3′</sup> and eGlu<sup>45</sup>. A, example clusters activated by different agonists. B, R/E analysis. Each point represents the average of three to five patches with its S.D. (Table 4). The x-axis represents the normalized log $f_2$, y-axis the normalized log $E_2′$, and the box the WT. The WT is boxed. The $\Phi$-value (lower right) was estimated as the linear slope of log $f_2$ versus log $E_2′$.

We created three eGlu<sup>45</sup>/eArg<sup>3′</sup> double-mutant constructs: Arg/Glu, Ala/Gln, and Ala/Lys. In the α-subunit, the side chains of αGlu<sup>45</sup> influence the expression of αArg<sup>3′</sup> mutants (14). The eR3′E construct alone did not express functional AChRs, and when we expressed this along with eE45R (a charge swap), we still did not observe the expression of functional AChRs currents. The other two double-mutant pairs (Ala/Gln and Ala/Lys) did express functional channels and produced AChRs having slightly larger $E_2$ values than predicted assuming independence (Fig. 6 and Table 3). The Ala/Gln and Ala/Lys double mutants exhibited very small coupling energies (−0.3 and −0.9 kcal/mol, respectively). The R/E plot for eArg<sup>3′</sup> on the eE45A background yielded a $\Phi$-value of 0.78 ± 0.06 (Fig. 6B), which is similar to its value on the WT background (Fig. 3).

**β- and δ-Subunit Linkers—**We also examined the kinetics of mutations at position 3′ in the β- and δ-subunits (Fig. 7 and Table 4). The mutation βR3′K had little effect on gating, whereas a Gln mutation here resulted in a 49-fold reduction of $E_2$. The estimated $\Phi$-value of position β3′ was 0.44 ± 0.06 (Fig. 7B). In the δ-subunit, Lys and Gln substitutions were assayed using two different agonists, acetylcholine and carbamylcholine. With both 6Arg<sup>3′</sup> mutations, the fold-changes in $E_2$ were similar for these ligands, even though acetylcholine is a full agonist and carbamylcholine is a partial agonist. The R/E plot for position δ3′ yielded a $\Phi$-value of 0.54 ± 0.07 and a range energy of ~2.0 kcal/mol.

**DISCUSSION**

In the *Torpedo* AChR (4) structure, the αArg<sup>3′</sup> side chain forms a salt bridge with αGlu<sup>45</sup> in loop 2 (and possibly with αGlu<sup>175</sup> in loop 9). In the ECD fragment of the mouse nicotinic acetylcholine receptor α-subunit (8), αArg<sup>3′</sup> interacts indirectly with αGlu<sup>45</sup> via a structural water. An electrostatic contact is present between the Arg<sup>3′</sup> and Glu<sup>31</sup> (in loop 2) side chains in the x-ray structure of GLIC (7). ELIC lacks this salt bridge because the loop 2 residue is a Thr that faces, but does not contact, Arg<sup>3′</sup> (which does appear to form a salt bridge with Asp<sup>122</sup> in loop 7 and Glu<sup>159</sup> in loop 9). Thus, the conserved pre-M1 linker Arg<sup>3′</sup> side chain is evidently involved in protein stability through electrostatic interactions with surrounding loops. However, analyses of function suggest that a perturbation of the salt bridge between αArg<sup>3′</sup> and αGlu<sup>45</sup> is not a critically important event in AChR gating (14).

One of our goals was to probe the degree of functional symmetry between subunits with regard to residues in the pre-M1 linker and loop 2. The non-α-linker contains three positive residues, whereas the α-linker contains only one. Recall that in the α-subunit linker, (i) many mutations at position 3′ reduce expression, (ii) only mutations at positions 3′ and 4′ affect $E_2$, and (iii) loop 2 residue αGlu<sup>45</sup> experiences a very large range energy change (~5 kcal/mol) early in the channel-opening process ($\Phi \sim 0.8$) (14). We can compare this basic pattern in the α-subunit with that we have found in the ε-subunit and, to a lesser extent, in the β- and δ-subunits.

Position 2′ is isoenergetic in both the α- and ε-subunits. All mutants tested here in both subunits expressed functional AChRs that had WT-like currents. It appears that in the mouse AChR, this position in the pre-M1 linker in the α- and ε-subunits is not essential for folding, expression, conductance, gating, or desensitization.

Position 3′ is much more interesting. In both the α- and ε-subunits, many substitutions here prevented the expression of functional AChRs. Single-channel currents were observed...
only with the Arg, Gln, His, and Lys side chains in both the α- and ε-subunits and also with Ala and Asn only in the ε-subunit. This difference in expression may simply reflect the fact that each AChR has two α-subunits but only one ε-subunit. The R3’Q substitution resulted in a reduction of $E_2$ in both α-subunits and all non-α-subunits, but the gating energy change was only moderate. The range energy (per subunit) was slightly larger in the ε-subunit (~3 kcal/mol) compared with the α-subunit (~2 kcal/mol); this result was influenced by the inclusion of the Ala and Asn substitutions only in the ε-subunit. Limiting this estimate to the Arg, Gln, His, and Lys side chains, the range energies were 1.8 and 1.0 kcal/mol/subunit for the α- and ε-subunits, respectively. The $\Phi$ value for position 3’ was the same in the α- and ε-subunits, but those in the β- and δ-subunits were somewhat smaller. Overall, this pattern for the central pre-M1 linker position suggests that this region is approximately symmetric between subunits with regard to protein expression and the magnitude and relative timing of the gating energy changes. However, a Lys substitution at Arg3 resulted in an $E_2$ increase in the α-subunits but a decrease in the δ- and ε-subunits and no change in the β-subunit, suggesting that the α-subunit may have a unique chemical environment near the pre-M1 linker.

Position 4’ is not conserved between the α- and ε-subunits (Leu versus Lys), and this residue behaved quite differently in these two subunits. The α4’K mutation increased $E_2$ by ~50-fold, whereas the εK4’V mutation (Leu was not tested) had no effect. Indeed, only small changes were observed for all tested mutations of εLys4’, whereas all mutations in the α-subunit modestly increased $E_2$ (range energy of ~2.3 kcal/mol). An even more interesting difference is the distinct $\Phi$ values for position 4’ in the α-subunit versus the ε-subunit (0.35 versus 0.63). This suggests that in the α-subunit, but not the ε-subunit, there is a boundary between pre-M1 linker positions 3’ and 4’ that defines the relative timing of the gating movements of the ECD and TMD.

The gating behavior of loop 2 residue Glu45 was also very different in the α-subunit compared with the ε-subunit. Substitutions in the ε-subunit decreased $E_2$ very slightly, whereas those in the α-subunit either increased or decreased $E_2$ substantially. Indeed, the εGlu45 range energy (~5 kcal/mol, His to Ile) is the third largest measured so far in the ECD (after αAla36 and αTy4127) (30). An important and common feature shared by Glu45 in
the α- and β-subunits is that double mutants of Arg3 and Glu45 in both subunits suggest weak energetic interactions between these side chains in gating.

In summary, the pre-M1 linker is mostly symmetrical between subunits at positions 2’ and 3’ with regard to expression, gating, and interactions with Glu45 in loop 2. Asymmetry between subunits was apparent at pre-M1 linker position 4’ and Glu45, where only the α-subunit plays a major role. It is of interest to identify the chemical details that define the distinct subunit environments at the ECD-TMD interface and to further explore how energy spreads between subunits in the AChR gating isomerization.

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REFERENCES

1. Hille, B. (2001) Ion Channels of Excitable Membranes, 3rd Ed., Sinauer Associates, Inc., Sunderland, MA.
2. Engel, A. G., Shen, X. M., Selcen, D., and Sine, S. M. (2010) J. Mol. Neurosci. 40, 143–153
3. Unwin, N. (1998) J. Struct. Biol. 121, 181–190
4. Unwin, N. (2005) J. Mol. Biol. 346, 967–989
5. Hilf, R. J., and Dutzler, R. (2008) Nature 452, 375–379
6. Bocquet, N., Nury, H., Baaden, M., Le Poupon, C., Changueux, J. P., Delarue, M., and Corringer, P. J. (2009) Nature 457, 111–114
7. Hilf, R. J., and Dutzler, R. (2009) Nature 457, 115–118
8. Dellisanti, C. D., Yao, Y., Stroud, J. C., Wang, Z. Z., and Chen, L. (2007) Nat. Neurosci. 10, 953–962
9. Brejc, K., van Dijk, W. J., Klaassen, R. V., Schuurmans, M., van Der Oost, J., Smit, A. B., and Sixma, T. K. (2001) Nature 411, 269–276
10. Auerbach, A. (2010) J. Physiol. 588, 573–586
11. Tamamizu, S., Todd, A. P., and McNamee, M. G. (1995) Cell. Mol. Neurobiol. 15, 427–438
12. Lee, W. Y., and Sine, S. M. (2005) Nature 438, 243–247
13. Xi, X., Hanek, A. P., Wang, J., Lester, H. A., and Dougherty, D. A. (2005) J. Biol. Chem. 280, 41655–41666
14. Purohit, P., and Auerbach, A. (2007) J. Gen. Physiol. 130, 559–568
15. Hu, X. Q., Zhang, L., Stewart, R. R., and Weight, F. F. (2003) J. Biol. Chem. 278, 46583–46589
16. Kash, T. L., Dizon, M. J., Trudell, J. R., and Harrison, N. L. (2004) J. Biol. Chem. 279, 4887–4893
17. Mercado, J., and Czajkowski, C. (2006) J. Neurosci. 26, 2031–2040
18. Castaldo, P., Stefanoni, P., Miceli, F., Coppola, G., Del Giudice, E. M., Bellini, G., Pascotto, A., Trudell, J. R., Harrison, N. L., Annunziato, L., and Tagliafalela, M. (2004) J. Biol. Chem. 279, 25598–25604
19. Mukhtasimova, N., and Sine, S. M. (2007) J. Neurosci. 27, 4110–4119
20. Chakrapani, S., Bailey, T. D., and Auerbach, A. (2003) J. Gen. Physiol. 122, 521–539
21. Purohit, P., and Auerbach, A. (2007) J. Gen. Physiol. 130, 569–579
22. Grosman, C., Salamone, F. N., Sine, S. M., and Auerbach, A. (2000) J. Gen. Physiol. 116, 327–340
23. Bafna, P. A., Purohit, P. G., and Auerbach, A. (2008) PLoS One 3, e2515
24. Mitra, A., Cymes, G. D., and Auerbach, A. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 15069–15074
25. Jha, A., Purohit, P., and Auerbach, A. (2009) Biophys. J. 96, 4075–4084
26. Lee, W. Y., Free, C. R., and Sine, S. M. (2009) J. Neurosci. 29, 3189–3199
27. Jha, A., Cadugan, D. J., Purohit, P., and Auerbach, A. (2007) J. Gen. Physiol. 130, 547–558
28. Auerbach, A., Sigurdson, W., Chen, J., and Akk, G. (1996) J. Physiol. 494, 155–170
29. Qin, F., Auerbach, A., and Sachs, F. (1996) Biophys. J. 70, 264–280
30. Cadugan, D. J., and Auerbach, A. (2010) Biophys. J. 99, 798–807
31. Cymes, G. D., Grosman, C., and Auerbach, A. (2002) Biochemistry 41, 5548–5555
32. Chakrapani, S., and Auerbach, A. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 87–92