Acetylcholine Receptor Gating at Extracellular Transmembrane Domain Interface: the “Pre-M1” Linker

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Charged residues in the β10–M1 linker region (“pre-M1”) are important in the expression and function of neuro-muscular acetylcholine receptors (AChRs). The perturbation of a salt bridge between pre-M1 residue R209 and loop 2 residue E45 has been proposed as being a principle event in the AChR gating conformational “wave.” We examined the effects of mutations to all five residues in pre-M1 (positions M207–P211) plus E45 in loop 2 in the mouse α1-subunit. M207, Q208, and P211 mutants caused small (approximately threefold) changes in the gating equilibrium constant (Keq), but the changes for R209, L210, and E45 were larger. Of 19 different side chain substitutions at R209 on the wild-type background, only Q, K, and H generated functional channels, with the largest change in Keq (67-fold) from R209Q. Various R209 mutants were functional on different E45 backgrounds: H, Q, and K (E45A), H, A, N, and Q (E45R), and K, A, and N (E45L). Φ values for R209 (on the E45A background), L210, and E45 were 0.74, 0.35, and 0.80, respectively. Φ values for R209 on the wt and three other backgrounds could not be estimated because of scatter. The average coupling energy between 209/45 side chains (six different pairs) was only −0.33 kcal/mol (for both α subunits, combined). Pre-M1 residues are important for expression of functional channels and participate in gating, but the relatively modest changes in closed- vs. open-state energy caused mutations, the weak coupling energy between these residues and the functional activity of several unmatched-charge pairs are not consistent with the perturbation of a salt bridge between R209 and E45 playing the principle role in gating.

INTRODUCTION

In acetylcholine receptor channels (AChRs), structural changes at two transmitter binding sites are linked with structural changes at a distant (~60 Å) “gate” in the transmembrane domain, by brownian movement along a linear sequence of intermediate steps (a “brownian conformational wave,” Auerbach, 2005; Zhou et al., 2005; Purohit et al., 2007). We, and others, seek to understand the structure and dynamics of the molecular events that connect the low affinity, nonconducting C(losed) conformation of the AChR with the high affinity, ion-conducting O(pen) conformation. This intermediate conformational ensemble forms the energy barrier that separates diliganded C and O, which is the transition region (TR) of the gating reaction.

The perturbation of a salt bridge near the interface of the extracellular domain (ECD) and the transmembrane domain (TMD) of the AChR has been suggested as being the key TR event in C→O gating. Lee and Sine (2005) studied the interaction between residues R209 at the C terminus of the ECD (at the base of loop C, in the pre-M1 segment) and E45 in loop 2, a segment that had previously been identified as playing an important and dynamic role in gating (Chakrapani et al., 2004). They found that charge-changing mutations alone (E45R or R209Q) reduced the diliganded gating equilibrium constant (Keq), but that these mutations in combination yielded AChR having almost wt-like Keq values. In addition, they found that the magnitude of the coupling energy between R209Q and E45R side chains was approximately that expected for a salt bridge. Given these results and the location of these closely apposed residues (Unwin, 2005; Dellisanti et al., 2007), about halfway between the transmitter binding sites and an equatorial gate and in the center of the α-subunit, they proposed that the “principle pathway” for the propagation of the gating conformational wave is as follows, in brief: agonist binding perturbs loop C, which perturbs the R209–E45 salt bridge, which perturbs other nearby elements that are somehow linked to the pore-lining transmembrane M2 helix and the gate (Lee and Sine, 2005). Several groups have also noted that charged residues located in the pre-M1 segment in AChRs and other pentameric, ligand-gated ion channels play a significant role in expression and gating, for example in 5-HT3A receptors (Hu et al., 2003), Gly receptors (Castaldo et al., 2004), GABA_A receptors (Kash et al., 2004; Keramidas et al., 2006; Mercado and Czajkowski, 2006), and ACh receptors (Tamamizu et al., 1995; Lee and Sine, 2005; Xiu et al., 2005). Recently, evidence for a salt bridge between residues in pre-M1 and loop 2 was

Abbreviations used in this paper: AChR, acetylcholine receptor; ECD, extracellular domain; REFER, rate-equilibrium free energy relationship; TMD, transmembrane domain.
reported for α1 GABA<sub>A</sub> and GABA<sub>C</sub> receptors, but mutation of these residues in 5HT<sub>3</sub> receptors mainly acts to reduce channel expression (Price et al., 2007; Wang et al., 2007).

We have modeled the conformational pathway between C and O as a linear sequence of intermediate steps, with the result that information about the relative timing of these steps can be gained from kinetic analyses (Zhou et al., 2005; Auerbach, 2007). The slope of a log–log plot of the opening rate constant vs. Keq for a mutational series of a single residue is called Φ and, according to this model, implies the relative timing of that residue’s gating motion (1 to 0, early to late). Lee and Sine (2005) combined the kinetic results for wt, R209Q, and E45K and E45R (but not E45A) to estimate Φ = 0.43 for this group, which places the R209–E45 perturbation near the middle of the gating reaction, after the movement of many M2 residues, including some near the gate (Φ = 0.65; Mitra et al., 2005; Purohit et al., 2007).

Here, we report the gating rate and equilibrium constants (and Φ) for AChRs having mutations in the α subunits of each of the five pre-M1 residues (position 207–211) or in E45. We made these measurements by using 68 different constructs, including AChRs having pairs of R209+E45 mutations (in both α subunits). The results show that only two of the pre-M1 residues move during gating. R209 moves relatively early, approximately at the same time as residues in loop 2 (including E45) and the cys-loop, and L210 moves relatively late, approximately in synchrony with the motion of M3 and the M2 equatorial (12’ and 9’) gate. However, the perturbation of pre-M1 residues R209 and L210 does not make a particularly large contribution to the overall energy of the gating reaction.

**MATERIALS AND METHODS**

For the details of mutagenesis, expression, electrophysiology, rate constant determination, and Φ-value analysis, see Jha et al. (on p. 547 of this issue). In brief, mouse AChR subunits were transiently expressed in human embryonic kidney fibroblast cells (HEK 293) and electrophysiological recordings were performed in cell-attached patch configuration (22°C, ~100 mV membrane potential, PBS in the bath and pipette). 0.1 μg/μl GFP was added as a marker in the transfection mixture. Agonist (acetylcholine or choline) was added to the pipette solution at a concentration that is approximately five times the closed-conformation equilibrium dissociation constant (K<sub>d</sub> (500 μM or 20 mM, respectively). Currents were analyzed by using QUB software (www.qub.buffalo.edu), with only the intervals within clusters of openings selected for quantitative analysis. Opening and closing rate constants were estimated from the selected interval durations by using a maximum-interval likelihood algorithm (Qin et al., 1997) after imposing a dead time of 25 μs. Φ was estimated as the slope of the rate-equilibrium free energy relationship (REFER), which is a plot of log k<sub>o</sub> vs. log Keq. Each point in the REFER represents the mean of at least three different patches. The coupling energy was calculated as ΔΔG = −RT ln(K<sub>eqwt*KeqDBI/Keqmutwt*Keqwtmut</sub>).

**RESULTS**

Fig. 1 shows the pre-M1 segment and loop 2 residue E45, in the Torpedo α-subunit (2bg9.pdb; Unwin, 2005). It is important to recall that this cryo-EM structure is of an unliganded-closed AChR, whereas we are investigating the diliganded-closed to diliganded-open gating reaction. There are significant conformational changes...
associated with ligand binding to AChBP (Brejc et al., 2001; Celie et al., 2004), and we therefore are uncertain the extent to which our functional results can be mapped, in detail, onto the 4-Å resolution Torpedo AChR structure. Also, in Torpedo the pre-M1 amino acid sequence is MQRIP but in mouse it is MQRLP. For both species loop 2 has the sequence EVNQ.

Fig. 2 shows a kinetic analysis of position E45 (wt plus 11 mutants). Five substitutions decreased $K_{eq}$ (A, G, P, K, and H), three increased $K_{eq}$ (Y, R, and I), and three had little or no effect on $K_{eq}$ (C, D, and L). We do not discern a simple pattern relating the chemical properties of the side chain and the magnitude or polarity of the change in $K_{eq}$. For example, the charge reversal mutations E→K and E→R decreased and increased $K_{eq}$, respectively.

Regardless of whether a substitution at position 45 increased or decreased $K_{eq}$, the effect was to change both the opening ($k_o$) and closing ($k_c$) rate constants (Table I). The L substitution modestly increased both $k_o$ and $k_c$ relative to the wt and therefore had little effect on $K_{eq}$. A REFER plot for the entire mutant series yields $\Phi = 0.80 \pm 0.06$ (Fig. 2 B), which is the same as that for other loop 2 residues ($\Phi = 0.81 \pm 0.05$, Chakrapani et al., 2004). Separate analyses of constructs activated either by ACh or choline gave similar $\Phi$ values ($0.81 \pm 0.08$ and $0.77 \pm 0.20$, respectively). (Note that the higher SEM with choline can be attributed to the smaller excursion in $K_{eq}$ realized with this agonist.)

Next, we measured $\Phi$ values for all of the residues in α-subunit pre-M1 linker. At three positions (207, 208, and 211) none of the 11 tested mutations changed $K_{eq}$ by greater than threefold (Table II). The specific constructs were M207→A, G, I, W, Q208→A, E, W, and P211→A, G, H, S. This result indicates that there is a little or no difference in energy between the C vs. O conformations of these residues, which implies that the atoms do not move in the gating reaction, that they move in register with their local microenvironments or that there are compensating energy changes in nearby residues associated with their motion. The lack of a change in $K_{eq}$ upon mutation of P211, at the top of the M1 helix, is particularly notable.

Some mutations of the two central pre-M1 residues, R209 and L210, changed $K_{eq}$ by greater than threefold. We examined 19 different substitutions at R209, plus a construct in which residue 209 was deleted. In 16 of these (A, C, D, E, F, G, I, L, M, N, P, S, T, V, W, Y, and the deletion) no single channel currents were observed (3–10 patches/mutant, ~20 min/patch). We did record single-channel currents from the constructs R209Q and R209H, but only with some difficulty (currents observed in ~20% of patches), whereas currents for the R209K mutant were observed in virtually every patch. Thus, a major effect of mutating R209 is to reduce or abolish the expression of functional AChRs.
Fig. 3 A and Table III show $k_o$, $k_c$, and $K_{eq}$ values for the Q, H, and K substitutions of R209. The kinetic pattern was complex. The Q mutation reduced $k_o$ by 7.6-fold and increased $k_c$ by 9.5-fold, leading to a 67-fold decrease in $K_{eq}$. This result is similar to the 46-fold increase in $K_{eq}$ previously reported by Lee and Sine (2005). The H substitution increased both $k_o$ and $k_c$ (each by 2.5-fold) so that there was essentially no change in $K_{eq}$. The K substitution also increased both $k_o$ (15-fold) and $k_c$ (only by 2.3-fold), leading to a modest 7-fold increase in $K_{eq}$. Overall, the REFER for R209 on the wt background showed a high degree of scatter and the $\Phi$-value could not be estimated with a high degree of precision ($0.72 \pm 0.16$).

We also examined the kinetics of R209 mutants using AChRs having a distant background mutation that specifically slows channel closing. $\delta L265$ is located in the M2 helix of the $\delta$-subunit, and when this residue is mutated to a T, $k_c$ decreases by 16-fold but $k_o$ remains unchanged (Table III; Cymes et al., 2002). We used this mutant as a background construct, in combination with αR209 mutants. As with the wt background, no currents were apparent with the R209A, C, W, N, L, Y, F, and E constructs, but, as was the case with the wt background, were present with H, K, and Q. This result suggests that it is unlikely that the lack of observable currents can be attributed to an ultrafast closing rate constant in expressed AChRs. Fig. 3 B shows the REFER plot for the R209 mutant series on the $\delta L265T$ background. The overall pattern was similar to that obtained with ACh on the wt background, with the exception that there was a smaller effect on $k_o$ for the R209H construct. The estimate of the slope of the REFER for R209 mutants on the $\delta L265T$ background was again imprecise ($\Phi = 0.48 \pm 0.19$) but, because of the large standard error, was not significantly different from that obtained using the wt background. The coupling interaction energy estimated for R209/L265 mutant pairs was small (average = 0.93 ± 0.07).

### Table I

**Kinetic Analysis of E45 Mutants**

| Construct | Agonist | $k_o$ (s$^{-1}$) | $k_{c\text{obs}}$ (s$^{-1}$) | $k_{c\text{cor}}$ (s$^{-1}$) | $K_{eq}$ ($k_o/k_{c\text{cor}}$) | Normalized $K_{eq}$ (mut/wt) | $n$ |
|-----------|---------|-----------------|-----------------------------|---------------------------|-----------------------------|-----------------------------|-----|
| wt ACh    | 48000   | –               | 1700                        | 28.2                      | 1                           | –                           | 1   |
| wt Cho    | 120     | –               | 2583                        | 0.046                     | 1                           | –                           | 1   |
| E45I Cho  | 7446 (377)| 2235 (383)    | 5967 (1772)                 | 1.3 (0.31)                | 28.3                        | 3                           |     |
| E45Y Cho  | 1756 (64) | 594 (54)       | 1585 (251)                  | 1.1 (0.25)                | 24                          | 3                           |     |
| E45K Cho  | 3901 (76) | 1957 (32)      | 5172 (170)                  | 0.76 (0.05)               | 16.5                        | 4                           |     |
| E45L Cho  | 201 (26) | 1420 (176)     | 3791 (665)                  | 0.05 (0)                  | 1.2                         | 2                           |     |
| E45C Cho  | 856 (83) | 7780 (1307)    | 20770 (6445)                | 0.043 (0.02)              | 0.93                        | 3                           |     |
| E45D Cho  | 409     | 4741            | 12650                       | 0.03                      | 0.65                        | 1                           |     |
| E45A ACh  | 17450 (715) | 5060 (625) | 6324 (1563)                 | 2.91 (0.8)                | 0.1                         | 4                           |     |
| E45G ACh  | 12760 (923) | 5158 (534)  | 6448 (1156)                 | 2.1 (0.65)                | 0.074                       | 3                           |     |
| E45P ACh  | 11040 (202) | 6212 (593)    | 7765 (982)                  | 1.43 (0.13)               | 0.05                        | 4                           |     |
| E45K ACh  | 4979 (352) | 15390 (1149)   | 19240 (2871)                | 0.27 (0.07)               | 0.01                        | 4                           |     |
| E45H ACh  | 214 (101) | 13210 (595)    | 16510 (744)                 | 0.013 (0.007)             | 0.005                       | 3                           |     |

All values are mean ± SEM. $k_o$, opening rate constant; $k_{c\text{obs}}$, observed closing rate constant; $k_{c\text{cor}}$, closing rate constant corrected for channel-block; $K_{eq}$ (= $k_o/k_{c\text{cor}}$), diliganded gating equilibrium constant, normalized $K_{eq}$ (mutant divided by the wt value for the salient agonist); $n$, number of patches.

### Table II

**Kinetic Analysis of M207, Q208, and P211**

| Construct | Agonist | $k_o$ (s$^{-1}$) | $k_{c\text{obs}}$ (s$^{-1}$) | $k_{c\text{cor}}$ (s$^{-1}$) | $K_{eq}$ ($k_o/k_{c\text{cor}}$) | Normalized $K_{eq}$ (mut/wt) | $n$ |
|-----------|---------|-----------------|-----------------------------|---------------------------|-----------------------------|-----------------------------|-----|
| M207A Cho | 288 (25.2) | 1748 (288)     | 4666 (1353)                 | 0.064 (0.02)              | 1.5                         | 3                           |     |
| M207G Cho | 175 (5.4) | 1094 (187)     | 2922 (863)                  | 0.065 (0.02)              | 1.4                         | 3                           |     |
| M207I Cho | 161     | 966             | 2579                        | 0.062                     | 1.3                         | 1                           |     |
| M207W Cho | 151 (15) | 495 (81)       | 1318 (435)                  | 0.12 (0.02)               | 2.6                         | 4                           |     |
| Q208A Cho | 121     | 423             | 1129                        | 0.11                      | 2.3                         | 1                           |     |
| Q208E ACh | ND      | ND              | ND                          | ND                        | ND                          | –                           |     |
| Q208W Cho | 40      | 800             | 2136                        | 0.02                      | 0.43                        | 1                           |     |
| P211G ACh | 31402   | 2883            | 3604                        | 8.7                       | 0.3                         | 1                           |     |
| P211H ACh | ND      | ND              | ND                          | ND                        | ND                          | –                           |     |
| P211A ACh | 29000 (999) | 2601 (257) | 3251 (453)                  | 8.9 (0.8)                 | 0.3                         | 2                           |     |
| P211S ACh | 365000  | 2450            | 3063                        | 11.9                      | 0.4                         | 1                           |     |

All of the mutants at positions M207, Q208, and P211 expressed and exhibited wt-like kinetic properties (change in $K_{eq}$ less than threefold). ND, no data at 20 mM choline or 500 μM ACh. At 30 μM ACh both Q208E and P211H had wt-like kinetic behaviors.
−0.40 kcal/mol), suggesting that the effects of the two mutations were essentially independent (Table III).

The second mutation-sensitive position in pre-M1 was L210 (Fig. 4 and Table IV). At this position all of the tested mutants (Y, A, F, W, and G) expressed functional AChRs that showed an increased $K_{eq}$ (by 2-, 6.1-, 6.3-, 10.7-, and 52.1-fold, respectively). The effect of the mutations was mainly to slow $k_c$, and a REFER plot for the L210 mutant series gives $\Phi = 0.35 \pm 0.12$ (Fig. 4 B). The A construct appeared to be an “outlier,” but eliminating this point from the fit only reduced the standard error and did not significantly change the estimate of $\Phi$ ($0.36 \pm 0.07$).

In the next set of experiments we measured $k_o$, $k_c$, and $K_{eq}$ in AChRs having two mutations in each of the two $\alpha$ subunits. Lee and Sine (2005) used this approach to detect a significant energetic interaction (−3.1 kcal/mol) between R209Q and E45R, which supported their proposal that these two side chains (Q/R) interact during gating. We measured $k_o$ and $k_c$ for R209 mutants on three different mutant E45 backgrounds, R, L, and A (Fig. 5). By themselves these background mutations increase, do not change, or decrease $K_{eq}$, respectively (Fig. 2).

Interestingly, the expression of functional R209 mutant channels was influenced by the side chain at the background position 45. The constructs R209A and R209N are totally silent in the wt E45 background, but give rise to functional channels in the E45R and E45L backgrounds. R209A, L, and N, however, still did not generate functional channels on the E45A background.

The coupling energies between the R209 and E45 mutants are shown in Table III. In four of the tested 209/45 mutant pairs (Q/A, H/A, Q/R, and K/L) the observed value for $K_{eq}$ was smaller than that predicted assuming independent energetic effects of the mutations. In the H/R and K/A pairs, $K_{eq}$ was larger than predicted from independence. The magnitude of the discrepancy varied with the side chains. The degree of coupling was largest for the K/A, Q/R, and H/R pairs, for which the observed value of the normalized $K_{eq}$ was 20.1-fold larger, 7.1-fold larger, and 6.1-fold smaller and than predicted from independence. These translate to coupling energies of −1.77, +1.16, and −1.07 kcal/mol, respectively. Overall, the average ($\pm$SD) coupling between all six different R209 and E45 mutant pairs was modest, −0.33 ± 1.02 kcal/mol.

The R209 REFERs on the E45 mutant backgrounds are shown in Fig. 5. As with the wt background, there was a large amount of scatter in the REFERs for the E45R background ($\Phi = 1.49 \pm 0.23$) and E45L background ($\Phi = 1.0 \pm 0.26$), and $\Phi$ values could not be estimated precisely. This result suggests that mutations of R209 on the wt and these backgrounds alter one or more of the TR barriers (a “catalytic” effect), that a point mutation of R209 changes the energies of multiple microstates of the TR to relative extents that depend on the specific side chain substitution, or both. Only the four-point REFER for the E45A background was clearly linear. For this construct, $\Phi = 0.74 \pm 0.02$, a value that...
TABLE III

Kinetic Analysis of R2

| Construct       | Agonist | $k_\text{o}$ (s$^{-1}$) | $k_\text{o}^{\text{obs}}$ (s$^{-1}$) | $k_\text{o}^{\text{corr}}$ (s$^{-1}$) | $K_\text{eq}$ ($k_\text{o}^{\text{corr}}$) | Normalized $K_\text{eq}$ (mut/wt) | $\Delta\Delta G$ (kcal/mol) | $n$ |
|-----------------|---------|-------------------------|-------------------------------------|-------------------------------------|-------------------------------------|----------------------------------|---------------------------|-----|
| R209Q           | ACh     | 6288 (694)              | 12860 (1688)                        | 16070 (5654)                       | 0.42 (0.17)                        | 0.015                            | –                         | 3  |
| R209K           | Cho     | 1820 (149)              | 2197 (260)                          | 5865 (980)                         | 0.32 (0.09)                        | 6.9                              | –                         | 2  |
| R209H           | Cho     | 222 (54)                | 2614 (193)                          | 6979 (894)                         | 0.037 (0.007)                      | 0.8                              | –                         | 3  |
| R209Q+δL265T    | Cho     | 92.2 (18.6)             | 1394 (294)                          | 3723 (1360)                        | 0.028 (0.02)                       | 0.63                             | 4.1                       | −5.4| 3  |
| R209K+δL265T    | Cho     | 1805 (196)              | 64.5 (6.5)                          | 172 (25)                           | 10.7 (3.13)                       | 233                              | 112.5                     | 2  |
| R209H+δL265T    | Cho     | 335.3 (24.5)            | 138.1 (9.3)                         | 369 (43)                           | 0.92 (0.2)                        | 0.17                             | –                         | −0.25|3  |
| L265T           | Cho     | 120                    | 60                                  | 160                                | 0.75                              | 16.3                             | –                         | 1  |
| R209A+E45L      | Cho     | 497.8 (64.8)            | 5590 (350)                          | 14930 (1869)                       | 0.034 (0.01)                      | 1.4                              | –                         | 4  |
| R209K+E45L      | Cho     | 976 (54)                | 1494 (152)                          | 3988 (572)                         | 0.25 (0.02)                       | 5.4                              | 7.9                       | −0.19|2  |
| R209N+E45L      | ACh     | 4536                   | 2005                                | 2506                               | 1.8                               | 0.06                             | –                         | 1  |
| R209Q+E45A      | ACh     | 1427 (181)              | 16230 (1293)                        | 20920 (3233)                       | 0.07 (0.02)                       | 0.002                            | 0.00152                   | −0.28|4  |
| R209K+E45A      | Cho     | 2061 (231)              | 1238 (192)                          | 3305 (886)                         | 0.66 (0.2)                        | 14.3                             | 0.71                      | −1.77|3  |
| R209H+E45A      | Cho     | 11410 (642)             | 7319 (469)                          | 9148 (587)                         | 1.25 (0.08)                       | 0.044                            | 0.083                     | 0.37 |3  |
| R209A+E45R      | ACh     | 26035                  | 761                                 | 951                                | 27.4                              | 0.97                             | –                         | 1  |
| R209H+E45R      | Cho     | 669.5 (135)             | 2660 (187)                          | 7102 (706)                         | 0.1 (0.03)                        | 2.17                             | 13.3                      | 1.1 |2  |
| R209N+E45R      | ACh     | 2105 (389)              | 354.5 (44.3)                        | 443 (111)                          | 4.7 (0.6)                         | 0.17                             | –                         | −4  |
| R209Q+E45R      | Cho     | 515 (73)                | 2383 (227)                          | 6362 (606)                         | 0.08 (0.01)                       | 1.74                             | 4.1                       | −1.16|3  |

R209A, C, W, N, L, Y, F, and E did not express functional AChRs in both the wt and δL265T backgrounds. For the doubly mutated (at positions 209 and 45) α-subunit constructs, “Predicted” is the expected, normalized $K_{\text{eq}}$ assuming that the effects of the mutations were independent. The coupling energy was calculated as described in the Materials and methods.

Discussion

Comparison with Previous Results

There are three major differences between our results and those of Lee and Sine (2005), who also studied mutants of αR209 and αE45. First, we find that E45 has a Φ-value (0.80) that is same as those for other loop 2 residues (0.81), as well as for many cys-loop residues (0.78). Using the rate constants reported in Lee and Sine (2005), we calculate an Φ-value for E45 (0.44 ± 0.12; for the wt and A, K, and R mutants) that is lower than our estimate. Second, with choline as the agonist we find that the charge-reversal mutation E45R causes an ∼16.5-fold increase in $K_{\text{eq}}$, whereas Lee and Sine used ACh as the agonist and found that this mutation caused a 6.6-fold decrease in $K_{\text{eq}}$ (Xiu et al., 2005) found that in mouse AChRs (expressed in Xenopus oocytes) the E45R mutation decreased the $EC_{\text{50}}$ for ACh (from 50 to 1.6 μM), which is consistent with our observation of an increase in $K_{\text{eq}}$. This result, and those shown in Fig. 2 B, indicates that the discrepancy cannot be attributed to different agonists. Third, we find the coupling between the R209Q and E45R side chains is −1.15 kcal/mol, whereas Lee and Sine report a larger (−3.1 kcal/mol) energetic interaction for this same mutant pair. This difference in coupling energy can be traced to the essential difference with regard to the effect of the E45R mutation alone on $K_{\text{eq}}$.

The sources of the differences in results regarding E45 in our hands (mouse AChRs, in HEK cells, exposed to extracellular Na$^+$) and those of Lee and Sine (human AChRs, in BOSC23 cells, exposed to extracellular K$^+$) remain unknown. Note that even if the E45R data point is eliminated from the E45 REFERs a significant difference in Φ for position E45 persists (0.80 vs. 0.44 vs. Lee and Sine). Our results indicate that αE45 moves relatively early in the channel opening process, at approximately the same time as all of the other residues in loop 2 and the neighboring cys-loop, and that a charge reversal mutation at this position increases $K_{\text{eq}}$.

Salt Bridge Hypothesis

Although R209 and E45 are close (<4 Å) to each other in the both the Torpedo and mouse ECD fragment...
structures, certain results are difficult to reconcile with
the hypothesis that the perturbation of a salt bridge
between these side chains is a principle event in AChR
gating. (a) Lee and Sine (2005) report that the mutation
R209Q (in both α subunits) causes only a 46-fold reduc-
tion in $K_{eq}$, which is similar to our measurement of a
67-fold reduction. This degree of change in $K_{eq}$ is sig-
ificant, but smaller than others caused by the mutation of
other residues that are near the base of the ECD/TMD
interface and elsewhere. For example, the following
mutations (also in both α subunits) all cause a larger
change in $K_{eq}$: D97A (170-fold; Chakrapani et al., 2003),
Y127D (4947-fold; Purohit and Auerbach, 2007), V46E
(233-fold; Chakrapani et al., 2003), F135A (134-fold;
Chakrapani et al., 2004), I274T and P272A (1613- and
218-fold, respectively; Jha et al., 2007). In addition, the
mutation of several δ- (Cymes et al., 2002) and α-sub-
unit (Mitra et al., 2004; Mitra et al., 2005; Cadugan and
Auerbach, 2007; Purohit et al., 2007) TMD residues also
causes a >100-fold change in $K_{eq}$. Although R209 and
E45 each play a significant role in gating, the unspec-
tacular changes in energy (O vs. C) caused by R209
mutations are not consistent with this residue playing
the principle role in AChR gating. (b) Charge-changing
mutations do not have the expected effects for the
perturbation of a salt bridge. In our hands, the charge-
reversal mutation E45R increases $K_{eq}$ while the charge-
reversal mutation E45K decreases $K_{eq}$. Substitution of a
neutral side chain at E45 can either increase (I), decrease
(A), or have essentially no effect (L). Likewise, at R209
the substitution of an H (which is probably uncharged
at pH 7.4 when buried in a protein) has almost no effect

\begin{table}
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Construct & Agonist & $k_o$ (s$^{-1}$) & $k_{obs}$ (s$^{-1}$) & $k_{cor}$ (s$^{-1}$) & $K_{eq}$ ($k_o/k_{cor}$) & \text{Observed} & \text{Predicted} & $\Delta\Delta G$ (kcal/mol) & \text{n} \\
\hline
L210G & Cho & 408 (91) & 78 (15) & 207 (70) & 2.4 (1.7) & 52.1 & – & – & 3 \\
L210W & Cho & 220 (21) & 176 (22) & 471 (117) & 0.49 (0.2) & 10.7 & – & – & 4 \\
L210A & Cho & 96 (8) & 124 (8.5) & 331 (39) & 0.29 (0.009) & 6.5 & – & – & 3 \\
L210F & Cho & 225 (30) & 516 (55) & 845 (201) & 0.28 (0.006) & 6.1 & – & – & 4 \\
L210V & Cho & 106 (28) & 424.4 (80) & 1125 (475) & 0.09 (0.04) & 2 & – & – & 5 \\
Y277H & Cho & 43 & 753.4 & 20130.2 & 0.0021 & 0.046 & & & & \\
L210G+Y277H & Cho & 73 (4) & 834 (1456) & 2226 (549) & 0.034 (0.01) & 0.74 & 2.39 & 0.69 & 2 \\
L210W+Y277H & Cho & 24.7 (4) & 1115 (175) & 2976 (809) & 0.009 (0.005) & 0.19 & 0.49 & 0.54 & 3 \\
\hline
\end{tabular}
\caption{Kinetic Analysis of L210}
\end{table}

The expression of all the mutants was approximately normal. The coupling energies between the mutant pairs (G/H and W/H at positions 210/277) were calculated as described in the Materials and methods.

*Cadugan and Auerbach (2007) and rate constant are translated for wt background.*

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**Figure 4.** L210 mutational series. (A) Example clusters elicited by choline. All of the mutations increased $K_{eq}$ and the cluster open probability, mainly by prolonging the open time (slowing the closing rate constant). (B) REFER plot (Table IV); wt is boxed. The Φ-value for L210 was Φ = 0.35 ± 0.12, which suggests that this residue moves “late” in the gating isomerization.
on $K_{eq}$. Further, the double mutant R209A+E45L, which does not have a charged side chain at either position, has a $K_{eq}$ that is nearly normal. Similarly, the R209/E45 constructs H/A, N/L, K/L, H/R, A/R, N/R, and Q/R cannot form such a salt bridge but nonetheless are functional. The relationship between $K_{eq}$ and the chemical nature of the 209/45 side chain is not consistent with the perturbation of a salt bridge being the chemical interaction between these two positions. (c) We observe weak energetic coupling between some 209/45 side chains. Specifically, the average coupling energy was not significantly different from zero for the six double mutant constructs. In summary, the magnitudes of the changes in $K_{eq}$, the magnitudes of the coupling energies, and the pattern of change as a function of side chain chemistry are not consistent with the perturbation of a salt bridge between R209 and E45 being an energetically dominant event in AChR gating.

Primary Roles for R209

Lee and Sine (2005) reported in human AChRs that R209Q, but not R209E, gave functional expression of surface channels. Tamamizu et al. (1995) found in Torpedo AChRs that R209K and H mutants produce currents but L, A, and E mutants do not, and that α-bungarotoxin binding is reduced by approximately threefold in these three nonfunctional constructs. Vicente-Agullo et al. (2001) found that there was no internal or surface expression of R209L bovine α7 receptors, and that the surface expression of bovine α3AChRs was attenuated by R209A and R209E (but not R209K) mutations. Xiu et al. (2005) also found that in the mouse α1 subunit the R209A construct did not express on the surface of oocytes. We examined 19 different mutants of R209 and 11 different mutants of E45 and a striking result was that only three of the R209 point mutants (Q, H, and K) gave rise to functional channels whereas all of the E45 mutants did so. Such a general failure to express functional channels following a point mutation is rare. We did not measure surface expression, but our results are consistent with the idea that R209 is important in AChR assembly and expression, as is the case with 5HT3 receptors (Price et al., 2007). Because we were able to record functional R209A and N mutant channels only on the E45 R and L mutant backgrounds, we speculate that R209–E45 interactions may be important in this regard.

$K_{eq}$ and $\Phi$

In the five pre-M1 residues that we examined none of the 26 mutants at positions M207, Q208, and P211 changed $K_{eq}$ (but all gave rise to functional channels). In contrast, of the constructs that produced functional channels, most at positions R209 and E45 changed $K_{eq}$ by greater than threefold. This result suggests that α-subunit residues 207, 208, and 211 probably do not move (or move with their environment) between C and O, and that in pre-M1 only positions R209 and L210 move in the TR of the diliganded gating reaction. Compared with other domains that are also in the vicinity of the ECD–TMD

![Figure 5](image-url)

**Figure 5.** Energy coupling between R209 and E45. Example currents and REFERs for R209 mutations series on three different E45 backgrounds: (A) E45R, (B) E45L, and (C) E45A (Table III). Mutations were made at both R209 and E45 (in both α subunits) and current was activated by choline (open circles) or ACh (filled circles). The R209A and R209N point mutants in a wt background did not yield functional AChRs but did so in the E45R and E45L backgrounds. The $\Phi$ values estimated for position 209 in E45R and E45L backgrounds are imprecise because of a large SD (±0.23 and ±0.26). A mutant cycle analysis for these constructs indicates that the coupling between R209K/E45A, R209Q/E45R, and R209H/E45R is greater than between R209Q/E45A and R209K/E45L (Table III). The REFER for R209 on an E45A background was linear over a greater than three order of magnitude range in $K_{eq}$, and is our best estimate of the $\Phi$-value for R209.
interface (for example, the M2–M3 linker, the cys-loop and loop 2), the pre-M1 segment does not appear to make major contribution to the energy difference between C and O. Rather, we think that the transfer of energy between the ECD and TMD is mediated to a greater extent by a combination of side chain interactions at multiple sites between loop 2 and M2, and between the cys-loop and the M2–M3 linker. Such a distributed nature for these interactions is consistent with the general observation that relationship between the chemical nature of a single amino acid and the change in $K_{eq}$ is rarely simple.

It is interesting that the REFERs for the two moving residues in pre-M1 (R209 and L210) were scattered. For R209, we measured $\Phi$ values (on five different backgrounds) ranging from 0.48 to 1.49, and with SEM values that ranged from 0.02 to 0.26. For L210, the standard error of the $\Phi$ estimate was 0.35 ± 0.12. For comparison, the SEM values for 14 different M2 residue REFERs (over a similar spread in $K_{eq}$) ranged from 0.01 to 0.16 (Purohit et al., 2007). We are not sure why the REFERs for R209 are so problematic. We note that R209 and L210 are in a region that is characterized by several different $\Phi$ values, including loop 2 and the cys-loop ($\Phi = 0.78$), the M2–M3 linker ($\Phi = 0.64$), and M3 ($\Phi = 0.31$). Given this location, at the juncture of three “nanotectonic plates,” we speculate that some mutations of R209 perturb multiple positions of the TR. If the relative extents of these energy perturbations vary with the side chain substitution, then the REFERs would be scattered.

The REFER for R209 on an E45A background was linear (over a greater than three order of magnitude range in $K_{eq}$) and showed little scatter, and thus gave rise to a reliable estimate for $\Phi$. This estimate, 0.74 ± 0.02, is similar to the one obtained on the wt background, 0.72 ± 0.16, so it is the best estimate of the $\Phi$-value for R209. This value places the gating motion of R209 relatively early in the gating reaction, approximately in synchrony with $\alpha$-subunit residues in loop 2 (including E45) and the cys-loop, and residue Y127. This value also places R209 at a $\Phi$-block boundary, because the $\Phi$-value of its neighbor, L210, is only 0.35.

The REFER for E45 ($\Phi = 0.77$; Fig. 2) spanned an $\sim$35,805-fold range in $K_{eq}$ value (E45H to E45R), which indicates that the movements of this residue (in two $\alpha$ subunits) is important insofar as this side chain substitution results in a significant (−6.18 kcal/mol) energetic contribution to the gating equilibrium constant. However, we can discern no simple pattern relating the value of $K_{eq}$ to the chemical nature of the side chain at this position.

Regarding position L210, all five of the tested mutants gave rise to functional channels in which $K_{eq}$ was increased relative to the wt. The $\Phi$-value for L210 (0.35) suggests that this residue is part of a domain that moves late in the reaction and whose motion links the ECD–TMD interface with those in M3 near the middle of the membrane. There is only a small degree of energetic coupling between L210 and Y277 side chains, even though these residues are closely apposed in the Torpedo structure. We do not yet understand the physiological significance of residues at the ECD–TMD interface moving late in the gating reaction, perhaps with or even after the change in ionic conductance at the gate (Purohit et al., 2007). We speculate that these late gating motions may serve as a “latch” that stabilizes the O conformation of the protein.

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