Acetylcholine Receptor Gating: Movement in the α-Subunit Extracellular Domain

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Acetylcholine receptor channel gating is a brownian conformational cascade in which nanometer-sized domains ("Φ blocks") move in staggering sequence to link an affinity change at the transmitter binding sites with a conductance change in the pore. In the α-subunit, the first Φ-block to move during channel opening is comprised of residues near the transmitter binding site and the second is comprised of residues near the base of the extracellular domain. We used the rate constants estimated from single-channel currents to infer the gating dynamics of Y127 and K145, in the inner and outer sheet of the β-core of the α-subunit. Y127 is at the boundary between the first and second Φ blocks, at a subunit interface. αY127 mutations cause large changes in the gating equilibrium constant and with a characteristic Φ-value (Φ = 0.77) that places this residue in the second Φ-block. We also examined the effect on gating of mutations in neighboring residues δ145 (Φ = 0.86), εN39 (complex kinetics), αI49 (no effect) and in residues that are homologous to αY127 on the ε, β, and δ subunits (no effect). The extent to which αY127 gating motions are coupled to its neighbors was estimated by measuring the kinetic and equilibrium constants of constructs having mutations in αY127 (in both α subunits) plus residues αD97 or εY127. The magnitude of the coupling between αD97 and αY127 depended on the αY127 side chain and was small for both H (0.53 kcal/mol) and C (−0.37 kcal/mol) substitutions. The coupling across the single α-δ subunit boundary was larger (0.84 kcal/mol). The Φ-value for K145 (0.96) indicates that its gating motion is correlated temporally with the motions of residues in the first Φ-block and is not synchronous with those of αY127. This suggests that the inner and outer sheets of the α-subunit β-core do not rotate as a rigid body.

INTRODUCTION

The diliganded gating isomerization of the acetylcholine receptor (AChR), between C(losed) and O(pen) structures, is a conformational “wave” that links a change in affinity for ligands at the transmitter binding sites with a change in the ionic conductance of the pore. In the α-subunit, the first group of amino acids to undergo a change in affinity for ligands at the transmitter binding site and the second group of residues near the base of the extracellular domain. We used the rate constants estimated from single-channel currents to infer the gating dynamics of Y127 and K145, in the inner and outer sheet of the β-core of the α-subunit. Y127 is at the boundary between the first and second Φ blocks, at a subunit interface. αY127 mutations cause large changes in the gating equilibrium constant and with a characteristic Φ-value (Φ = 0.77) that places this residue in the second Φ-block. We also examined the effect on gating of mutations in neighboring residues δ145 (Φ = 0.86), εN39 (complex kinetics), αI49 (no effect) and in residues that are homologous to αY127 on the ε, β, and δ subunits (no effect). The extent to which αY127 gating motions are coupled to its neighbors was estimated by measuring the kinetic and equilibrium constants of constructs having mutations in αY127 (in both α subunits) plus residues αD97 or δ145. The magnitude of the coupling between αD97 and αY127 depended on the αY127 side chain and was small for both H (0.53 kcal/mol) and C (−0.37 kcal/mol) substitutions. The coupling across the single α-δ subunit boundary was larger (0.84 kcal/mol). The Φ-value for K145 (0.96) indicates that its gating motion is correlated temporally with the motions of residues in the first Φ-block and is not synchronous with those of αY127. This suggests that the inner and outer sheets of the α-subunit β-core do not rotate as a rigid body.
Φ-Value Analysis of Y127, K145, and Nearby Residues

The structure of Y127 is located at or near the C terminus of β-strand 6, one position from the C128–C142 disulfide bond that defines the cys-loop (loop 7) of the eukaryote pentameric receptor superfamily (Fig. 1). Y127 also is at a subunit interface and faces either the ε (γ in embryonic AChRs) or δ subunit, and for this reason the structure of this residue is poorly resolved in the monomeric ECD fragment (Dellisanti et al., 2007). Mukhtasimova and Sine (2007) found that the mutation αY127T substantially decreases Keq, as do the mutations εN39A and δN41A in nearby residues in these non–α subunits. Moreover, the effects of these perturbations were not independent, which suggests that these positions are coupled energetically and are a link for the intersubunit propagation of the gating conformational cascade.

In both the Torpedo AChR and ECD fragment structures, αK145 is <4 Å from two residues whose mutation significantly changes Keq: αD97 (in loop C) and αY93 (in loop A) (Akk et al., 1996; Akk, 2001). Although rate constants for only a few mutations of each of these positions have been measured, the values are consistent with a Φ-value near 1, which places these neighboring amino acids in the first, Φ = 0.93 block. M144, next to K145 in sequence, was measured to have a Φ-value of 0.84 ± 0.05. Mukhtasimova et al. (2005) found that substitution of A, Q, and E side chains at αK145 all reduce Keq substantially and that the effect of αK145E and αD200N mutations are not energetically independent, and proposed that interactions between αK145–αD200 vs. αK145–αY190 (based on structure) stabilize the C vs. O conformation, respectively.

We have extended these studies regarding αY127 and αK145 by more extensive Φ-value analysis, and have related the results to the ECD rotation hypothesis for gating. First, we measured rate constants from single-channel currents and estimated Φ for αY127 (all 20 natural amino acid side chains) and its neighbor in the δ subunit, I43. Moreover, the effects of these perturbations were not independent, which suggests that these positions are coupled energetically and are a link for the intersubunit propagation of the gating conformational cascade.

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Figure 1. Location of αY127 in Torpedo AChRs. (A) A Cartoon of the αε/δ subunits viewed from the exterior of the AChR. Only the αε (left) and ε subunits are shown; the horizontal lines mark, approximately, the membrane. In αε the three Φ blocks that link the transmitter binding site with the gate are color coded as purple (Φ = 0.93, W149, K145), orange (Φ = 0.78, Y127), green (Φ = 0.65, S269), and red (Φ = 0.31, L251). (B and C) Expansion of boxed region in A. αK145 (purple) is on β-strand 7 and αY127 (orange) at the αε/ε (B) and αε/δ (C) subunit interface. αY127 is <4 Å from residues αD97 and αN94 in loop A (purple), αQ48 in loop 2 (orange), and εN39/δI43 in β-strand 1 (black). Structures were displayed by using PYMOL (DeLano Scientific).
these two residues do not move synchronously in 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 page 547 of this issue. In brief, mouse AChR subunits were transiently expressed in HEK 293 cells and recordings were from cell-attached patches (22°C, −100 mV membrane potential). Agonist was added to the pipette solution (500 μM ACh, 5 mM carbamylcholine, or 20 mM choline). Currents were analyzed with QUB software (www.qub.buffalo.edu). Opening and closing rate constants were estimated from 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 k₀ vs. log Kₑq. Each point in the plot represents the mean of at least three different patches.

RESULTS

Mutations of αY127 and its Homologues

In vertebrate α₁ subunits, position 127 is always a Y but in non-α₁ subunits it is never a Y (but is, rather, S, A, T, or V). A tyrosine at position 127 is a specific marker for the vertebrate neuromuscular α₁-subunit. The location of Y127 in the Torpedo AChR structure is shown in Fig. 1.

Fig. 2 and Table I show the results of single-channel kinetic analyses of wild-type AChRs plus all 19 natural amino acid substitutions at αY127. 16 of the mutations decreased Kₑq (D by ~4,900-fold) while the three aromatic

Figure 2. Example single-channel traces for 19 different side chains at αY127. (A) Continuous, low time resolution view of Y127F single-channel currents elicited by 20 mM choline (low pass filtered at 2 kHz for clarity; calibrations: 4 s, 2 pA). In the continued presence of such a high concentration of agonist, openings occur in clusters (open is down) separated by long nonconducting sojourns in “desensitized” states. Each cluster reflects C ↔ O gating of a single AChR. (B) Three gain-of-function constructs (F, W, and H) were activated by 20 mM choline and the current elicited for all other mutants were by 500 μM ACh. Example cluster for WT is shown for both the agonists. Calibration bars: (horizontal = 100 ms for choline and ACh, vertical scale bar = 2 pA, choline and 6 pA, ACh). (B) There was no apparent correlation of the side chain hydrophobicity or volume with the change in the diliganded gating equilibrium constant (Kₑq). The r values were 0.26 (hydrophobicity) and 0.28 (volume).
side chains H, W, or F increased $K_{eq}$ (F by $\sim$59-fold). There was no correlation between side chain hydrophobicity or volume and the change in $K_{eq}$. The change in $K_{eq}$ in AChRs having D vs. F at position 127 (in both α subunits) was $\sim$290,000-fold, which represents an energy difference of $\sim$7.4 kcal/mol. For comparison, the maximum fold-changes in $K_{eq}$ caused by mutations of some other α-subunit residues are shown in Table II. In our hands, Y127 is the most sensitive position ever reported for a point side chain substitution in both α subunits. The substantial changes in $K_{eq}$ indicate that the energetic consequences of the mutations are substantially different in C vs. O, which implies that αY127 changes its structure, environment, or both (i.e., moves) in the gating reaction.

The mutation-induced changes in $K_{eq}$ at αY127 arose mainly from changes in the channel opening rate constant ($k_o$). Fig. 3 shows a REFER analysis (a log–log plot of $k_o$ vs. $K_{eq}$) of the mutational series at αY127. Each $\sim$10-fold change in $K_{eq}$ arose, on average, from an $\sim$6.2-fold change in $k_o$ and an $\sim$1.6-fold change in $k_c$. The slope of this relationship, $\Phi$, was $0.77 \pm 0.02$. Notice that the results for AChRs activated by different agonists scatter about the same line and that the $\Phi$ estimate was similar regardless of whether the AChRs were activated by acetylcholine (0.85 $\pm$ 0.04), carbamylcholine (0.75 $\pm$ 0.04) or choline (0.75 $\pm$ 0.04) (Fig. 3).

The $\Phi$-value for αY127 is the same as those for several residues in loop 2 and the cys-loop ($\Phi = 0.80 \pm 0.05$ and 0.78 $\pm$ 0.03) (Jha et al., 2007) and R209 in the pre-M1 linker (0.74 $\pm$ 0.02, on an E45A background) (Purohit and Auerbach, 2007), but is different from those for the transmitter binding site (0.93 $\pm$ 0.02) (Grosman et al., 2000) and residue αD97 in loop A (0.93 $\pm$ 0.03) (Chakrapani et al., 2003). This result suggests that position 127 moves relatively early in the diliganded channel-opening process and that its gating motions are correlated temporally with other residues in the second ($\Phi = 0.78$) gating block, but that these occur after those in the first ($\Phi = 0.93$) gating block.

### Table I

**Kinetic Analyses of AChR Mutants**

| Construct | Agonist | $k_o$ (s$^{-1}$) | $k_{obs}$ (s$^{-1}$) | $k_c$ (s$^{-1}$) | $K_{eq}$ ($k_o/k_c$) | Normalized $K_{eq}$ (mut/wt) | n |
|-----------|---------|----------------|---------------------|-----------------|---------------------|-------------------------------|---|
| wt        | Cho$^a$ | 120            | –                   | 2583            | 0.046              | 1 –                           | – |
| wt        | ACh$^b$ | 48000          | –                   | 1700            | 28.2               | 1 –                           | – |
| Y127F     | Cho     | 2853 (221)     | 390 (20.3)          | 1041.3 (107)    | 2.7 (0.28)         | 58.7                          | 4 |
| Y127W     | Cho     | 1518 (170)     | 353 (122)           | 943 (254)       | 1.6 (0.4)          | 34.8                          | 2 |
| Y127H     | Cho     | 520 (68)       | 577 (86)            | 1541 (295)      | 0.33 (0.01)        | 7.2                           | 4 |
| Y127P     | ACh     | 3008            | 5498                | 6872.5          | 0.43               | 0.015                         | 1 |
| Y127L     | ACh     | 2351 (217)     | 6277 (536)          | 7846 (670)      | 0.29 (0.05)        | 0.01                          | 4 |
| Y127G     | ACh     | 2009 (95)      | 5676 (247)          | 7095 (309)      | 0.28 (0.05)        | 0.01                          | 4 |
| Y127E     | ACh     | 1166 (153)     | 3411 (306)          | 4264 (382)      | 0.27 (0.05)        | 0.01                          | 4 |
| Y127A     | ACh     | 1726 (97)      | 5447 (332)          | 6809 (416)      | 0.25 (0.03)        | 0.009                         | 3 |
| Y127Q     | ACh     | 1570 (70)      | 5126 (104)          | 6408 (130)      | 0.24 (0.01)        | 0.009                         | 5 |
| Y127N     | ACh     | 1574 (36)      | 5506 (353)          | 6883 (441)      | 0.22 (0.01)        | 0.008                         | 3 |
| Y127C     | ACh     | 862 (119)      | 4674 (248)          | 5845 (310)      | 0.14 (0.05)        | 0.005                         | 4 |
| Y127S     | ACh     | 1076 (94)      | 5953 (522)          | 7442 (653)      | 0.14 (0.03)        | 0.005                         | 2 |
| Y127M     | ACh     | 555 (101)      | 4952 (340)          | 6190 (425)      | 0.089 (0.02)       | 0.003                         | 4 |
| Y127T     | ACh     | 550 (7)        | 7619 (569)          | 9524 (711)      | 0.057 (0.04)       | 0.002                         | 4 |
| Y127R     | ACh     | 336 (25)       | 5273 (363)          | 6591 (454)      | 0.051 (0.003)      | 0.0018                        | 3 |
| Y127I     | ACh     | 208 (44)       | 5763 (533)          | 7204 (667)      | 0.028 (0.006)      | 0.001                         | 2 |
| Y127K     | ACh     | 238 (57)       | 8556 (585)          | 10690 (732)     | 0.022 (0.005)      | 0.0008                        | 3 |
| Y127V     | ACh     | 112 (4)        | 8230 (1388)         | 10288 (1374)    | 0.010 (0.001)      | 0.0004                        | 3 |
| Y127D     | ACh     | 29 (12)        | 3966 (435)          | 4958 (543)      | 0.0057 (0.002)     | 0.0002                        | 3 |
| Y127P     | CCh     | 2956            | 2386                | 5843            | 0.51               | 0.075                         | 1 |
| Y127A     | CCh     | 603 (38)       | 2600 (91)           | 6162 (517)      | 0.098 (0.01)       | 0.014                         | 3 |
| Y127E     | CCh     | 175 (21)       | 1092 (274)          | 2617 (651)      | 0.07 (0.01)        | 0.01                          | 2 |
| I49C      | ACh     | 39200 (1593)   | 1828 (116)          | 2285 (145)      | 17.2 (0.42)        | 0.6                           | 3 |
| I49Y      | ACh     | 39280 (4000)   | 3574 (316)          | 4468 (395)      | 8.8 (0.45)         | 0.3                           | 3 |

All values pertain to fully liganded AChRs. $k_o$, apparent opening rate constant; $k_{obs}$, observed closing rate constant; $k_c$, closing rate constant corrected for channel block; $K_{eq}$, diliganded gating equilibrium constant; normalized $K_{eq}$ (divided by the wt value for the salient agonist); n, number of patches. The mutant/wt ratio is with regard to the diliganded gating equilibrium constant.

$^a$From Mitra et al. (2005).

$^b$From Chakrapani and Auerbach (2005).
We measured the single-site association and dissociation rate constants (k$_+$ and k$_-$) and equilibrium dissociation constant (k$_+$/k$_-$ = K$_d$) for ACh binding to the closed conformation in one mutant construct, Y127C (Fig. 4). In this mutant K$_d$ = 144 μM, which is in the range of previous measurements for wild-type AChRs exposed to 140 mM NaCl (100–150 μM) (Akk and Auerbach, 1996; Chakrapani et al., 2003). Similarly, the association and dissociation rate constants in the mutant, k$_+$ = 2.108 M$^{-1}$s$^{-1}$ and k$_-$ = 3.0 × 10$^4$ s$^{-1}$, were similar to wt values.

We also probed the effects on gating of mutations to residues in the β, ε, and δ subunits that are homologous to αY127. In the non-α subunits, which are homologous in both sequence and structure to the α subunits in the vicinity of αY127, the residue in question (βS127, εT127, or δS129) immediately preceded in sequence the extracellular disulfide bond. Seven mutations of these three positions all yielded AChRs having wt-like gating behaviors (Table IV).

αI49, δI43, and εN39

We next examined the gating properties of AChRs having mutations of residues that are close to αY127 (Fig. 1 B). αI49 is at the N terminus of β-strand 2, ~5 Å from αY127. The gating kinetics for three mutants of this position, C, V, and Y, did not change K$_d$ by greater than threefold (Table I). Thus, we have no evidence that the αI49 side chain moves relative to its local environment between C and O conformations.

εN39 or δI43 are neighbors of αY127 in the companion, non–α subunit. A REFER analysis of position δI43 is shown in Fig. 5. All four of the tested substitutions decreased K$_{eq}$, with Φ = 0.86 ± 0.10. Although this result indicates that δI43 moves early in the reaction, we are unable to distinguish this Φ-value from those of the first (0.93; agonist and loops A, B, and C) and second (0.77; Y127, loop 2, and cys-loop) blocks of the α-subunit. At εN39, F and D substitutions caused a small (less than threefold) change in K$_{eq}$, and the substitution of an Ile at this position also generated currents having wt-like kinetic behavior (when activated by 30 μM ACh). The substitution of an H increased the cluster open probability relative to the wt, but the kinetics of these intracluster intervals was complex, with at least two conducting and two nonconducting states apparent. Therefore, unambiguous values of k$_+$ and k$_-$ could not be estimated. These results suggest that εN39 moves during gating, but we were unable to estimate a Φ-value for this position.

Coupling of αY127 Gating Motions within and between Subunits

In the α-subunit, two residues in loop A, part of which contributes to the transmitter binding site, may be close to αY127: αD97 and αN94. Mutation of αD97 causes a substantial change in K$_{eq}$ and has a Φ-value that is different from that of αY127 (0.93 vs. 0.77). We therefore tested whether an interaction between αY127 and αD97 couples the gating motions (energy transfer) between the transmitter binding site (in the first Φ-block) and the cys-loop (in the second Φ-block).

We probed a D97↔Y127 interaction by measuring the gating kinetics of AChRs having a mutation (in both

### TABLE II

| Constructs | Location    | Fold-change | ΔΔG (kcal/mol) | Reference          |
|------------|-------------|-------------|----------------|--------------------|
| Y93W       | Loop A      | 129         | 2.9            | Akk et al., 1999   |
| D97A       | Loop A      | 167         | 3.0            | Chakrapani et al., 2003 |
| V46E       | Loop 2      | 208         | 3.1            | Chakrapani et al., 2003 |
| V46A       | Loop 2      | 474         | 3.6            | Lee and Sine, 2005  |
| E45K       | Loop 2      | 120         | 2.8            | Lee and Sine, 2005  |
| E45H       | Loop 2      | 2,170       | 4.5            | Purohit and Auerbach, 2007 |
| S269I      | M2–M3 linker| 115         | 2.8            | Mitra et al., 2005  |
| I274T      | M2–M3 linker| 2,014       | 4.4            | Jha et al., 2007    |
| P272G      | M2–M3 linker| 159         | 3.0            | Lee and Sine, 2005  |
| V132F      | Cys-loop    | 2,820       | 4.5            | Jha et al., 2007    |
| F135A      | Cys-loop    | 3,125       | 4.7            | Chakrapani et al., 2003 |
| Q140A      | Cys-loop    | 658         | 3.8            | Chakrapani et al., 2003 |
| D200N adult| β10-strand  | 368         | 5.5            | Akk et al., 1996    |
| D200N embryonic | β10-strand | 1,283       | 4.2            | Akk et al., 1996    |
| R209Q      | Pre-M1      | 46          | 2.3            | Lee and Sine, 2005  |
| εI121L     | ββ-β' strand| 290         | 3.3            | Ohno et al., 1996   |
| εD175N     | ββ-β' strand| 79          | 2.6            | Akk et al., 1999    |
| εE184D     | ββ-β' strand| 56          | 2.4            | Akk et al., 1999    |

Fold-change in gating equilibrium constants are from the literature and for diliganded gating using single channel analysis. ΔΔG = −RT ln(K$_{eq}^{wt}$/K$_{eq}^{mut}$).
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α subunits) at both of these positions (Table III). Six pairwise combinations were tested, with two different side chains at Y127 (H and C) and three different side chains at D97 (M, Y, and H). By themselves, the mutations at position 127 either reduced $K_{eq}$ (C, by 201-fold) or increased $K_{eq}$ (H, by 7.3-fold), while those at position 97 always increased $K_{eq}$ (M, Y, or H, by 5.5-, 20-, and 7.3-fold, respectively). The hallmark of energetic coupling between $\alpha Y127$ and $\alpha D97$ is a fold-change in $K_{eq}$ with both sites mutated that is not equal to the product of the fold-changes for each site mutated.

With Y127H (activated by choline), the observed values of $K_{eq}$ for the three D97 mutants were, on average, modestly (~2.5-fold) smaller than predicted assuming independence (Table III). With the Y127C constructs (activated by ACh), the observed values of $K_{eq}$ for the three D97 mutants were close to those predicted assuming independence. The average coupling energy was 0.53 kcal/mol for the Y127H background and $-0.37$ kcal/mol for the Y127C background. These results suggest that the magnitude of the coupling energy can vary with the side chain substitution. However, the coupling energy was small for both of the two tested backgrounds, especially when one considers that this coupling energy is spread between two Y127–D97 pairs (two $\alpha$ subunits).

Overall, the results suggest that although a large magnitude of energy change is associated with positions D97 and Y127 when examined individually, a D97 ↔ Y127 perturbation in combination is not an important component of energy transfer within the transition state of diliganded gating. The assumption that the residues may be interacting at the Φ-block boundaries, however, is based on the proximity of the two residues in the Torpedo AchR structure. Two problems with this assumption are that Y127 and D97 are >9 Å apart in the mouse $\alpha$-subunit fragment structure, and that neither structure reflects a ligand-bound AchR. There is a reason to suspect that

Figure 3. REFER analyses for $\alpha Y127$. Each point represents the mean of greater than two patches (Table I). Φ-Value was estimated as the slope of an unweighted linear fit to a log-log plot of normalized $k_{on}$ vs. normalized $K_{eq}$ for all 19 mutants. The slope $\Phi = 0.77 \pm 0.02$ makes $\alpha Y127$ a member of the second Φ-block that includes the cys-loop and loop 2. The open circles, filled circles, and open squares are choline, ACh, and carbamylcholine data points, respectively.

Figure 4. The mutation $\alpha Y127C$ does not alter the closed-channel equilibrium dissociation constant. Left, open and closed interval duration histograms at different ACh concentrations. The solid lines are calculated from the globally optimized rate constants. Number of events analyzed at various concentrations of ACh were: 100 μM, 3391; 300 μM, 2244; and 500 μM, 6940. Right, example clusters from each concentration. The optimal rate constants were: $k_+$(single-site association) = 205 μM s$^{-1}$, $k_-$ (single-site dissociation) = 29604 s$^{-1}$, $k_o = 2089$ s$^{-1}$, and $k_c = 5032$ s$^{-1}$. We calculate $K_d (k_-/k_+) = 144$ μM for the mutant. For comparison, the wt estimates are $k_+ = 167$ μM s$^{-1}$ and $k_- = 24,745$ s$^{-1}$, $K_0 = 148$ μM (Chakrapani and Auerbach, 2005). There is no significant effect of this mutation on ACh binding to closed AchRs and we speculate that $\alpha Y127$ mutations that change $K_{eq}$ do so by changing the unliganded gating equilibrium constant rather than the closed/open affinity ratio. Calibration bars for single channel traces: (horizontal scale bar = 100 ms, vertical scale bar = 6 pA).
loop A moves as a consequence of agonist binding (in addition to channel gating), so we do not know the separation between these residues in fully liganded AChRs.

We next measured the extent of coupling between $\alpha$Y127H (7.3-fold increase in $K_{eq}$) and $\delta$I43H (13.8-fold decrease). Together, these mutations caused a 2.2-fold increase in $K_{eq}$, whereas if they were independent we would expect a 1.9-fold decrease in $K_{eq}$. This approximately fourfold effect indicates that there is modest degree of coupling between the $\alpha$Y127 and $\delta$I43 side chains (+0.84 kcal/mol; Table IV). Note that this interaction occurs at a single subunit interface and should therefore be considered to be substantially greater than the $\alpha$Y127–$\alpha$D97 interaction.

$\alpha$K145

We measured the gating rate constants for four different mutations of $\alpha$K145, which is on $\beta$-strand 6 (Fig. 1). In the unliganded Torpedo structure, this residue is within 4 Å $\alpha$D200 and loop A residue $\alpha$Y93, two residues that have been shown to move during diliganded C-O gating. K145 is also likely to be close to moving-residue $\alpha$Y190 (Chen et al., 1995) when the transmitter binding site is occupied by an agonist (Celie et al., 2004). Finally, $\alpha$K145 is near $\alpha$T202, a residue that has not yet been probed at the rate constant level.

All four of the mutations of K145 (C, A, R, and D) decreased $K_{eq}$, by up to 282-fold (Table V). The causes of these decreases were, in all cases, almost exclusively due to decreases in $k_o$. Fig. 6 shows the REFER for $\alpha$K145. The $\Phi$-value was 0.96 ± 0.04.

**DISCUSSION**

Comparison with Previous Results

Mukhtasimova and Sine (2007) studied the kinetic behavior of two $\alpha$Y127 mutants (F and T) plus $\varepsilon$N39A and $\delta$N41A. Further, they measured the coupling between three pairs and two triplet combinations of these mutants. Although they studied human AChRs activated by ACh in 142 mM KCl and we studied mouse AChRs activated by ACh or choline in 140 mM NaCl, both sets of results are in general agreement. Mutations to Y127 have a profound effect on channel gating ($K_{eq}$), and this residue is a site where gating motions are coupled between subunits.

The main difference in the two sets of results is in relation to the $\alpha$Y127F mutation. We measured a much larger increase in $K_{eq}$ for Y127F (58.7-fold vs. 2.2-fold increase). We speculate that this difference can be traced to an immeasurably fast opening rate constant for this construct in the experiments where the mutant AChRs were activated by ACh. In wt AChRs the difference in

| Construct | Agonist | $k_o$ (s$^{-1}$) | $k_{o,obs}$ (s$^{-1}$) | $k_{o,corr}$ (s$^{-1}$) | $K_{eq}$ ($k_o/k_{corr}$) | Normalized $K_{eq}$ (mut/wt) | $\Delta\Delta G$ | $n$ |
|-----------|---------|----------------|------------------------|--------------------------|-----------------------------|-----------------------------|-------------------|-----|
| wt Cho    | 120     | –              | 2583                   | 0.046                    | 1.0                         | –                           | –                 | –   |
| wt ACh    | 48000   | –              | 1700                   | 28.2                     | 1.0                         | –                           | –                 | –   |
| D97H      | Cho     | 1364 (19)      | 480                    | 1282 (80)                | 1.06 (0.05)                 | 23                          | 2                 | –   |
| D97Y      | Cho     | 1420 (29)      | 563                    | 1503 (48)                | 0.95 (0.03)                 | 20.5                        | 3                 | –   |
| D97M      | Cho     | 462            | 680                    | 1816                     | 0.25                        | 5.5                         | –                 | 1   |
| V127H     | Cho     | 520 (68)       | 577 (88)               | 1541 (295)               | 0.33 (0.01)                 | 7.3                         | –                 | 4   |
| V127C     | ACh     | 862 (119)      | 4674 (248)             | 5843 (310)               | 0.14 (0.03)                 | 0.005                       | –                 | 4   |
| V127H+D97H| Cho     | 5066 (166)     | 649 (66)               | 1734 (176)               | 2.98 (0.3)                  | 65                          | 167.9             | 0.55 |
| V127H+D97Y| Cho     | 6033 (540)     | 904 (19)               | 2414 (52)                | 2.5 (0.3)                   | 54                          | 150               | 0.61 |
| V127H+D97M| ACh     | 3338 (228)     | 1437 (234)             | 3837 (624)               | 0.93 (0.19)                 | 18.9                        | 40.2              | 0.44 |
| V127C+D97H| ACh     | 13400 (1249)   | 2694 (181)             | 3568 (226)               | 4.00 (0.64)                 | 0.14                        | 0.04              | −0.80|
| V127C+D97Y| ACh     | 16220 (204)    | 4601 (111)             | 5751 (159)               | 2.82 (0.03)                 | 0.1                         | 0.1               | 0.01 |
| δI43N     | ACh     | 5935           | 1472                   | 1840                     | 2.93                        | 0.1                         | –                 | 1   |
| δI43H     | ACh     | 3820 (227)     | 1546 (159)             | 1932 (198)               | 2.04 (0.31)                 | 0.07                        | –                 | 3   |
| δI43T     | ACh     | 4778 (278)     | 2768 (160)             | 3460 (200)               | 1.4 (0.16)                  | 0.05                        | –                 | 3   |
| δI43A     | ACh     | 2424 (266)     | 1948 (458)             | 2434 (572)               | 1.1 (0.36)                  | 0.04                        | –                 | 2   |
| αY127H+δI43H| Cho | 530 (33)       | 1980 (38)              | 5285 (100)               | 0.1 (0.004)                 | 2.16                        | 0.5               | 0.84 |
| εN39F     | ACh     | 27776          | 2210                   | 2763                     | 10.1                        | 0.4                         | –                 | 1   |
| εN39D     | Cho     | 40 (2)         | 882 (87)               | 2554 (231)               | 0.017 (0.001)               | 0.4                         | –                 | 3   |
| εN39H     | Cho     | ND             | ND                     | ND                       | ND                          | ND                          | ND                | ND   |
| εN39I     | ACh     | ND             | ND                     | ND                       | ND                          | ND                          | ND                | ND   |

Mutations of $\alpha$D97 (M, H, and Y) on V127H or V127C constructs generally showed a fold-change in $K_{eq}$ approximately half that predicted from the product of the single-mutant fold-change. The coupling energies for both double mutant series are small, suggesting that the coupling energy is distributed across multiple sites along between the first and second $\Phi$ blocks.
K<sub>eq</sub> for different agonists is manifest almost exclusively as a difference in the opening rate constant (Φ = 0.93; Grosman et al., 2000). Assuming that this pattern pertains to the Y127F mutant, then k<sub>o</sub> with ACh should be ~400 times larger than k<sub>o</sub> with choline (Chakrapani and Auerbach, 2005). In this case, our measurement for k<sub>o</sub> with choline (2853 s<sup>-1</sup>) translates to an opening rate of k<sub>o</sub> with ACh of >10<sup>6</sup> s<sup>-1</sup>, which is too fast to be detected experimentally. Perhaps the brief gaps observed in the experiments with human AChRs (Fig. 2 and Table II in MS) did not arise from C↔O gating but rather from channel block by the agonist or some other process.

Our results do not agree with the proposal that aromatic side chains can be substituted at position α<sub>Y127</sub> without consequence. Mukhtasimova et al. (2005) also measured the gating rate constants for E, Q, and A mutants of α<sub>K145</sub>. They report that these mutations decrease k<sub>o</sub> but leave k<sub>c</sub> essentially unchanged is consistent with our estimated Φ value of 0.96 for this position.

Structure–Function

A D-to-F side chain substitution at α<sub>Y127</sub> changes Keq by nearly ~290,000-fold. The magnitude of this change is substantially greater than that caused by any other ECD side chain substitution observed so far, even considering the fact that both α subunits carried the mutation. (The change in Keq would be ~540-fold if the energy difference between C and O was equally distributed between the two α subunits).

The relationship between a change in structure and the magnitude of the change in Keq is complex. Although we measured Keq for all 20 natural side chains at α<sub>Y127</sub> and for four side chains at α<sub>K145</sub>, we are nonetheless unable to draw strong conclusions about the chemical natures of the forces behind the αY127 gating motions. We note, however, that the mutations of α<sub>Y127</sub> that increased Keq are aromatic and flat. There is no apparent correlation between side chain volume or hydrophobicity and the magnitude of the change in Keq. Also, the charged side chains D, K, R, and E all reduced Keq at α<sub>Y127</sub> (by 4847-, 1282-, 553-, and 104-fold, respectively), and D and R reduced Keq at α<sub>K145</sub> (by 282- and 60-fold, respectively), so the sign of the charge at both of these positions appears not to be an important determinant of Keq.

The gating motion of α<sub>K145</sub> (as evidenced by the mutation-induced change in Keq) occurs approximately synchronously (same Φ-value) as other residues near the

**Table IV**

| Construct | Agonist | k<sub>o</sub> (s<sup>-1</sup>) | k<sub>o</sub><sup>obs</sup> (s<sup>-1</sup>) | k<sub>c</sub><sup>obs</sup> (s<sup>-1</sup>) | K<sub>eq</sub> (k<sub>o</sub>/k<sub>c</sub>) | Normalized K<sub>eq</sub> (mut/wt) | n |
|-----------|---------|-----------------|-----------------|-----------------|----------------|----------------|---|
| βS127A    | Cho     | 53              | 1296            | 3460.3          | 0.015          | 3.1            | 1  |
| βS127V    | Cho     | 108             | 1305            | 3484.3          | 0.030          | 1.5            | 1  |
| βS127Y    | Cho     | 70              | 850             | 2269.5          | 0.030          | 1.5            | 1  |
| δS129Y    | ACh     | 23253           | 1874            | 2342.5          | 9.92           | 0.35           | 1  |
| εT127A    | Cho     | 138             | 1484            | 3962.3          | 0.034          | 0.74           | 1  |
| εT127V    | Cho     | 52              | 420             | 1121.4          | 0.046          | 1.0            | 1  |
| εT127Y    | ACh     | 30370           | 2550            | 3187.5          | 9.52           | 0.34           | 1  |

In β, δ, or ε subunit, none of the mutants at residues homologous to Y127 show fold-change in Keq greater than threefold. These residues may not be moving during AChR gating. The abbreviations used here are the same as indicated earlier.
transmitter binding site, in loops A, B, and C. The movement of $\alpha_{K145}$ is correlated temporally with the movement of its close neighbors $\alpha_{D200}$ and $\alpha_{Y93}$. The movement of $\alpha_{Y127}$ occurs after the movement of $\alpha_{K145}$, and approximately synchronously with residues in the cys-loop and loop 2.

**Rotation Hypothesis**

The mutation-induced changes in $K_{eq}$ at positions $\alpha_{K145}$ and $\alpha_{Y127}$ are consistent with the proposal that gating entails a rotation of the $\alpha$-subunit $\beta$-sandwich core (Unwin et al., 2002). However, some observations of AChR function appear to be inconsistent with this hypothesis. (a) A substituted cysteine accessibility study of residues between L36 and I53 in strands $\beta_1$ and $\beta_2$ in the $\alpha_7$ AChR showed that the rates of reaction with MTSEA in the presence of ACh varied significantly (McLaughlin et al., 2007). However, the rate of reaction decreased and increased, respectively, for the closely apposed residues M40 and N52, a result that is unexpected for a rigid body rotation of the $\beta$-core. (b) The effects of mutations on $K_{eq}$ have been measured for seven different residues that are in the inner $\beta$ strands of the ECD core: $\alpha_{L40A}$ (in strand 1), $\alpha_{I49C}$, V, and Y, $\alpha_{V54L}$, $\alpha_{R55A}$ and W (in strand 2), and $\alpha_{A122L}$, $\alpha_{S126V}$ and A, and $\alpha_{Y127}$ (in strand 6). Of these constructs, only the $\alpha_{Y127}$ mutants changed $K_{eq}$ by greater than threefold and, hence, gave a clear indication of motion. Although the lack of change in $K_{eq}$ does not unequivocally indicate a lack of gating motion, it would be surprising if a rotation altered the energetic environment only around $\alpha_{Y127}$. More residues (Celie et al., 2004) and mutations in both the inner and outer leaflets of the $\beta$-core need to be tested to test the energetic consequences of such a rotation. (c) The asynchrony of motion (different $\Phi$ values) for $\alpha_{Y127}$ and $\alpha_{K145}$ is unexpected if the $\beta$-core rotation was that of a rigid body motion. In summary, the results suggest that the hypothesis of a $\beta$-sandwich core rotation in the gating reaction is, at best, incomplete.

**$\Phi$ Map**

Fig. 7 shows the map of $\Phi$ superimposed on the mouse $\alpha$-subunit fragment structure (2qc1.pdb, Dellisanti et al., 2007). The $\Phi$ values for the purple residues are $\sim$0.93, those for the orange residues are $\sim$0.77, and the white residues show no indication of a gating motion ($\Delta K_{eq} <$ threefold). This pattern suggests that the diliganded gating motions in the $\alpha$-subunit mainly propagate along the $\alpha$-$\epsilon$ (or $\alpha$-$\delta$) subunit interface.

$\Phi$ changes significantly (by $\sim$0.16 units) between $\alpha_{D97}$ and $\alpha_{Y127}$ (which are within 4 Å in 2bg9.pdb and 9 Å in 2qc1.pdb), whereas $\Phi$ is the same for residues that

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**Table V**

| Construct | Agonist | $k_0$ ($s^{-1}$) | $k_{obs}$ ($s^{-1}$) | $k_{max}$ ($s^{-1}$) | $K_{eq}$ ($k_0/k_{max}$) | Normalized $K_{eq}$ (mut/wt) |
|-----------|---------|-----------------|-----------------|------------------|----------------|-----------------------------|
| K145C     | ACh     | 2030 (50)       | 1200 (126)      | 1500 (157)       | 1.38 (0.14)   | 0.05                        | 3                           |
| K145A     | ACh     | 1111 (102)      | 1585 (126)      | 1732 (157)       | 0.65 (0.08)   | 0.02                        | 3                           |
| K145R     | ACh     | 1061 (13)       | 1808 (85)       | 2260 (106)       | 0.47 (0.02)   | 0.016                       | 3                           |
| K145D     | ACh     | 210 (42)        | 1603 (4)        | 2003 (002)       | 0.10 (0.02)   | 0.004                       | 2                           |

All the mutants tested at position K145 produced loss of function constructs. The abbreviations used here are the same as indicated earlier.
αD97 even though these side chains are close, are mutation-sensitive, and have different Φ values (Fig. 1). It is therefore unlikely that an interaction between these two residues is an important link in the propagation of the AChR gating conformational wave.

Mukhtasimova and Sine (2007) found large coupling coefficients between the intersubunit pairs αY127T/εN39A (1.7 kcal/mol) and αY127T/εN41A (3.8 kcal/mol). Our estimate of coupling for the αY127H/εI43H pair was somewhat smaller (0.84 kcal/mol) but still larger than for the αY127/εD97 pair. Our results support the idea that αY127 is a site where the gating conformational cascade in the α-subunit is linked to that in the δ or ε subunits. The Φ value of εI43 (0.86 ± 0.10) cannot be distinguished from those of either αD97 (0.93 ± 0.01) or αY127 (0.77 ± 0.02). Thus, we are unable to use Φ-value analysis to determine if the δ-subunit motions are synchronous with those of α, or if not, which subunit precedes the other.

The Framework for AChR Gating

The results presented here and in the two companion papers support the idea that the framework for understanding the mechanism of diliganded AChR gating is that it is “brownian conformational wave.” All of the 29 newly probed positions have Φ values that are similar to those previously reported for other amino acids in the extracellular region of the AChR α-subunit, and with magnitudes as expected based on location. There is little doubt that in the AChR, the map of Φ is highly organized and that residues are clustered into Φ blocks. Whatever mechanisms are proposed for AChR gating, and whatever physical interpretation is applied to Φ (relative timing, fractional side chain structure, multiple pathways), these must account for this highly ordered map of Φ values that has been derived from an extensive array of experiments.

The results do not support the notion that there is a single, rate-limiting structural transition that is the intersection of the C and O conformational ensembles. If there is a rotation of the α-subunit β-core, it is unlikely to be as a rigid body because αK145 on the outer sheet and αY127 on the inner sheet belong to two different Φ blocks. Although R209 and E45 both move and make a substantial energy contribution to the TR, these energy changes apparently do not arise from the perturbation of a salt bridge between this pair. The movement of the M2–M3 linker is an important TR event, but a full, cis-trans isomerization of the P272 or G275 backbone is not necessary for efficient gating. Rotations, electrostatic forces, changes in backbone bond angles, and hydrophobic interaction may occur in various regions of the protein, but each of these structural transitions contributes only a fraction to the total energy to the TR barrier.

Rather than conceiving of the energy barrier separating C from O as the point intersection of two parabolas,
the experimental results suggest that this TR barrier is a broad, corrugated, flat plateau (Auerbach, 2005). The map and range of \( \Phi \) values, the spatially distributed effects of mutations on \( K_{eq} \), and the rather weak coupling energies that we have observed between specific pairs of moving residues all suggest that the barrier for diliganded gating arises from the motions of many different metastable intermediate structures that are separated, sequentially, by small energy barriers. This energy distribution is certainly not isotropic, because some moving residues make larger energy contributions than others.

Several important regions of the AChR have not yet been mapped for \( \Phi \), including most of M1, the upper half of M2, and some regions of the ECD in the \( \alpha \)-subunit, and many regions of the non-\( \alpha \) subunits. This map of the TR, along with high resolution structures of the diliganded C and O end state ensembles, should serve as a guide for understanding the details of the structural transitions that constitute AChR gating.

We would like to thank Mary Merritt and Mary Teeling for technical assistance.

Olaf S. Andersen served as editor.

Submitted: 17 July 2007
Accepted: 8 November 2007

REFERENCES

Akk, G. 2001. Aromatics at the murine nicotinic receptor agonist binding site: mutational analysis of the \( \alpha Y93 \) and \( \alpha W149 \) residues. J. Physiol. 535:729–740.

Akk, G., and A. Auerbach. 1996. Inorganic, monovalent cations compete with agonists for the transmitter binding site of nicotinic acetylcholine receptors. Biochim. Biophys. Acta 1280:243–255.

Akk, G., S. Sine, and A. Auerbach. 1998. Binding sites contribute unequally to the gating of mouse nicotinic \( \alpha 2 \beta 0 \)N acetylcholine receptors. J. Physiol. 496:185–196.

Akk, G., M. Zhou, and A. Auerbach. 1999. A mutational analysis of the acetylcholine receptor channel transmitter binding site. Biochim. Biophys. Acta 1408–1412.

Auerbach, A. 2003. Gating of acetylcholine receptor channels: Brownian motion across a broad transition state. Proc. Natl. Acad. Sci. USA. 100:245–247.

Auerbach, A. 2007. Acetylcholine receptor channel gating at extracellular-transmembrane domain interface: the cyst-loop and M2–M3 linker. J. Gen. Physiol. 130:547–558.

Lee, W., and S.M. Sine. 2005. Principal pathway coupling agonist binding to channel gating in nicotinic receptors. Nature, 438:245–247.

McLaughlin, J.T., J. Fu, and R.L. Rosenberg. 2007. Agonist-driven conformational changes in the inner \( \beta \)-sheet of \( \alpha 7 \) nicotinic receptors. Mol. Pharmacol. 71:1312–1318.

Mitra, A., G.D. Cymes, and A. Auerbach. 2005. Dynamics of the acetylcholine receptor pore at the gating transition state. Proc. Natl. Acad. Sci. USA. 102:13506–13507.

Mukhtasimova, N., C. Free, and S.M. Sine. 2005. Initial coupling of binding to gating mediated by conserved residues in the muscle nicotinic receptor. J. Gen. Physiol. 126:25–39.

Mukhtasimova, N., and S.M. Sine. 2007. An intersubunit trigger of channel gating in the muscle nicotinic receptor. J. Neurosci. 27:4110–4119.

Ohno, K., H.-L. Wang, M. Milone, N. Bren, J.M. Brengman, S. Nakano, P. Quiram, J.N. Pruitt, S.M. Sine, and A.G. Engel. 1996. Congenital myasthenic syndrome caused by decreased agonist binding affinity due to a mutation in the acetylcholine receptor \( \alpha 1 \) subunit. Neuron, 17:157–170.

Purohit, P., and A. Auerbach. 2007. Acetylcholine receptor gating at extracellular-transmembrane domain interface: the "pre-M1" linker. J. Gen. Physiol. 130:559–568.

Qin, P., A. Auerbach, and F. Sachs. 1997. Maximum likelihood estimation of aggregated Markov processes. Proc. Biol. Sci. 264:375–383.

Unwin, N. 2005. Refined structure of the nicotinic acetylcholine receptor at 4 Å resolution. J. Mol. Biol. 346:967–989.

Unwin, N., A. Miyazawa, J. Li, and Y. Fujisaki. 2002. Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the \( \alpha \) subunits. J. Mol. Biol. 319:1165–1176.