Gating at the Mouth of the Acetylcholine Receptor Channel: Energetic Consequences of Mutations in the αM2-Cap

Pallavi A. Bafna, Prasad G. Purohit, Anthony Auerbach*

Department of Biophysics and Physiology, State University of New York at Buffalo, Buffalo, New York, United States of America

Abstract
Gating of nicotinic acetylcholine receptors from a C(losed) to an O(open) conformation is the initial event in the postsynaptic signaling cascade at the vertebrate nerve-muscle junction. Studies of receptor structure and function show that many residues in this large, five-subunit membrane protein contribute to the energy difference between C and O. Of special interest are amino acids located at the two transmitter binding sites and in the narrow region of the channel, where C→O gating motions generate a low←→high change in the affinity for agonists and in the ionic conductance, respectively. We have measured the energy changes and relative timing of gating movements for residues that lie between these two locations, in the C-terminus of the pore-lining M2 helix of the α subunit (‘αM2-cap’). This region contains a binding site for non-competitive inhibitors and a charged ring that influences the conductance of the open pore. αM2-cap mutations have large effects on gating but much smaller effects on agonist binding, channel conductance, channel block and desensitization. Three αM2-cap residues (β260, β265 and α268) appear to move at the outset of channel-opening, about at the same time as those at the transmitter binding site. The results suggest that the αM2-cap changes its secondary structure to link gating motions in the extracellular domain with those in the channel that regulate ionic conductance.

Introduction
In the acetylcholine receptor-channel (AChR), the M2-cap lies at the junction of the extracellular vestibule and the narrow region of the ion permeation pathway (Fig. 1). In the muscle α subunit, the αM2-cap sequence is IVELIPSTSSA (residues 260–270; Table 1). There is a 4 Å cryo-EM structure of closed and unliganded Torpedo AChRs [1], a 1.94 Å resolution x-ray structure of a toxin-bound fragment of the mouse α subunit [2], and a 3.3 Å resolution structure of a prokaryotic member of the pentameric, ligand-gated channel superfamily [3]. However, as yet there are no high resolution structures of an intact AChR in either end state of the fully-ligated gating reaction, A2C or A2O (where A is the agonist). Here we report the channel opening (k o) and closing (k c) rate constants for 64 different mutations of nine αM2-cap residues in the mouse neuromuscular AChR (αM2-αS268), as well as the effects of these mutations on channel conductance, channel blockade and an approximate rate constant for entry into long-lived desensitized states.

Estimates of the energetic consequences of individual side chain movements can be gained from measuring mutation-induced changes in the diliganded gating equilibrium constant (K eq(C→O)) which is the ratio k o/k c. K eq depends on the difference in free energy between the entire protein in the C vs. O conformation. Therefore, a change in K eq consequent to a mutation indicates that the perturbation caused the AChR to change this free energy difference, and, hence, the relative structure or dynamics (entropy) in the vicinity of the mutation, in the A2C→A2O reaction. The extent to which a change in K eq is determined by a change in k o vs. k c (given by the parameter Φ) may reflect mutation-induced changes in the transmission coefficient of the reaction [4], in which case Φ is a measure of the relative time within the reaction when the perturbed side chain flips from a C-like to an O-like conformation [5,6].

The information regarding changes in energy and the transmission coefficient (K eq and Φ, respectively) can be mapped onto the available structures to generate a framework for understanding AChR gating. These parameters (derived from experimental measurements of k o and k c) have been estimated for dozens of residues (hundreds of mutations) in the adult form of the mouse neuromuscular AChR. At most positions, at least one side chain substitution causes a substantial change in K eq with the majority of these sensitive sites residing in the α subunit and falling between the transmitter binding site (TBS) and the cytoplasmic limit of the transmembrane domain (TMD). In the extracellular domain (ECD) of the α subunit, the ‘moving’ residues are located mainly along the “+4” side of the subunit interface (adjacent to the δ or ε subunit) and are involved in a transmembrane domain (TMD) in the extracellular domain (ECD) of the α subunit and the cytoplasmic limit of the transmembrane domain (TMD). In the extracellular domain (ECD) of the α subunit, the ‘moving’ residues are located mainly along the “+4” side of the subunit interface (adjacent to the δ or ε subunit) and as throughout the interface with the TMD. In the TMD of the α subunit, at least one residue in all four membrane spanning helices is mutation-sensitive, including most of those in M2. These results suggest that the energy changes realized in gating are widespread, with no one structural transition standing out as being the single ‘on-off switch’ that separates A2C from A2O. With regard to Φ, values are clustered into domains that, as a first
approximation, follow a coarse-grained and decreasing gradient along the long axis of the protein. This pattern suggests that the overall framework for the gating mechanism is that of an approximately linear sequence of stochastic domain motions (a ‘Brownian conformational wave’) that connects structural changes that regulate transmitter affinity with those that regulate conductance [7]. However, as described below, the timing of the αM2-cap gating motions do not neatly fit this pattern.

The M2-cap contains a high affinity binding site for non-competitive inhibitors (NCIs) that stabilize D (esensitized) conformations of the AChR, where the affinity for agonists is high (like in O) but the conductance of the channel is essentially zero (like in C) [8,9]. Some NCIs have a high affinity specifically for D AChRs, while others may also act as traditional channel blockers that bind to the open pore [10,11,12]. A second function of the M2-cap is to regulate ionic conductance. All cyst-loop receptors have a charged residue (opposite sign of the conducting ion) in the M2-cap domain (189) which corresponds to a non-α subunit in AChRs. Forman et al. [19] studied mutants of αE262 by using a combination of photo-modification (by 3-azioctanol) and fast patch perfusion. Most constructs decreased the EC_{50} for Ach, possibly by increasing K_{eq}.

The results presented below show that αM2-cap residues have higher Φ-values than do the flanking residues in αM2, the αM2-M3 linker and loop 2. This pattern is discussed with respect to the overall framework for AChR gating and the conformational changes occurring at the mouth of the channel in the gating isomerization.

**Results**

For alignment purposes, the amino acids of the entire M2 helix can be numbered sequentially from N- to C-terminus (intracellular-to extracellular, 1’–28; M243–A270 in α subunit). Table 1 shows an alignment for the M2-cap (189–28) for all mouse AChR subunits plus representative subunits of other ‘Cys-loop’ receptors.

Several AChR αM2-cap amino acids have previously been studied with respect to the effects of mutation on the kinetics of gating [17]. Mutations at positions α267–α269 significantly changed K_{eq} (indicating a gating motion) mainly by changing the channel-opening rate constant, but had little or no effect on the equilibrium dissociation constant for agonist binding to the G conformation (K_{G}). Φ and changes in K_{eq} and K_{G} have also been estimated for the M2-M3 linker (α270–α276) [18]. Forman et al. [19] studied mutants of αE262 by using a combination of photo-modification (by 3-azioctanol) and fast patch perfusion. Most constructs decreased the EC_{50} for Ach, possibly by increasing K_{eq}.

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Table 1. M2-cap Sequence Alignment of Cys-loop receptors.

| Residue | Domain | n mutants | $\Phi$ | Fold-change in $K_{eq}$ |
|---------|--------|-----------|-------|-----------------------|
| AChR α1 | V       | 1         | 0.25  | -5.06                 |
| AChR α2 | V       | 2         | 0.50  | -0.07                 |
| AChR α3 | V       | 3         | 0.75  | -0.06                 |
| AChR α4 | V       | 4         | 0.50  | -0.08                 |
| AChR α5 | V       | 5         | 0.75  | -0.06                 |

The entire sequence for the AChR α1 subunit (VELIPSTSSA) is conserved in all vertebrates. Position 23 is a proline (bold) in all cys-loop receptors. The superscripts on the conserved Pro represent the residue number.

Table 2. $K_{eq}$ and $\Phi$ for M2 and the M2-M3 linker.

| Residue | Domain | n mutants | $\Phi$ | Fold-change in $K_{eq}$ |
|---------|--------|-----------|-------|-----------------------|
| T244 (2') | M2 (filter) | 3 | - | - |
| L245 (3') | M2 | 2 | 0.58 | 0.03 |
| S246 (4') | M2 | 7 | 0.67 | 0.17 |
| L247 (5') | M2 | 3 | - | - |
| S248 (6') | M2 | 2 | 0.67 | 0.14 |
| V249 (7') | M2 | 4 | 0.52 | 0.05 |
| L250 (8') | M2 | 3 | 0.63 | 0.02 |
| L251 (9') | M2 (gate) | 5 | 0.26 | 0.04 |
| S252 (10') | M2 | 3 | - | - |
| L253 (11') | M2 | 5 | 0.55 | 0.08 |
| T254 (12') | M2 | 4 | 0.35 | 0.08 |
| V255 (13') | M2 | 3 | 0.51 | 0.01 |
| F256 (14') | M2 | 3 | 0.72 | 0.26 |
| L257 (15') | M2 | 3 | 0.68 | 0.09 |
| L258 (16') | M2 | 4 | 0.59 | 0.13 |
| V259 (17') | M2 | 6 | 0.63 | 0.18 |
| D260 (18') | M2 CAP | 5 | 0.89 | 0.04 |
| V261 (19') | M2 CAP | 6 | 0.78 | 0.11 |
| E262 (20') | M2 CAP (ring) | 9 | 0.82 | 0.15 |
| L263 (21') | M2 CAP | 9 | 0.66 | 0.12 |
| D264 (22') | M2 CAP | 7 | 0.78 | 0.15 |
| P265 (23') | M2 CAP | 5 | 0.90 | 0.10 |
| S266 (24') | M2 CAP | 6 | 0.64 | 0.13 |
| T267 (25') | M2 CAP | 3 | 0.71 | 0.09 |
| S268 (26') | M2 CAP | 6 | 0.97 | 0.11 |
| S269 (27') | M2 CAP | 3 | 0.65 | 0.06 |
| A270 (28') | M2 CAP | 3 | 0.65 | 0.07 |
| V271 | M2-M3 Linker | 4 | - | - |
| P272 | M2-M3 Linker | 3 | 0.62 | 0.05 |
| L273 | M2-M3 Linker | 3 | - | - |
| I274 | M2-M3 Linker | 4 | 0.62 | 0.04 |
| G275 | M2-M3 Linker | 3 | 0.65 | 0.06 |
| K276 | M2-M3 Linker | 4 | - | - |

At four cap positions [xI260 (18'), xV261 (19'), xS266 (24') and xT267(25')] all side chain substitutions decreased $K_{eq}$ and at five positions [xE262 (20'), xL263 (21'), xI246 (22'), xP265 (23') and xS268(26')] substitutions either increased or decreased $K_{eq}$. There was no striking correlation between side chain chemistry and the change in $K_{eq}$ at any position. Note that G, A, S, T, and K side chains were tolerated at the conserved P265.

For all positions, the cap mutations changed $K_{eq}$ mainly by changing $\Phi$, (resulting in high $\Phi$ values). The average $\Phi$ value for the entire region (x260–x270), calculated from the $\Phi$ estimate for each residue, was 0.77±0.12 (mean±s.d.), which is somewhat higher than for the flanking regions, the M2-M3 linker (x272–x275; 0.63±0.02) and M2 13'–17' (x253–x259; 0.63±0.08). Three cap residues had particularly high $\Phi$ values, xS268, xP265 and xI260 (0.92±0.04). This result suggests that the M2-cap moves early in A2C–A3O gating.

There are two $\Phi$-subunits per AChR. To address the possibility that an M2 mutation in each subunit might contribute unequally to the fold change in $K_{eq}$ or moves at a different point in the gating reaction as does its partner, we expressed hybrid AChRs having one mutated and one wt $\alpha$ subunit (Fig. 4 and Methods). In cells that were transfected with both wt and $\alpha$P265K subunit cDNAs (along with wt $\beta$, $\delta$, and $\epsilon$), three genetically distinct populations of clusters were apparent. One had a $K_{eq}$ similar to wt AChRs (38), one had a $K_{eq}$ similar to the $\alpha$P265K double mutant (0.015), and the remaining group had a $K_{eq}$ that was intermediate (0.76). We attribute this intermediate population to hybrid AChRs that contain one wt and one mutated $\alpha$ subunit. This pattern, a single hybrid class with a fold-change in $K_{eq}$ (50.3) that is approximately equal to the square root of the fold-change of the double mutant (2542), indicates that each $\alpha$P265K mutation makes an approximately equal and energetically-independent contribution to $K_{eq}$. Further, the $\Phi$ value for the $\alpha$P265K hybrid was similar to that of...
the double mutant (Fig. 4D), which suggests that at this position the two α subunits move approximately synchronously in the reaction.

Population analyses of α subunit W-values are shown in Fig. 5. Considering all 55 residues for which W has been measured, there are most likely five W-populations, with mean (s.e.m.) values of 0.94 (0.03), 0.78 (0.05), 0.64 (0.03), 0.54 (0.02), and 0.31 (0.04). In the αM2-cap, three residues [αI260 (18°), αP265 (23°) and αS268 (26°)] belong to the highest, four [αV261 (19°), αE262 (20°), αI264 (22°) and αT267 (23°)] to the next-highest and the rest [αL263 (21°), αS266 (24°), αS269 (27°) and αA270 (28°)] to the middle W-population. αM2-cap residues exhibit higher W-values than their flanking segments. αI260, αP265 and αS268 have W-values that are similar to those for amino acids located at the transmitter binding sites (Fig. 5A) [20,21,22].

The single-site association and dissociation rate constants (k⁺ and k⁻) and equilibrium dissociation constant (k⁺/k⁻ = Kᵦ) for ACh binding to the closed conformation were determined for one mutant construct, αE262L (Fig. 2). In this mutant, Kᵦ = 155 μM, which is similar to measurements for wild-type AChRs exposed to 140 mM NaCl (100–150 μM [20,23]). The association and dissociation rate constants in the mutant, k⁺ = 102 μM⁻¹s⁻¹ and k⁻ = 15,873 s⁻¹, were also not greatly different from the wt values (k⁺ = 167 μM⁻¹s⁻¹ and k⁻ = 24,745 s⁻¹; [21]). The failure of this mutation to change Kᵦ agrees with similar measurements for three other αM2-cap mutants, αT267I and A, and αS268I [17].

The substitution of a Q at position αE262 (the charged ring) was previously shown to reduce the single-channel conductance by ~50% [14]. For all constructs, we estimated both the single-channel current amplitude in the absence of channel block...
(measured at a low agonist concentration) as well as the equilibrium constant for channel block by the agonist ($K_B$) (Table S2). Excluding lysine substitutions, the average effect of the mutations on the single-channel current amplitude was substantial for only two positions, $aI264$ (22\%) and $aP265$ (23\%). At four positions the effects were moderate [$aE262$ (20\%), $aS266$ (24\%), $aT267$ (25\%) and $aS268$ (26\%)], while at three the effects were insignificant [$aI260$ (18\%), $aV261$ (19\%), and $aL263$ (21\%)]. Positively-charged side chains were substituted at four positions and caused a large decrease (by ~75\%) in the current at $aE262$ (K and R) and $aP265$ (K), had a moderate effect at $aL263$ (K) and had no effect at $aS266$ (K). Note that the average consequence of a charge-removal mutation (A, C, F, G, L or V) at $aE262$ (in both $\alpha$ subunits) was a modest 32\% reduction in the current amplitude.

Agonist molecules can bind to the pore and block ionic conduction. In our experimental conditions, the equilibrium dissociation constant for this blockade ($K_B$) in wt AChRs is ~1.9 mM for ACh [8] and ~13 mM for choline [24]. We estimated the effects of mutations on $K_B$ at 5 different cap residues (see Methods and Table S2). Only three mutations had a significant effect: $aE262T$ (9-fold increase for ACh), $aI264L$ (16-fold decrease for choline) and $aP265T$ (5.8-fold decrease for choline). These results suggest that the side chains of the $\alpha$M2 cap domain do not have a strong effect on equilibrium block by agonist molecules.

Occupancy of the cap domain by certain ligands stabilizes desensitized AChRs. For all constructs, we estimated an apparent rate for entry into long-lived desensitized states, $k_{+1D}$ (Table S3). Surprisingly, most of the mutations had little, if any, effect on this rate. The biggest effects on $k_{+1D}$ were in $aI264$L and $aS266K$ (~10-fold increase) and $aL263E$ (~2-fold decrease). Although the rate of recovery from desensitization and the number of channels in the patch both contribute to the overall frequency of clusters, we observed no striking change in this parameter for the mutants. Overall, the effects of $\alpha$M2-cap mutations on desensitization are quite modest, especially when compared to their substantial effects on gating. This result suggests that NCIs increase equilibrium desensitization mainly by perturbing regions of the AChR other than the $\alpha$M2-cap, and that point side chain substitutions in this region do not mimic these perturbations. We hypothesize that the
previously-reported effects of cap mutations on the macroscopic desensitization rate \[19,25\] arise from their effects on \(K_{eq}\) rather than on microscopic desensitization rate constants.

Overall, \(\alpha M2\)-cap mutations have substantial effects on gating but comparatively small effects on agonist binding, channel conductance, channel block and desensitization. The insertion of a positively charged side chain at \(\alpha E262\) (20') and \(\alpha P265\) (23') significantly reduces the single-channel current amplitude, which is consistent with the notion that these residues face the open pore and that there is a charged ring in this domain that influences ionic conductance.

**Discussion**

The residues of the pore-lining \(\alpha M2\) helix, along with the M2 segments from non-\(\alpha\) subunits, form several important functional elements. These include NCI binding sites, a charged ring, residues in the pore that control conductance and an ion selectivity filter (Fig. 5C). All 27 \(\alpha M2\) residues (\(\alpha T244-\alpha A270\)) have been examined with respect to the effects of mutations on \(K_{eq}\) and \(\Phi\) (Table 2). We cannot, from our experiments and the available AChR structures, correlate the magnitude of the observed changes in \(K_{eq}\) with the magnitudes of the gating motions. However, the large excursions in \(K_{eq}\) caused by side chain substitutions at most positions show that most of \(\alpha M2\) changes its structure, dynamics or both between \(\alpha A2C\) and \(\alpha A2O\). Residues of the \(\alpha M2\)-cap show particularly large excursions in \(K_{eq}\) while those in the cytoplasmic portion of \(\alpha M2\) show relatively smaller changes (Fig. 5B). This pattern supports the notion that the most significant \(C\rightarrow O\) conformational changes in \(\alpha M2\) (and \(\delta M2\) [26]) occur at and above the equator [5]. \(\alpha M2\)-cap \(\Phi\) values are higher than for the rest of \(\alpha M2\), which is consistent with the “conformational wave” framework for AChR gating insofar as this domain is near the extracellular limit of the helix and moves prior to the (low-\(W\)) equatorial zone in channel-opening. The pattern of \(\Phi\) in the \(\alpha M2\)-cap is, however, surprising in two respects. First, \(\alpha M2\)-cap \(\Phi\) values are higher than those of residues in the M2-M3 linker, cytoplasmic loop and loop 2, all of which are located between the cap and the TBS. Three \(\alpha M2\)-cap residues (\(\alpha I260\) (18'), \(\alpha P265\) (23') and \(\alpha S268\) [26']) have \(\Phi\)-values that cannot be distinguished from those of TBS residues. If \(\Phi\) reflects the relative timing of gating motions, this result indicates that the gating movements in these two apparently-unconnected regions are approximately synchronous and occur at the outset of the

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**Figure 4. Analysis of \(\alpha P265K\) hybrid AChRs.** Hybrids are AChRs in which only one of the two \(\alpha\)-subunits has been mutated. (A) Low time-resolution view of a continuous current trace showing wild-type, hybrid, and double mutant clusters activated by 500 \(\mu M\) ACh. (B) Expanded view of clusters boxed in A, plus interval duration histograms. (C) Cluster open probability (\(P_o\)) for the patch shown in panel A. The clusters with the highest \(P_o\) correspond to wild-type receptors, those of the intermediate population correspond to hybrid receptors, and those with the lowest \(P_o\) are doubly-mutated AChRs. The total number of clusters was 402. (D) REFER analysis shows that the fold-change in \(K_{eq}\) for the hybrid is approximately equal to the square root of the fold change for the double mutant, thus the effect of each mutation with regard to \(\alpha A2C\) vs. \(\alpha A2O\) energy changes is equal and independent. The slope of the REFER (\(\Phi\)) is similar for single- and double-mutant constructs, suggesting that the gating motions of P265 in each \(\alpha\)-subunit are approximately synchronous. doi:10.1371/journal.pone.0002515.g004
channel-opening process. Second, the map of the entire $\alpha$M2 segment is complex, with all five $W$-values represented (Fig. 5B). With the temporal interpretation, this suggests that the gating movements in this helix are highly asynchronous, whereas we might expect that side chain motions of such a secondary structural element would either be synchronous, or, perhaps, constitute a continuous, top-to-bottom sequential conformational cascade.

Although we cannot resolve these two conundrums, we can offer some possible explanations.

1) **Unknown linkage elements.** There is no obvious structural connection between the TBS and the $\alpha$M2-cap in the Torpedo AChR structure, where the tip of loop A (residue $\alpha$L297 [20]; $\Phi = 0.93 \pm 0.02$) and cap residue $\alpha$S268 ($\Phi = 0.97 \pm 0.11$) are separated by $\sim 17$ Å. It is difficult to imagine that agonist-triggered gating structural changes at the TBS could propagate, by direct steric interactions, to the $\alpha$M2-cap. It is possible that the TBS and the $\alpha$M2-cap are directly linked by high $\Phi$ amino acids that have yet to be probed, or that there is a physical connection between these two domains that is invisible in electron density maps (e.g., is electrostatic or arises from the water). For example, gating motions of the $\alpha$M1 segment, or perturbation of the aqueous milieu consequent to TBS binding or gating motions, might serve to generate the high $W$-values in the $\alpha$M2-cap.

2) **Incomplete structural information.** Protein movement consequent to agonist binding may move the two high-$W$ domains (loop A and the $\alpha$M2-cap) closer than they are in the unliganded-closed Torpedo AChR structural model. This highlights our lack of high resolution structural information regarding the ground states of the $\alpha_2$C3$\rightarrow\alpha_2$O reaction.

3) **Independent gating motions.** Perhaps the motions at the TBS and the cap are completely independent, and these two regions just happen to move early and approximately at the same time.

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**Figure 5. $K_{eq}$ and $\Phi$ of the $\alpha$ subunit.** (A) Population analysis of $\Phi$ in the $\alpha$ subunit. $\Phi$-values of 55 different residues plotted as a function of sequence position ($\geq$2 mutants and >5-fold range in $K_{eq}$). Subunit domains are shown along the $x$-axis. Each residue was assigned to a $\Phi$ population by using a statistical algorithm (see below and Methods). The population means are: purple, 0.94; blue, 0.78; green, 0.64; orange, 0.54 and red, 0.31. $\Phi$-values (Table 2) may reflect the relative timing of gating movements: purple/blue is early, green is intermediate and orange/red is late. High-$\Phi$ residues in the TBS are circled. Inset, The number of $\Phi$ populations ($n$) was estimated from the sum-squares deviation (SSQ). SSQ decreases significantly as $n$ is increased from $n = 2$–5, but decreases more slowly between $n = 6$–20. The most likely number of $\Phi$ populations is 5. (B) Map of $\Phi$ in the $\alpha$ subunit. Residues are colored according to $\Phi$ value (see panel A for color code). The TBS and $\alpha$M2-cap (purple) move at the outset, and the equatorial residues (red) move near the end, of the channel-opening process. (C) Functional maps of $\alpha$M2 and $\alpha$M2-$\alpha$M3 linker ($\alpha$244–$\alpha$276). $\alpha$M2 residues T244, L251 and E262 face the lumen of the pore. Left, residues colored according to the range for the fold-change in $K_{eq}$: >1000-fold (blue), 10–1000 fold (cyan) and <10-fold (grey) (Table 2). $\alpha$M2-cap residues experience large energy differences (‘move’) between C and O, whereas many mutants of residues near the cytoplasmic limit of the channel are iso-energetic, which may indicate relatively smaller structural changes. The three biggest excursions in $K_{eq}$ were observed for $\alpha$P272, $\alpha$P265 and $\alpha$V255. Right, residues colored according to $\Phi$ value (see panel A for color code). Most of the residues in the $\alpha$M2-cap move ‘early’ in gating (purple and blue), before those in the $\alpha$M2-$\alpha$M3 linker and much of $\alpha$M2 (green). Three cap residues ($\alpha$I260, $\alpha$P265 and $\alpha$S268) have the same $\Phi$ value as those for residues at the transmitter binding sites (see panel A). In $\alpha$M2, residues near the equator have the lowest $\Phi$ values and, therefore, move last in C$\rightarrow$O gating. Arrow, we speculate that when the channel opens, $\alpha$P265 rotates to position its side chain in the lumen of the channel.

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relative time in the gating reaction in the absence of any direct interactions to couple these motions. This would mean that the microscopic structural transitions that separate C and O are not strictly sequential. There are precedents for such apparently independent-but-synchronous gating movements. Large distances separate the two z subunits. For example, in both the loop A and M4, residues on the two z subunits are separated by ~26 Å (zD97) and ~58 Å (zC418), respectively. Nonetheless, hybrid constructs of these two amino acids have approximately the same z value [20,27], as do those of zP265 in the zM2-cap (~24 Å). Given the complexity of the AChR conformational change, it is not unreasonable to think that separate domains can move independently but approximately at the same time, and will thus have similar experimental z values. The zM2 cap and the agonist-occupied TBS may be inherently unstable structures that deform early in the C-»O isomerization.

4) The interpretation of z. z may not reflect time in the zM2-cap domain. The central assumption of the temporal interpretation of z is that mutations alter the C-»O rate constant by changing the transmission coefficient, but the magnitude of z, also reflects transition state (TS) energy and, perhaps, heterogeneity. Further, the weights given to these various factors (with regard to z) could be different for different regions of a protein or for different individual residues. Another assumption of the temporal interpretation is that a side chain undergoes only a single, instantaneous, all-or-nothing gating movement. It is, however, possible that some side chain atoms (we do not mutate the backbone) are jostled more than once within the reaction, in which case the apparent z value will be a weighted average of the relative times and energy changes of such multiple motions. We can imagine that the transition region energy changes of the three cap high-z residues (z260, z265 and z268; z = 0.92) occur mainly early in the reaction, those in the M2-M3 linker and in much of M2 occur mainly near the middle of the reaction (z = 0.64), and that the ‘intermediate’ residues of the cap (z261, z262, z264 and z267; z = 0.77) move twice, along with each of these other groups. The possibility of multiple side chain motions is physically plausible but further complicates the interpretation of z values.

The resolution of the electron density map of the zM2 cap in the Torpedo AChR is not sufficiently high to assess the potential for, or chemical nature of, the specific structural changes in this domain that accompany C-»O gating. Also, there are as yet no published structures of a ligand-occupied intact AChR, although there are structural differences between occupied and vacant AChBP [28,29,30] and the ECDs of z vs. non-z AChR subunits that may reflect C vs. O conformations, respectively [31]. In the absence of high resolution structures of the wt and mutant AChRs it is difficult to infer specific structural events based on the functional effects of mutations.

The basic features of the zM2 cap are as follows. It is a ~9 residue (260–268, which subbinds the high-z (z = 9) segment) that is at the C-terminus of a long z-helix. Some cap side chains face the water-accessible, ion permeation pathway while others are close to M1 and M3. There is a conserved Pro near the middle of the segment. In 2bg9.pdb, the modeled (z + P) backbone bonds for zP265 and zL264 are ~89°/30° and ~84°/12°, which are outside the typical values for proline (55°/50°) [32] and pre-proline (60°/45°) [32,33] residues. We speculate that the central proline (zP265) of the zM2-cap distorts and destabilizes the C-terminal portion of M2, which enables the cap to readily switch its secondary structure during the C-»O conformational change. This hypothesis accounts for the observations that most cap residues experience large energy changes in gating, and that some appear to move at the outset of channel-opening. The change in the backbone cannot be a full, cis-trans isomerization, because many different side chain substitutions at zP265 support efficient gating. The fact that the effect of a K substitution on the single-channel current amplitude was similar at zE262 (20°) and zP265 (23°) (Table S2) suggests that these two residues are aligned along the pore axis when the AChR is in an open-channel conformation (Fig. 5C). Although the specific structural changes are not revealed in our experiments, we hypothesize that the backbone angles of the central proline and preceding isoleucine change in C-»O gating, and that this switch in the secondary structure of the zM2-cap permits the translation of ECD motions into the rest of M2 and, thence, to other M2 residues that regulate ionic conductance, including the late-moving 9° and 12° residues [5]. This is similar to the suggestion that channel-opening involves an outward tilt of the M2-cap [3], although our experiments suggest this motion may involve a twist. Interestingly, a different experimental approach indicates that there are only minor movements in the M2 helix of the δ subunit in C-»O gating [34].

We now describe a sequence of events in the z subunit opening cascade, based on z values and the assumption that mutations mainly affect the transmission coefficient of z. In the following framework, all of the gating motions are stochastic (are characterized by back-and-forth, Brownian dynamics). Also, the reverse sequence describes channel-closing.

- i) Conformational changes consequent to agonist binding destabilize at least two domains of each z subunit, the TBS (loops A, B and C) and the zM2-cap. Residue zK145 in the outer β sheet of the ECD is also destabilized [22]. The gating motions of the TBS residues increase the affinity for ACh by a factor of ~10,000 [22,35], but the conductance of the channel remains low. The motion of the TBS announces the exit from the C structural ensemble and entry into the TS ensemble. The trigger for the change in structure at the TBS is the presence of the agonist itself, but that for the cap region remains obscure.

- ii) The motions of the TBS and zM2-cap trigger those in adjacent domains, including loop 2, the cytosolic loop and residue zY127 in the inner β sheet of the ECD. These motions are then followed by the movement of residues in the M2-M3 linker and in M2, both within the zM2-cap and below, to the equator and beyond. These intermediate events reflect structural changes that occur within the TS ensemble of the reaction, where the TBS affinity remains high but the channel conductance is still low.

- iii) The above gating motions in zM2 destabilize residues zL251(9°) and zT254(12°). It is possible that the movement of these residues serves to change ionic conductance (they act as a ‘gate’), but it is also possible that ions begin to cross the channel rapidly when the protein is still in the short-lived TS ensemble (they act as a ‘latch’). At this point in opening the TBS still has a high affinity for agonists, and the movement of the zM2 cap reflects entry into the O structural ensemble.

To confirm and complete this gating scenario we will need high resolution structures of intact AChRs in both A2C and A2O conformations, more extensive estimates of the energy changes in
αM1 and the M2 segments of the non-α subunits, and more sophisticated theories for, and analyses of, the transition state of the gating reaction.

Methods

Detailed methods are given in Jha et al, (2007) [18]. Briefly, mutant AChRs (64 different mutants of 9 different amino acid positions) were transiently expressed in HEK cells, and single channel currents were recorded in the cell-attached patch configuration at 23°C. The bath and pipette solutions were Dulbecco’s phosphate buffered saline containing (in mM): 137 NaCl, 0.9 CaCl2, 2.7 KCl, 1.5 KH2PO4, 0.5 MgCl2, and 8.1 Na2HPO4 (pH 7.3). The currents were filtered at 20 kHz and digitized at a sampling frequency of 50 kHz. Agonist (acetylcholine or choline) was added to the pipette solution. For rate constant measurements, the agonist concentration was approximately five times K<sub>n</sub> (500 μM ACh or 20 mM choline). Choline was used to activate constructs in which K<sub>n</sub> was similar to or larger than in the wt (gain-of-function mutants), and ACh was used to activate constructs in which K<sub>n</sub> was smaller than in the wt (loss-of-function mutants). Rate constant estimation (12 kHz bandwidth) was done by using QUB software (www.qub.buffalo.edu). Clusters of individual-channel, diliganded G<sub>α</sub> activity were usually selected by eye or by using a critical time of 50 ms. Typically, ~50 clusters were selected in each record. The opening and closing rate constants were estimated from the interval durations by using a maximum likelihood algorithm [36] after imposing a dead time correction of, typically, 25 μs. Φ was estimated as the slope of the rate-equilibrium free energy relationship (REFER), which is a plot of (τP<sub>o</sub>)<sup>-1</sup> vs. log K<sub>n</sub> (Fig. 3). Each point in the REFER represents the mean of at least three different patches for a single mutant construct.

We could not determine the gating rate constants for αP2635F and αP265L because no currents were detected (8 patches each, 10 min/patch). Also, rate constants could not be measured for the constructs αP266F, αS266L, αS266Y and αT267F because the openings were not organized into well-defined clusters at 500 μM ACh, most likely because these constructs had exceeding small values of K<sub>n</sub>. Clusters from αS266C showed two distinct kinetic patterns, and k<sub>o</sub> and k<sub>c</sub> were estimated separately for each. αS2680Y showed multiple kinetics patterns so no rate constants were estimated for this mutant. In total, rate constants were estimated for 57 of the 64 constructs that were examined (Table S1).

The K<sub>n</sub> for acetylcholine was estimated only for the αP262L mutant (Fig. 2). Open and closed interval durations were obtained at three different ACh concentrations (30, 50 and 100 μM). Two agonist binding sites were assumed to be equivalent and independent [37] and the interval durations at all three concentrations were fitted together by using a G<sub>α</sub>→A<sub>γ</sub>→A<sub>δ</sub>→A<sub>ε</sub>→A<sub>ø</sub> kinetic model (A = agonist) that had four rate constants as free parameters: single-site association (k<sub>a</sub>, scaled by [A]), single-site dissociation (k<sub>d</sub>), k<sub>c</sub>, and k<sub>o</sub>.

In the REFERs (Fig. 3), the wt values used to normalize k<sub>c</sub> and K<sub>n</sub> were 120 s<sup>-1</sup> and 0.046 for AChRs activated by choline and 48,000 s<sup>-1</sup> and 28.2 for AChRs activated by ACh. The slope of the REFER was estimated by an unweighted, linear fit in Origin Pro 7.0. All structures were displayed by using PYMOL (DeLano Scientific).

The number of Φ populations (Fig. 5A) was estimated statistically by using a cluster-detection algorithm (SKM), which assumes each population had a Gaussian distribution with an independent mean and s.d [5]. The overall sum-square deviation (SSQ) was estimated assuming n = 2 to 20 populations. 300 random starting assignments were used for each value of n.

In the experiments concerning hybrid AChRs (Fig. 4), cells were transfected with both wild-type and mutant [P263K] α subunit cDNAs in a 1:3 ratio, together with wild-type β, δ, and ε subunit cDNAs. All recordings showed populations of clusters that could be distinguished statistically according to the cluster open probability (P<sub>o</sub>), corresponding to wild-type, hybrid (containing one wild-type and one mutant α subunit) or double-mutant AChRs. Clusters were either selected by eye or defined using a critical time of 50 ms and were segregated statistically (segmentation k-means algorithm; SKM) into separate populations for subsequent kinetic analyses with only the cluster P<sub>o</sub> as the discrimination criterion. Clusters that had P<sub>o</sub> values that were >1 SD from the corresponding population mean were rejected from these analyses.

In neuromuscular AChRs desensitization appears to proceed mainly from the α<sub>2</sub>O state [8] or from a transition micro-state that is near α<sub>2</sub>O [4,7]. An approximate rate of entry into long-lived desensitized states was determined by computing the inverse of the product of the cluster duration times the cluster open probability: K<sub>B</sub> = (τP<sub>o</sub>)<sup>-1</sup> (Table S3). This parameter is a rough estimate of the net rate of exiting α<sub>2</sub>O into a long-lived D state.

An estimate of the equilibrium constant for channel block by the agonist (K<sub>B</sub>) was determined for each construct from the relationship K<sub>B</sub> = [A]i<sub>o</sub>/i<sub>n</sub>−i<sub>o</sub>, where [A] is the agonist concentration, i<sub>o</sub> is the current amplitude in the absence of channel block (30 μM ACh or 200 μM choline), and i<sub>n</sub> is the current amplitude at high [A] (Table S2). For normalization, the wt parameters were K<sub>B</sub> = 1.9 mM for ACh [8] and 13 mM for choline [24]. The fractional reduction in amplitude at 500 μM ACh was small (~20% in the wt), and, because of errors in the estimate of the membrane voltage, the K<sub>B</sub> estimates for such ACh-activated currents were imprecise. Therefore, only mutants that showed a >50% decrease in current amplitude at 500 μM ACh were used for K<sub>B</sub> estimation. For choline-activated constructs, the fractional reduction in the wt current amplitude at 20 mM is more substantial (~60%) so K<sub>B</sub> could be estimated for all.

Supporting Information

Table S1 Rate and equilibrium constant estimates for the αM2-cap Mutants (260–268) Found at: doi:10.1371/journal.pone.0002515.s001 (0.16 MB DOC)

Table S2 Conductance and Channel Block for αM2-cap Mutants (260–268) Found at: doi:10.1371/journal.pone.0002515.s002 (0.14 MB DOC)

Table S3 Apparent Desensitization Rates for αM2-cap Mutants (260–268) Found at: doi:10.1371/journal.pone.0002515.s003 (0.12 MB DOC)

Figure S1 Single-channel current traces of various αM2 cap mutants Found at: doi:10.1371/journal.pone.0002515.s004 (0.82 MB TIF)

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Author Contributions

Conceived and designed the experiments: AA PB. Performed the experiments: PB PP. Analyzed the data: PB. Contributed reagents/materials/analysis tools: AA. Wrote the paper: AA PB.
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