Due to an editorial error, a $\theta$ value in the right column appears as a $\tau$ value. The corrected sentence is given here:

Application of Eq. 4, assuming that only $\theta_0$ changes upon mutation and using a $\sigma$ value of 200 s$^{-1}$, yields $\tau_0$ values of 3.7 and 2.2 ms for the $\beta$54I and $\epsilon$54I mutant receptors, respectively.
We describe the functional consequences of mutations in the linker between the second and third transmembrane segments (M2–M3L) of muscle acetylcholine receptors at the single-channel level. Hydrophobic mutations (Ile, Cys, and Phe) placed near the middle of the linker of the α subunit (αS269) prolong apparent openings elicited by low concentrations of acetylcholine (ACh), whereas hydrophilic mutations (Asp, Lys, and Gln) are without effect. Because the gating kinetics of the αS269I receptor (a congenital myasthenic syndrome mutant) in the presence of ACh are too fast, choline was used as the agonist. This revealed an ~92-fold increased gating equilibrium constant, which is consistent with an ~10-fold decreased EC₅₀ in the presence of ACh. With choline, this mutation accelerates channel opening ~28-fold, slows channel closing ~3-fold, but does not affect agonist binding to the closed state. These ratios suggest that, with ACh, αS269I acetylcholine receptors open at a rate of ~1.4 × 10⁶ s⁻¹ and close at a rate of ~760 s⁻¹. These gating rate constants, together with the measured duration of apparent openings at low ACh concentrations, further suggest that ACh dissociates from the diliganded open receptor at a rate of ~140 s⁻¹. Ile mutations at positions flanking αS269 impair, rather than enhance, channel gating. Inserting or deleting one residue from this linker in the α subunit increased and decreased, respectively, the apparent open time approximately twofold. Contrary to the αS269I mutation, Ile mutations at equivalent positions of the β, ε, and δ subunits do not affect apparent open-channel lifetimes. However, in β and ε, shifting the mutation one residue to the NH₂-terminal end enhances channel gating. The overall results indicate that this linker is a control element whose hydrophobicity determines channel gating in a position- and subunit-dependent manner. Characterization of the transition state of the gating reaction suggests that during channel opening the M2–M3L of the α subunit moves before the corresponding linkers of the β and ε subunits.

Keywords: nicotinic receptors • allosteric proteins • single-channel kinetics • transition state
ings started calcium-phosphate precipitate, and electrophysiological re-
cord-al., 1999). The medium was changed sequencing. HEK-293 cells were transiently transfected by cal-
mutations were confirmed by restriction mapping and dideoxy 
(Salamone et al., 1999). Mutations were engineered using either
the QuickChange™ Site-Directed Mutagenesis kit (Stratagene)
(Sine, 1993). All engineered a set of mutations in this region of the dif-
result in channel opening the M2–M3L of the α subunit moves before the corresponding linkers of the β and ε subunits.

METHODS

Mutagenesis and Expression

Mouse cDNA clones were obtained as described by Grosman and Auerbach (2000a,b). The α-subunit clone contained an inciden-
tal M4 mutation (V433A) that has no functional consequences (Salamone et al., 1999). Mutations were engineered using either
the QuickChange™ Site-Directed Mutagenesis kit (Stratagene) protocol or the cassette mutagenesis method (Sine, 1993). All
mutations were confirmed by restriction mapping and dideoxy sequencing. HEK-293 cells were transiently transfected by cal-
cium-phosphate precipitation (Ausubel et al., 1992; Salamone et al.,1999). The medium was changed ~24 h after addition of the calcium-phosphate precipitate, and electrophysiological record-
ings started ~24 h later.

Patch-Clamp Recordings and Analysis

Recordings were performed in the cell-attached patch configura-
tion (~22°C). The pipette solution con-
tained (mM): 142 KCl, 5.4 NaCl, 1.8 CaCl2, 1.7 MgCl2, 10 HEPES/
KOH, pH 7.4. In most experiments using ACh, the bath solution
was the same as the pipette solution. In all other experiments, the
bath solution was Dulbecco’s phosphate-buffered saline (mM):
137 NaCl, 0.9 CaCl2, 2.7 KCl, 1.5 K2HPO4, 0.5 MgCl2, and 8.1
NaH2PO4, pH 7.3. Single-channel currents were amplified using a
patch-clamp amplifier (PC-505; Warner Instrument Corp. or
200B; Axon Instruments, Inc.) and were digitized at 100 kHz.

To separate the more rapid binding and gating steps from the slower desensitization steps, clusters of single-channel activity
were identified and used to estimate open probability (Popen), time constants, and gating rate constants. Clusters were defined
as a series of openings separated by closuresshorter than a criti-
cal time, τe (Sakmann et al., 1980). In the presence of ACh or low
concentrations of choline, τe was determined as described by
Salamone et al. (1999). In the case of currents elicited with satu-
rating choline, τe was the longest time interval that defined clus-
ters still best fitted with a C ↔ O kinetic model, as described by
Grosman and Auerbach (2000b). Clusters of raw data were ideal-
ized according to either a half-amplitude threshold-crossing cri-
terion (program IPR0C; Sachs et al., 1982) at a bandwidth of
~4 kHz or a hidden-Markovmodel-based algorithm (program
SKM) at a bandwidth of ~18 kHz. Only clusters whose overall
mean open time was within ±2 SD of the mean of the corre-
sponding patch were retained for further analysis (typically
98% of the original data; programs LPROC or SELECT).

Equilibrium concentration–response curves were analyzed in
the framework of Scheme I:

\[
C \rightarrow 2k_A \rightarrow CA \rightarrow k_A \rightarrow CA_2 \rightarrow \beta_2 \rightarrow OA_2 \rightarrow k_\alpha \rightarrow CA_* \rightarrow OA_2B
\]

(SCHEME I)

where C and O denote the closed and open channel, respecti-
vely. A denotes the agonist, OA2B represents the open channel
blocked by the agonist itself, and CA* is a shut state whose mean
lifetime is independent of the agonist concentration (Salaman-
e et al., 1999). In the presence of acetylcholine (ACh), the latter
has a mean duration of ~1 ms and is therefore distinct from the
long-lived desensitized state(s) that gives rise to the longer-lived closed intervals between clusters. Popen values were estimated
from the idealized current traces as the fraction of time the chan-
nel is open within a cluster. Plots of Popen vs. agonist concentra-
tion were fitted with Eq.1 to estimate the microscopic agonist-dis-
sociation equilibrium constant (Kb = kα/kβ) and the diliganded-
gating equilibrium constant (θ2 = β2/α2):

\[
P_{\text{open}} = \frac{1 + \frac{cKd}{θ2A} + \frac{ck^2}{θ2A} + CKg}{1 + \frac{cKd}{θ2A} + \frac{ck^2}{θ2A} + CKg}^{-1}
\]

where c = Kd/(A + Kd) and Kd is the dissociation equilibrium constant for channel block (Kd = 0.05 mM for ACh and 20 mM
for choline), and Kb is the equilibrium constant between OA2 and
CA2* (Kd = 0.05 mM for ACh, as in Salamone et al. (1999), and
ignores the presence of choline). Eq. 1 corresponds to the classi-
cal sequential model (del Castillo and Katz, 1957; Magleby and
Stevens, 1972) modified for the case of two equivalent binding
sites, fast blockade by the agonist, and a short-lived desensitized
state (Salamone et al., 1999).

The opening rate constants of the various constructs in the
presence of ACh were estimated by fitting plots of β' vs. agonist
concentration (A) with the following empirical equation (Hill
equation, Eq. 2):

\[
β' = \frac{β_A}{1 + (A/A_0)^n}
\]

where β' (the “effective” opening rate) is defined as the recipr-
cal of the slowest component of the closed-time distribution, β2 is
the opening rate constant, A0 is the agonist concentration at
which β’ is half maximal (i.e., β'/2), and n is the Hill coefficient.
β' at each ACh concentration was estimated from the fit of log-
binned (Sigworth and Sine, 1987) closed-time histograms with
sums of exponential densities. Likewise, mean open times in the
presence of ACh were estimated from the exponential fit of log-
binned open-time histograms. Both closed- and open-interval his-
tograms were compiled from data idealized (half-amplitude cri-
teron) at an effective bandwidth of 4 kHz. The opening and clos-
ing rate constants in the presence of saturating 20 mM choline
were estimated, after idealization (program SKM), by applying a
full-likelihood algorithm that includes a correction for missed
events (program MIL; Qin et al., 1996, 1997). We used a one-step
C ↔ O kinetic scheme, an effective bandwidth of ~18 kHz, and a
dead time of ~25 μs, as described by Grosman and Auerbach.
Because of the lengthening effect of fast blockade by choline on the duration of openings, the closing rate constants determined in this way are underestimations by a factor no greater than 2 (Grosman and Auerbach, 2000b). As the single-channel current amplitude was reduced to similar extents in both wild-type and mutants (i.e., the affinity for choline as a blocker is largely unaffected by the mutations), the prolongation of the openings is expected to be the same for all tested constructs.

The distribution of open times in the presence of low concentrations of Ach was analyzed in the context of an MWC-like kinetic model (Scheme II; Monod et al., 1965). This model incorporates the dissociation of agonist from the open channel as well as openings of un- and mono-ligated receptors.

\[
\begin{align*}
\alpha_0 & \xrightarrow{k_0} \beta_0 \\
\beta_0 & \xrightarrow{k_1} \alpha_1 \\
\alpha_1 & \xrightarrow{k_2} \beta_2 \\
\beta_2 & \xrightarrow{k_3} \alpha_3 \\
\alpha_3 & \xrightarrow{k_4} \beta_4 \\
\beta_4 & \xrightarrow{k_5} \alpha_5
\end{align*}
\]

(Scheme II)

Where \( k_i \) \( k_i \), \( j_i \), \( j_i \) are the microscopic agonist-dissociation equilibrium constants from the closed and open channels, respectively, \( \beta_i/\alpha_0 \) \( \beta_i/\alpha_0 \), \( \beta_i/\alpha_1 \) \( \beta_i/\alpha_1 \), and \( \beta_i/\alpha_2 \) \( \beta_i/\alpha_2 \) are the gating equilibrium constants of un-, mono-, and dili-ganded receptors, respectively, \( k_{g}/k_{-g} \) \( k_{g}/k_{-g} \) is the desensitization equilibrium constant, and \( k_{g}/k_{-g} \) \( k_{g}/k_{-g} \) is the equilibrium constant between \( \alpha_2 \) and \( \alpha_2^* \). Because this model was used to interpret data recorded at low concentrations of Ach, the probability that sojourns in shut states other than \( \alpha_2 \) were shorter than 45 \( \mu \)s and, therefore, are not true estimates of the reciprocal of the diliganded closing rate constant. Taking the sequential model (Scheme I) as an approximation of the channel’s kinetic behavior, and neglecting the (short) time spent in the closed diliganded state \( \alpha_2 \), the mean duration of the slowest component of these apparent openings is given by:

\[
\tau_0 = \frac{1}{\alpha_2 + \beta_2/2k_2},
\]

where \( 1 + \beta_2/2k_2 \) is the mean number of openings per burst. In the experiments at low concentrations of Ach, the probability that sojourns in shut states other than \( \alpha_2 \) were shorter than the time resolution of 45 \( \mu \)s (and thus that were included within an apparent opening) is negligible. Likewise, the probability that a sojourn in \( \alpha_2 \) was longer than 45 \( \mu \)s is vanishingly small. In conclusion, an “apparent opening,” defined as a series of openings separated by closures shorter than 45 \( \mu \)s, is likely to reflect a sojourn in the combined set of states \( \alpha_2 + \alpha_2^* \).

From Eq. 3, it can be seen that the prolonged open-time constant of \( \alpha S5I \) AChRs may arise from one or a combination of the following: an increased opening rate constant \( \beta_2 \), a decreased closing rate constant \( \alpha_2 \), or a decreased agonist-dissociation rate constant \( 2k_2 \). To distinguish between these possibilities, we studied the effect of the \( \alpha S5I \) mutation on gating by using choline as the agonist. Choline slows channel gating so open and closed intervals can be fully resolved (Grosman and Auerbach, 2000b). Fig. 2 shows clusters of single-channel currents of \( \alpha S5I \) AChRs recorded in saturating 20-mM choline. The opening rate constant \( \beta_2 \) was 7.098 \( \pm \) 1.227 \( \text{s}^{-1} \), and the closing rate constant \( \alpha_2 \) was 1.487 \( \pm \) 0.134 \( \text{s}^{-1} \). These are, respectively, 27.7\( \times \) faster and 3.33\( \times \) slower than those of wild-type AChRs activated by choline (Grosman and Auerbach, 2000b). Thus, with choline as the agonist, the \( \alpha S5I \) mutation increases the gating equilibrium constant \( \sim 92 \) fold, mostly due to an increase in \( \beta_2 \).

Next, we sought to determine whether the \( \alpha S5I \) mutation affects binding affinity. Single-channel currents elicited over a range of choline concentrations were re-
Table I

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<td>V</td>
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<tr>
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<td>A</td>
<td>V</td>
<td>D</td>
<td>I</td>
<td>Y</td>
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</table>

Position -2 marks the putative beginning of the M2–M3 linker for ACh and serotonin (5HT3) receptors. In glycine and GABA receptors, this domain presumably starts one residue before (position -3) being an R in GlyRα1-4 and all GABAα receptor subunits, an A in the GlyRβ subunit, and an N in both GABAβ2 subunits. A Pro residue occupies the fourth or fifth position in all members of the superfamily and is assigned the number 1, according to our numbering system. The putative end of this linker is at position 12 in Ach, 6 in 5HT3, 10 in glycine, and 7–8 in GABA receptors. Whenever available, the human (h) sequences are given; otherwise, the sequences of either chicken (ch), mouse (m), or rat (r) are included. The M2–M3 linker sequences of the human α, β, δ, and ε AChR subunits are almost identical to those of mouse (the clones used in this paper). Torpedo (t) sequences are also included.

corded, and intracluster $P_{\text{open}}$ values were estimated and compared with that of ACh (Fig. 2). The lower efficacy (i.e., smaller $\theta_2$) of choline allowed us to fit the $P_{\text{open}}$-vs-concentration data with Eq. 1, yielding $\theta_2 = \beta_2/\alpha_2 = 2.5 \pm 0.9$ and $K_d = k_1/k_4 = 3.3 \pm 1.4$ mM. This value of $\theta_2$ is consistent with the rate constants obtained by single-channel kinetic modeling of currents elicited by 20-mM choline with a $K_d$ of 20 mM and assuming a sequential model for channel block. The $K_d$ for wild-type AChRs activated by choline is not known with certainty because the extremely low efficacy of this agonist makes this dose-response curve difficult to measure. However, the wild-type $K_d$ is in the millimolar range (Zhou et al., 1999). Thus, the choline dose-response results suggest that the αS5I mutation does not substantially alter the affinity of the closed-channel transmitter binding site for choline, but rather predominantly affects channel gating.

The gating ($\theta_2$) and agonist dissociation ($K_d$) equilibrium constants of ACh-activated receptors could not be directly estimated from the dose-response data (by fitting Eq. 1) because of the high ACh $\theta_2$ value. However, the $P_{\text{open}}$-vs-concentration relationship of the αS5I mutant activated by ACh revealed an EC$_{50}$ of ~4.0 μM.
which is \(\sim 10 \times\) lower than that of the wild type determined under identical conditions (Salamone et al., 1999). According to Eq. 1, when the gating equilibrium constant is large \((\sqrt{\theta_2} \gg 1)\), \(K_{50} = K_{d}/\sqrt{\theta_2}\). Therefore, the \(\sim 10\)-fold decrease in ACh \(E_{50}\) can be accounted for by an \(\sim 100\)-fold increase in \(\theta_2\), which is similar to the 92-fold increase in \(\theta_2\) measured in the presence of choline. These results indicate that \(\theta_2\) is increased to the same extent in the presence of ACh or choline. An increase in \(\theta_2\) can arise from an increase in the unliganded gating equilibrium constant, \(\theta_0 = (\beta_0/\alpha_0)\) and/or an increase in the closed/open agonist-affinity ratio, \(K_{d}/J_{d}\) (Scheme II). The agonist-insensitive effect of the \(\alpha 5I\) mutation on \(\theta_2\) suggests that this mutation increases the unliganded gating equilibrium constant with little, if any, effect on the affinity ratio, which is expected to be sensitive to the nature of the agonist. Several SCCMS mutations, including \(\alpha 5I\), have been shown to increase unliganded gating, thus turning choline, a normally inert molecule, into a significant agonist (Zhou et al., 1999).

Table I

<table>
<thead>
<tr>
<th>Mutant</th>
<th>(\tau_o) (ms)</th>
<th>SD</th>
<th>Mutant</th>
<th>(\tau_o) (ms)</th>
<th>SD</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>0.75</td>
<td>0.21</td>
<td>(\alpha 5K)</td>
<td>0.84</td>
<td>0.03</td>
</tr>
<tr>
<td>ADD</td>
<td>1.46</td>
<td>0.05</td>
<td>(\alpha 5Q)</td>
<td>0.60</td>
<td>0.10</td>
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<tr>
<td>DEL</td>
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<td>0.05</td>
<td>(\alpha 5I)</td>
<td>4.50</td>
<td>0.30</td>
</tr>
<tr>
<td>(\alpha T3A)</td>
<td>0.16</td>
<td>0.04</td>
<td>(\alpha 5C)</td>
<td>7.50</td>
<td>0.30</td>
</tr>
<tr>
<td>(\alpha T3I)</td>
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<td>0.03</td>
<td>(\alpha 5F)</td>
<td>7.40</td>
<td>0.80</td>
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<tr>
<td>(\alpha 5A)</td>
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<td>0.20</td>
<td>(\alpha 6A)</td>
<td>0.71</td>
<td>0.05</td>
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<tr>
<td>(\alpha 5I)</td>
<td>0.36</td>
<td>0.03</td>
<td>(\alpha V7A)</td>
<td>0.74</td>
<td>0.04</td>
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<tr>
<td>(\alpha 5A)</td>
<td>0.79</td>
<td>0.08</td>
<td>(\beta 5A)</td>
<td>9.10</td>
<td>0.90</td>
</tr>
<tr>
<td>(\alpha 5D)</td>
<td>0.61</td>
<td>0.06</td>
<td>(\beta 5S)</td>
<td>1.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Although gating of \(\alpha 5I\) receptors in the presence of ACh is exceedingly fast for the rate constants to be measured directly, the closing and opening rate constants can be predicted based on the results in the presence of choline. This is because the structure of the AChR at the transition state of the gating reaction is likely to be the same regardless of the particular ligand bound (Grosman et al., 2000). Thus, like in the presence of choline, we estimate that ACh-bound \(\alpha 5I\) receptors open \(\sim 27.7\)× faster, and close \(\sim 3.33\times\) slower, than the wild type; i.e., \(\beta_2 = 1.4 \times 10^6 \text{ s}^{-1}\) and \(\alpha_2 = 750 \text{ s}^{-1}\).

Even though the predicted value of \(\beta_2\) cannot be confirmed experimentally from the duration of shut intervals, the effect that such a fast opening rate constant would have on the apparent open-time distribution can be calculated. In particular, we focused on the time constant of the slowest component of the distribution (\(\tau_{32}\)), which, for Scheme I, is approximately given by Eq. 3 at any concentration of agonist. According to this equation, \(\tau_{32}\) for the \(\alpha 5I\) mutant should be 48 ms, which is more than \(10\times\) longer than the observed \(\tau_{32}\) of \(\sim 4.5\) ms.

However, as \(\beta_2\) increases and \(\alpha_2\) decreases, the sequential-model simplification (Scheme I and Eq. 3) becomes inappropriate because the probability that a burst of openings terminates from the open state (i.e., by agonist dissociation from the open state followed by closing or by directly entering a desensitized state) is no longer negligible. Accordingly, an MWC-like kinetic model (Monod et al., 1965; Scheme II) was used to interpret \(\tau_3\) values. Considering Scheme II, it can be shown that \(\tau_3\) (the slowest component of the open-time distribution) is an increasing function of agonist concentration whose value at infinite agonist concentration is given by Eq. 3, and at zero agonist concentration (our conditions, approximately) by Eq. 4:
where $\sigma$ is the sum of all the rate constants leading away from $OA_2$ other than $\alpha_2$. These are the dissociation rate constant from the diliganded open channel ($2k_2$), the rate constant leading to the long-lived desensitized state ($k_1D$), and the rate constant leading to $CA_2^*$ ($k_1g$).

Using the wild-type value of $40,000 \text{ s}^{-1}$ for $2k_2$ (Akk and Auerbach, 1996; Wang et al., 1997; Salamone et al., 1999), it can be calculated from Eq. 4 that a $\sigma$ value of 200 $\text{s}^{-1}$ accounts for all three experimental observations in the presence of ACh: (a) a $\tau_o$ value of 0.75 ms for the wild type; (b) a $\tau_o$ value of 4.5 ms for the mutant at low ACh concentration, and (c) an $\sim10$-fold smaller $EC_{50}$ for the mutant. We were unable to find other combinations of $\beta_2$, $\alpha_2$, $2k_2$, and $\sigma$ values that could account simultaneously for these three findings.

Mutational Analysis of Position $\alpha_5$

The $\alphaS5I$ mutation increases both the hydrophobicity and size of the side chain. To further characterize the structure–function relationships of this position, we engineered a series of polar and hydrophobic side chains at $\alpha_5$ (Fig. 1 and Table II). Using ACh as the agonist, hydrophobic substitutions (Phe, Cys) increased $\tau_o$ nearly 10-fold, while polar substitutions (Asp, Gln, and Lys) were without effect. Ala, which has the highest mutational probability with Ser, also yielded wild-type-like $\tau_o$ values.

Some of these $\alphaS5$ mutants were also studied using choline as the agonist (Fig. 3 A). The $\alphaS5C$ mutation had essentially the same effect as the $\alphaS5I$ mutation; that is, it produced a large increase in the opening rate constant (26-fold) and a modest decrease in the closing rate constant (3.7-fold). The $\alphaS5Q$ mutant was similar to the wild type, with essentially no change in the opening ($\sim1.3$-fold increase) or closing ($\sim1.2$-fold increase) rate constants. For both mutations, $\tau_o$ values were calculated using Eq. 4 with $2k_2 = 40,000 \text{ s}^{-1}$ and $\sigma = 200 \text{ s}^{-1}$, assuming that only $\beta_2$ and $\alpha_2$ change upon mutation. For $\alphaS5C$ and $\alphaS5Q$ receptors, the calculated values were $\sim4.5$ and 0.77 ms, respectively, in reasonable agreement with the experimental values of 7.5 and 0.6 ms (Table II).

The similarity of the effects of S/Q and C/I residues suggests that the volume of the $\alpha_5$ sidechain ($\sim89$ and $\sim144 \AA^3$ for S and Q, and $\sim109$ and $\sim167 \AA^3$ for C and I, respectively; Zamyatnin, 1972) is not a significant factor with regard to channel gating. The overall results suggest that a hydrophobic residue in position $\alpha_5$ increases the AChR gating equilibrium constant.

Mutational Analysis of the $\alpha$-subunit M2–M3L Domain

To further explore the role of the M2–M3L in gating, we made additional mutations at different positions of the $\alpha$ subunit (Fig. 4 and Table II). Both $T \rightarrow A$ and $T \rightarrow I$ mutations at position $\alpha_3$ shortened $\tau_o$ of ACh-gated currents by a factor of $\sim4.5$ (0.16–0.17 vs. 0.75 ms in the wild type).
For these mutations, kinetic modeling of single-channel clusters elicited by ACh was possible because the opening rate constant was slower than the wild type's and, therefore, the use of choline was not needed. $\beta_2$ was reduced from $50,000 \text{ s}^{-1}$ in the wild type to 2,127 s$^{-1}$ and 1,730 s$^{-1}$ in the Ala and Ile mutants (Fig. 5), whereas $\alpha_2$ increased from 2,500 s$^{-1}$ in the wild type to $6,000 \text{ s}^{-1}$ in both mutants. Thus, the consequences of these $\alpha T3$ mutations are nearly opposite to those of the $\alpha S5$ mutation; i.e., $\beta_2$ decreases $\sim 26$-fold and $\alpha_2$ increases $\sim 2.4$-fold. The net effect of these mutations is to decrease $u_2$ by $\sim 62$-fold.

Returning to Eq. 4, the shorter $\tau_o$ in $\alpha T3A$ and $\alpha T3I$ AChRs mainly reflects a reduced number of openings per burst (because of a much smaller $\beta_2$). Like mutations at position $\alpha 5$, mutations of $\alpha 3$ residues also had a larger effect on channel opening than on channel closing.

The dose–response properties of $\alpha T3I$ and $\alpha T3A$ activated by ACh are shown in Fig. 5. The $K_d$ values of these two mutants for ACh were similar to that of the wild type, while the $\tau_o$ values were smaller. This result is consistent with the suggestion that the nearby mutation $\alpha S5I$ does not alter the $K_d$. Together, these results suggest that the M2–M3L of the $\alpha$ subunit is not an important determinant of agonist affinity.

We next studied the effect of $S \rightarrow A$ and $S \rightarrow I$ mutations of the $\alpha 4$ residue. With ACh as the agonist, $\alpha S4A$ AChRs had a $\tau_o$ of $\sim 1.2$ ms, which is not very different from that of the wild type, while the $\alpha S4I$ mutant had a $\tau_o$ of $\sim 0.36$ ms (Fig. 4 and Table II). Further kinetic and dose–response analyses of the $\alpha S4I$ mutant in the presence of ACh are shown in Fig. 5 and Table II. In this regard, $\alpha S4I$ resembles the $\alpha T3I$ mutant. Unlike the $\alpha 4$ mutant, however, $\alpha_2 = 2,777 \text{ s}^{-1}$, which is similar to that of the wild type. Again, mutations at the $\alpha 4$ position mostly affect the channel opening rate constant. Dose–response analysis of $\alpha S4I$ indicates that this mutation decreases the $K_d$, but less than approximately threefold (Fig. 5).

In summary, positions 3, 4, and 5 of the M2–M3L of the $\alpha$ subunit were probed using mutagenesis. Muta-
tions in this region mainly affect channel gating with little effect on agonist binding. Hydrophobic substitutions at position 5 increase, and at positions 3 and 4 decrease, the channel opening rate constant.

The M2–M3L Domain in Other Subunits

In the β and ε subunits, an S → I mutation in position 4 produced channels having prolonged τ₀ values. With ACh as the agonist, τ₀ was ~9.1 ms for βS4I and 6.6 ms for εS4I AChRs, respectively (Fig. 6). Again, we quantified the effects of the mutations on the opening and closing rate constants using choline as the agonist (Fig. 7). For βS4I, β₂ was ~3.9-fold faster and α₂ was ~6.1-fold slower than the wild type (θ₂ increased ~23.5-fold). The effect of εS4I was less pronounced, as β₂ was only ~2.8-fold faster and α₂ was ~2.2-fold slower than in the wild type (θ₂ increased approximately sixfold). Application of Eq. 4 assuming that only τ₀ changes upon mutation and using a σ value of 200 s⁻¹ yields τ₀ values of 3.7 and 2.2 ms for the βS4I and εS4I mutant receptors, respectively.

The δS4I mutation did not affect channel gating. We scanned nearby residues in δ by mutation to Ile, and none yielded AChRs having prolonged τ₀ values (Fig. 6 and Table II).

Change in the Length of the M2–M3L

To explore the effect of changes in length, we engineered α-subunit mutants having longer or shorter M2–M3L regions. Decreasing the linker size by one residue

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<Figure 5. Equilibrium concentration–response curves for wild-type and representative M2–M3L mutant AChRs in the presence of ACh. (A) Intracellular P_open as a function of ACh concentration was fitted with Eq. 1. (B) The effective opening rate (β') as a function of ACh concentration was fitted with Eq. 2. (●) wild type, ( (*) αS5D, (▲) αS4I, (●) αT3A, (+) αT3I.

<Figure 6. Open-interval properties of β, δ, and ε-subunit M2–M3L mutants. Currents were recorded in the presence of 0.2 (β, δ, and ε-subunit mutants) or 1 (wild-type) μM ACh. The slowest open-time constant of each construct is indicated as the mean ± SD of three patches.
shortened $\tau_o$ (\~0.28 ms), while adding one residue lengthened it (\~1.46 ms) (Fig. 4 and Table II). The briefer $\tau_o$ of the deletion mutant suggests that $\beta_2$ is slower and $\alpha_2$ is \~1.5-fold faster when the M2–M3L is longer. The $\tau_o$ of the addition mutant suggests that $\beta_2$ is faster, and/or $\alpha_2$ is slower, when the M2–M3L is longer.

Mapping the Gating Transition State at the M2–M3L

To probe the closed-like-vs.-open-like character of the M2–M3L at the gating transition state, we examined the correlation between rate and equilibrium constants of the gating reaction for the side-chain series at positions $\alpha_3$ (S, A, and I), $\alpha_4$ (S and I) and $\alpha_5$ (S, Q, C, and I). A linear correlation ("linear free-energy relationship;" Leffler and Grunwald, 1963) is expected to hold when the conformation of the mutated region of the protein at the transition state is intermediate between the conformations in the open and closed states (Grosman and Auerbach, 2000b; Grosman et al., 2000). The slope of the Brønsted plot ($\log$ rate constant vs. $\log$ equilibrium constant), $\Phi$, is a measure of how "open" the probed region is when the transition state is reached, with $\Phi = 0$ being fully closed and $\Phi = 1$ being fully open.

For the $\alpha_3$ position, $\Phi = 0.789 \pm 0.010$ in the presence of ACh (the correlation coefficient between $\log \beta$ and $\log \alpha$ is 0.985). For the $\alpha_4$ position, $\Phi = 0.971$ in the presence of ACh as well (two-point relation). For the $\alpha_5$ position, $\Phi = 0.694 \pm 0.028$ in the presence of choline (Fig. 3 B; the correlation coefficient between $\log \beta$ and $\log \alpha$ is 0.988). These results suggest that the conformation of the region around $\alpha_5$–S3–5 at the transition state of the gating allosteric transition is \~70% open-like (\~30% closed-like). It is important to note that $\Phi$ values are likely to be insensitive to the nature of the particular ligand used as the agonist (Grosman et al., 2000) and, therefore, that $\Phi$ values estimated in the presence of choline are good estimates of the corresponding values in the presence of ACh.

$\Phi$ values of the $\beta$ and $\epsilon$ positions were calculated from two-point relations. With choline as the agonist, these values were 0.431 in $\beta$ and 0.568 in $\epsilon$. The $\Phi$ value corresponding to the $\delta$ subunit, however, could not be measured because none of the tested mutations in the M2–M3L of this subunit caused a significant change in the gating equilibrium constant.

DISCUSSION

$\alpha$5I is a Typical SCCMS Mutant

Like many other SCCMS mutants (Sigurdson and Auerbach, 1994; Ohno et al., 1995), $\alpha$5I AChRs have a prolonged apparent open time (Table II), an increased sensitivity to the metabolite choline (Fig. 3 A and Zhou et al., 1999), and an increased spontaneous activity (Zhou et al., 1999). Our results suggest that the affinity ratio is largely unaffected by the $\alpha$5I mutation and, therefore, that the increased gating in the presence of ACh can be accounted for by a more favorable isomerization step (an increased unliganded gating equilibrium constant). Although the term "slow channel" that describes the SCCMS phenotype refers to the slow decay of the endplate current, the predominant underlying mechanism of the disease is the accelerated channel opening: $\alpha$5I AChRs are hyperactive channels that open too fast. Along similar lines, "fast-channel" congenital myasthenic syndromes (e.g., $\epsilon$P121L; Ohno et al., 1996) are caused by sluggish AChRs that open too slowly.

Mechanistic Basis of the SCCMS Phenotype of $\alpha$5I Receptors

It is difficult to analyze the kinetic and dose–response properties of AChRs that open extremely rapidly, such as $\alpha$5I. The brevity of sojourns in the closed diligated state results in many missed closures, and this distortion of the data makes it impossible to estimate binding and gating rate constants directly from the dwell-time series. Moreover, in these cases, the gating equilibrium constant is very large, hence the estimates of gating ($\theta_2$) and agonist-dissociation ($K_a$) equilibrium constants obtained by fitting $P_{\text{open}}$–vs–concentration curves are ill-determined (i.e., have large coefficients of variation).

Another consequence of a very fast opening rate constant is that the EC50 value may no longer depend on the agonist affinity for the closed state of the receptor. According to Scheme II, when $\sqrt{\theta_2} > 1$, (EC50 $\equiv$ $J_d$/$\sqrt{\theta_0}$), where $J_d$ is the microscopic dissociation equilibrium constant from the open state.

To overcome the limitations imposed by using ACh as the agonist, we analyzed the single-channel behavior of the $\alpha$5I mutant in the presence of choline, a low-
efficacy agonist that opens the channel relatively slowly. In the presence of choline, we found that the mutation affects gating (28-fold increase in $\beta_2$, 3.3-fold decrease in $\alpha_2$) while having little effect on agonist affinity for the closed state. The latter was also suggested by the analysis of $P_{\text{open}}$ vs. ACh concentration data of nearby mutations that decrease $\theta_2$ ($\alpha T 3 I, \alpha T 3 A,$ and $\alpha S 5 I$). Because the mutation increases the gating equilibrium constant to the same extent regardless of whether ACh or choline is bound to the receptor, we also suggest that the agonist affinity for the open channel is largely unaffected. These results clearly indicate that the predominant effect of the $\alpha S 5 I$ mutation is to favor the channel’s isomerization step.

The observed prolongation of $\tau_0$ in the presence of ACh by the $\alpha S 5 I$ mutation was similar to the value predicted using Eq. 4, with $\sigma$ equal to 200 s$^{-1}$. Considering that open channels desensitize at a rate <10 s$^{-1}$ (Auerbach and Akk, 1998) and enter the $CA^s$ state (see Scheme II) at a rate of ~50 s$^{-1}$ (Salamone et al., 1999), then ligand dissociation from the diliganded open state would occur at a rate of ~140 s$^{-1}$; that is, ~70 s$^{-1}$ from each binding site if they were equivalent. This value is ~300 slower than the dissociation rate constant from the closed conformation, and (only) about three times faster than the dissociation rate constant from the desensitized state (Auerbach and Akk, 1998).

Previous estimates of the agonist-dissociation rate constant from diliganded open AChRs consisted of calculations in the context of cyclic kinetic schemes, assuming microscopic reversibility and identical association rate constants of the agonist to the closed and open conformations. For ACh, values of 0.2 s$^{-1}$ for adult-type ($\alpha_2\beta_2\epsilon$; Edelstein et al., 1997) and 17.3 s$^{-1}$ for fetal-type receptors ($\alpha_2\beta_2\gamma$; Edelstein et al., 1996) can be found in the literature. Values of 0.02 s$^{-1}$ for suberyldicholine bound to adult-type AChRs (Colquhoun and Sakmann, 1985) and 40 s$^{-1}$ for carbamylcholine bound to fetal-type AChRs (Jackson, 1988) have also been reported.

Applying a similar approach, we can calculate the dissociation rate constant from diliganded open channels using published values of the agonist-dissociation rate constant from diliganded closed channels and the equilibrium constants of mono- and diliganded gating. For ACh bound to adult-type AChRs, values include 3.7 s$^{-1}$ (from data in Ohno et al., 1996) and 96.5 s$^{-1}$ (from data in Wang et al., 1997).

The reason for the disparity of these estimates is not clear, but is most likely related to inaccuracies in the identification of monoligated openings (Lingle et al., 1992). Our approach, although having its own caveats, is an alternative one that not only avoids this pitfall, but also relieves the constraint of equal ACh-association rate constants to the closed and open forms of the channel.

Taken together, our results suggest that $\alpha S 5 I$ receptors can open as fast as at ~$1.4 \times 10^5$ s$^{-1}$. Experiments with other very-fast-opening mutants, as well as confirmation of the $\sigma$ value estimated here, are needed to explore the issue of a speed limit for AChR gating. An upper limit of $10^5$ s$^{-1}$ has been proposed for the $T \rightarrow R$ conformational change of hemoglobin (Eaton et al., 1991) and of $10^6$ s$^{-1}$ for protein folding (Hagen et al., 1996).

**Mutational Analysis of the M 2-M 3L Domain**

The results indicate that the hydrophobic amino acids Phe, Cys, and Ile at the $\alpha 5$ position increase the diliganded gating equilibrium constant and produce the SCCMs phenotype. The less hydrophobic residues Asp, Lys, Gln, Ala, and Ser result in wild-type–like gating. As noted before, the large variation in the size of these side chains indicates that residue volume is not a significant factor with regard to gating. It is also interesting that the $\alpha 5$ mutants have either a wild-type or an SCCMs-like phenotype, in spite of the variety of residues tested. Taken alone, this pattern would suggest that the local environment of $\alpha S 5$ changes from polar to nonpolar upon channel opening.

It is remarkable that similar modifications to adjacent residues in the M2-M3L have such diverse effects on gating. As opposed to the effect of $S \rightarrow I$ and $S \rightarrow A$ mutations at position $\alpha 5$, similar substitutions at positions $\alpha 3 (T \rightarrow I$ and $T \rightarrow A)$ or $\alpha 4 (S \rightarrow I)$ impair gating (i.e., decrease $\theta_2$), making the interpretation of the structure-function results less straightforward. This suggests a marked dependence of the effect of mutations on the position in the primary sequence of the M2-M3L. This pattern is different from that of other regions of the AChR like M2, where, regardless of the position, mutations have either little effect or favor (rather than impair) gating (e.g., Labarca et al., 1995; Grosman and Auerbach, 2000a,b).

The effect of M2-M3L mutations was different in the different subunits even though they were at positions that were homologous in terms of sequence. Even though mutations in $\beta$ and $\epsilon$ yielded AChRs with increased $\theta_2$ values, characteristic of the SCCMs phenotype, position 4 was more sensitive to mutation than position 5, as opposed to the situation in the $\alpha$ subunit. Also, the $\tau_0$ values for the $\beta$- and $\epsilon$-subunit mutants predicted by Eq. 4, assuming that only $\theta_0$ changes upon mutation and a value of 200 s$^{-1}$ for $\sigma$, differ from the experimentally derived $\tau_0$ values. More experiments are needed to determine the agonist-binding properties of the $\beta$ and $\epsilon$ M2-M3L mutants. Interestingly, mutations at positions $\delta 3-6$ did not have any effect on channel gating whatsoever. Thus, we conclude that, in the M2-M3L, homology in sequence does not coincide with homology in function. It is unlikely that this interpretation is caused by the vagaries of primary-structure
alignment because the M2-M3L motif is very well conserved (Table I). That mutations in the δ subunit do not affect gating is yet additional evidence for the distinct role of this subunit in the receptor’s function (Grosman and Auerbach, 2000a,b).

Physical Mechanism

It is remarkable that the gating behavior of the α-subunit M2–M3L mutants was trimodal, being either that of the wild type (αSSD, αSSK, and αSSA), of the SCCMS mutant αSSl (αSSl, αSSC, and αSSF), or of the αT3 mutants (αT3A, αT3L, and αS4l). Considering that these side chains cover a rather wide range of physicochemical properties, we would have expected to observe a continuum of gating phenotypes if the local environment of the side chains themselves had been a critical determinant of the gating equilibrium constant. Instead, the modality of the results suggests that the mutations cause the M2–M3L as a whole to adopt one of at least three conformations, each leading to a discrete phenotype.

M2–M3L Mutations in Other Receptors

Mutations of the M2–M3L domain have been studied in other ionotropic receptors. In glycine receptors, these have been shown to selectively impair gating (according to our numbering system: α5, R-3L, α5, R-3Q, α5, K2E, and α5, Y5C; Lynch et al., 1997), a conclusion that was confirmed by single-channel analysis of the α5, K2E mutant (Lewis et al., 1998). These results are similar to ours for the α3 position of muscle AChRs. In GABA<sub>C</sub> receptor β1 homomers, the R2A mutation (Kusama et al., 1994) increases the diliganded gating equilibrium constant, a result that is similar to ours for the S → I mutations at positions α5, β4, and e4 of the muscle AChR. Mutation of position 5 of the neuronal AChR subunits α3, α6, or position 6 of β3, impair gating without affecting binding (Campos-Caro et al., 1996; Rovira et al., 1998, 1999).

In summary, the M2–M3L is an important domain with regard to channel gating. However, whether it acts as the crucial transduction element between the binding sites and the pore, as it has often been suggested (e.g., Lynch et al., 1997), remains an open question. Mutagenesis data indicate that substitutions almost everywhere in the protein (including the binding sites) can affect the gating allosteric transition in the same way as those in the M2–M3L. This supports the notion that regions throughout the entire receptor are involved in the gating conformational change.

The M2–M3L and the Gating Reaction Pathway

Through Φ value analysis (Fersht, 1999; Grosman et al., 2000), we estimated the position of the transition state along the gating-reaction pathway measured at the M2-M3L of the α, β, and ε subunits. In the α subunit, Φ ≈ 0.7. This suggests that, during the opening reaction, the closed → open conformational rearrangement of this domain is >70% complete at the transition state (or <30% complete when going in the open → closed direction). In the ε and β subunits, however, Φ was found to be ~0.57 and ~0.43, respectively. Φ values have been suggested to reflect the sequence of conformational rearrangements with the movement of regions having larger Φ values preceding those with smaller ones (Itzhaki et al., 1995; Villegas et al., 1998; Ternström et al., 1999; Grosman et al., 2000). Insofar as the M2–M3L residues of all subunits occupy similar locations in the quaternary structure of the AChR, this result suggests that the α subunit leads the conformational change in the opening direction, being followed by ε, and then by β, at least as far as the M2–M3L domains are concerned.

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