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Mutations at Lipid-Exposed Residues of the Acetylcholine Receptor Affect Its Gating Kinetics

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ABSTRACT

The firmest candidate among the transmembrane portions of the nicotinic acetylcholine receptor (AChR) to be in contact with the lipid bilayer is the fourth segment, M4. To explore the contribution of αM4 amino acid residues of mouse AChR to channel gating, we combined site-directed mutagenesis with single-channel recordings. Two residues in αM4, Cys418 and Thr422, were found to significantly affect gating kinetics when replaced by alanine. AChRs containing αC418A and αT422A subunits form channels characterized by a 3- and 5-fold reduction in the mean open time, respectively, suggesting an increase in the closing rate due to the mutations. The calculated changes in the energy barrier for the channel closing process show unequal and coupled contributions of both positions to channel gating. Single-channel recordings of hybrid wild-type α/αT422A AChR show that the closing rate depends on the number of α subunits mutated. Each substitution of threonine to alanine changes the energy barrier of the closing process by ~0.5 kcal/mol. Recordings of channels activated by high agonist concentration suggest that these mutations also impair channel opening. Both Cys418 and Thr422 have been postulated to be in contact with the lipid milieu and are highly conserved among species and subunits. Our results support the involvement of lipid-exposed residues in αM4 in AChR channel gating mechanism.

The nicotinic AChR from muscle and electric organ is an integral membrane protein composed of four homologous subunits in the stoichiometry $\alpha_2\beta\gamma\delta$. In muscle, the ϵ subunit replaces γ during development, and AChRs composed of $\alpha_2\beta\epsilon\delta$ are found in all normal adult muscles. Based on hydrophobicity profiles and immunochemical and biochemical studies, the occurrence of four transmembrane regions (M1–M4) has been postulated for each subunit, flanked by extracellular amino and carboxyl termini. Of these four transmembrane regions, M2 has been indicated to line the walls of the channel (Hucho *et al*., 1996; Bouzat and Barrantes, 1997). Accumulated evidence on the influence of the lipid environment on AChR function suggests that the ability of the protein to "sense" the lipid is located at the lipid/protein interface (Barrantes, 1993, 1997). On the basis of the classic four-helix model of the AChR, it has been argued that the firmest candidate among the transmembrane portions to be in contact with the lipid is M4 (residues 409–426 in the *Torpedo californica* α chain). The M4 transmembrane region (1) is not part of the ion channel proper, (2) is the least conserved among the putative transmembrane segments of the AChR, (3) is the most hydrophobic, and (4) has been labeled at specific amino acid residues by hydrophobic probes (Blanton and Cohen, 1992, 1994). Labeling by use of the photoactivatable hydrophobic probe [^{125}I]TID occurs at Cys412, Met415, Cys418, Thr422, and Val425 of the α subunit. The labeling pattern in *T. californica* suggests that this segment adopts an α-helical structure and has substantial contacts with the lipid (Blanton and Cohen, 1994).

Experimental data suggest that M4 is involved in AChR channel gating kinetics. Mutation of αCys418 of *T. californica* AChR to tryptophan greatly prolongs channel open time (Li *et al*., 1990). Leu458 and Met460 of the mouse γ subunit contribute to the long duration of single-channel events (Bouzat *et al*., 1994). Replacement of Gly421 in the *T. californica* α subunit by phenylalanine or tryptophan produces a substantial increase in the open time constant (Lasalde *et al*., 1996). The mechanistic contribution of this segment to channel gating is still unknown.

Here, we explore in detail the involvement of mouse α subunit M4 residues in channel gating by combining site-directed mutagenesis with single-channel recordings. Our strategy is based on examination of the gating behavior of mutant AChR in which different amino acids located in αM4 are replaced by alanine. Two residues in αM4, Cys418 and

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ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine; TID, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine; HEK, human embryonic kidney; BTX, bungarotoxin; CCh, carbamoylcholine.

Thr422, are found to significantly affect gating kinetics when replaced by alanine. We also show that M4 segments of both α subunits make nonadditive contributions to the stabilization of the open state. This study provides new insights into the role of lipid-facing residues in the AChR channel function and further supports the participation of the M4 segment in channel gating mechanisms.

Materials and Methods

Construction of mutant subunit. Mouse cDNAs were subcloned into the cytomegalovirus-based expression vector pRBG4 (Sine, 1993). Mutant α subunits were constructed by bridging the naturally occurring sites *Bst*X-1 and *Bsp*M-1 with synthetic double-stranded oligonucleotides (Bio-Synthesis, Lewisville, TX), essentially as described previously (Bouzat *et al*., 1994). Single-stranded oligonucleotides were purified by polyacrylamide gel electrophoresis and annealed before ligation. Restriction mapping and dideoxy sequencing on polyacrylamide gels confirmed all constructs.

Expression of AChR and ligand binding measurements. HEK 293 cells were transfected with α (wild-type or mutant), β, δ, and ε cDNA subunits using calcium phosphate precipitation at a subunit ratio of 2:1:1:1 for α/β/δ/ε, respectively, essentially as described previously (Bouzat *et al*., 1994). For expression of embryonic-type AChRs, the ε subunit was replaced by the γ subunit. For transfections, cells at 40–50% confluence were incubated for 8–12 hr at 37° with the calcium phosphate precipitate containing the cDNAs in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Cells were used for single-channel measurements 1 or 2 days after transfection.

Surface AChR expression was determined by [^{125}I]α-BTX binding. Cells were incubated with 10 nM [^{125}I]α-BTX for 60 min at room temperature, and unbound toxin was removed by centrifugation. Nonspecific binding was determined in the presence of 5 mM CCh.

Binding of CCh was measured by competition against the initial rate of [^{125}I]α-BTX as described previously (Sine and Taylor, 1979; Sine *et al*., 1994). Cells were resuspended in potassium Ringer's solution (140 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, 25 mM HEPES, pH 7.4, containing 30 mg/liter bovine serum albumin) and divided into aliquots for ligand binding measurements. Cells first were incubated for 30 min with different concentrations of CCh; [^{125}I]α-BTX was subsequently added to a final concentration of 5 nM, and the cells were incubated for an additional 20 min. The total number of binding sites was determined by incubating cells with 5 nM [^{125}I]α-BTX for 2 hr in the absence of agonist. Binding was finished by the addition of potassium Ringer's solution containing 30 mM CCh. Fractional occupancy by CCh was fitted by the Hill equation:

$$1 - \text{fractional occupancy} = [1/(1 + ([\text{CCh}]/K_d)^{n_H}] \quad (1)$$

where K_d is the apparent dissociation constant, and n_H is the Hill coefficient.

Patch-clamp recording and analysis. Recordings were obtained in the cell-attached configuration at 20°. The bath and pipette solutions contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, and 10 mM HEPES, pH 7.4. Patch pipettes were pulled from 7052 capillary tubes (Garner Glass, Claremont, CA) and coated with Coat D (M-Line Accesories, Measurements Group, Raleigh, NC). Pipette resistances ranged from 5 to 7 MΩ, and ACh was added to the pipette solution. In most of the experiments, the final concentration of ACh was 1 μM. For recordings at a high agonist concentration, 100 μM ACh was used. Single-channel currents were recorded using an Axopatch 200 B (Axon Instruments, Burlingame, CA) patch-clamp amplifier, stored using a video cassette recorder (Panasonic) and a modified pulse-code modulator (Sony), and transferred in digital form at 50 kHz to a Macintosh Centris 650 computer using the program Pulse (HEKA Elektronics, Lambrecht, Germany). Channel events were detected using the program MacTAC (Skalar Instruments, Seattle, WA; purchased from HEKA Elektronics) with the threshold set at 0.5× amplitude, the digital filter set at 5 kHz, and a decimation ratio of 4. Open-time histograms were plotted using a logarithmic abscissa and a square root ordinate (Sigworth and Sine, 1987) and fitted to the sum of exponential functions by maximum likelihood using the program TACFit. Bursts were defined as a series of opening events separated by less than a specified closed time corresponding to the intersection between the first and second briefest components in the closed-time histogram.

Results

Single-channel measurements of αM4 mutant AChRs. To explore at the single-channel level the involvement of amino acid residues of the M4 domain in AChR gating kinetics, we constructed a series of α subunits mutated at positions postulated to be in contact with the lipid (Blanton and Cohen, 1994); single-channel currents then were recorded from the resulting mutant AChRs. A quadruple-point mutant α subunit ($M4_{x4}$) first was constructed through the alanine substitution of amino acids Leu411, Met415, Cys418, and Thr422. If one assumes an α-helix structure, all these residues should be oriented toward the same face. Blanton and Cohen (1994) further postulated that this face of the helix is in contact with the membrane lipid. HEK cells transfected with α, β, ε, and δ subunit cDNAs (wild-type AChR) exhibit channel openings typical of adult AChRs (Fig. 1a). Open-time distributions show a major component of ~1 msec with a relative amplitude of >0.8 in all recordings (Table 1). As described previously, a brief component of ~100–300 μsec or a longer component of ~3 msec was observed in <40% of the recordings (Bouzat *et al*., 1994). As shown in Fig. 1b, the quadruple-mutant α subunit formed channels characterized by a significant reduction in the duration of the main open state when coexpressed with wild-type β, ε, and δ subunits. The mean open time calculated for these mutant channels was about four times briefer than that of wild-type channels (Table 1). For wild-type AChRs, closed-duration histograms were well described by the sum of two exponentials: a minor component of ~50–100 μsec and a major component (relative area of >0.90) of 10–50 msec. No apparent changes were observed in closed-time histograms obtained with 1 μM ACh-activated mutant channels. The burst duration of the mutant AChR decreased in parallel with the open time, and no change was observed in the number of openings per burst (Table 1). As expected, the conductance of the mutant AChR channel was identical to that of wild-type channels as judged from single-channel current amplitude at the standard holding potential of −70 mV (Table 1).

To determine which of the four amino acids was responsible for affecting the gating kinetics observed in the $\alpha M4_{x4}$ mutant, we next dissected the mutated residues into four individual point mutations. As shown in Table 1, two of the four amino acids, αCys418 and αThr422, seem to determine the reduction in the open-channel duration when substituted by alanine. In the case of the mutants αL411A and αM415A, the channels were indistinguishable from wild-type channels (Table 1). In the case of αC418A and αT422A, briefer openings were accompanied by briefer burst durations, with no significant changes in the closed-time components and number of openings per burst (Table 1). Differences in mean open

times between αC418A and αT422A AChRs were statistically significant ($p < 0.05$), suggesting that alanine substitution at the different positions has quantitatively different effects. The results suggest that the main effect of alanine substitution at positions αCys418 and αThr422 is an increase in the closing rate.

We also carried out alanine substitution at position 414 (Phe414) of the α subunit M4 segment. The αF414A-containing channels were similar to wild-type AChRs. Single-channel measurements revealed only a slight increase in the duration of the open state, paralleled by an increase in the burst duration (Table 1). No other significant changes in channel properties were observed with this mutant (not shown).

Estimation of changes in energy barrier for the closure of the channel produced by alanine substitution. We explored the contribution of each individual amino acid (Cys418 and Thr422) to the energy barrier for the closing process. We considered the classic activation scheme for the AChR:

$$A + R \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} AR + A \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} C \underset{\alpha}{\overset{\beta}{\Leftrightarrow}} O \qquad (2)$$

where two agonists A bind to the AChR with association rates k_1 and k_2, respectively, and dissociate from the receptor with rates k_{-1} and k_{-2}, respectively. Fully occupied AChR (C) opens with rate β, and open AChR (O) closes with rate α. We determined the closing rate, α, as the reciprocal of the mean open time and calculated the differences in free energy for the closing reaction introduced by the mutations as:

$$\Delta(\Delta G) = RT \ln(\alpha_{wt}/\alpha_m) \qquad (3)$$

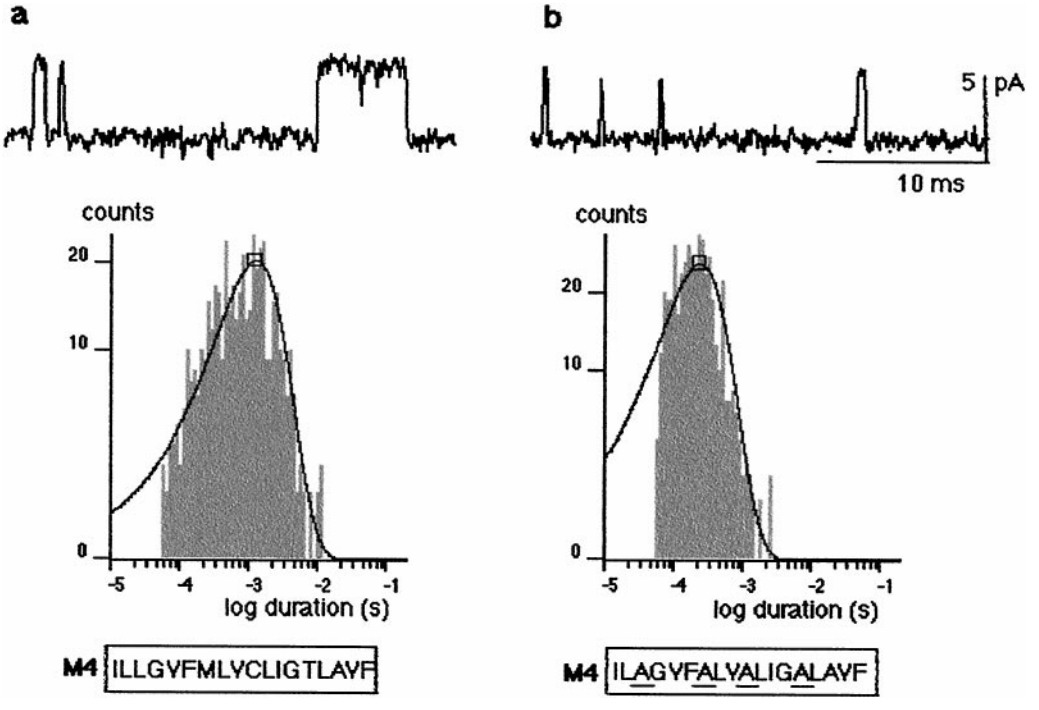

Fig. 1. Single-channel recordings of wild-type and alanine-substituted mutant AChR channels. Recordings were obtained from cells expressing wild-type adult AChRs (a) and mutant AChRs containing the α subunit in which positions 411, 415, 418, and 422 of the M4 segment were replaced by alanine (b) (see corresponding M4 sequence at the *bottom*). *Top traces*, representative current traces for each AChR (−70 mV membrane potential; filtered at 5 kHz). *Upward deflections*, openings. The histograms show the open-time distribution. Mean open time and relative areas are (a) wild-type: τ_{on} = 1.16 msec (1), 541 events; and (b) mutant: τ_{on} = 0.23 msec (1), 460 events.

TABLE 1

Channel properties of adult- and embryonic-type AChR carrying mutations in the α subunit M4 segment

Recordings were obtained at a membrane potential of −70 mV from HEK cells transfected with α (wild-type or mutants), β, δ, and either ε or γ subunit cDNAs. Values for the mean open time (τ_{on}) and mean burst duration (τ_{burst}) were obtained from the corresponding open-time histograms and correspond to the major component (relative area > 0.80). A burst was defined as a series of opening events separated by <1 msec. n/b is the number of openings per burst. In the case of $\alpha M_{4\times4}$, 50% of the open-time histograms showed a minor component (relative area, 0.14 ± 0.04) of 1.09 ± 0.32 msec. A minor component of 1.03 ± 0.16 msec (relative area, 0.20 ± 0.03) was observed in 40% of the recordings from C418A-containing channels. In all recordings corresponding to αT422A-containing AChRs, open-time histograms were well fitted by only one component.

α Subunit	τ_{on}	τ_{burst}	n/b	Current	n
	msec			*pA*	
Adult (ε-containing) AChR					
Wild-type	0.98 ± 0.23	1.25 ± 0.19	1.08 ± 0.10	5.70 ± 0.30	6
$M4_{\times4}$	0.23 ± 0.02[a]	0.29 ± 0.10[a]	1.09 ± 0.05	5.50 ± 0.20	6
L411A	0.93 ± 0.16	1.06 ± 0.15	1.12 ± 0.10	5.60 ± 0.10	5
M415A	1.30 ± 0.25	1.49 ± 0.21	1.10 ± 0.02	5.80 ± 0.12	3
C418A	0.31 ± 0.07[a]	0.36 ± 0.07[a]	1.03 ± 0.02	5.80 ± 0.10	5
T422A	0.18 ± 0.03[a]	0.20 ± 0.04[a]	1.04 ± 0.03	5.70 ± 0.24	6
F414A	1.34 ± 0.30	1.49 ± 0.40	1.08 ± 0.06	5.80 ± 0.25	8
Embryonic (γ-containing) AChR					
Wild-type	5.51 ± 0.51	9.20 ± 1.41	1.70 ± 0.14	3.85 ± 0.13	6
C418A	2.82 ± 0.56[a]	4.03 ± 0.90[a]	1.28 ± 0.10	3.67 ± 0.14	6
T422A	1.55 ± 0.03[a]	1.80 ± 0.05[a]	1.20 ± 0.10	4.00 ± 0.20	6

[a] Statistically different from the wild-type value ($p < 0.001$).

where $\Delta(\Delta G)$ is the change between mutant and wild-type AChRs in the energy that the channel must overcome to make the transition from the open to the closed state, expressed in kcal/mol, and α_{wt} and α_m are the closing rates for wild-type and mutant AChRs, respectively. The values obtained for $\Delta(\Delta G)$ were −0.84 kcal/mol for $\alpha M4_{x4}$, −0.67 kcal/mol for αC418A, and −0.98 kcal/mol for αT422A mutant AChR. All values are negative, indicating that the energy barrier for the closing of the channel decreases when the specified amino acids are replaced by alanine. The energy values calculated for the individual point mutant α subunits are different. In addition, these energy values are nonadditive when the two mutations are combined in the quadruple-point mutant α subunit. Energetic coupling between amino acids can be studied by thermodynamic mutant cycles (Horovitz and Fersht, 1990; Hidalgo and MacKinnon, 1995). We applied this type of analysis to our data by constructing the double-mutant cycle:

$$\begin{array}{ccc} & X1 & \\ C_{418}T_{422} & \rightarrow & A_{418}T_{422} \\ \downarrow & & \downarrow \\ C_{418}A_{422} & \rightarrow & A_{418}A_{422} \\ & X2 & \end{array} \qquad (4)$$

where C418T422 corresponds to wild-type AChR and A418T422 and C418A422 correspond to single-mutant channels, and data for the double-mutant channel (A418A422) are taken from the $\alpha M4_{x4}$ mutant AChR. Based on this cycle, we calculated the value of the coupling coefficient $\Omega = X1/X2$, where X1 is τ_{on} (C418T422)/τ_{on} (A418T422) = 3.16, and X2= τ_{on} (C418A422)/τon (A418A422) = 0.78. The value determined for Ω is 4.0, which corresponds to a coupling energy given by RT ln Ω of ~0.8 kcal/mol.

Expression of hybrid wild-type/mutant AChR channels. To determine whether the two α subunits contribute independently to channel gating, we coexpressed wild-type and T422A α subunits (cDNA ratio 1:1) together with wild-type β, ϵ, and δ. In some recordings, all wild-type AChR kinetics were detected; in others, channels typical of the αT422A mutation were observed. We were able to record from patches in which different channel populations were apparent. Because mutation T422A does not interfere with normal assembly of the AChR oligomer (see below), cells expressing both wild-type and mutated α subunits should show three (or four) different AChR populations: wild-type channels, mutant channels, and hybrid channels composed of wild-type and mutant α subunits. As shown in Fig. 2b, open-time distributions in these recordings could be dissected into three components, in which the briefest component corresponded to the mutant channels (Fig. 2c), the longest to wild-type channels (Fig. 2a), and the intermediate one to channels expressing both types of α subunits.

Fig. 3 shows the effect of the three possible combinations of two different α subunits assembled in a pentameric AChR on the closing rate, ranging from wild-type AChRs (0) to all αT422A-containing, mutant AChR channels. It can be seen that τ_{on} depends on the number of α subunits that are mutated and that each substitution of threonine for alanine makes a similar contribution to the energy barrier of the closing process, decreasing it by ~0.5 kcal/mol.

Expression of mutant AChRs containing the γ subunit. Muscle development involves a change in the subunit composition of the AChR from the $\alpha_2\beta\gamma\delta$ to the $\alpha_2\beta\epsilon\delta$ combination. Inspection of the amino acid residues in the M4 region of γ and ϵ subunits indicates a remarkable degree of conservation of certain residues among species (Cockroft *et al.*, 1992). We also measured the mean open time of AChR channels containing the mutant α subunits assembled in embryonic (γ)-type oligomers. As described previously (Bouzat *et al.*, 1994), open-time histograms of wild-type γ-type AChRs show a main component of ~5–7 msec (relative area > 0.7) (Table 1). When T422A or C418A mutant α

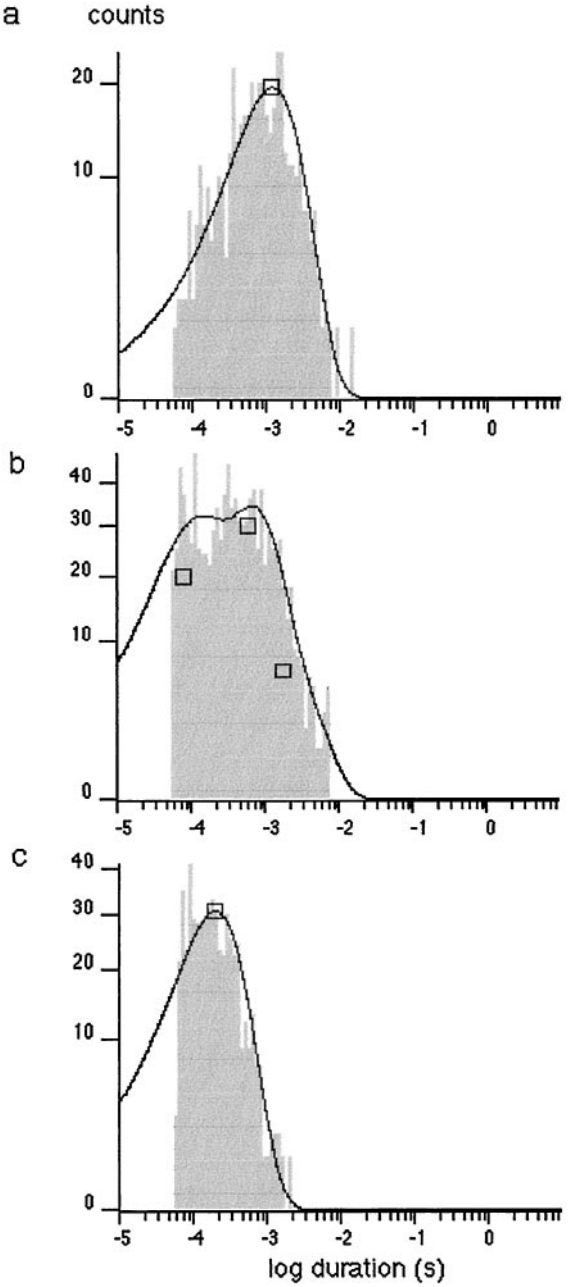

Fig. 2. Single-channel recordings of hybrid AChRs composed of wild-type α/αT422A mutant subunits. Open-time histograms were obtained from patch-clamp recordings in cells transfected with wild-type β, ϵ, and δ subunits together with a single wild-type α subunit (a), wild-type and mutant α subunits (b), and T422A α subunit (c). Recordings were obtained at −70 mV. Mean open-time and relative areas are (a) 1.21 msec (1), 440 events; (b) 1.30 msec (0.27), 0.51 msec (0.38), and 0.1 msec (0.35), 1021 events; and (c) 0.18 msec (1), 545 events.

subunit replaced wild-type, a reduction in the mean open time was evident (Table 1). Thus, the effect of alanine substitution at these positions in γ-type AChRs mimicked the channel-gating behavior observed with wild-type α subunits coexpressed with the ϵ subunit in adult AChRs (Table 1). However, the extent of the reduction in the mean open time differed from that of the adult AChR. Calculation of the ratios of the mean open time of mutant channels/wild-type channels (τ_{on} mutant AChR/τ_{on} wild-type AChR) yielded values of 0.50 ± 0.05 and 0.31 ± 0.01 for αC418A AChRs containing γ and ϵ subunits, respectively. For αT422A channels, the calculated ratios were 0.28 ± 0.02 and 0.19 ± 0.01 for γ- and ϵ-containing AChRs, respectively. Thus, the reduction in the mean open time produced by alanine substitution at positions 418 and 422 seems to be more significant in ϵ-containing AChR than in the embryonic-type AChR.

Mutant αAla418 and αAla422 AChR channels activated by high agonist concentration. We next studied the effect of αC418A and αT422A mutations on AChR channel properties activated by high agonist concentration. At 100 μM ACh, wild-type channels open in long clusters of well defined activation episodes, clearly illustrated in the closed-time histogram (Fig. 4a). The closed-time distribution is well fitted by three or four components; the main one corresponds to brief durations due to closings within a cluster, and the longer one corresponds to desensitization of the AChR. Clusters corresponding to mutant AChR showed longer closed intervals. The increase in the duration of the closing episodes within a cluster is reflected in the closed-time histograms (Fig. 4, b and c). The change is more evident for the αT422A mutant. The reduction in the mean open time induced by alanine substitutions also was evident at high agonist concentrations: from 1.29 ± 0.20 msec in wild-type AChR, there was a 4-fold reduction (0.37 ± 0.10 msec) for αCys418, and; a 6-fold reduction (0.23 ± 0.50 msec) was observed with the αT422A mutant. The slight increase in the mean open time of all types of channels activated by 100 μM ACh with respect to 1 μM ACh is only apparent because of the loss of resolution for the very brief closings.

Effects of αC418A and αT422A mutations on agonist binding. To determine whether the mutations in αM4 introduced changes in equilibrium agonist binding, we compared the inhibition of α-BTX binding by the agonist CCh in the two αM4 mutants, C418A, and T422A. Similar profiles were observed in all cases, with a slight increase in the apparent affinity constant (K_d) in the case of the T422A-mutant AChR (Fig. 5). No significant changes in the Hill coefficient (n_H) were introduced by the point mutations, indicating that cooperative interactions in ligand binding were not affected.

Surface expression of mutant AChRs. We proceeded to determine the cell surface expression of AChR-containing α subunit mutants by measuring [^{125}I]α-BTX binding. Fig. 6 shows that alanine substitution at strategic positions decreased surface expression of AChR. In all cases, expression was not lower than 30% of that of the control. The largest decrease was observed with the C418A mutant (Fig. 6).

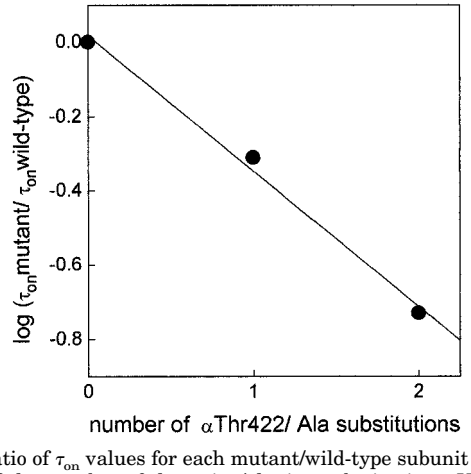

Fig. 3. Ratio of τ_{on} values for each mutant/wild-type subunit AChR as a function of the number of threonine/alanine substitutions. Values were obtained from the corresponding open-time histograms. Ratios plotted on a log scale display the data on a linear free energy scale.

Discussion

In the current work, mutagenesis and heterologous expression studies were carried out to explore the involvement of αM4 residues in AChR function. We mutated six different residues (Table 1), five of them postulated to be oriented mainly toward the lipid bilayer (Fig. 7). Two of them, Cys418 and Thr422, were found to affect gating kinetics, indicating that they are located at strategic positions in αM4. The main effect of alanine substitution at these positions was a reduction in the duration of the open state, ~5-fold for Thr422 and ~3-fold for Cys418. Double-mutant cycle analysis suggests that the two residues (Cys418 and Thr422) are somehow coupled in their contribution to the closing rate given that the calculated coupling coefficient, Ω, differed from unity (Hidalgo and MacKinnon, 1995). This coupling between Cys418 and Thr422 seems to be weak (~0.8 kcal/mol) and could be direct or not (Horovitz and Fersht, 1990).

In *T. californica* AChRs, αCys418 has been reported to affect the channel closing rate (Lee *et al.*, 1994; Lasalde *et al.*, 1996). Here, we found that Thr422 has a more significant effect on channel kinetics than Cys418, at least when both positions are mutated to alanine. This could be due to the fact that alanine substitution of a threonine involves a more drastic change in the hydrophobicity than substitution of cysteine [hydrophobicity changes of −2.5 and 0.7 for threonine and cysteine, respectively, in accordance with the hydropathicity index of Kyte and Doolittle (1982)].

αThr422 has been labeled by TID and thus is a firm candidate to have a lipid-facing location (Blanton and Cohen, 1994). An inspection of M4 sequence among species indicates that Thr422 is highly conserved in all muscle α subunits, as well as in β, δ, and γ subunits (see Fig. 7), and even in neuronal α2, α3, α4, β2, and β4 subunits. Thr422 is replaced by serine in mouse ϵ subunit (Cockroft *et al.*, 1992). The high degree of conservation of Thr422 suggests that this position is structurally or functionally (or both) important for the AChR.

Although a more detailed kinetic analysis (Zhang *et al.*, 1995) would be necessary to determine the contribution of individual microscopic steps to the changes observed in mutant AChRs, our results clearly suggest that the rate of channel closure (α in eq. 2) is the main step involved, in accordance with data obtained from αCys418 mutations of *T. californica* AChR (Lasalde *et al.*, 1996). Alanine substitution at both 418 and 422 positions favors the closing process.

Cotransfection of cells with wild-type and T422A mutant α

subunits resulted in the expression of hybrid AChR channels. Because α subunits are asymmetrically assembled in the pentameric AChRs (for review, see Prince and Sine, 1998), two different hybrid channels should be formed, although they might be kinetically indistinguishable. Open-time histograms of recordings containing mixed-channel populations were well fitted by three components, with the intermediate one corresponding to the hybrid channel. Calculation of changes in free energy in the hybrid channels shows that both α subunits contribute independently to the energy barrier for the closing process. The change in free energy due to a single threonine/alanine substitution is in the order of 0.5 kcal/mol. This is in the range of the energies calculated for hydrogen bonds between two uncharged residues (0.5–1.5 kcal/mol; Fersht *et al*., 1985) and for other weak interactions such as Van der Waals and dipole interactions. Threonine is highly capable of hydrogen bonding due to its hydroxyl group. Thus, one possible explanation for the effect of αThr422 substitution is that this residue is involved in AChR channel gating through stabilization of the open state by hydrogen bonds. A mechanism involving polar interactions was postulated for the contribution of Leu9′ of the M2 segment to channel gating (Filatov and White, 1995). The detailed contribution of hydrogen bonds or other weak interactions in αM4 is still unclear. Further structural information will be needed to settle this issue, particularly because the secondary structure of the M4 domain remains controversial. Both β sheet (Unwin, 1993) and all-α-helix models (Blanton and

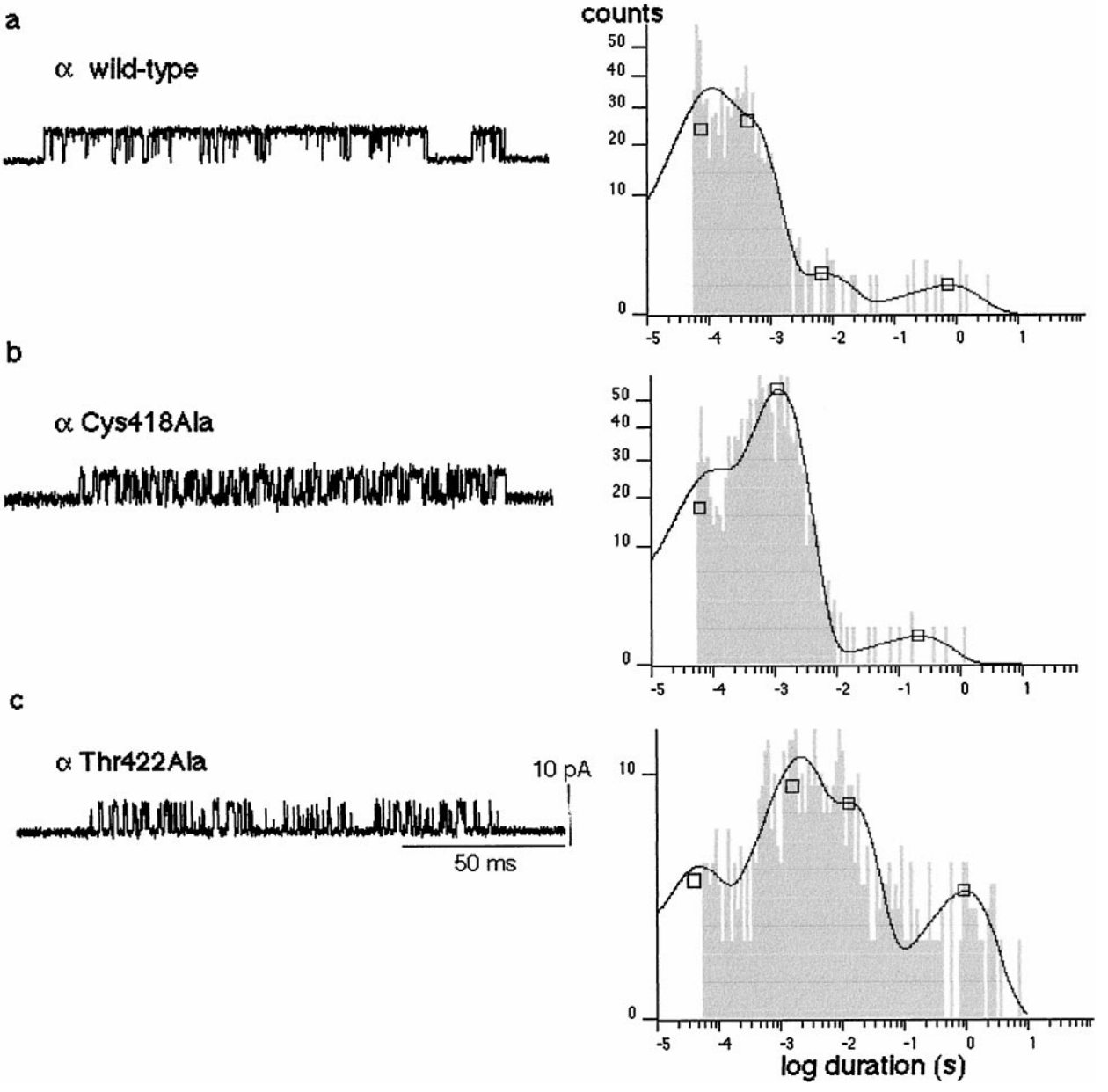

Fig. 4. Recordings at high agonist concentration of wild-type, αC418A, and αT422A AChR. Cells were transfected with β, ϵ, and δ subunits together with wild-type α (a), αC418A (b), and αT422A (c) subunits. Channels activated by 100 μM ACh were recorded at −70 mV. *Traces*, representative AChR currents. The histograms correspond to the closed durations. Mean closed-time components and areas are (a) c1 = 101 μsec (0.45), c2 = 422 μsec (0.51), c3 = 6.37 msec (0.024), c4 = 0.73 sec (0.011); 838 events; (b) c1 = 62.5 μsec (0.24), c2 = 1.16 msec (0.75), c3 = 0.208 sec (0.01); 1413 events; and (c) c1 = 39.7 μsec (0.14), c2 = 1.64 msec (0.39), c3 = 13.3 msec (0.34), c4 = 0.93 sec (0.13); 566 events.

Cohen, 1992, 1994; Baenziger and Méthot, 1995) have been proposed. One possibility is that αThr422, and M4 in general, contributes through strategic positions to channel gating by mediating allosteric contacts with the other transmembrane segments. An alternative, nonexcluding explanation is that certain residues in M4 might be involved in maintaining appropriate interactions with lipids, as suggested by labeling studies with the hydrophobic probe TID (Blanton and Cohen, 1992, 1994). αThr422 is one of the residues presumably located at the lipid/protein interface. The sensitivity of AChR function to its lipid environment (see reviews in Barrantes 1993, 1997), as well as to the presence of natural (Marsh and Barrantes, 1978; Bouzat and Barrantes, 1996) or synthetic (Bouzat and Barrantes, 1993) steroids, has been demonstrated. If αM4 were an α-helix perpendicular to the plane of the membrane, Thr422 would be located at ~10 Å from the polar head region of the extracellular membrane leaflet. At this position, exposure of the hydroxyl group to the phospholipid acyl chain region would be energetically unfavorable. However, if the helix were not perpendicular to the plane of the membrane, then Thr422 would be able to fulfill its potential H bond-forming ability with the phospholipid polar head groups. In fact, current models of the AChR transmembrane region (Ortells and Lunt, 1996, Ortells *et al.*, 1997, 1998) that closely match the overall shape of this region as observed by cryoelectron microscopy (Unwin, 1993, 1995) place M4 with a tilt of ~30 degrees with respect to the membrane, thus providing a structural basis to this contention.

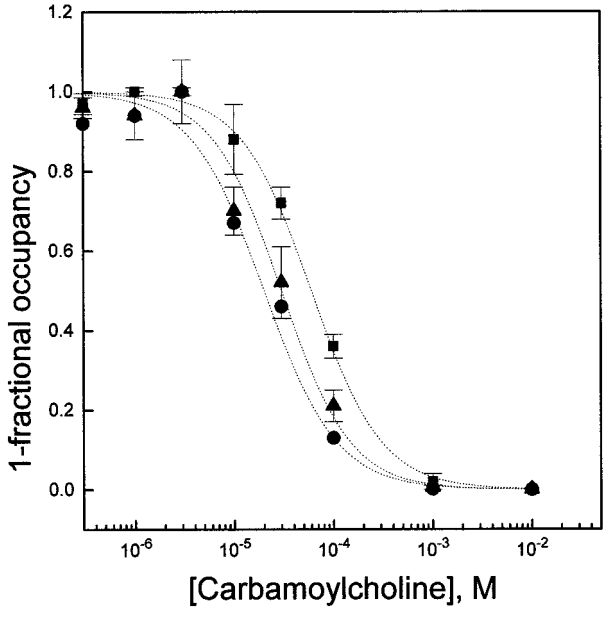

Fig. 5. CCh binding to cell-surface AChR. The curves correspond to data obtained from cells transfected with β, ϵ, and δ subunits together with wild-type α (●), αC418A (▲), and αT422A subunits (■). The curves are fits to the Hill equation with the following parameters: $\alpha_2\beta\epsilon\delta$: $K_d = 2 \times 10^{-5}$ M, $n_H = 1.18$; α_2(C418A)$\beta\epsilon\delta$: $K_d = 2 \times 10^{-5}$ M, $n_H = 1.10$; α_2(T422A)$\beta\epsilon\delta$: $K_d = 6 \times 10^{-5}$ M, $n_H = 1.23$. In all curves, values are mean of at least three experiments.

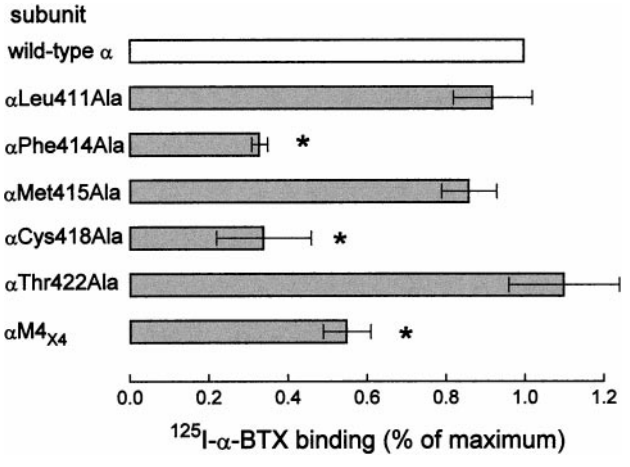

Fig. 6. Surface expression of AChRs containing mutant α subunits. HEK 293 cells were transfected with β, ϵ, and δ plus the specified α subunit. [^{125}I]α-BTX binding is expressed in terms of total number of toxin sites, and normalized to the level of expression observed in wild-type AChR. *, Statistically significant ($p < 0.05$).

The increase in channel closing rate observed in the αM4 mutant AChR could be due either to the fact that the mutations destabilize the open state or to the fact that the energy barrier for the closing reaction is reduced (Jackson, 1993). In the presence of a high ACh concentration (100 μM), wild-type AChRs open in clusters of many closely spaced openings. These correspond to the repetitive activation of a single AChR molecule, separated by prolonged silent periods due to desensitization. The open probability is high at such ACh concentrations, as judged from the proportion of time spent in open periods within clusters. In the T422A mutant, the majority of closings within clusters are in long-lived components, with a mean time of >1 msec. The increase in the duration of these closed intervals could arise from a slower opening rate (β in eq. 2). Single-channel recordings at saturating concentrations are needed to unequivocally settle this issue. What is beyond doubt is that both mutations impair opening of the channel, with this effect being more pronounced for the αT422A mutation.

To help elucidate the mechanistic contribution of αM4 to binding kinetics, we also measured equilibrium agonist binding by competition against the initial rate of [^{125}I]α-BTX binding. At equilibrium, binding of CCh includes contributions of resting, open-channel, and desensitized AChR states, each of which binds agonist with a different affinity. A change in the contribution of one or more of these states therefore can result in a change in apparent affinity for CCh. A slight (3-fold) increase in apparent K_d was observed in the αT422A mutant, which could be explained by a small change in the extent of desensitization.

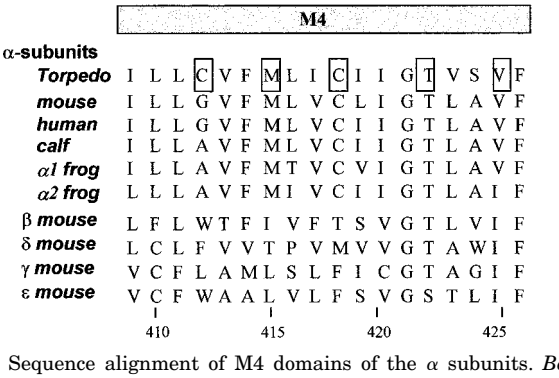

Fig. 7. Sequence alignment of M4 domains of the α subunits. *Boxed*, amino acids labeled by the hydrophobic probe TID (Blanton and Cohen, 1992, 1994).

In all muscle AChRs, the γ and ϵ subunits are postulated to be located between the two α subunits in both embryonic and adult receptors (see Prince and Sine, 1998). The decrease in the reduction of the mean open time exerted by alanine substitutions at positions 418 and 422 of the α subunit is slightly more pronounced in adult (ϵ) than in embryonic (γ) AChR. In *T. californica* AChR, little if any effects were observed on the maximum normalized response to ACh upon substitution of αCys418 by alanine (Lee *et al*., 1994). One possible explanation for the influence of non-α subunits on the effects produced by mutations in the α subunit is the occurrence of interactions between the M4 domain of the α subunit and the ϵ or γ subunit.

In summary, our results clearly demonstrate that conserved residues at strategic positions in αM4 play a significant role in muscle-type AChR gating. We demonstrate that the highly conserved Thr422, as well as the previously reported Cys418, contributes to channel kinetics. The major effect of alanine substitution at these positions seems to be a decrease in the energy that the channel must overcome to make the transition from the open to closed state, probably mediated by disruption of hydrogen bonds or other weak interactions. The findings support the involvement of the AChR αM4 domain in channel kinetics. Given the high degree of homology among ligand-gated ion channels (Ortells and Lunt, 1995), it would be worth exploring whether this property also is found in other members of the superfamily. In this respect, the position occupied by Thr422 seems to be highly conserved among other ligand-gated ion channels. A threonine at an homologous position is found in 5-hydroxytryptamine$_3$ receptor subunits; in γ-aminobutyric acid$_A$ receptor subunits, it is replaced by a highly conserved tyrosine residue (Cockroft *et al*., 1992).

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References

Baenziger JE and Méthot N (1995) Fourier transform infrared and hydrogen/deuterium exchange reveal an exchange-resistant core of α-helical peptide hydrogens in the nicotinic acetylcholine receptor. *J Biol Chem* **270:**29129–29137.

Barrantes FJ (1993) Protein-lipid interactions, in *New Comprehensive Biochemistry* 26 (Watts A, ed) pp 231–257, Elsevier, Amsterdam.

Barrantes FJ (1997) Physical state of the nicotinic acetylcholine receptor membrane and modulation of the receptor channel by the lipid environment, in *From Ion Channels to Cell-to-Cell Conversations* (Latorre R and Saenz JC, eds) pp 199–216, Plenum Press, New York.

Blanton MP and Cohen JB (1992) Mapping the lipid-exposed regions in the *Torpedo californica* nicotinic acetylcholine receptor. *Biochemistry* **31:**3738–3750.

Blanton MP and Cohen JB (1994) Identifying the lipid-protein interface of the *Torpedo* nicotinic acetylcholine receptor: secondary structure implications. *Biochemistry* **33**:2859–2872.

Bouzat C and Barrantes FJ (1993) Acute exposure of nicotinic acetylcholine receptors to the synthetic glucocorticoid dexamethasone alters single-channel gating properties. *Mol Neuropharm* **3:**109–116.

Bouzat C, Bren N, and Sine S (1994) Structural basis of the different gating kinetics of fetal and adult acetylcholine receptors. *Neuron* **13:**1395–1402.

Bouzat C and Barrantes FJ (1996) Modulation of muscle nicotinic acetylcholine receptors by the glucocorticoid hydrocortisone: possible allosteric mechanism of channel blockade. *J Biol Chem* **271:**25835–25841.

Bouzat C and Barrantes FJ (1997) Assigning functions to residues in the acetylcholine receptor channel region. *Mol Membr Biol* **14:**167–177.

Cockroft VB, Osguthorpe DJ, Barnard EA, Friday AE, and Lunt GG (1992) Ligand-gated ion channels: homology and diversity. *Mol Neurobiol* **4:**129–169.

Fersht AR, Shi J-P, Knill-Jones J, Lowe DM, Wilkinson AJ, Blow DM, Brick P, Carter P, Waye MMY, and Winter G (1985) Hydrogen bonding and biological specificity analyzed by protein engineering. *Nature (Lond)* **314:**235–238.

Filatov GN and White MM (1995) The role of conserved leucines in the in the M2 domain of the acetylcholine receptor in channel gating. *Mol Pharmacol* **48:**379–384.

Hidalgo P and MacKinnon R (1995) Revealing the architecture of a K^+ channel pore through mutant cycles with a peptide inhibitor. *Science (Washington DC)* **268:**307–310.

Horovitz A and Fersht AR (1990) Strategy for analysing the co-operativity of intramolecular interactions in peptides and proteins. *J Mol Biol* **214:**613–617.

Hucho F, Tsetlin VI, and Machold J (1996) The emerging three-dimensional structure of a receptor. *Eur J Biochem* **239:**539–557.

Jackson M (1993) *Thermodynamics of Membrane Receptors and Channels*. CRC Press, Boca Raton, FL.

Kyte J and Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157:**105–132.

Lasalde JA, Tamamizu S, Butler DH, Vibat CRT, Hung B, and McNamee MG (1996) Tryptophan substitutions at the lipid-exposed transmembrane segment M4 of *Torpedo californica* acetylcholine receptor govern channel gating. *Biochemistry* **35:**14139–14148.

Lee Y-H, Li L, Lasalde J, Rojas L, McNamee M, Ortiz-Miranda SI, and Pappone P (1994) Mutations in the M4 domain of *Torpedo californica* acetylcholine receptor dramatically alter ion channel function. *Biophys J* **66:**646–653.

Li L, Schuchard M, Palma A, Pradier L, and McNamee MG (1990) Functional role of the Cys-451 thiol group in the M4 helix of the γ subunit of *Torpedo californica* acetylcholine receptor. *Biochemistry* **29:**5428–5436.

Marsh D and Barrantes FJ (1978) Immobilized lipid in acetylcholine receptor-rich membranes from *Torpedo marmorata*. *Proc Natl Acad Sci USA* **75:**4329–4333.

Ortells MO, Barrantes GE, Wood C, Lunt GG, and Barrantes FJ (1997) Molecular modelling of the nicotinic acetylcholine receptor transmembrane region in the open state. *Prot Eng* **10:**511–517.

Ortells MO, Barrantes GE, and Barrantes FJ (1998) Molecular modelling of the nicotinic acetylcholine receptor, in *The Nicotinic Acetylcholine receptor: Current Views and Future Trends* (Barrantes FJ, ed.). pp 85–108, Neuroscience Intelligence Unit, Landes Publishing, Austin, TX.

Ortells MO and Lunt GG (1995) Evolutionary history of the ligand gated ion channel superfamily. *Trends Neurosci* **18:**121–127.

Ortells MO and Lunt GG (1996) A β-sheet/α-helix model for the transmembrane region of the nicotinic acetylcholine receptor. *Prot Eng* **9:**51–59.

Prince R and Sine SM (1998) The ligand binding domains of the nicotinic acetylcholine receptor, in *The Nicotinic Acetylcholine receptor: Current Views and Future Trends* (Barrantes FJ, ed.). pp 31–59, Neuroscience Intelligence Unit, Landes Publishing, Austin, TX.

Sigworth F and Sine SM (1987) Data transformations for improved display and fitting of single-channel dwell time histograms. *Biophys J* **52:**1047–1052.

Sine SM (1993) Molecular dissection of subunit interfaces in the acetylcholine receptor: identification of residues that determine curare selectivity. *Proc Nat Acad Sci USA* **90:**9436–9440.

Sine SM, Quiram P, Papanikolaou F, Kreienkamp HJ, and Taylor P (1994) Conserved tyrosines in the α subunit of the nicotinic acetylcholine receptor stabilize quaternary ammonium groups of agonist and curariform antagonists. *J Biol Chem* **269:**8808–8816.

Sine SM and Taylor P (1979) Functional consequences of agonist-induced state transitions in the cholinergic receptor: studies in culture muscle cells. *J Biol Chem* **254:**3315–3325.

Unwin N (1993) The nicotinic acetylcholine receptor at 9 Å resolution. *J Membr Biol* **229:**1101–1124.

Unwin N (1995) Acetylcholine receptor channel imaged in the open state. *Nature (Lond)* **373:**37–43.

Zhang Y, Chen J, and Auerbach A (1995) Activation of recombinant mouse acetylcholine receptors by acetylcholine, carbamylcholine and tetramethylammonium. *J Physiol (Lond)* **486.1:**189–206.

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