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Evidence That the M2 Membrane-Spanning Region Lines the Ion Channel Pore of the Nicotinic Receptor

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Site-directed mutagenesis and expression in *Xenopus* oocytes were used to study acetylcholine receptors in which serine residues (i) were replaced by alanines (α, δ subunits) or (ii) replaced a phenylalanine (β subunit) at a postulated polar site within the M2 transmembrane helix. As the number of serines decreased, there were decreases in the residence time and consequently the equilibrium binding affinity of QX-222, a quaternary ammonium anesthetic derivative thought to bind within the open channel. Receptors with three serine-to-alanine mutations also displayed a selective decrease in outward single-channel currents. Both the direction of this rectification and the voltage dependence of QX-222 blockade suggest that the residues mutated are within the aqueous pore of the receptor and near its cytoplasmic (inner) surface.

THE NICOTINIC ACETYLCHOLINE RECEPTOR (AChR) contains both binding sites for agonists and an aqueous cation-selective channel or pore that opens for times on the order of milliseconds as a result of agonist binding. The receptor from skeletal muscle is an integral membrane protein comprised of four homologous polypeptide subunits, in the stoichiometry $\alpha_2\beta\gamma\delta$. Hydrophobicity analysis of the translated cDNA sequences indicates that each subunit probably contains at least four membrane-spanning regions (MSRs) denoted M1 through M4. There are many important questions about the relation of primary sequence to (i) the three-dimensional structure of the receptor and (ii) gating and permeation through the channel. The MSRs are likely to be crucial for these functions; the present study uses a combination of site-directed mutagenesis and electrophysiological measurements to locate a site on the M2 MSR that forms part of the lining of the pore.

Low-resolution structural data on the AChR, deduced by electron microscopy, reveal that the five individual subunits are arranged with radial pseudosymmetry in a roughly cylindrical structure; the transmembrane pore is at the axis (*1*). In most structural models, homologous MSRs from each subunit associate to form the lining of the pore. The available structural data do not yet reveal the identities of the particular MSRs involved. An atomic resolution structure by x-ray diffraction is not yet available but will eventually provide a detailed structure and will aid in interpreting changes in the function of mutants with altered amino acid sequence. However, at present, for the AChR as for many other proteins, site-directed mutagenesis is being combined with functional measurements to identify the roles of various domains and of individual amino acids (*2, 3*). For membrane channels in particular, electrophysiological measurements are an appropriate way to obtain high-resolution data on properties that define the function of the protein: gating and blockade on the millisecond time scale, single-channel conductance, and voltage sensitivity.

We have therefore constructed and studied receptors with substitutions at a postulated site (*4*), containing polar but uncharged amino acids on the M2 regions of three subunits. For both the α and δ subunits, the existing serine was changed to an alanine; in the β subunit, the existing phenylalanine was changed to serine. Electrophysiological measurements of two types on these mutated receptors strongly indicate that the M2 MSRs of these subunits form part of the lining of the pore. (i) As serines are removed, there are consistent effects on the kinetics and equilibria of receptor blockade by the cationic open-channel blocker, QX-222, which is thought to bind within the pore near its cytoplasmic (inner) end. (ii) For receptors with three fewer serines than normal, outward currents through the channel are selectively decreased; this rectification could be caused by an increased energy barrier for ion flux within the pore and near its cytoplasmic (inner) end. These results thus identify specific serine residues in the M2 helices that interact both with permeant cations and with an open-channel blocker. Because most structural models of the AChR also place these residues near the cytoplasmic (inner) end of the M2 MSR, we refer to them as the inner polar site (IPS).

The hypothesis that the M2 MSRs, which are amphiphilic helices, form part of the lining of the ion pore was suggested by reports that noncompetitive inhibitors attach covalently to serines at the IPS in at least α, β, and δ *Torpedo* AChR subunits (*4*). There is a 1:1 stoichiometry of label to receptor, but serines are labeled in the M2 region of several subunits. [However, labeling was reported within the M1 region of the α subunit with the use of a different compound (*5*).] Also, experiments with chimeric calf-*Torpedo* δ subunits suggest that a region containing the M2 helix and the adjacent bend between M2 and M3 is involved in determining the rate of ion transport in the absence of external divalent cations (*3*).

Mouse AChRs from BC3H-1 cells contain serine residues at the postulated IPS on only the α and δ subunits (*6*). Residues Ser^{248} in the α subunit and Ser^{262} in the δ subunit were therefore replaced (*7*) by alanine (subunits bearing the mutation are denoted by the subscript A). In addition, residue Phe^{259}, which occupies an equiva-

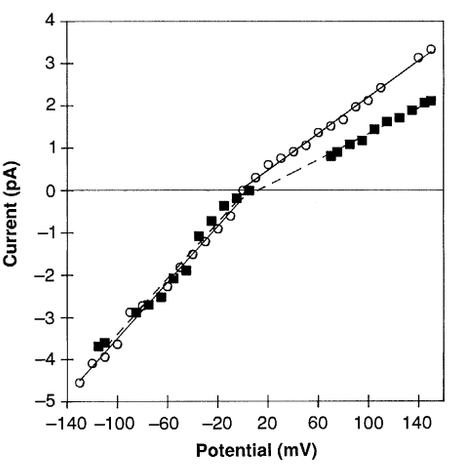

Fig. 1. Single-channel current-voltage relations for representative patches containing $\alpha\beta\gamma\delta$ (○) or $\alpha_A\beta\gamma\delta_A$ (■) AChRs. Data were obtained and analyzed as described in Table 1.

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lent position in the β subunit, was replaced by Ser (denoted by the subscript S). In vitro RNA synthesis then generated mRNA encoding either mutated or normal receptor subunits. These mRNAs, along with that encoding the γ subunit, were injected into *Xenopus* oocytes to form various hybrid AChRs containing combinations of normal and mutated subunits (*2, 8–10*). Because the subunit stoichiometry is $\alpha_2\beta\gamma\delta$, this procedure enabled the construction of receptors containing one Phe → Ser or zero, one, two, or three Ser → Ala mutations at the IPS.

The expressed receptors were studied with single-channel (*11*) and macroscopic techniques (*12*) (Table 1). As reported previously, the normal AChRs expressed in oocytes have properties that agree closely with those of AChRs in mouse muscle and in BC3H-1 cells (*10, 13, 14*). Furthermore, the mutated receptors also display the major functional hallmarks of correctly assembled AChRs, as judged by the following results: (i) the Hill coefficients were normal; (ii) macroscopic currents were 64% to 78% of normal levels; and (iii) for inward currents, the single-channel conductance was unchanged in all the mutants (*15*). However, the $\alpha_A\beta\gamma\delta_A$ receptor (containing three Ser → Ala mutations) displayed abnormally small outward single-channel currents both in outside-out and in cell-attached patches (Table 1 and Fig. 1).

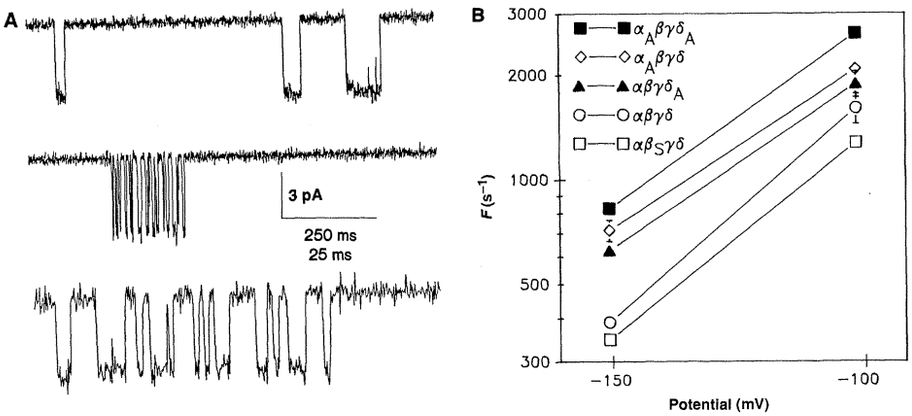

Fig. 2. (**A**) Single-channel analysis of QX-222 effects on normal and mutated receptors. Three discontinuous data segments from the same patch (1 μ*M* ACh, −150 mV, 12°C) are displayed to illustrate the kinetics of the openings (downward deflections) before (upper sweep) and during (lower sweeps) exposure to 20 μ*M* QX-222. Note the tenfold difference in time scale between the lower two traces. (**B**) Effect of mutations on rate constant *F* for dissociation of QX-222 at two voltages. Distributions of closed times briefer than 20 ms were fit to single exponentials; the time constant $1/F$ is the average residence time for QX-222. Note the logarithmic scale of the ordinate. SEM are shown where they exceed the size of the symbols.

Table 1. Effects of M2 Ser → Ala and Phe → Ser mutations on properties of AChRs expressed in oocytes. Note the selective decrease in conductance for outward currents displayed by $\alpha_A\beta\gamma\delta_A$ receptors. Messenger RNAs were transcribed in vitro from cDNA clones of α, β, γ, and δ subunits of mouse BC3H-1 AChR and injected into *Xenopus* oocytes. Mixtures of mRNAs encoded either normal α or mutated α_A (Ser^{248} replaced by Ala), either normal β or mutated β_S (Phe^{259} replaced by Ser), normal γ, and either normal δ or mutated δ_A (Ser^{262} replaced by Ala). Depending on the mixture, a given oocyte thus produced AChRs with one of five genotypes. For $\alpha\beta_S\gamma\delta$, one Phe was replaced by Ser. For $\alpha\beta\gamma\delta$, all were "normal" mouse BC3H-1 subunits. For $\alpha\beta\gamma\delta_A$, one Ser was replaced per AChR. For $\alpha_A\beta\gamma\delta$, two Ser were replaced per AChR. For $\alpha_A\beta\gamma\delta_A$, three Ser were replaced per AChR. Electrophysiological measurements (*33*) at 1 μ*M* ACh and at 12° to 13°C were made 36 to 72 hours after injection of in vitro transcribed subunit mRNAs. Single-channel conductance was determined on both cell-attached and excised outside-out patches, with voltage ramps that swept from −150 to +150 mV in 400 ms; straight lines were fit separately to the limbs at positive and negative voltages. Single-channel open times were measured at −100 mV. Values were obtained from the time constants of exponential decays fit to duration distributions of channel openings. All resolvable sojourns at the baseline level (greater than ~ 100 μs) were treated as bona fide closings. Time constants τ of voltage-jump relaxations are given for jumps from +50 to −110 mV. Data are means ± SEM for the number of observations in parentheses.

mRNAs	Single channel			Macroscopic		
	Conductance (pS)		Open time (ms)	Current (nA)	τ (ms)	Hill coefficient
	Outward	Inward				
$\alpha\beta_S\gamma\delta$	22 ± 2 (6)	33 ± 1 (6)	2.8 ± 0.18 (1)	857 ± 13 (8)	8.3 ± 0.6 (6)	1.6 ± 0.3 (5)
$\alpha\beta\gamma\delta$	24 ± 1 (4)	36 ± 1 (4)	9.2 ± 1.8 (4)	1341 ± 92 (11)	31 ± 2 (7)	1.6 ± 0.15 (4)
$\alpha\beta\gamma\delta_A$	21 ± 1 (6)	32 ± 1 (6)	18.6 ± 3.3 (5)	1043 ± 132 (18)	36 ± 3 (21)	1.7 ± 0.05 (14)
$\alpha_A\beta\gamma\delta$	24 ± 2 (6)	36 ± 1 (6)	12.4 ± 1.1 (2)	1031 ± 119 (14)	26 ± 2 (10)	1.8 ± 0.05 (7)
$\alpha_A\beta\gamma\delta_A$	13 ± 1 (5)	33 ± 2 (7)	19.9 ± 1.8 (3)	950 ± 159 (14)	30 ± 2 (11)	1.6 ± 0.05 (7)

This change in channel conductance occurs only for receptors with three Ser → Ala mutations at the IPS. We have found that an open-channel blocker provides a more sensitive structural probe for changes resulting from single–amino acid changes at the IPS. QX-222, a permanently charged quaternary ammonium derivative of lidocaine, was originally synthesized to provide a model local anesthetic that acts from only one side of the membrane (*17*). QX-222 was subsequently exploited as a "pure" open-channel blocker, a drug that enters the pore and binds at the blocking site only when the channel is in the open state (*18–21*). The bound molecule of QX-222 thus occludes current flow during its residence time according to the following reaction scheme:

$$R_{closed} \rightleftharpoons R_{open} \underset{F}{\overset{G\cdot[Q]}{\rightleftharpoons}} (R_{open}\cdot Q)_{blocked} \quad (1)$$

where R = AChR and Q = blocker (*22*). Equation 1 was developed on the basis of data from neurally evoked postsynaptic currents, fluctuations, voltage-jump relaxations, light-flash relaxations, and single-channel

measurements (*18–21, 23–25*). The latter recordings are especially decisive: the blocker produces transient interruptions of the current through an open channel, and each interruption represents an individual occupancy of the channels by a blocker molecule (*20, 21, 25*). For blockers that act in the charged form, both the forward and reverse rate constants (G and F of Eq. 1) depend on transmembrane voltage; this dependence implies that the QX-222–receptor interaction occurs in a region of the receptor sensitive to the membrane field, presumably within the pore itself. According to these concepts, the binding and the blockade are the same molecular event. We therefore studied the forward and reverse rate constants (G and F) for binding of QX-222 to the open channel.

A pure open-channel blocker such as QX-222 (*26*) is more appropriate for studies of the pore itself than are other noncompetitive inhibitors such as the tertiary amine chlorpromazine, which can also enter closed channels and has a large component of voltage-independent block (*27–29*). Furthermore, the mouse AChR is more appropriate than the *Torpedo* AChR for kinetic studies with open-channel blockers, because the relatively long average open duration allows one to resolve the flickers associated with the binding events.

Experiments with QX-222 and AChRs expressed in oocytes confirmed that channel openings were interrupted by transient periods of blockade (Fig. 2A), lasting on the order of milliseconds (*20, 21, 25*). The time constant of these interruptions, which equals the lifetime of the blocked state ($1/F$ in Eq. 1), decreased monotonically with each successive Ser → Ala mutation. The rate F changed by a factor of 3 to 4 over the range of mutations (Fig. 2B). These data strongly suggest an interaction between QX-222 and the serine residues of the IPS in the M2 MSR.

The forward rate constant, G, for QX-222 binding was determined from the concentration-dependent decrease in the mean channel open time, t,

$$G = \Delta(1/t)/\Delta[\text{QX-222}] \qquad (2)$$

where t is mean open time at a given QX-222 concentration (*20*). We find $G = 1.9 \times 10^7\ M^{-1}\ s^{-1}$ at −100 mV; within the limits of our measurements (about 15%), there was no difference between normal and mutated receptors.

The single-channel measurements on QX-222 blockade were confirmed and extended with macroscopic voltage-jump relaxations, which can be obtained with less tedium and over a broader range of conditions. One expects two exponential components in such relaxations (*24*). The faster "inverse relaxation" (*24*) is too rapid to be resolved by the two-microelectrode voltage clamp. The slower component (*30*) was well resolved in our experiments; its time constant is increased (*24*) in the presence of QX-222:

$$\tau_Q = \tau(1 + [\text{QX-222}]/K_Q) \qquad (3)$$

where τ_Q and τ are the time constants in the presence and absence of QX-222, respectively, and K_Q is the equilibrium constant for the binding of QX-222 and is equal to F/G.

Voltage-jump relaxations were prolonged, as expected, by QX-222 (Fig. 3A); furthermore, the extent of prolongation depended on the number of serine residues at the IPS of each mutated receptor (Fig. 3B). From the voltage-jump data, K_Q was calculated (Fig. 3C), and we made three observations from the results: (i) The effect of mutations on K_Q was nearly the same as on the blocked duration $1/F$, amounting to a four- to fivefold difference over the entire series of mutations. This implies that the mutations affect primarily the residence time $1/F$, although we cannot rule out small effects on the bimolecular binding rate G. In all cases where K_Q was measured from both single-channel and macroscopic experiments, the values agreed to within 10%. (ii) K_Q increased by about 42% for each serine removed. This dependence implies a change in binding free energy of 0.21 kcal/mol of QX-222 per Ser → Ala or Phe → Ser mutation. (iii) The mutations did not substantially affect the voltage dependence of K_Q (Fig. 3C); like previous measurements (*20*), our data suggest that the binding site senses 70% to 80% of the membrane field. Thus the mutations affect only the affinity of the binding but not its location.

Residues with ·OH groups are attractive candidates for the lining of ion channel pores (*31*), because they participate as donors or (less often) acceptors in hydrogen bonds with water. The serines studied here might form hydrogen bonds primarily (i) with water molecules that are contacting other water molecules or (ii) with those that are hydrating a permeant ion. Either possibility suggests that removal of the serines could affect the stability of the open channel, perhaps leading to the subtle changes in gating kinetics noted in our experiments (Table 1). The second possibility might also produce changes in single-channel conductance, as measured for the $\alpha_A\beta\gamma\delta_A$ receptor. That there were no detectable conductance changes for either the δ- or α-mutants alone, however, indicates that ion-pore interactions proceed well enough to support nearly normal permeation even if only a single

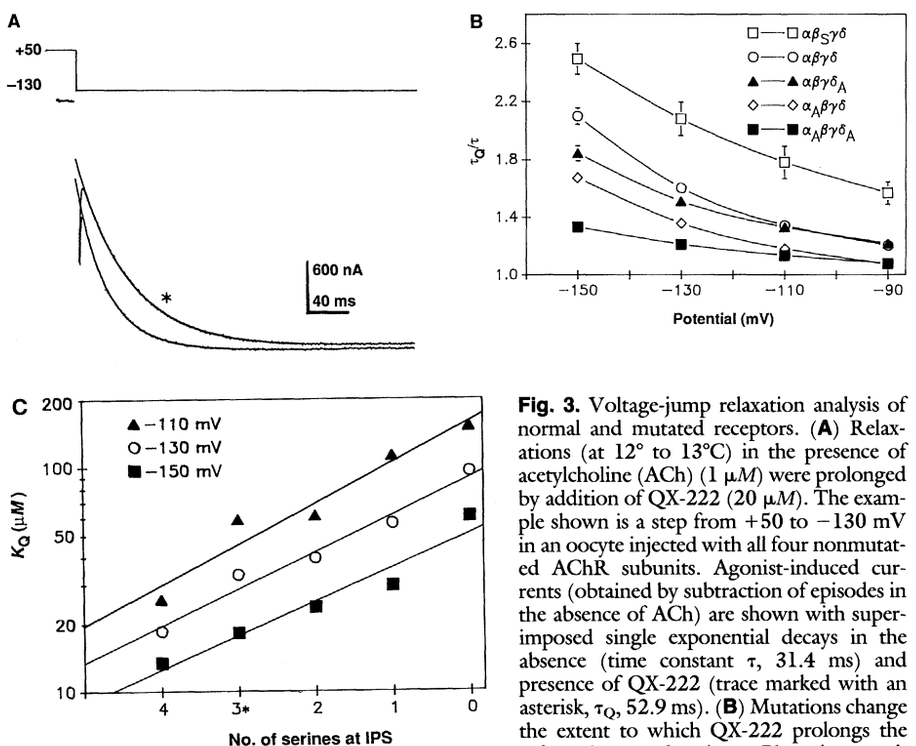

Fig. 3. Voltage-jump relaxation analysis of normal and mutated receptors. (**A**) Relaxations (at 12° to 13°C) in the presence of acetylcholine (ACh) (1 μ*M*) were prolonged by addition of QX-222 (20 μ*M*). The example shown is a step from +50 to −130 mV in an oocyte injected with all four nonmutated AChR subunits. Agonist-induced currents (obtained by subtraction of episodes in the absence of ACh) are shown with superimposed single exponential decays in the absence (time constant τ, 31.4 ms) and presence of QX-222 (trace marked with an asterisk, τ_Q, 52.9 ms). (**B**) Mutations change the extent to which QX-222 prolongs the voltage-jump relaxations. Plotted at each potential for each genotype is the fractional change in relaxation time constant caused by QX-222. Increasing values indicate greater prolongation. For genotype nomenclature, see Table 1. Points are averaged from five to eight cells; SEM are shown when they exceed the size of the symbol. (**C**) The data from (B) at three voltages were transformed according to Eq. 3. Asterisk denotes normal ($\alpha\beta\gamma\delta$) receptor. Lines are semilog fits. SEM were smaller than the graph symbols.

serine is present at the site we mutated (an asparagine residue is also contributed by the γ subunit). It will be of interest to test permeation and gating with the mutated channels under varying ionic conditions. Such experiments, as well as further mutations both within the putative pore and at its mouth (*3*), should provide a more complete description of ion permeation through membrane channels.

Further mutations, outside the IPS, will be necessary to describe the rest of the local anesthetic binding site. For instance, other anesthetics may interact preferentially with different (nonpolar) regions of the pore (*5*). Some blockers interact with AChR channels in both the open and closed states (*19, 23, 25, 27*). Many of the same local anesthetics and their derivatives also block electrically excitable Na^+ channels (their clinical target) and K^+ channels (*32*); therefore, similar experiments may yield insights into the structure of these channels.

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15. The mutated receptors do display subtle changes in channel gating: (i) Receptors containing Ser → Ala mutations displayed unchanged voltage-jump relaxations but channel open times that were up to twofold as great as normal receptors. Further analysis showed that the mutated receptors display fewer brief (<1 ms) closings per burst of openings. (ii) The $\alpha\beta_S\gamma\delta$ receptor displayed substantially briefer voltage-jump relaxations and open times. The apparent decrease in macroscopic conductance of the $\alpha\beta_S\gamma\delta$ receptor can be accounted for by the brief burst duration. In contrast, receptors with a Thr → Ala mutation in M2 at position 256 of the γ subunit produced little or no response to acetylcholine (ACh) in oocytes.
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26. QX-222 is a pure open-channel blocker in rat muscle, at concentrations ≤40 μ*M* (*20*). We verified this condition in our experiments with the following tests: (i) For low ACh concentration, QX-222 had little or no effect on the macroscopic conductance (less than 10% change, see Fig. 2). (ii) During a single "burst" of channel openings, the time integral of charge transferred was not decreased by QX-222 (again, within 10%, R. J. Leonard, C. G. Labarca, P. Charnet, N. Davidson, H. A. Lester, unpublished observations), although the bursts were lengthened in time by the brief interruptions. These results, taken together, suggest that there is no direct pathway between the open-blocked state (with QX-222 bound) and any closed state. The only exception to this conclusion was provided by the $\alpha\beta_S\gamma\delta$ receptor at −150 mV: 20 μ*M* QX-222 decreased macroscopic currents by 30%, presumably because the relatively large rate constant for normal channel closing (Table 1) increases the probability that the channel closes with QX-222 bound. Our measurements on the $\alpha\beta_S\gamma\delta$ receptor at −150 mV are nonetheless approximately correct with respect to the characteristics of open-channel blockade.
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