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Structure of the γ-less Nicotinic Acetylcholine Receptor: Learning from Omission

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SUMMARY

The nicotinic acetylcholine receptor can be expressed in *Xenopus* oocytes by injection of *in vitro* synthesized RNA for the α, β, γ, and δ mouse muscle subunits. However, detectable responses can also be obtained by injection of α, β, and δ subunit RNA only. The receptors expressed in this case (γ-less receptors) share many of the properties of the normal receptor, including relaxation time constants, Hill slope, and relative permeability for Na^+, K^+, Cs^+, and $Tris^+$. The major single-channel conductances of αβγδ and αβδ receptors are similar (34.2 ± 2.9 and 38.5 ± 0.6 pS, respectively) but clearly different from the major conductances seen after the combined injection of αβδ mouse subunit RNA and *Xenopus* γ subunit RNA. Mutations in the second transmembrane segment of the α and β subunits, known to affect open time and blockade by QX-222, are equally effective in the γ-less receptor. These data strongly suggest that the γ-less receptor has the same pore diameter as the normal receptor and that α, β, and δ subunits participate in its formation. Injection of αβγδ as well as αβδ RNA produced additional subconductance states of around 25 pS. The low conductance state was sensitive to mutations introduced in the α or β subunits with or without the γ subunit, indicating that this channel did not need the γ subunits but required at least the α and β subunits to be produced. Injection of αβδ and the adult-type ε subunit RNA gave rise to channels with conductances of 35 and 55 pS when the stoichiometry of the injection was 2:1:1:1, but only the 55-pS channel was recorded when the ε subunit RNA concentration was increased by 10-fold (stoichiometry of 2:1:1:10). The γ-less receptor can thus be expressed even when the adult ε subunit is present. Whether γ-less receptors are expressed at normal adult neuromuscular junctions remains unknown.

Functional aspects of the nAChR have been extensively studied using electrophysiological and biochemical techniques. Cloning and expression of the different subunits forming the pentameric structure of the nAChR have enabled a biophysical approach to structure-function studies, using mutated receptors. Site-directed mutagenesis has focused on sites of important functions of the receptor, such as ligand binding (1, 2), permeation (3–5), gating (6), and binding of open-channel blockers (3, 7). An important assumption of such studies is that injected RNAs are properly translated and assembled into proteins in *Xenopus* oocytes and that endogenous oocyte RNA does not interfere. However, multiple studies have reported expression of functional receptors responding to ACh even when one of the subunits (α, γ, or δ) was omitted from the RNA mixture used for oocyte injection. Buller and White (8) have shown that in some cases endogenous RNA could participate in the formation of multisubunit proteins; this participation was eliminated by actinomycin D, which suppresses endogenous RNA transcription. It has also been reported that expression of nAChR missing the γ or δ subunit could be obtained without the involvement of the endogenous *Xenopus* RNA. In such cases it was suggested (9–11) that the pentameric structure was preserved in the deficient receptor, with one of the remaining subunits being duplicated. Such deficient receptors have provided valuable information about the role of the respective subunits in different functions of the receptor (9, 11).

Oocytes injected with αβγδ RNA display multiple single-channel conductances, some of them being due to imperfect receptor assembly (αβδ or αβγ) (12). It has also been suggested that effects of some mutations could be due to this type of improper assembly and not to the mutation by itself (8). However, no systematic comparisons have been made among wild-type nAChR, subunit-deficient receptors, and hybrid receptors containing mouse and *Xenopus* subunits, although it is well known that such hybrid receptors can be obtained by mixing mouse or calf and *Torpedo* subunits (13–16).

We have investigated in detail the biophysical properties of

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; M2, second transmembrane segment; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; 4AP, 4-aminopyridine.

nAChR expressed after injection of $\alpha\beta\delta$ subunits only, and we compare this channel (i) with the normal wild-type nAChR and (ii) with the hybrid receptor formed by α, β, and δ mouse subunits and γ from *Xenopus*. Our results strongly suggest that *Xenopus* RNA does not play any role in the formation of $\alpha\beta\delta$ receptors and that the channel possesses a pentameric structure. Moreover, when $\alpha\beta\delta$ and the adult-type ϵ subunit RNA are injected into oocytes, two single-channel conductances (35 and 55 pS) result. The channel of lower conductance is due to expression of $\alpha\beta\delta$ receptors.

Materials and Methods

A full-length *Xenopus* γ subunit cDNA was generously provided by S. Burden (Massachusetts Institute of Technology), and a full-length mouse ϵ subunit cDNA was generously provided by P. Gardner (Dartmouth Medical School) (30). Mutations were generated by oligonucleotide-primed synthesis on single-stranded uracil-containing template DNA from M13mp19 or pBluescript (Stratagene, San Diego, CA) vectors, by means of the Mutagene kit (Bio-Rad, Richmond, CA). Mutated inserts that were in M13mp19 were subcloned into pGEM2 or pBluescript for *in vitro* RNA synthesis performed with either SP6 or T7 RNA polymerase. Ten nanograms of RNA mixture encoding α, β, γ, and δ subunits were injected into *Xenopus* oocytes. Electrophysiological measurements were made 2 (wild-type) to 7 (γ-less receptor) days later, at an ACh concentration of 0.1–10 μM, in a chamber perfused with physiological saline at 12–13°.

Macroscopic measurements were performed in a solution containing 96 mM NaCl, 1 mM KCl, and 5 mM HEPES, with a two-electrode voltage-clamp circuit (Axoclamp 2A; Axon Instruments, Foster, CA) under the control of pCLAMP software (Axon Instruments). The voltage was stepped from a holding level of −30 mV to +50 mV for 50 msec, followed by a step to a test potential between −150 and −50 mV. ACh-induced currents were separated from other endogenous conductances by substracting the currents recorded in the presence of ACh from currents obtained using the same paradigm but in the absence of ACh. Voltage ramps were applied from −120 mV to 100 mV at a speed of 260 mV/sec. Time constants of relaxation were obtained from nonlinear regression routines of the pCLAMP analysis program.

Single-channel recordings were performed on both cell-attached and, mostly, outside-out patches (using 100 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, pH adjusted to 7.4 with KOH). Data were recorded with a 8900 amplifier (Dagan, Mineapolis, MN) and stored on videocassettes tapes. Signals were played back, digitized by a Labmaster interface, and analyzed using pCLAMP (version 5 or 5.5). Single-channel open times were measured from the time constant of exponential decays fitted to duration distributions of channel opening. Single-channel conductances were determined by fitting Gaussian functions to amplitude histograms.

Results

Expression of $\alpha\beta\delta$ channels. When 15 ng of $\alpha\beta\gamma\delta$ nAChR RNA were injected into oocytes, ACh (1 μM) induced currents as early as 24 hr later, with a mean amplitude at −110 mV of 1.3 ± 0.09 μA. However, omitting the γ subunit from the RNA mixture (γ-less receptor) produced a much smaller current, and reliable responses could be obtained only 3–6 days after injection. In such recordings the current increased with agonist concentration (Fig. 1A) and displayed desensitization for concentrations greater than 5 μM. The current reached 1–3 μA in amplitude at −110 mV and 1 μM ACh (Table 1). A double-logarithmic plot of current amplitude versus agonist concentration yielded a Hill number of 1.8 ± 0.15, close to values obtained with wild-type receptors (1.6 ± 0.2; see Table 1). A Hill coefficient greater than 1 indicates a significant cooperativity in the action of ACh and the presence of more than one binding site.

Stepping the voltage from positive to negative values induced a relaxation in the agonist-induced current with normal nAChR. This effect is related to the voltage dependence of the burst duration (17–19). Similar relaxations were also obtained from oocytes injected with γ-less mRNA mixtures (Fig. 1B; Table 1). The current decay can be well fitted, in both cases, with a single exponential (Fig. 2, *top*) (4, 7), giving time constants that became slower for larger hyperpolarizations (Fig. 2, *bottom*). When plotted against step potential, the time constant is exponentially related to the voltage according to the relation

$$\tau = (A \cdot \exp(-B \cdot V))$$

and least-squares fits gave voltage dependences of the relaxation that were similar for the wild-type and the γ-less nAChR (slope of −0.0052 and −0.0064, repectively; see Table 1). Relaxation time constants were reduced by *e*-fold for 156 mV and 192 mV for $\alpha\beta\delta$ and $\alpha\beta\gamma\delta$ nAChR, respectively. It thus appeared that the macroscopic gating characteristics of the receptor were not altered by the omission of the γ subunit.

Further characterization of the γ-less receptor has been achieved by examining the relative permeability of different cations. Such experiments are useful in determining the narrowest section of the channel pore (20) and, consequently, could give information on the channel structure. The reversal potential for Na^+, measured from recordings similar to those in Fig. 1C, was slightly smaller than those for Cs^+ and K^+ and larger than that for Tris. The calculated permeability ratios (Table 1) showed no major changes between the normal and the γ-less nAChR and attributed, in both cases, a relative permeability to Tris close to 0.3. Because Tris is one of the biggest permeant cations, this clearly shows that the narrowest part of the channel is not changed by the omission of the γ subunit and, as a consequence, that the structure of the pore is likely to be a pentamer.

Thus, when the γ subunit is removed from the RNA mixture, the expressed receptors resemble the normal receptors in macroscopic properties. Although functional γ-less receptors appeared more slowly on the surface of the membrane after RNA injection, robust responses could be obtained, allowing analysis of the single-channel properties. The results presented in the following sections represent data recorded on 24 different patches from eight separate injections.

Structure and properties of the $\alpha\beta\delta$ channel. The fact that the $\alpha\beta\delta$ and $\alpha\beta\gamma\delta$ receptors display almost identical electrophysiological properties and that the γ-less and $\alpha\beta\gamma\delta$ receptors have a similar pentameric structure suggests that another subunit filled the space of the γ subunit. Two possibilities can be put forward, that (i) a γ subunit endogenous to the oocyte is inserted in the final receptor or (ii) the channel is composed of only α, β, and δ subunits but one of these subunits is duplicated. In experiments designed to decide between these possibilities, we compare single-channel recordings obtained from oocytes injected with $\alpha\beta\gamma\delta$, $\alpha\beta\delta$, or $\alpha\beta\gamma_X\delta$, where X denotes a *Xenopus* subunit and an unsubscripted subunit is from mouse. Typical single-channel openings recorded for each type of receptor are depicted in Fig. 3. The similarity between normal and γ-less nAChR for macroscopic responses extends to the single-channel level. The two channels (Fig. 3, *left* and *middle*) had similar inward conductance, closing rate constant,

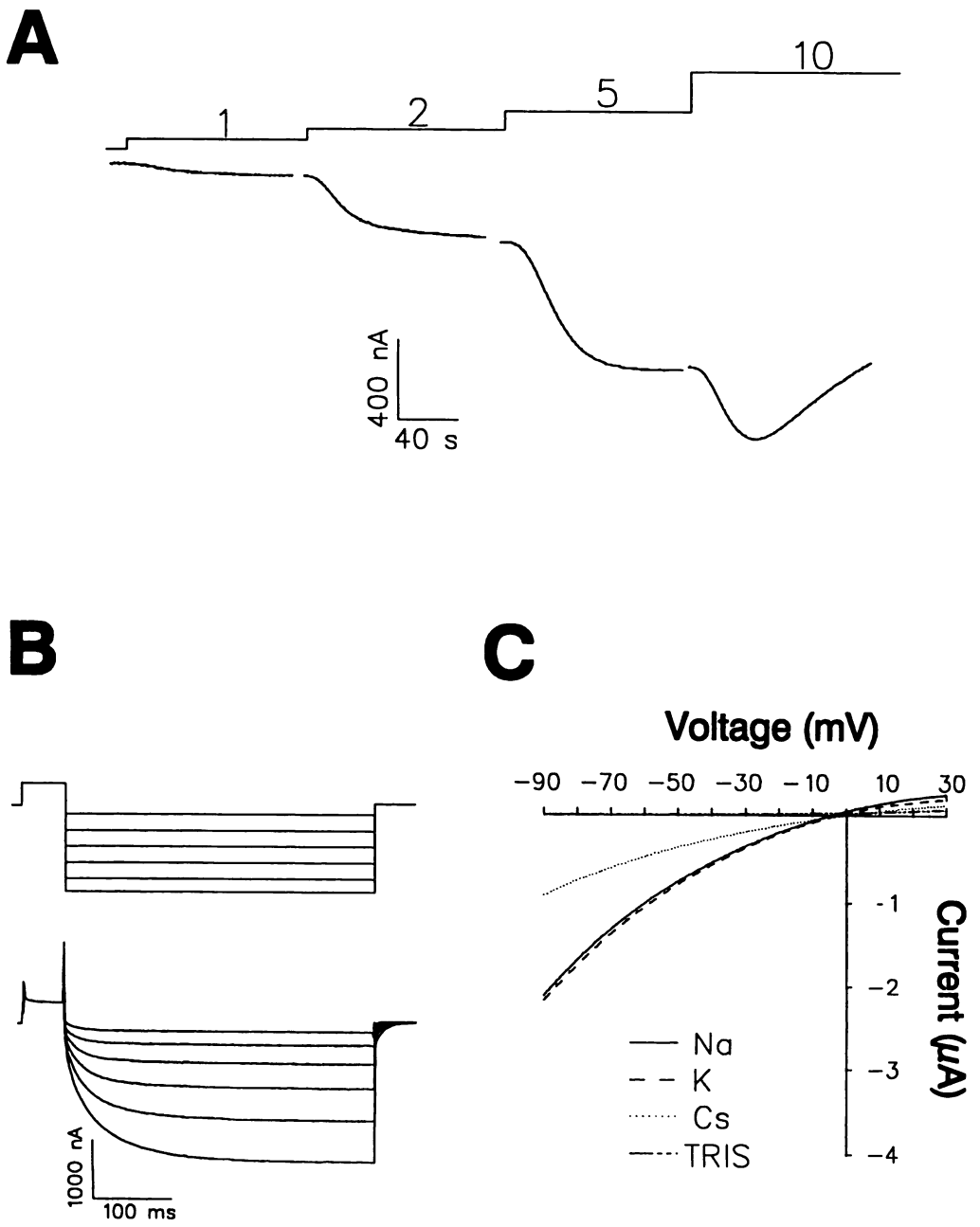

Fig. 1. A, Response of $\alpha\beta\delta$ RNA-injected oocyte to 1, 2, 5, and 10 μM ACh. The membrane potential was held at −60 mV. B, Voltage-jump relaxation in the presence of 1 μM ACh. The voltage protocols used are shown at the *top*. The membrane potential was stepped from a holding level of −30 mV to +50 mV and then to various test potentials between −150 and −50 mV. Relaxation time constants, calculated from least square regression, were 49.2 ± 9.2, 42.1 ± 7.2, 36.4 ± 5, 32.4 ± 6.7, 27.1 ± 5, and 26.6 ± 6.5 msec for −150, −130, −110, −90, −70, and −50 mV, respectively. C, Current-voltage relationships for the same oocyte in the presence of 1 μM ACh, using Na^+, K^+, Cs^+, and Tris as permeant cation. The reversal potentials were −5.8, −4.6, −1.8, and −30.8 mV, respectively.

TABLE 1

Comparison of electrophysiological properties of $\alpha\beta\gamma\delta$ and $\alpha\beta\delta$ nAChR

Recordings were obtained from oocytes injected with RNA 2–7 days before experiments, using a two-microelectrode voltage-clamp, with ACh concentration of 1 μM, at −110 mV.

	Wild-type	γ-Less
Current (pA)	1341 ± 92	1037 ± 321
Time constant (msec)	31.0 ± 2	36.4 ± 2.8
Hill slope	1.6 ± 0.2	1.8 ± 0.15
Permeability	K = Cs (1.16) > Na (1) > 4AP (0.83) > Tris (0.37)	K = Cs (1.19) > Na (1) > Li (0.8) > 4AP (0.75) > Tris (0.25)

and voltage dependence of the closing rate constant. These voltage dependences (0.0078 and 0.0087, respectively) were similar to the voltage dependences of the macroscopic relaxations. Moreover, the mean number of openings/burst (1.18 ± 0.03, $n = 3$, for $\alpha\beta\gamma\delta$ and 1.26 ± 0.13, $n = 3$, for $\alpha\beta\delta$, at −100 mV, using 10 msec as critical close time to leave a burst) (see Ref. 11) was not affected by voltage between −50 and −150 mV, suggesting that most of the relaxation was due to the effect of voltage on the closing rate constant. Similar results have been found by others (11). It should be noted, however, that the γ-less receptor displayed a slightly higher outward conductance than the wild-type (28.1 ± 1.1 and 24 ± 3.1 pS,

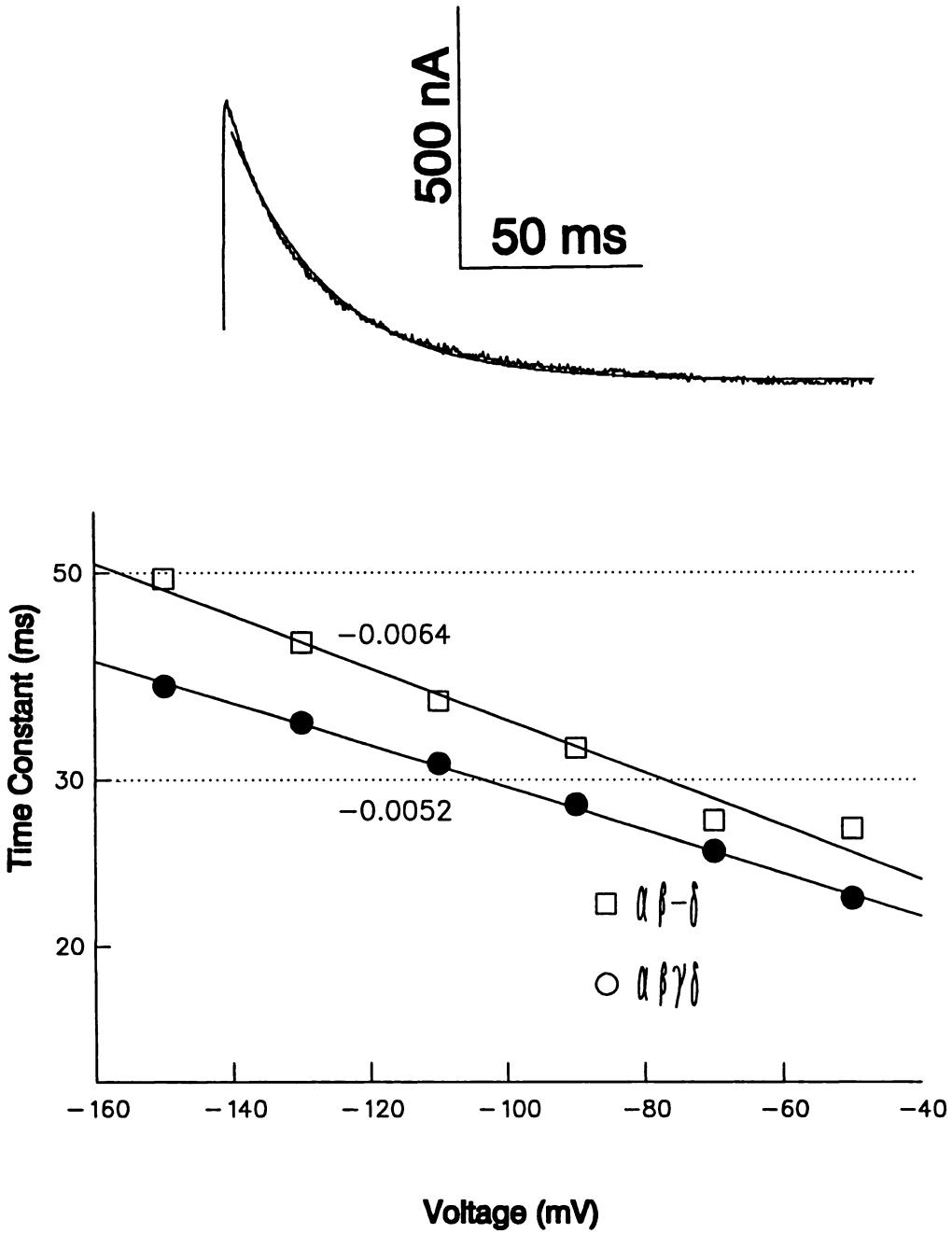

Fig. 2. Voltage dependence of the relaxation time constant in the presence of 1 μM ACh. *Top*, typical relaxation obtained for a voltage jump at −150 mV, with 1 μM ACh. Superimposed is a single exponential fit to the trace. The protocol used was similar to that in Fig. 1B. *Bottom*, semilogarithmic plot of time constants of similar relaxations, obtained using voltage jumps at −150, −130, −110, −90, −70, and −50 mV, versus the amplitude of the jump. *Lines*, linear regressions, giving an *e*-fold decrease for 156 and 192 mV for αβγδ and αβδ nAChR, respectively. Regression coefficients were >0.95.

respectively), which suggests an assymetric change in the charge distribution on the two sides of the membrane between the normal and γ-less receptors.

Interestingly, when the *Xenopus* γ_X subunit RNA was injected in combination with the α, β, and δ subunit RNAs from mouse (Fig. 3, *right*), the inward channel conductance was significantly larger, compared with wild-type and γ-less receptors (42.9 ± 1.2, 35.2 ± 2.9, and 38.5 ± 0.6 pS, respectively), but the closing rate constants and the voltage dependence of the closing rate remained similar (see Fig. 3). In view of these results, a contribution of the *Xenopus* subunits in the formation of the γ-less nAChR can be ruled out, and it seems likely from the similar functional characteristics that the γ-less channel has a replacing subunit that can fulfill the role of the γ subunit in the essential functions of the receptor, i.e., gating and permeation. The difference in conductances can, however, be explained by the data of Imoto and co-workers (3, 21). These authors demonstrated that the channel conductance and rectification are partly determined by the number of negative charges located at the putative internal and the external termini of the M2 region (boxed residues in Fig. 4). Indeed, when the sequence of the mouse γ subunit is compared with its *Xenopus* counterpart (Fig. 4), an additional positive charge (arginine) is found in the mouse subunit; this could produce the larger inward conductance obtained with the hybrid receptor, with other changes in the pore-lining segment being conservative.

Stoichiometry of αβδ receptors. We then attempted to determine the identity of the duplicated subunit by using some of the mutations that have been shown to affect QX-222 binding. QX-222 is a charged derivative of lidocaine that blocks the nAChR channel, by simply plugging the pore (21). This blockade is affected by voltage, which led to the conclusion that

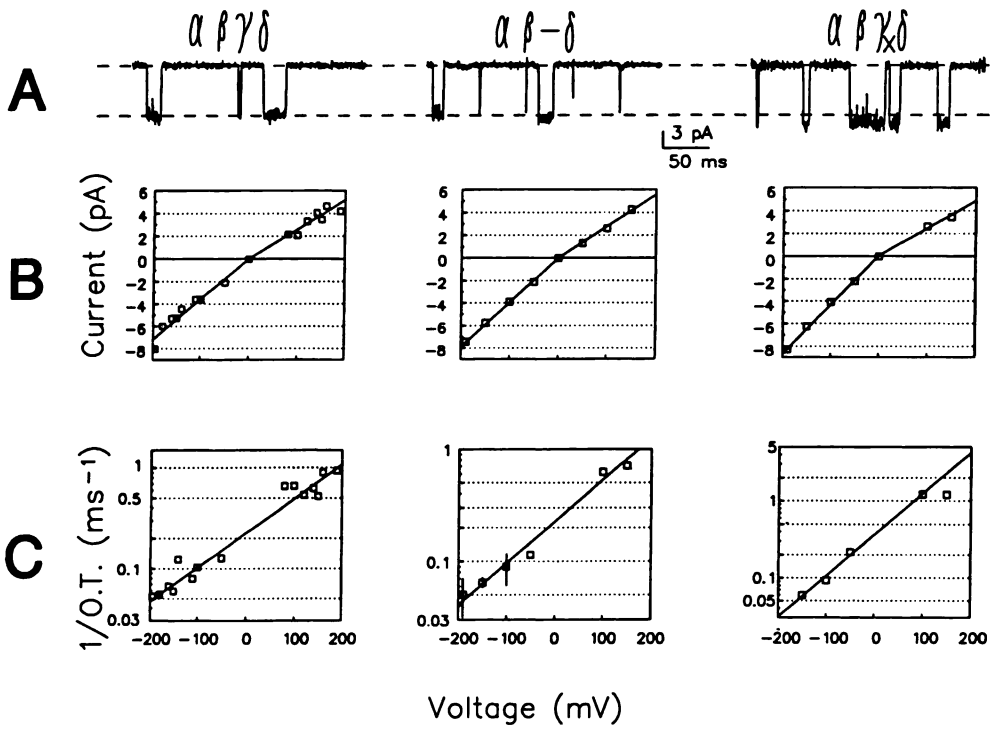

Fig. 3. Single-channel properties of $\alpha\beta\gamma\delta$ (*left*), $\alpha\beta\delta$ (*middle*), and $\alpha\beta\gamma_x\delta$ (*right*). A, Typical traces recorded at −150 mV with 1 μM ACh for each channel type, in outside-out mode. Note the higher current for *Xenopus* γ subunit-containing channels. B, Mean inward and outward current-voltage relationship for the main conductance class of each type. Inward conductances were 35.2 ± 2.9, 38.5 ± 0.6, and 42.9 ± 1.2 pS and outward conductances were 24 ± 3.1, 28.1 ± 1.1, and 23.6 ± 2.2 pS for $\alpha\beta\gamma\delta$, $\alpha\beta\delta$, and $\alpha\beta\gamma_x\delta$, respectively. C, Voltage dependence of the closing rate constant. *Lines*, e-fold change for 128, 114, and 81.3 mV; open times at 0 mV are 4.4, 4.1, and 2.75 msec for $\alpha\beta\gamma\delta$, $\alpha\beta\delta$, and $\alpha\beta\gamma_x\delta$, respectively.

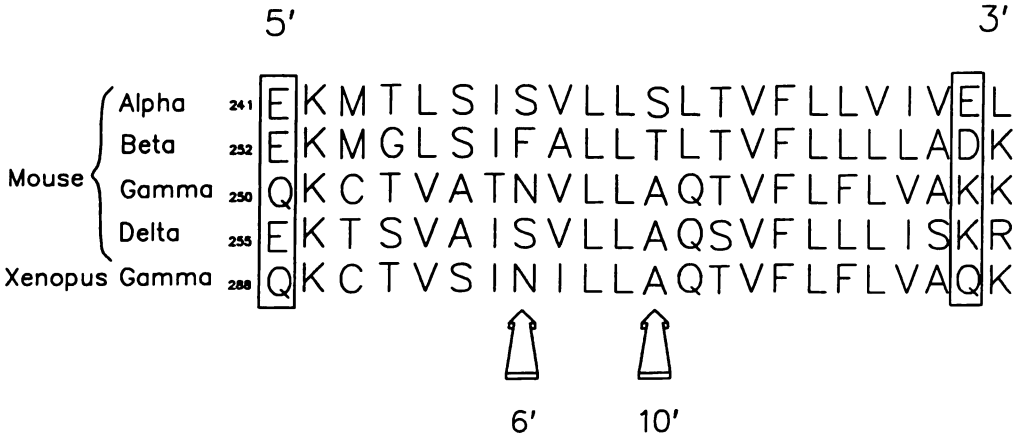

Fig. 4. Amino acid sequence (one-letter code) showing the differences between mouse and *Xenopus* γ subunit. One external positive charge in the mouse (K) is replaced by an uncharged residue (Q) in *Xenopus*, leading to the increase in the inward current. The γ-replacing subunit should have identical external charges and additional negative intracellular charges, to account for the changes in conductance. Only the δ subunit fits these criteria. Positions 6′ and 10′ (*arrows*) affect QX-222 binding in normal mouse nAChR (4, 7).

the binding site for QX-222 is located inside the pore. It has been shown (4, 7) that replacing serine or threonine at position 6′, in the M2 of each subunit, increases the affinity of the QX-222-receptor interaction, whereas similar changes decrease this affinity when they are made at position 10′ (see Fig. 4 for the sequence of the M2 region and our numbering system). Effects of removing these residues or adding them to a particular subunit were roughly multiplicative with the number of copies of that subunit in the channel, i.e., a change in the α subunit was twice as effective as an equivalent change in another subunit (7).

We, therefore, studied the relative effect of two mutations on the complete and the γ-less nAChR (Figs. 5 and 6). The effects of (i) changing a phenylalanine to a serine in the β subunit at position 6′ ($\beta_{F6'S}$) and (ii) changing a serine to an alanine in the α subunit at position 10′ ($\alpha_{T10'A}$) were analyzed at the single-channel level. The $\beta_{F6'S}$ mutation resulted in a shorter open-time duration for both the complete and γ-less receptor. This shortening was from 15.5 ± 4.7 msec (n = 5) to 3.5 ± 2.2 msec (n = 5) at a holding potential of −150 mV for the complete receptor and from 14.9 ± 1.5 msec (n = 5) to 3.3 ± 2.0 msec (n = 9) for the $\alpha\beta\delta$ receptor. As already reported (7), the $\alpha_{T10'A}$ mutation did not significantly modify the open time for the $\alpha\beta\gamma\delta$ nAChR; there was also little change for $\alpha\beta\delta$ (15.2 ± 4.7 msec, n = 6, and 13.2 ± 3.8 msec, n = 3, respectively; see Fig. 5). The voltage dependence of the closing rate and the conductance at positive and negative voltages were not significantly changed by the two mutations for either hybrid receptor (data not shown). Once again, these results, which confirm those of Lo *et al.* (11), favor a pore with a minor change in the structure; all the elements are present and functional.

When QX-222 (20 μM) was present in the ACh-containing solution bathing outside-out patches, typical open-channel block was recorded with all the mutant hybrids studied. As for the wild-type receptor, the block was characterized by the presence of brief gaps, which have been interpreted as an open-blocked state (22). Duration histogram of these gaps gave an estimate of the QX-222 dissociation rate constant, related to the binding energy of the QX-222 in the channel. The γ-less nACHR diplayed a 25.9% greater QX-222 dissociation rate

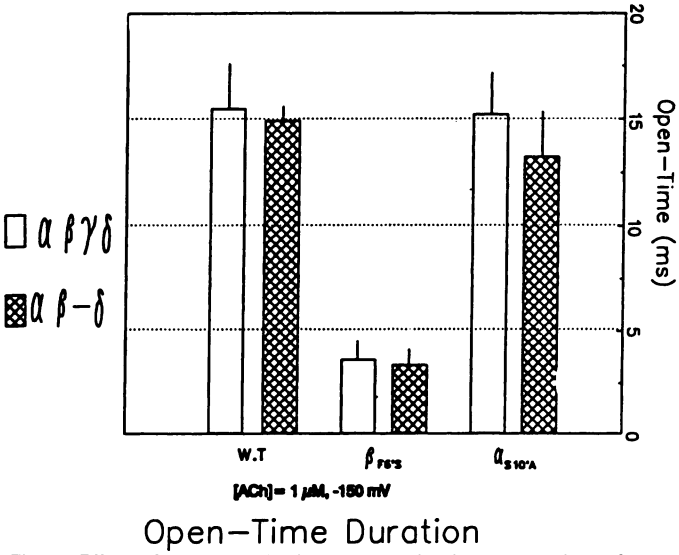

Fig. 5. Effect of mutations in the α or β subunit on open time of αβγδ and αβδ receptors. Both receptors have the same open time and voltage dependence of the open time. Moreover, mutations located at positions 6′ and 10′, on the β and α subunits, respectively, have the same effects on both channels. *W.T.*, wild-type.

constant than the wild-type, suggesting that the duplicated subunit possesses differences in the distribution of the amino acids forming the QX-222 binding site (7). However, the voltage dependence of the dissociation rate constant was not altered; this parameter increased by *e*-fold for 39.1 mV and 39.5 mV for normal and γ-less receptors, respectively (data not shown). This implies that the fraction of the membrane electric field sensed by the blocker was not changed in the deficient receptor.

Dissociation rate constants obtained for complete and γ-less nAChR of the wild-type and with the two mutations ($\beta_{F6'S}$ and $\alpha_{T10'A}$) are shown in Fig. 6. The $\beta_{F6'S}$ mutation induced a decrease of 23.6% in the dissociation rate constant when introduced into the complete receptor (Fig. 6, *left*) and of 19.8% in the γ-less receptor. Similarly, for the $\alpha_{T10'A}$ mutation the decrease was 27.9% and 21.3% for complete and γ-less nAChR, respectively. Thus, the effects of these two mutations were quite similar for the two types of receptor. A decisive experiment would be to analyze similar mutations in the δ subunit. Unfortunately, $\delta_{A10'S}$ was not expressed in the complete receptor, and $\delta_{S6'A}$, which increased the dissociation rate constant when expressed in the normal receptor, blocked functional expression when expressed in the γ-less receptor. We do not know whether this was due to improper assembly or a drastic effect on conductance of the channel, because alteration of the number of serines at that particular position has been shown to alter channel conductance (4). Other mutations in the δ subunit have been studied. None of them affected significantly the inward conductance when expressed in complete γ-containing receptors (Table 2). However, when expressed in γ-less receptors, the amplitude of the macroscopic current elicited by 1 μM ACh was far too small for us to record single-channel events and to look at the effects of QX-222.

Multiple conductance states. As recently pointed out by others (10), injection of all four nAChR subunit RNAs into oocytes gave rise to three single-channel conductances. In our case the main classes were 39 and 23 pS (chord conductance at −100 mV; see Fig. 7A) and a smaller one, which has not been studied further. However, oocytes injected with only the α, β, and δ RNA also possessed all three of these conductance classes.

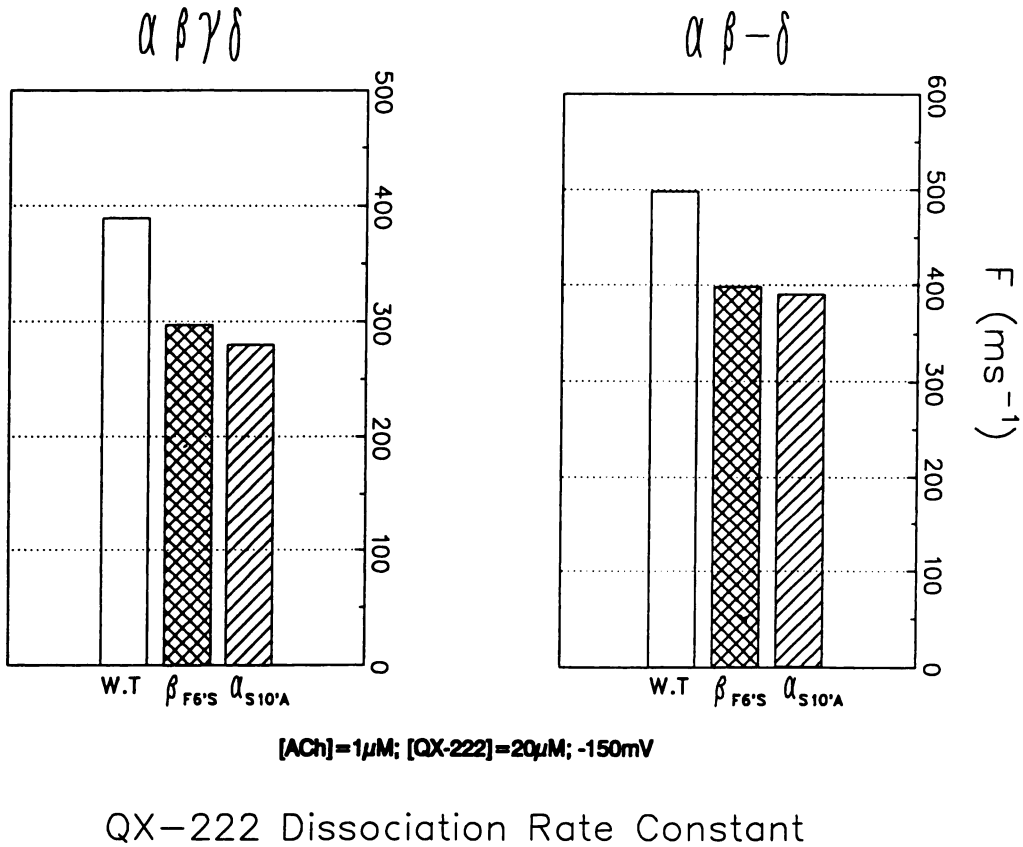

Fig. 6. Blockade by QX-222 of the αβδ nAChR. Receptors expressed without the γ subunit have a higher dissociation rate constant than the normal receptor (*W.T*). Mutations at sites 6′ and 10′, $\beta_{F6'S}$, and α_{S10A}, respectively, had similar relative effects on αβγδ and αβδ channels. Note the differing *vertical scales* for the two panels.

TABLE 2

Effects of mutations in the δ subunit on channel conductances of fully assembled receptors

Chord conductances (mean ± SE (no of patches)) were obtained from outside-out patches at −100 mV.

δ Mutation	Conductance
	pS
S2′A	33 ± 1 (9)
S6′A	31 ± 1 (6)
S6′F	33 ± 1 (5)
S6′F	36 ± 3 (9)
F14′Y	36 ± 2 (5)

The two main classes were in both cases activated by ACh and blocked by QX-222. However, QX-222 blockade was less pronounced with the 23-pS channel; the dissociation rate constants were 3800 and 1800 sec^{-1} for 23 and 39 pS γ-less channels, respectively (Fig. 7B). Moreover, the $\beta_{F6'S}$ mutation, which affected the open time in normal and γ-less receptors, also affected the open time of the low conductance γ-less channel; the mean open time decreased from 7.8 to 2.4 msec when oocytes were injected with αβδ and $\alpha\beta_{F6'S}\delta$ RNA (Fig. 7C). This suggests that the low conductance channel was formed both with and without the γ subunit and that it included at least the α and the β subunit. Kullberg *et al.* (10) reported the disappearance of this channel when the δ subunit was missing, suggesting that δ is also needed. The γ-less channel is then similar to the complete receptor not only for the main conductance classes but also for the secondary conductances.

Background expression of the αβδ nAChR. It may also be asked whether expression of the deficient receptor can occur under normal conditions, i.e., when all the subunits (αβγδ) are coinjected. This seems a difficult problem to solve, because both receptors share the same characteristics. We addressed this by expressing the subunits that normally form a channel in the adult membrane, with properties clearly different from those of the αβδ nAChR. At the molecular level, the adult and fetal receptors differ by the replacement of the γ subunit for the ε subunit. Moreover, expression of αβδε nAChR has been already reported in *Xenopus* oocytes (9, 23). We injected the cloned mouse ε subunit together with the α, β, and δ subunits and recorded single channels. From oocytes injected with equal amounts of α, β, δ, and ε subunit RNAs, two types of channel conductance could be detected (see Fig. 8, *left*) in about 80% of the patches. Additional smaller conductances were also some-

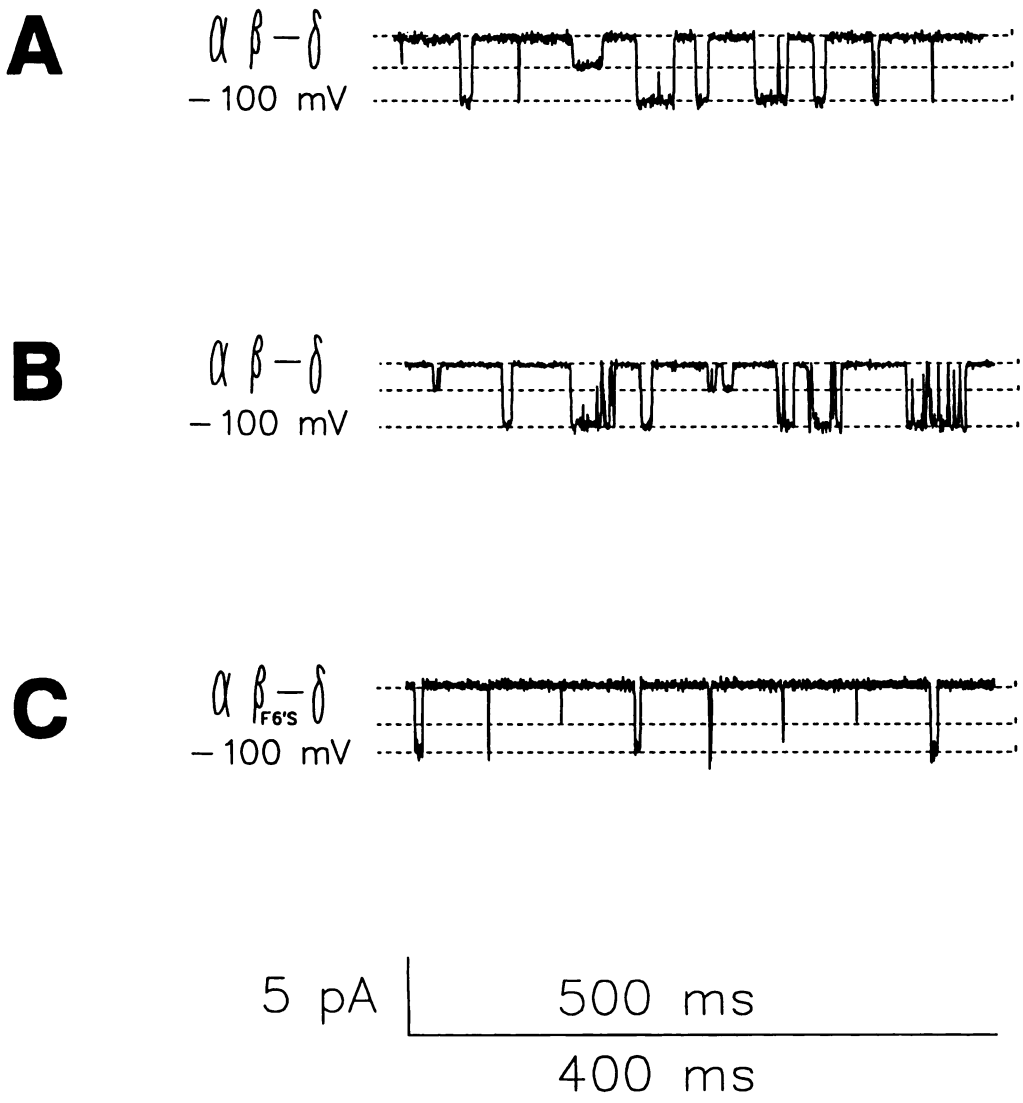

Fig. 7. Multiple conductance states expressed after the injection of αβδ subunit RNAs in oocytes. Traces are from outside-out patches, with 1 μM ACh, at −100 mV. A, Without QX-222; B, 20 μM QX-222; C, current recorded after the injection of $\alpha\beta_{F6'S}\delta$ subunit RNA; otherwise, same conditions as in A. Note that the low conductance state is blocked by QX-222 and displays altered kinetics with a mutated β subunit. *Scale bar*, 500 msec for A and C and 400 msec for B.

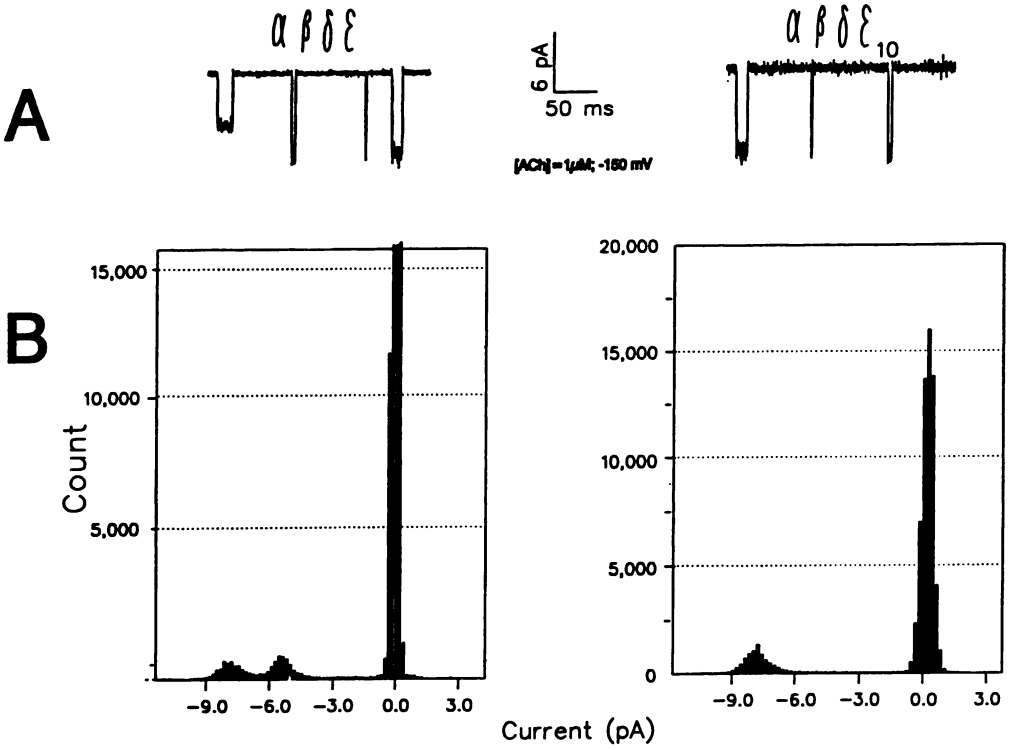

Fig. 8. Expression of $\alpha\beta\delta\epsilon$ receptors. A, Typical current traces recorded after injection of $\alpha\beta\delta\epsilon$ subunit RNAs with a stoichiometry of 2:1:1:1 (*left*) and 2:1:1:10 (*right*). B, All-point amplitude histograms of traces shown in A. The low conductance openings (around 35 pS) are not present when the ϵ RNA concentration is increased 10-fold. Under these conditions, only the larger (55 pS) channel is recorded. Outside-out patches, holding potential of −150 mV, [ACh] = 1 μM, 13°.

times recorded. The two main conductance states were 35 and 55 pS in 100 mM KCl (13° and nominally Ca^{2+}-free solutions). The 55-pS channel is undoubtedly related to expression of $\alpha\beta\delta\epsilon$ receptors, because its conductance and open-time duration are similar to those recorded at adult end-plate (23). We believe that the lower conductance channel (35 pS) is the result of the expression of incomplete ($\alpha\beta\delta$) receptors that can be formed even in the presence of the ϵ subunit. Increasing the relative amount of ϵ subunit RNA by a factor 10 (i.e., α, β, and δ RNAs were mixed at 0.2 μg/μl, with ϵ at 2 μg/μl) induced a decrease in the probability of finding the low conductance channel (present in only 30% of the patches; see Fig. 8, *right*).

Discussion

Previous reports from this laboratory showed that all four nAChR subunit mRNAs are required for robust responses (15, 24). However, the rather small responses obtained in the absence of the γ subunit RNA were nearly normal when expressed per picomole of α-bungarotoxin bound to the oocyte, suggesting that fewer receptors were formed at the membrane surface but they had functional efficiency similar to that of the complete receptor. The present report and that of Lo *et al.* (11) confirm and extend these observations. Kurosaki *et al.* (16) and Mishina *et al.* (23) also reported the detectable but inefficient formation of γ-less bovine and *Torpedo* receptors, respectively. Previous studies have reached no conclusions about the possible structure of these channels. Because the γ subunit plays an important role in channel gating and participates at the agonist binding site (25), it is important to know the structure of these channels that display the same gating characteristics as the complete receptor. The expression of the γ-less nAChR after 4–6 days of translation was adequate to allow reproducible single-channel measurements and study of effects of some mutations; data were obtained from 24, 9, 16, and 11 outside-out patches from the $\alpha\beta\delta$, $\alpha\beta\gamma_X\delta$, $\alpha\beta_{F6'S}\delta$, and $\alpha_{S10'A}\beta\delta$ nAChR, respectively, at potentials ranging from −190 to 190 mV. Therefore, effects of these mutations could be compared in the γ-less and the complete nAChR.

Effects of γ subunit omission. The sole effect of omission of the γ subunit was, at first glance, a decrease in the ACh-induced current. This decrease could be caused by a decrease in the number of functional channels, in the single-channel conductance or gating chararcteristics, or in the agonist affinity. Macroscopic recording showed no differences in Hill slope and relaxation time constants and gave similar permeability ratios for monovalent cations. This suggests that (i) two α subunits were present in the receptor, (ii) the gating of the channel was only slightly affected, and (iii) the overall structure was preserved. Blount and Merlie (26) showed that $\alpha\delta$ subunit hybrids formed high affinity binding sites for both tubocurarine and carbamylcholine and the $\alpha\gamma$ subunit pair gave agonist affinities 1 order of magnitude smaller. Because the γ-less receptor gave (i) reliable expression and (ii) Hill slopes close to 2, it seems reasonable to conclude the presence of two agonist binding sites. Because the β subunit does not participate in binding, the only candidate for the formation of the second site (in association with α) is the δ subunit. This was partially confirmed by single-channel recordings obtained with and without γ RNA and with γ_X. It is clear from Fig. 3 that the participation of the *Xenopus* RNA can be ruled out on the basis of single-channel conductance. Such participation is known to occur when the α subunit is omitted (8, 26), but the expression level was >3 orders of magnitude smaller with the α-deficient receptor. We have also demonstrated that α and β subunits were participating in the formation of the γ-less receptor, by the use of mutations in these two subunits.

Role of other subunits. The conductance of the γ-less nAChR suggests that the subunit replacing γ has the same

peripheral charges (3). Given the homology between the subunits, the δ subunit seems a prime candidate. Mutations in α or β subunits affected the open time for both the complete and the γ-less nAChR. This proves that these two subunits also participate in the formation of the γ-less nAChR. If we assume, according to the reversal potential measurement, that the pentameric structure is preserved, a possible stoichiometry would be $\alpha_2\beta\delta_2$. Unfortunately, the mutated δ subunit RNAs injected in the present study were not expressed well when injected without γ, so that a definitive statement on the participation of the δ subunit cannot be given. However, the very fact that these mutations were not expressed suggested that, indeed, the δ subunit is present in the $\alpha\beta\delta$ receptor. Furthermore, the $\alpha\beta\gamma\delta_{S6'A}$ hybrid receptor was expressed well (4, 7) but the $\alpha\beta\delta_{S6'A}$ receptor was not expressed, as though the presence of two δ subunits allowed any possible effects of this mutation to block function completely. This might also be explained by the particular role of the γ subunit in the channel (27), which seems to be arranged asymmetrically in the channel pore of *Torpedo* receptors.

QX-222 blockade was slightly weaker for γ-less nAChR than for complete receptors. Charnet *et al.* (7) have analyzed the effects of serine differences in amino acid residues at positions 6′ and 10′. The γ and δ subunits have identical residues at positions 10′ (alanine) and 14′ (phenylalanine). At position 6′ the asparagine in the γ subunit is replaced by a serine in the δ. When more detailed structural information is eventually available about the interaction between QX-222 and the receptor channel, it will be of interest to understand how this interaction changes slightly with the serine-for-asparagine substitution implied by our suggestion that δ replaces γ.

Mutations at positions 6′ and 10′ in β and α subunits, respectively, had similar effects on the γ-less and complete receptors. The location of the binding site is thus probably not changed, which is also suggested by the fact that the voltage dependence of the QX-222 dissociation rate constant is similar for both channels. Moreover, the fractional change in the QX-222 dissociation rate constant was quantitatively similar on complete and γ-less receptors (Fig. 6), which supports the idea that these two subunits are present with equal stoichiometry in the two receptors. Thus, it is highly likely that the γ-less receptor is in fact a pentamer, and it is also highly likely that the open-channel blocker QX-222 penetrates the same distance from the extracellular surface in the γ-less pore as in the normal nAChR.

Fragmentary data on conductance/fmol of α-bungarotoxin binding (15, 24) and agonist blockade of α-bungarotoxin binding (16) suggest that agonist affinity, which is related to the opening rate, differs by <10-fold between the γ-less and the $\alpha\beta\gamma\delta$ receptors. Altered agonist affinity has also been found on bovine γ-less receptors (9). In that case, the γ subunit appeared essential for the stability of the closed unliganded channel, and removal of γ induced spontaneous openings. This was not the case for mouse receptors, where γ-less receptors displayed unaltered gating properties (Ref. 10 and this work). Indeed, some mutations in the γ subunit induced drastic changes in the gating or the conductance of the channel (3, 6), but such changes can be obtained with mutations in other subunits as well (7). It is now generally agreed that particular functions of the channel, like gating or conductance, are not restricted to a single subunit but involve the whole pentamer. It is interesting to note that, whereas the δ subunit could replace the γ subunit quite effectively, the converse was not true; δ-less *Torpedo* receptors have substantially altered gating properties (13).

The possibility that δ could substitute for γ is interesting in view of the fact that the replacement of ϵ for γ is thought to account, at least partially, for the progression from embryonic to adult end-plates (23). Our data show that, in the presence of ϵ RNA, γ-less channels can be formed in oocytes, with properties similar to those of embryonic channels. It is not known (i) whether adult end-plates do have such $\alpha_2\beta\delta_2$ channels, (ii) whether adult muscle has mechanisms to suppress such presumably aberrant receptors, or (iii) whether the γ and ϵ subunits play additional roles in assembly of the channel or targeting to the end-plates.

However, it is noteworthy that the removal of one subunit leads to a channel in which the replacing subunit might not have an equivalent position, or might not play an equivalent role, as the original missing subunit or the replacing subunit at its right place. As a consequence, the results obtained with mutations on normal receptors might not be able to be extrapolated directly to subunit-deficient receptors.

In conclusion, expression of deficient receptors is useful when combined with single-channel measurements and site-directed mutagenesis, to explore the functional role of the nAChR subunits.

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References

1. Changeux, J. P. The nicotinic acetylcholine-receptor: an allosteric protein prototype of ligand-gated ion channels. *Trends Pharmacol. Sci.* **11**:485–492 (1990).
2. Karlin, A. Exploration of the nicotinic acetylcholine receptor. *Harvey Lect.* **85**:71–107 (1991).
3. Imoto, K., C. Busch, B. Sakmann, M. Mishina, T. Konno, J. Nakai, H. Bujo, Y. Mori, K. Fukuda, and S. Numa. Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance. *Nature (Lond.)* **335**:645–648 (1988).
4. Leonard, R. J., C. Labarca, P. Charnet, N. Davidson, and H. A. Lester. Evidence that the M2 membrane-spanning region lines the ion-channel pore of the nicotinic receptor. *Science (Washington D. C.)* **242**:1578–1581 (1988).
5. Villarroel, A., S. Herlitze, M. Koenen, and B. Sakmann. Location of a threonine residue in the *alpha*-subunit M2 transmembrane segment that determines the ion flow through the acetylcholine receptor channel. *Proc. R. Soc. Lond. B Biol. Sci.* **243**:69–74 (1991).
6. Lo, D. C., J. L. Pinkham, and C. F. Stevens. Role of a key cysteine residue in the gating of the acetylcholine receptor. *Neuron* **6**:31–40 (1991).
7. Charnet, P., C. Labarca, R. J. Leonard, N. J. Vogelaar, L. Czyzyk, A. Gouin, N. Davidson, and H. A. Lester. An open-channel blocker interacts with adjacent turns of *alpha*-helices in the nicotinic acetylcholine receptor. *Neuron* **4**:87–95 (1990).
8. Buller, A. L., and M. M. White. Control of *Torpedo* acetylcholine receptor biosynthesis in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **85**:8717–8721 (1988).
9. Jackson, M. B., K. Imoto, M. Mishina, T. Konno, S. Numa, and B. Sakmann. Spontaneous and agonist-induced openings of an acetylcholine receptor channel composed of bovine muscle α-, β-, and δ-subunits. *Pflugers. Arch.* **417**:129–135 (1990).
10. Kullberg, R., J. L. Owens, P. Camacho, G. Mandel, and P. Brehm. Multiple conductance classes of mouse nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **87**:2067–2071 (1990).
11. Lo, D. C., J. L. Pinkham, and C. F. Stevens. Influence of the *gamma* subunit and expression system on acetylcholine receptor gating. *Neuron* **5**:857–866 (1990).
12. Buller, A. L., and M. M. White. Functional acetylcholine receptors expressed in *Xenopus* oocytes after injection of *Torpedo* β, γ, and δ subunit RNAs are a consequence of endogenous oocyte gene expression. *Mol. Pharmacol.* **37**:423–428 (1990).
13. White, M. M., K. Mixter Mayne, H. A. Lester, and N. Davidson. Mouse-*Torpedo* hybrid acetylcholine receptors: functional homology does not equal sequence homology. *Proc. Natl. Acad. Sci. USA* **82**:4852–4856 (1985).

14. Sakmann, B., C. Methfessel, M. Mishina, T. Takahashi, T. Takai, M. Kurasaki, K. Fukuda, and S. Numa. Role of acetylcholine receptor subunits in gating of the channel. *Nature (Lond.)* **318**:538–543 (1985).
15. Yoshii, K., L. Yu, K. M. Mayne, N. Davidson, and H. A. Lester. Equilibrium properties of mouse-*Torpedo* acetylcholine receptor hybrids expressed in *Xenopus* oocytes. *J. Gen. Physiol.* **90**:553–573 (1987).
16. Kurosaki, T., K. Fukuda, T. Konno, Y. Mori, K. Tanaka, M. Mishina, and S. Numa. Functional properties of nicotinic acetylcholine receptor subunits expressed in various combinations. *FEBS Lett.* **214**:253–258 (1987).
17. Adams, P. R. Drug blockade of open end-plate channels. *J. Physiol. (Lond.)* **260**:531–552 (1976).
18. Sheridan, R. E., and H. A. Lester. Relaxation measurements on the acetylcholine receptors. *Proc. Natl. Acad. Sci. USA* **72**:3496–3500 (1975).
19. Neher, E., and B. Sackmann. Voltage-dependence of drug-induced conductance in frog neuromuscular junction. *Proc. Natl. Acad. Sci. USA* **72**:2140–2144 (1975).
20. Hille, B. Ionic channels in electrical membrane. Sinauer, Sunderland, MA (1984).
21. Konno, T., C. Busch, E. VonKitzing, K. Imoto, F. Wang, J. Nakai, M. Mishina, S. Numa, and B. Sackmann. Rings of anionic amino acids as structural determinants of ion selectivity in the acetylcholine receptor channel. *Proc. R. Soc. Lond. B Biol. Sci.* **244**:69–79 (1991).
22. Neher, E., and J. H. Steinbach. Local anesthectics transiently block currents through single acetylcholine-receptor channels. *J. Physiol. (Lond.)* **277**:153–176 (1978).
23. Mishina, M., T. Takai, K. Imoto, M. Noda, T. Takahashi, S. Numa, C. Methfessel, and B. Sackmann. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. *Nature (Lond.)* **321**:406–411 (1986).
24. Mixter Mayne, K., K. Yoshii, L. Yu, H. A. Lester, and N. Davidson. Expression of mouse-*Torpedo* acetylcholine receptor subunit chimeras and hybrids in *Xenopus* oocytes. *Mol. Brain Res.* **2**:191–197 (1987).
25. Pedersen, S. E., and J. B. Cohen. *D*-Tubucurarine binding sites are located at *alpha-gamma* and *alpha-delta* subunit interfaces of the nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **87**:2785–2789 (1990).
27. Imoto, K., T. Konno, J. Nakai, F. Wang, M. Mishina, and S. Numa. A ring of uncharged polar amino acids as a component of channel constriction in the nicotinic acetylcholine receptor. *FEBS Lett.* **289**:193–200 (1991).

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