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The Effects of Isoflurane on Acetylcholine Receptor Channels: 3. Effects of Conservative Polar-to-Nonpolar Mutations within the Channel Pore

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ABSTRACT

We performed macroscopic and single-channel current measurements on wild-type (WT) and two mutant muscle-type nicotinic acetylcholine (ACh) receptor channels transiently expressed in HEK-293 cells. The mutants contained polar-to-nonpolar substitutions at the 10′ (α_2S10′AβT10′A$\gamma\delta$) and 6′ positions (α_2S6′A$\beta\gamma\delta$S6′A) in the M2 pore region of the channel. We studied the behavior of these channels in the absence and presence of the volatile general anesthetic isoflurane. Both mutations changed the gating behavior of the channel. A comparison of the α_2S10′AβT10′A$\gamma\delta$ mutant to WT receptors revealed faster desensitization kinetics, increased sensitivity to ACh, a higher efficacy for activation by the partial nicotinic agonist decamethonium, and a greater number of openings per burst. A comparison of the α_2S6′A$\beta\gamma\delta$S6′A mutant to WT receptors also revealed increased sensitivity to ACh and an increased burst duration at the single-channel level with ACh as agonist. The α_2S10′AβT10′A$\gamma\delta$ mutation increased the sensitivity of the ACh receptor to isoflurane, whereas the α_2S6′A$\beta\gamma\delta$S6′A mutation did not. These changes were probably not caused by the differential effects of the mutation on channel gating and desensitization. The increased sensitivity of the α_2S10′AβT10′A$\gamma\delta$ receptor to isoflurane is state-dependent; the mutation changes the affinity of the closed state but not that of the open state of the channel.

In recent years, there has been increased support for the idea that general anesthetics can act directly on membrane proteins, especially ion channels (Mihic et al., 1997; Franks and Lieb, 1998). The muscle-type nicotinic acetylcholine receptor (AChR) is the site of action of muscle relaxants and may play a role in relaxant effects of some anesthetics. In addition, this receptor is a useful prototype for studying the effects of anesthetics on ligand-gated channels (Barann et al., 1998; Raines and Zachariah, 1999, 2000).

Previously, we described how the volatile anesthetic isoflurane causes AChR channel activity to occur in bursts of briefer-than-normal openings (Dilger et al., 1992). We showed how this behavior was well described by a model in which isoflurane blocks both the open and closed states of the receptor pore (Dilger et al., 1993). We also found evidence for cooperative interactions between the channel-blocking local anesthetic QX-222 and diethyl ether, the parent compound of isoflurane (Dilger and Vidal, 1994). Forman and his colleagues (Forman et al., 1995; Forman, 1997) studied site-directed mutants of the AChR in which polar amino acids in the second membrane-spanning region of the receptor (M2) were replaced with large hydrophobic residues. These mutations affected the affinity of the receptor for isoflurane and some alcohols. This was interpreted as evidence for anesthetic binding within the pore of the AChR. However, the kinetic predictions of the model were not tested in this study. It was also observed that the mutations affected channel desensitization. The consequences of this finding for the interpretation of the data were not completely addressed.

In this article, we consider more conservative mutations at two sites within the pore of the channel. We studied wild-type ($\alpha_2\beta\gamma\delta$, WT) and two mutant receptors [α_2S10′AβT10′A$\gamma\delta$ (10′$\alpha\beta$) and α_2S6′A$\beta\gamma\delta$S6′A (6′$\alpha\delta$)] (Fig. 1). In the experiments conducted by Charnet et al. (1990), the binding of QX-222 was found to be affected by mutations at both the 10′ and 6′ regions of M2. Polar-to-nonpolar mutations at 10′ resulted in tighter binding of QX-222. In contrast, polar-to-nonpolar mutations at 6′ resulted in less tight binding of QX-222. The authors reasoned that the amphipathic QX-222 molecule binds with its charged nitrogen near the 6′ level and its ring moiety near 10′. Our hypothesis is that the hydrophobic general anesthetic

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ABBREVIATIONS: ACh, acetylcholine; AchR, acetylcholine receptor; WT, wild-type; QX-222, *N*′-(trimethyl-amino-methyl)-2′,6′-xylidide; ECS, extracellular solution; 10′$\alpha\beta$, α_2S10′AβT10′A$\gamma\delta$; 6′$\alpha\delta$, α_2S6′A$\beta\gamma\delta$S6′A.

isoflurane binds near 10′. Thus, inhibition by isoflurane will be sensitive to mutations at 10′ but not at 6′.

In any study involving site-directed mutagenesis of proteins, it is essential to determine whether the mutation is simply causing a fairly localized change in the protein or a global change that affects multiple functions of the protein. The first part of this study concerns the behavior of WT and the two mutant receptors in the absence of isoflurane. We found that the mutations affect both channel gating and desensitization, and these changes are different for the two mutants. The second part of the study deals with the effects of isoflurane. We find that the 10′$\alpha\beta$ mutation increases the sensitivity of the ACh receptor to isoflurane, whereas the 6′$\alpha\delta$ mutation does not. These changes are probably not caused by the differential effects of the mutation on channel gating and desensitization. Finally, we found that the increased sensitivity of the 10′$\alpha\beta$ receptor to isoflurane is state-dependent; the mutation changes the affinity of the closed state but not the open state of the channel. Preliminary results have been presented in abstract form (Barann et al., 1996).

Materials and Methods

Mouse nicotinic acetylcholine receptors were expressed transiently in HEK-293 cells (American Type Culture Collection, Manassas, VA) using a calcium-phosphate transfection protocol (Sine, 1993). Cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Solutions of 2× HEPES (275 mM NaCl, 1.5 mM $Na_2HPO_4{\cdot}7H_2O$, 55 mM HEPES, pH 7.05) and calcium/DNA (123 mM $CaCl_2$ plus cDNA for each AChR subunit) were prepared. To transfect three 30-mm dishes, we used 7.5, 3.75, 3.75, and 3.75 μg of cDNA (subcloned into the pRGB4 expression vector) for the α (WT or mutant), β (WT or mutant), γ, and δ (WT or mutant) subunits, respectively, and 1 μg of cDNA for CD8 (gift from Dr. Brian Seed), a T-cell antigen used as a marker. A precipitate was formed by adding 0.5 ml of the calcium/DNA solution dropwise into the 0.5 ml of the 2× HEPES buffer solution. After 30 min, the precipitate was distributed over each dish of cells. After 8 to 12 h, the transfection solution was replaced with Dulbecco's modified Eagle's medium plus 10% fetal calf serum. Experiments were performed on cells between 1 and 4 days after transfection.

We prepared a stock solution of saturated (15 mM; Firestone et al., 1986) isoflurane (1-chloro-2,2,2-trifluroethyl difluoromethyl ether, Forane; Anaquest, Madison, WI) in extracellular solution (ECS) by adding isoflurane to a glass bottle filled with ECS, closing the bottle with a cap providing a Teflon seal, and stirring the solution overnight with a Teflon-coated magnetic stirring bar. The stock solution was then diluted with ECS to obtain the desired concentration of isoflurane. The solution was quickly transferred to a solution reservoir, an intravenous drip bag that was made air-tight with a dialysis bag clamp.

To prepare cells for patch-clamp recording, the culture medium was replaced with an ECS containing 150 mM NaCl, 5.6 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM HEPES, pH 7.3. To help identify transfected cells, we added 5 to 10 μl of polysterene beads coated with a monoclonal antibody specific for the CD8 antigen (Dynabeads; Dynal, Lake Success, NY). After 15 min of equilibration with beads, ECS was flowed into the culture dish to remove loose beads. Cells with two or more beads attached (about 10%) were considered likely to express AChR as well as CD8 (Jurman et al., 1994). We found that patches from some of the cells with beads did not have AChR channel activity; perhaps these cells expressed such a low density of channels that most excised patches had no channels. We also used morphological clues to help determine which of those cells with beads also contained AChR channels. These clues included large, spread-out cells, cells with a granular appearance, and cells having areas that appeared as plateaus emerging from the surface.

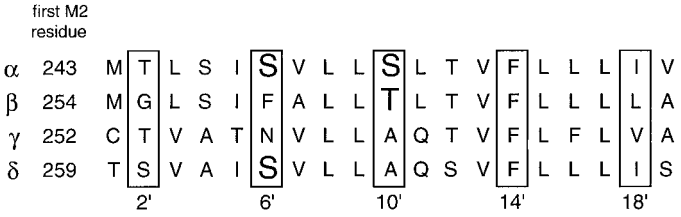


Fig. 1. The amino acid sequences of the M2 region of the four subunits of the embryonic muscle AChR.

Patch pipettes were filled with a solution consisting of 140 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, and 10 mM HEPES, pH 7.3, and had resistances of 3 to 6 MΩ. An outside-out patch (Hamill et al., 1981) with a seal resistance of 10 GΩ or greater was excised from a cell and moved into position at the outflow of a perfusion system. The perfusion system consisted of solution reservoirs, manual switching valves, a solenoid-driven pinch valve, and two Teflon tubes inserted into the culture dish (Liu and Dilger, 1991). One tube contained ECS without agonist (normal solution); the other contained ECS with a known concentration of ACh (test solution). In the resting position of the pinch valve, normal solution perfused the patch. The solenoid was then triggered to stop the flow of normal solution and start the flow of test solution. After the patch was exposed to the test solution for 0.1 to 0.25 s, the pinch valve was returned to its resting position for several seconds. In this way, we exposed the patch to a series of timed exposures to the agonist-containing solution while minimizing the desensitizing effects of prolonged exposure to ACh. The perfusion system allows a rapid (0.1–1 ms) exchange of the solution bathing the patch. During single-channel recording, we used a similar but slower perfusion system that was adequate for the relatively nondesensitizing conditions of 0.2 μM ACh.

The currents flowing during exposure of the patch to ACh were measured with a patch-clamp amplifier (EPC-7; List-Medical Instruments, Darmstadt, Germany), filtered at 3 kHz (−3-db frequency, 8-pole Bessel filter; Frequency Devices, Haverhill, MA), digitized, and stored on the hard disk of a laboratory computer (PDP11; Digital Equipment Corporation, Maynard, MA). Data analysis was performed off-line as described previously (Dilger et al., 1997). Experiments were performed at room temperature (20–23°C).

For macroscopic currents, we recorded current responses (at −50 mV) during 200-ms applications (at 5-s intervals and sampled at 100–200 μs per point) of ECS containing 100 μM ACh (test solution), a concentration that activates about 90% of the AChR channels from BC_3H-1 cells (Dilger and Brett, 1990). We subsequently used this test solution to quantify the loss of channel activity. Both the normal and test perfusion solutions were then switched to isoflurane-containing solutions. Responses of the patch to 100 μM ACh applications during continuous exposure to isoflurane were recorded. The drug-free solutions were then re-introduced, and recovery currents were measured. Data were accepted when the initial and recovery currents changed by less than 10%. Some experiments were performed with other perfusion protocols. We used the following nomenclature: (−/−) = control (no drug in either normal or test solutions), (+/+) = equilibrium (drug in both normal and test solutions), and (−/+) = onset (drug in test solution only—simultaneous application of agonist and drug).

The ensemble mean current was calculated from 10 to 30 individual current traces. Mean currents were fit to single- or double-exponential functions to obtain peak and steady-state current values and a time constant of the decay caused by desensitization (Dilger and Liu, 1992). The 2-exponential fit was considered better if χ_1^2 was at least 3-fold greater than χ_2^2. Relative peak-response amplitudes are calculated as the ratio of the peak current response to application of a given concentration of ACh to that obtained in response to application of 100 μM ACh. Fractional inhibition of the peak mean

current, the maximum inward current obtained during perfusion of ACh, was calculated as the ratio of current in the presence of drug to current in the absence of drug.

Single-channel recordings were made while the patch was transiently exposed to ECS containing 0.2 μM ACh at a patch potential of −100 mV. We digitized data in 5-s segments at a rate of 50 μs per point. The patch was perfused with normal ECS for at least 10 s in between recordings. This differs from our previous protocol and was necessary because we found that mutant AChRs desensitize quickly and extensively when exposed to this low concentration of ACh. We obtained enough segments of data to provide 300 to 1000 single-channel events. Data recording was repeated with ECS containing isoflurane and then again with normal ECS (recovery). Data were accepted if, after analysis, channel kinetics during recovery were within 20% of the values obtained during the initial data-collection segments. Data analysis, including correction for missed events, was performed off-line as described previously (Dilger et al., 1997). The kinetic parameters of Scheme 2 (see *Results*) were determined by fitting the open duration-versus-concentration data to obtain values for α and f (eq. 3). These values were used to determine b/α' from the number of openings per burst versus concentration data (eq. 5). Finally, $b + \alpha'$ was determined from the gap duration averaged for all concentrations (eq. 4). Thus, we calculated both b and α' values. Next, 90% confidence limits were obtained from the fits, and these confidence limits were propagated in quadrature, e.g.

$$\frac{\Delta b}{b} = \sqrt{\left(\frac{\Delta \alpha'}{\alpha'}\right)^2 + \left(\frac{\Delta (b/\alpha')}{b/\alpha'}\right)^2}, \qquad \frac{\Delta (b/f)}{b/f} = \sqrt{\left(\frac{\Delta b}{b}\right)^2 + \left(\frac{\Delta f}{f}\right)^2}.$$

Data are expressed as mean ± S.D. Statistical comparisons were made using the Student's t test. A level of $p < 0.05$ was considered to be significant.

Results

Comparison of WT and Mutant AChR Currents and Channels. When an outside-out patch is rapidly perfused with 3 to 100 μM ACh, a macroscopic current develops as channels are opened by ACh within less than 1 ms and subsequently desensitizes within tens of ms in the continued presence of ACh. Figure 2 shows examples of macroscopic currents from WT, 10′$\alpha\beta$-mutant, and 6′$\alpha\delta$-mutant AChRs activated by rapid perfusion of 3, 10, and 100 μM ACh. In these examples, both of the mutant AChR currents desensitize more quickly and seem to be more sensitive to ACh than do WT. As was seen previously with the AChR in the clonal murine BC_3H-1 cell line (Dilger and Brett, 1990) and in embryonic receptors expressed in *Xenopus* oocytes (Bufler et al., 1993), there is a large patch-to-patch variability in the rate of fast desensitization for AChRs expressed in HEK-293 cells (compare currents in Fig. 2 with those in Fig. 4). Table 1 summarizes the data on the desensitization of the three types of receptor by 100 μM ACh. When the decay phase (desensitization) of the current is fit to a single exponential function, the desensitization time constant, τ, for the 10′$\alpha\beta$ mutant receptors is 60% faster than that for WT receptors ($p < 0.03$); the 26% lower value of τ for 6′$\alpha\delta$ compared with that for WT receptors was not significant ($p > 0.1$). The time course of desensitization is sometimes better fit by a 2-exponential decay function. Using the criterion $\chi_1^2 \geq 3\ \chi_2^2$, a 2-exponential fit was better in 15, 47, and 17% of the patches containing WT, 10′$\alpha\beta$, and 6′$\alpha\delta$ receptors, respectively. Table 1 compares the two time constants, τ_{fast}, τ_{slow}, and the ratios of the amplitudes of the two components, a_{fast}/a_{slow}, for WT and mutant receptors. The 10′$\alpha\beta$-mutant receptors differ from WT in that both components of desensitization are faster. In addition, both components have approximately the same amplitude in 10′$\alpha\beta$ receptors, whereas the slow component dominates in WT receptors. Comparisons of these parameters between 6′$\alpha\delta$ mutant and WT show no significant differences ($p > 0.5$). The data for WT receptors are similar to published data for the native fetal mouse AChR in BC_3H1 cells (Dilger and Brett, 1990); these data are included in Table 1.

Figure 3 shows the plots of the relative peak-response amplitude versus [ACh] for the three types of AChR. The solid curves are fits of the data to a Hill equation, $p = [ACh]^{n_H}/(EC_{50}^{n_H} + [ACh]^{n_H})$ where EC_{50} is the concentration of ACh producing half-maximal activation, and n_H is the Hill coefficient. The EC_{50} value is lower for the mutant receptors, but the steepness of the curves does not differ among the three receptors (Table 2). Both the 10′$\alpha\beta$ and 6′$\alpha\delta$ concentration-response curves are significantly different from that of the WT ($p < 0.001$, F-test). The data for WT receptors are similar to published data for the native fetal mouse AChR in BC_3H1 cells (Dilger and Brett, 1990).

Macroscopic Current Response to Perfusion of Decamethonium. The shift in ACh sensitivity induced by the mutations (Fig. 3) could arise from either a change in agonist affinity or agonist efficacy. Scheme 1 is the standard 4-state model for AChR channel activation.

Acetylcholine (A) binds sequentially to two sites on the receptor (R) to form monoligand (AR) and double-ligand (A_2R) closed states. A_2R undergoes a conformational change to the open state (A_2R^*). Mutant receptors could have a smaller equilibrium dissociation constant (K_{eq}) or a larger

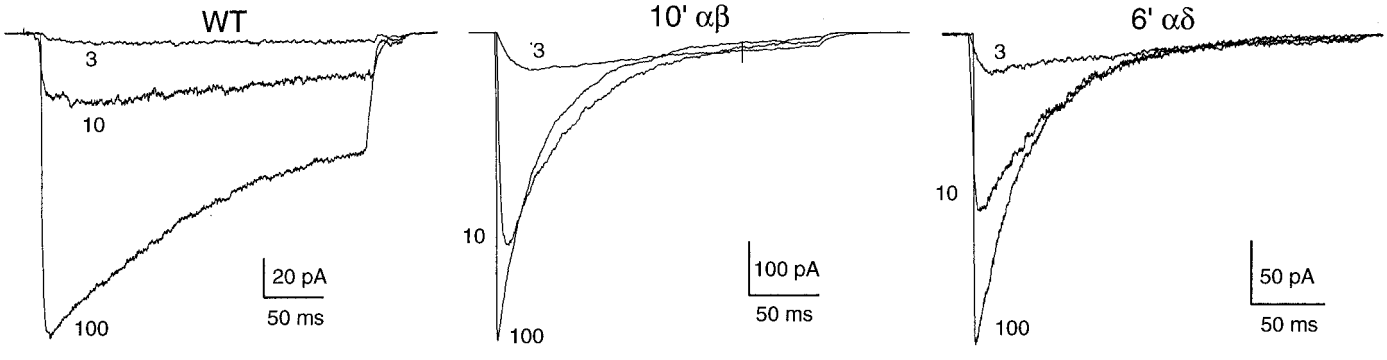


Fig. 2. Macroscopic current recordings from WT, 6′$\alpha\delta$, and 10′$\alpha\beta$ AChR in response to rapid perfusion with 100, 10, and 3 μM ACh. The applied potential was −50 mV.

isomerization constant, (β/α, where β and α are the opening and closing rate constants, respectively). Because ACh is a very efficacious agonist [$\beta/\alpha \geq 50$ for WT AChR (Dilger and Brett, 1990; Maconochie and Steinbach, 1998)], β/α cannot be accurately determined from equilibrium measurements on macroscopic currents; high-resolution, single-channel current measurements (Zhang et al., 1995) or macroscopic current kinetic measurements (Maconochie and Steinbach, 1998) are necessary. We obtained lower limits on β by measuring the 20 to 80% onset time (t_{20-80}) in response to rapid perfusion of 1 mM ACh: $t_{20-80} = 0.11 \pm 0.05$ ms ($n = 6$) for $10'\alpha\beta$ receptors and 0.10 ± 0.03 ($n = 15$) for $6'\alpha\delta$ receptors. Thus, $\beta > 14$/ms for both mutant receptors. This is the resolution limit for our rapid perfusion system (Liu and Dilger, 1991), so we cannot use this approach to determine whether the mutant receptors have a value of β/α that is greater than that of WT.

We previously used the partial nicotinic agonist decamethonium as a tool to test for drug-induced changes in efficacy (Liu et al., 1994). For AChR from BC_3H1 cells, the maximal open-channel probability for activation by decamethonium is 0.016 (Liu and Dilger, 1993). Because decamethonium has such a low efficacy, the peak-response amplitude is very sensitive to differences in β/α. We measured the response of the three receptor types to applications of 100 μM decamethonium (a concentration that produces a maximal current in BC_3H1 cells) compared with 100 μM ACh. We found the relative currents to be 0.019 ± 0.007 ($n = 4$) for WT, 0.069 ± 0.029 ($n = 5$) for $10'\alpha\beta$, and 0.031 ± 0.008 ($n = 6$) for $6'\alpha\delta$. There was no evidence of an overshoot upon washout of 100 μM decamethonium for any of the receptors, suggesting that channel block by decamethonium is insignificant. The data are summarized in Table 3. Using this as an assay for efficacy, decamethonium is a more efficacious agonist on $10'\alpha\beta$ ($p < 0.02$) but not $6'\alpha\delta$ ($p = 0.056$) receptors compared with WT receptors.

Single-Channel Experiments. The results of single-channel experiments with WT, $10'\alpha\beta$, and $6'\alpha\delta$ AChR are summarized in Table 4. Single channels are activated by transient exposure to 0.2 μM ACh. As observed with AChRs from BC_3H1 cells, the three types of expressed AChRs exhibit two-component open, burst, and closed duration histograms (see Fig. 7). A brief closed component with a time constant of ≤ 50 μs is expected to arise from repeated transitions of a single channel between A_2R and A_2R^* before the agonist dissociates from its binding site (Zhang et al., 1995). This component is not fully resolved in our experiments, so the value of the observed fast time constant does not indicate the lifetime of the A_2R state. This also suggests that the observed open duration overestimates the actual value. The long closed time corresponds to the time between activation of separate channels in the patch and depends on the number of active channels in the patch and on the agonist concentration. For both WT and mutant receptors, the long closed time varied from patch-to-patch, between 30 and 700 ms. The origin of the two open and burst components is not certain, but a fraction of the brief duration openings is believed to originate from the opening of single-ligand receptors whereas, the long duration openings represent transitions to the more stable, double-liganded open state, A_2R^* (Dilger et al., 1992). The single-channel current at -100 mV for both $10'\alpha\beta$ and $6'\alpha\delta$ AChRs was the same as for WT. This corresponds to a conductance of 40 pS.

The $10'\alpha\beta$ receptors differ from WT in that the duration of brief closures is larger. This is seen to a smaller degree in the $6'\alpha\delta$ receptors as well. This component does not have a direct association with steps in the normal AChR activation process (Scheme 1) and has been observed in other studies (Sine and Steinbach, 1986). In addition, the duration of both brief and long bursts is longer in the $6'\alpha\delta$ receptors than in WT. An increased burst duration can arise from changes in either gating (increased opening rate or decreased closing rate) or agonist binding (decreased dissociation rate).

One other difference was apparent at the single-channel level. Exposure of either $10'\alpha\beta$ or $6'\alpha\delta$ AChRs to 0.2 μM ACh for 30 s greatly reduced the frequency of openings, whereas there was no obvious change in the opening frequency of WT AChRs under these conditions. In the case of the $10'\alpha\beta$ mutation, this reduced frequency of openings may be a manifestation of the accelerated desensitization seen with higher concentration of ACh at the macroscopic level with mutant AChRs. Because of this phenomenon, we used a perfusion protocol that limited the time of exposure to ACh during single-channel recording to <10 s.

Table 4 includes single-channel conductance and kinetic parameters for the native fetal mouse AChR found in BC_3H1 cells. These values are similar to those for WT receptors transiently expressed in HEK-293 cells.

The Effects of Isoflurane on Macroscopic AChR Currents. Isoflurane inhibits macroscopic currents elicited by rapid perfusion of ACh (Dilger et al., 1993). Figure 4 presents examples of currents elicited by 100 μM ACh in controls and in the presence of 1 mM isoflurane for WT (Fig. 4A), $10'\alpha\beta$ mutant (Fig. 4B), and $6'\alpha\delta$ mutant (Fig. 4C) AChRs. Whereas 1 mM isoflurane produces the same inhibition of WT and $6'\alpha\delta$ currents (46 and 47%, respectively), it produces more inhibition of the $10'\alpha\beta$ channel current (88%). Inhibition

TABLE 1

Desensitization time constants for native, WT, and mutant AChRs

τ is the time constant of a single-exponential fit to the decay in the macroscopic current response to application of 100 μM ACh at -50 mV. For those cases in which a double-exponential fit was superior (see *Materials and Methods*), the average values of τ_{fast}, τ_{slow}, and a_{fast}/a_{slow} are given. Results are expressed as mean ± S.D. (number of patches). Data for receptors in BC_3H1 cells are from Dilger and Brett, 1990.

	BC_3H1	WT	$10'\alpha\beta$	$6'\alpha\delta$
τ (ms)	67±17(8)	110 ± 34 (49)	44 ± 14 (34)[a]	81 ± 41 (24)
τ range (ms)	46 –95	32 –184	14 –76	29 –170
τ_{fast} (ms)	N/A	26 ± 11 (7)	17 ± 9 (16)[a]	18 ± 11 (4)
τ_{slow} (ms)	N/A	169 ± 68 (7)	85 ± 35 (16)[b]	216 ± 199 (4)
a_{fast}/a_{slow}	N/A	0.47 ± 0.31 (7)	1.05 ± 0.75 (16)[b]	0.75 ± 0.71 (4)

N/A, data not available.

[a] Significantly different from WT, t test, $p < 0.04$.

[b] Significantly different from WT, t test, $p < 0.02$.

curves for the three types of receptors are given in Fig. 5. These data were fit to a Hill equation (Fig. 5, solid lines):

$$\frac{I_p(\text{iso})}{I_p} = \frac{K_i^n}{K_i^n + [\text{iso}]^n} \quad (1)$$

where K_i is the drug concentration for 50% inhibition and n is the Hill coefficient.

Isoflurane is four times more potent at inhibiting 10′$\alpha\beta$ receptors than WT and 6′$\alpha\delta$ receptors (Table 5). The affinity for isoflurane inhibition of WT receptors transiently expressed in HEK cells is the same as for isoflurane inhibition of ACh receptors found in BC_3H1 cells (Dilger et al., 1993). The Hill slope suggests that more than one molecule of isoflurane is involved in the inhibition. With this in mind, we investigated whether the data shown in Fig. 5 could be fit to a model with two drug binding sites. Because the Hill coefficients are larger than 1.25, a model with two independent binding sites does not suffice. However, if we assume that two isoflurane molecules bind sequentially (the second site is not revealed until the first site is occupied), the large Hill coefficients can be accounted for. Using K_1 and K_2 to describe the affinities of the two sites,

$$\frac{I_p(\text{iso})}{I_p} = \frac{K_1K_2}{K_1K_2 + K_2[\text{iso}] + [\text{iso}]^2} \quad (2)$$

The two-site model fits the data as well as does the Hill equation (Fig. 5, dotted line shown for 10′$\alpha\beta$ receptors). For each receptor type, the fit to eq. 2 suggests that there is positive binding cooperativity, and the two isoflurane binding affinities are within a factor of 2.5 of each other (Table 5).

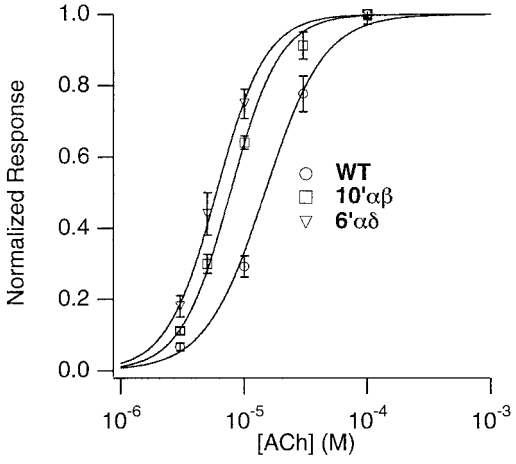


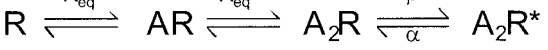
Fig. 3. ACh concentration-response curves for WT and mutant AChR. Solid lines are drawn according to $p = [ACh]^{n_H}/(EC_{50}^{n_H} + [ACh]^{n_H})$; the best fit parameters are given in Table 2.

TABLE 2

Activation of native, WT, and mutant AChRs by ACh

Concentration-response data (Fig. 3) were fit to the Hill equation (see *Results*). Results are presented as best fit parameter ±90% confidence limit. Data for receptors in BC_3H1 cells are from Dilger and Brett, 1990, and consider a wider concentration range (0.3–1000 μM) than the other experiments.

Fit Parameter	BC_3H1	WT	10′$\alpha\beta$	6′$\alpha\delta$
EC_{50}(μM)	20	14.5 ± 3.0	8.4 ± 1.8	5.7 ± 1.3
n_H	1.6	1.8 ± 0.4	2.0 ± 0.4	1.9 ± 0.8



Scheme 1

The Kinetics of Inhibition of Macroscopic Currents by Isoflurane. One way to test the idea that the apparent high affinity of 10′$\alpha\beta$ receptors for isoflurane is caused by an increase in binding to open channels is to examine the state dependence of block using different rapid application protocols (Dilger et al., 1993, 1997; Barann et al., 2000). For BC_3H1 cells, we found that simultaneous rapid perfusion of high concentrations of ACh and 1 mM isoflurane (−/+ onset protocol) resulted in a macroscopic current that began close to the control level and decayed to close to the equilibrium level within a few hundred microseconds (Dilger et al., 1993). The situation for WT and 6′$\alpha\delta$ receptors is similar (Fig. 6 top, left and right, respectively). In contrast, the −/+ perfusion protocol performed on the 10′$\alpha\beta$ receptors revealed comparatively less inhibition of the current (Fig. 6, top, center). For these figures, we used a concentration of isoflurane that produced nearly 50% inhibition of each mutant receptor (1.8

TABLE 3

Activation of native, WT, and mutant AChRs by 100 μM decamethonium

Results are expressed as mean ± S.D. (number of patches) in the presence of 100 μM decamethonium normalized to 100 μM ACh. Data for receptors in BC_3H1 cells are from Liu and Dilger, 1993.

	BC_3H1	WT	10′$\alpha\beta$	6′$\alpha\delta$
Relative amplitude	0.016	0.019 ± 0.007 (4)	0.069 ± 0.029 (5)[a]	0.031 ± 0.008 (6)

[a] Significantly different from WT, t test, $p < 0.02$.

TABLE 4

Single-channel properties of native, WT, and mutant AChRs

Results are presented as mean ± S.D. for nine patches of WT, ten patches of 10′$\alpha\beta$, and seven patches of 6′$\alpha\delta$ (all expressed in HEK-293 cells). Data for receptors in BC_3H1 cells are from Dilger et al., 1992, except for the brief long burst durations, which were not previously published (but are based on the same experiments with 20 patches) 0.2 μM ACh, −100 mV.

Property	BC_3H1	WT	10′$\alpha\beta$	6′$\alpha\delta$
Single-channel current (pA)	−4.0 ± 0.2	−4.0 ± 0.3	−4.0 ± 0.2	−3.9 ± 0.2
Brief burst duration (ms)	0.23 ± 0.13	0.20 ± 0.10	0.18 ± 0.04	0.38 ± 0.10[a]
Fraction of brief bursts	0.30 ± 0.15	0.37 ± 0.17	0.49 ± 0.16	0.34 ± 0.08
Long burst duration (ms)	4.6 ± 1.4	5.9 ± 0.9	7.4 ± 3.2	13.5 ± 6.8[a]
Brief closed duration (ms)	0.09 ± 0.05	0.11 ± 0.05	0.54 ± 0.24[a]	0.20 ± 0.10[b]

[a] Significantly different from WT, t test, $p < 0.01$.
[b] Significantly different from WT, t test, $p < 0.05$.

mM for WT, 1.6 mM for 6′$\alpha\delta$, 0.6 mM for 10′$\alpha\beta$).[1] We estimated the degree of inhibition of open channels by comparing the current after the fast decay process during a −/+ protocol with the control peak current. The results of −/+ protocol experiments over a range of isoflurane concentrations were fit to eq. 1 to determine the potency of isoflurane to inhibit open channels [K_i = 2.5 ± 0.8 mM for the 6′$\alpha\delta$ mutant (23 patches) and K_i = 1.6 ± 0.2 mM for the 10′$\alpha\beta$ mutant (19 patches)]. Thus, for the 10′$\alpha\beta$ mutant, isoflurane is much more effective when applied to closed channels (0.3 mM) then when applied to open channels (1.6 mM). There is also a marked difference between the mutants when the +/− protocol is used (Fig. 6, bottom panels). The WT and 6′$\alpha\delta$ mutant receptors begin to recover from inhibition by isoflurane within 1 ms, but the 10′$\alpha\beta$ receptors do not.

The Effects of Isoflurane on AChR Single-Channel Currents. If the difference in affinity for isoflurane between WT and 10′$\alpha\beta$ receptors is, indeed, caused by a difference in the binding of isoflurane within the closed but not open pore of the channel, then we expect there to be no differences in the behavior of single AChR channels in the presence of the drug. Single-channel recordings allow us to examine both the association and dissociation rates of drugs interacting with open channels.

Scheme 2 has been used to describe the inhibitory effects of general anesthetics and alcohols on AChR channels (Murrell et al., 1991; Dilger et al., 1993):

The upper limb of Scheme 2 represents the operation of the AChR channel in the absence of isoflurane (see Scheme 1). Isoflurane (B) binds to both closed and open states of the channel. None of these isoflurane-bound states is conducting. The closed and open states may bind isoflurane with different affinities (b/f and b'/f'). Although this scheme is incomplete (see *Discussion*), we can use the far-right loop to evaluate effects on single channels. Because the time resolution of these experiments does not provide information about the true open duration, we will consider the control long-burst duration as the apparent open duration, $\tau_{open,\ app}$. If the drug association rate, f, is increased, the concentration dependence, [B], of the channel open time (long open time component)

$$\tau_{open,app} = \frac{1}{\alpha_{app} + f[B]} \quad (3)$$

would shift to lower concentrations of isoflurane. In eq. 3, α_{app} is the apparent closing rate, which is the reciprocal of the apparent open duration in control experiments. If the drug dissociation rate, b, is decreased, the duration of closed gaps within bursts (short closed time component) would increase

$$\tau_{gap} = \frac{1}{b + \alpha'} \quad (4)$$

and the number of openings per burst (see *Appendix 1*) would decrease

$$N_{o/b} = \frac{\alpha_{app} + f[B]}{\alpha_{app} + f[B]\left(1 + \frac{b}{\alpha'}\right)^{-1}}. \quad (5)$$

A combination of the two effects is also possible.

Figure 7 shows examples of single-channel currents for WT, 10′$\alpha\beta$, and 6′$\alpha\delta$ AChR in the absence and presence of 1 mM isoflurane. There are no obvious differences among the channels in their response to isoflurane. Isoflurane causes channel activity to occur in bursts of brief openings. The brief component in the closed time histograms becomes much more pronounced in the presence of isoflurane.

Figure 8 shows the isoflurane concentration dependencies of $\tau_{open,app}$, τ_{gap}, and $N_{o/b}$ for WT, 6′$\alpha\delta$, and 10′$\alpha\beta$ receptors. There are no differences in $\tau_{open,app}$ versus [isoflurane] (Fig. 8A) between WT and 10′$\alpha\beta$ receptors. The open duration of 6′$\alpha\delta$ receptors is larger than that of WT at all concentrations of isoflurane, and the slope of the $1/\tau_{open,app}$ versus [isoflurane] relationship (Fig. 8A, inset) is less steep than that of the others. The 10′$\alpha\beta$ receptors also have a longer gap duration than either WT or 6′$\alpha\delta$ receptors (Fig. 8B). The $N_{o/b}$ for 6′$\alpha\delta$ receptors increases more steeply with drug concentration than does that for either WT or 10′$\alpha\beta$ receptors (Fig. 8C). From these comparisons, we could conclude that in 10′$\alpha\beta$ receptors, the drug association rate is similar to that of WT receptors. However, the status of the drug dissociation rate is not apparent because the $N_{o/b}$ and τ_{gap} data cannot both be explained by a simple change in dissociation rate.

The results of fitting the data to eqs. 3 to 5 are presented in Table 6. The main difference between WT and 10′$\alpha\beta$ receptors is a smaller value for α', the closing rate of blocked channels, whereas the isoflurane dissociation rate b is only 50% smaller than that of WT receptors. Because of the large uncertainties associated with the determination of the pa-

[1] A rapid decay is not obvious when inhibition is low in the −/+ application mode with the 10′ mutant. It becomes apparent at higher concentrations of isoflurane.

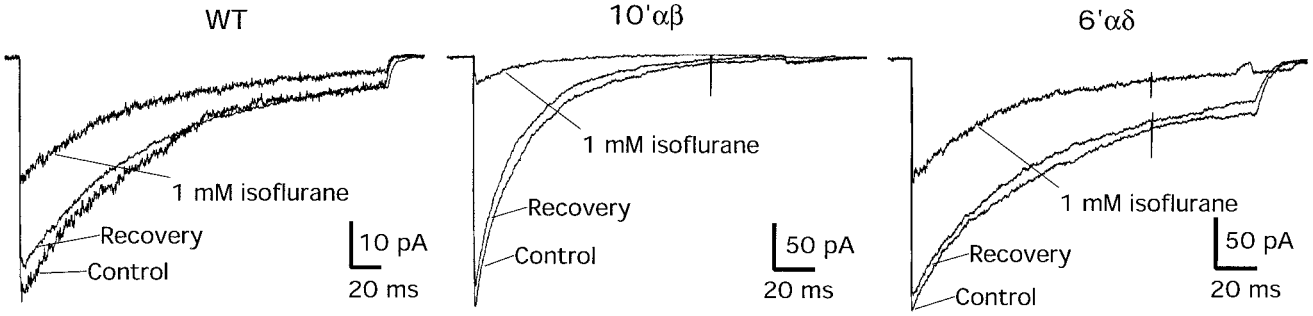


Fig. 4. Macroscopic current recordings from WT, 6′$\alpha\delta$, and 10′$\alpha\beta$ AChR. Activation by 100 μM ACh in the absence and presence of 1 mM isoflurane (equilibrium, +/+ protocol). Applied potential was −50 mV.

rameters, we compared our results with the prediction that the high affinity of 10′$\alpha\beta$ receptors for isoflurane is caused by a drug dissociation rate that is 4-fold lower than with WT with the WT value of α'. The predicted gap duration agrees with the measurements (0.3 ms), but the predicted number of openings per burst is considerably less than the observed number (Fig. 8c, broken line). The best fit parameters for 6′$\alpha\delta$ receptors are also unexpected given the similarity between these receptors and WT receptors in the effect of isoflurane on macroscopic currents (Fig. 5). The drug association rate, f, is 3-fold lower in 6′$\alpha\delta$ receptors compared with WT receptors.

We performed some single-channel experiments with 30 μM QX-222 (at $V = -100$ mV) to confirm that the 10′$\alpha\beta$ mutation had the expected effect on gap duration produced by this local anesthetic. For WT receptors, the gap duration was 0.68 ± 0.20 ms ($n = 2$). For 10′$\alpha\beta$ receptors, the gap duration was 1.7 ±0.41 ms ($n = 6$). This is in agreement with the results of Charnet et al. (1990).

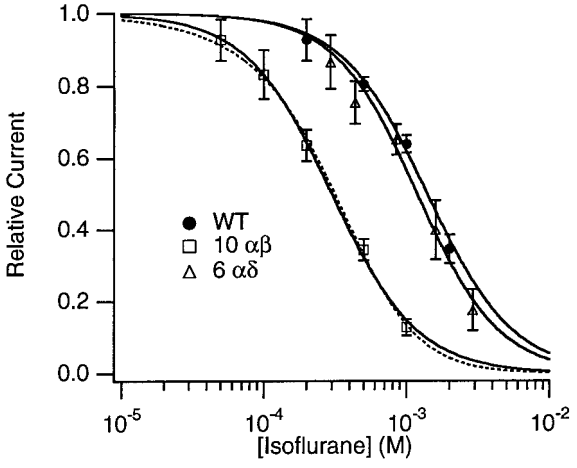


Fig. 5. Isoflurane concentration-response curves for WT, 6′$\alpha\delta$, and 10′$\alpha\beta$ AChR. Solid lines are fits to the Hill equation (eq. 1); the broken line for the 10′$\alpha\beta$ AChR represents the fit to the two-site model (eq. 2). Fitting parameters are given in Table 3.

Discussion

We found no significant differences between WT ACh receptors expressed in HEK-293 cells and the native channel found in murine BC_3H1 cells either in the absence or presence of isoflurane. This suggests that the membrane and intracellular environment provided by the nonmuscle host cell line do not induce biophysical changes in receptor function.

Comparison of WT and Mutant AChR Currents and Channels. The mutant receptors studied here were chosen because they affect the interaction of QX-222 with the AChR channel (Charnet et al., 1990). Substitution of the small, nonpolar alanine residue for the polar serine and threonine

TABLE 5

Parameters used in the fitting of the inhibition data in Fig. 5

K_i and n are used in the Hill Equation fit (eq. 1); K_1 and K_2 are used in the two-site model fit (eq. 2).

Property	BC_3H1	WT	10′$\alpha\beta$	6′$\alpha\delta$
K_i (mM)	0.9 ± 0.1	1.4 ± 0.1	0.30 ± 0.02	1.2 ± 0.1
n	1.7 ± 0.2	1.45 ± 0.14	1.50 ± 0.14	1.52 ± 0.09
K_1 (mM)	3.9 ± 1.8	3.0 ± 0.8	0.59 ± 0.19	2.6 ± 0.5
K_2 (mM)	0.36 ± 0.27	1.2 ± 0.7	0.36 ± 0.21	1.1 ± 0.4

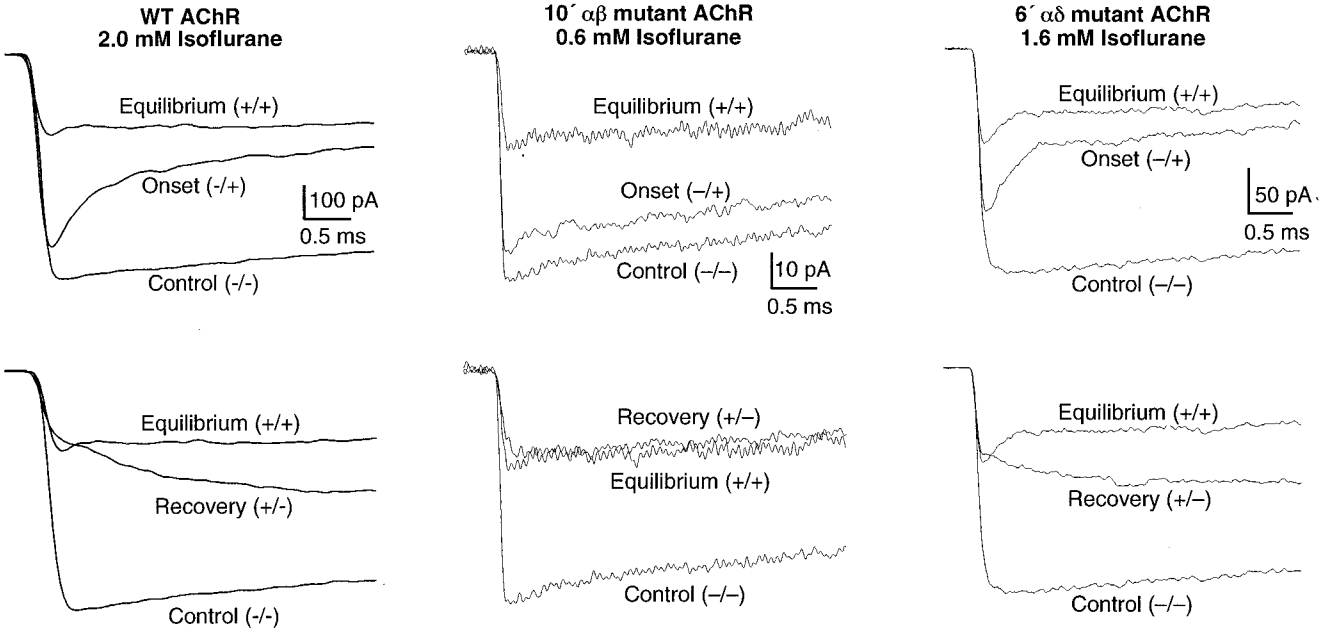
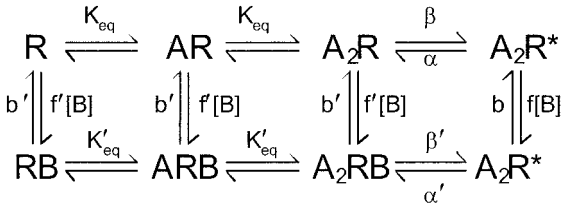


Fig. 6. Macroscopic current responses to 1 mM ACh obtained under different perfusion protocols with equipotent concentrations of isoflurane. Left, WT and 1.8 mM isoflurane; center, 6′$\alpha\delta$ AChR and 1.6 mM isoflurane; right, 10′$\alpha\beta$ AChR and 0.6 mM isoflurane. The top panels show onset kinetics and compare the simultaneous perfusion of ACh and isoflurane (−/+) with control (−/ −) and equilibrium (+/+) application protocols. The bottom panels illustrate recovery kinetics and compare currents during rapid removal of isoflurane (+/−) with control and equilibrium application protocols.

residues at the 6′ and 10′ regions of M2 was considered to have an effect that is localized to the pore of the channel. However, our experiments show that these point mutations are not benign; they affect the global behavior of the channel.



Scheme 2

Other mutations in M2 have also been shown to affect the gating and/or binding behavior of the AChR (Filatov and White, 1995; Forman et al., 1995; Labarca et al., 1995; Chen and Auerbach, 1998; Grosman and Auerbach, 2000).

The process of fast desensitization proceeds mainly from the double-liganded open state (Auerbach and Akk, 1998). The desensitization rate can be approximated by $1/\tau$ measured at saturating agonist concentrations (Fig. 3). The desensitization rate is not affected by several mutations at the ACh binding site of the receptor (Auerbach and Akk, 1998). However, for the 10′$\alpha\beta$ receptor, we found the rate of fast desensitization to be 2.5-fold higher than for the WT receptor (Table 1).

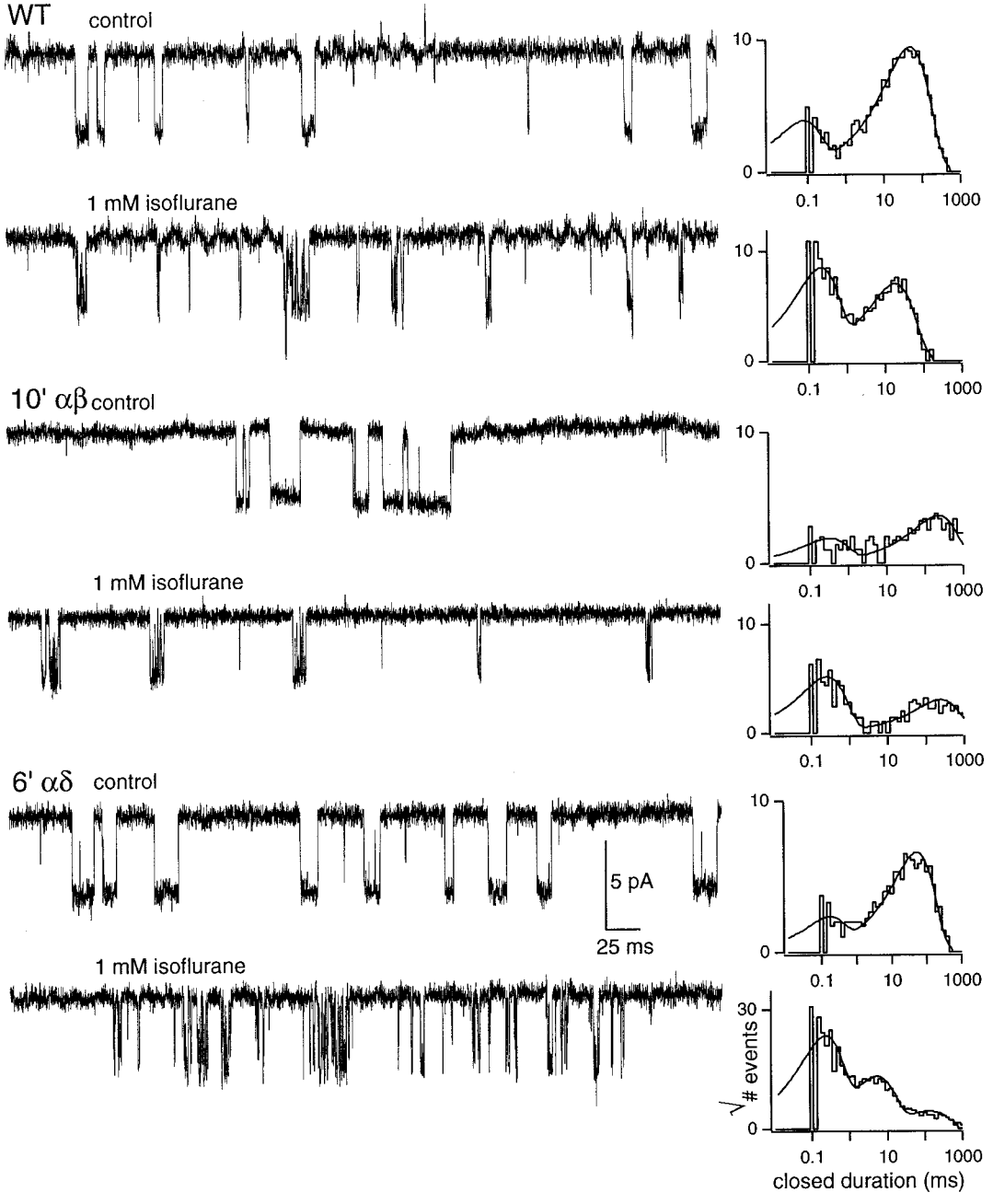


Fig. 7. Single-channel records (500-ms each) obtained from WT, 6′$\alpha\delta$, and 10′$\alpha\beta$ AChR in the absence and presence of 1 mM isoflurane. Applied potential = −100 mV, 0.2 μM ACh. Closed time distributions compiled from 30 to 60-s recording epochs are shown to the right of each record. The distributions are fit to two-exponential probability distribution functions, except for the distribution for 6′$\alpha\delta$ AChR in the presence of isoflurane, which required a three-exponential distribution function.

In addition, for both mutant receptors, the EC_{50} value for activation by ACh occurred at lower concentrations than for WT receptors (Fig. 3; Table 2). Macroscopic current measurements made with the partial agonist decamethonium indicate that the 10′$\alpha\beta$ mutation affects the isomerization equilibrium of the receptor (β/α), whereas the 6′$\alpha\delta$ mutation does not affect β/α to a significant degree. The shift in apparent sensitivity to ACh by the 6′$\alpha\delta$ mutation is probably caused by a change in the agonist binding affinity. This would be consistent with the observed increase in the long-burst duration (Table 4) if agonist dissociation were slowed. Although many mutations within M2 cause a change in gating rather than in agonist binding, at least one, in the δ-subunit at 12′, is believed to have a significant effect on binding (Chen and Auerbach, 1998). It is more difficult to construct a consistent picture of the effects of the 10′$\alpha\beta$ mutation. A 3-fold increase in β/α would be expected to produce a 3-fold increased burst duration, and this was not observed. It is possible that an increase in burst duration is countered by an increase in the agonist dissociation rate. However, this would have to be accompanied by an increase in the agonist association rate to account for the observed EC_{50} value for ACh on 10′$\alpha\beta$ receptors. Additional experiments with partial agonists (Grosman and Auerbach, 2000) or slowly opening mutants (Chen and Auerbach, 1998) are needed to provide a complete picture of the effects of mutations at the 10′ residue.

Interactions of Isoflurane with WT and Mutant AChR. Isoflurane inhibited ACh-activated macroscopic currents in the three channel types studied. The 10′$\alpha\beta$ mutant channel had a 4.5-fold greater affinity for isoflurane than did WT receptors; the affinity of 6′$\alpha\delta$ receptors was not different from that of WT (Fig. 5; Table 5). In all cases, the isoflurane concentration dependence was steep (n_H = 1.4–1.5). When the concentration dependence was fit by a two-site cooperative inhibition model, both sites on the 10′$\alpha\beta$ receptor exhibited greater affinity for isoflurane. These results agree with our hypothesis that polar-to-nonpolar mutations at the 10′ level, but not at the 6′ level, would increase the binding affinity for a hydrophobic drug such as isoflurane. However, the kinetic and single-channel data suggest that this simple interpretation is incomplete.

Although Scheme 2 is useful for examining the inhibitory effect of isoflurane, it does not provide a complete description. The steepness of the macroscopic concentration-response curve suggests the presence of more than one binding site. Isoflurane also increases the ACh binding affinity and the rate of desensitization (Dilger et al., 1993; Raines and Zachariah, 1999, 2000). Our experimental approach helps to ensure that our measurements reveal the inhibitory effect of isoflurane primarily. Responses at saturating concentrations of ACh are unaffected by changes in apparent ACh binding affinity. Peak currents are measured to avoid effects on desensitization. This does not circumvent the problem of multiple isoflurane binding sites. In the remaining discussion, we focus on two conceptual aspects of Scheme 2: the assessment of drug binding to open and closed channels, and the interpretation of single-channel bursting behavior.

Our previous study on AChR from BC_3H1 cells concluded that isoflurane inhibited both open and closed states of the receptor with equal affinity. This was deduced from data such as those shown for the WT and 6′$\alpha\delta$ receptors in Fig. 6. The degree of inhibition achieved during equilibrium exposure to isoflurane (+/+ protocol) was nearly the same as that achieved within 1 ms of simultaneous application of agonist and isoflurane (−/+ protocol). The transient observed in the −/+ protocol current trace represents the kinetics of isoflurane inhibition. In contrast, the 10′$\alpha\beta$ mutant receptor undergoes little inhibition during simultaneous application of

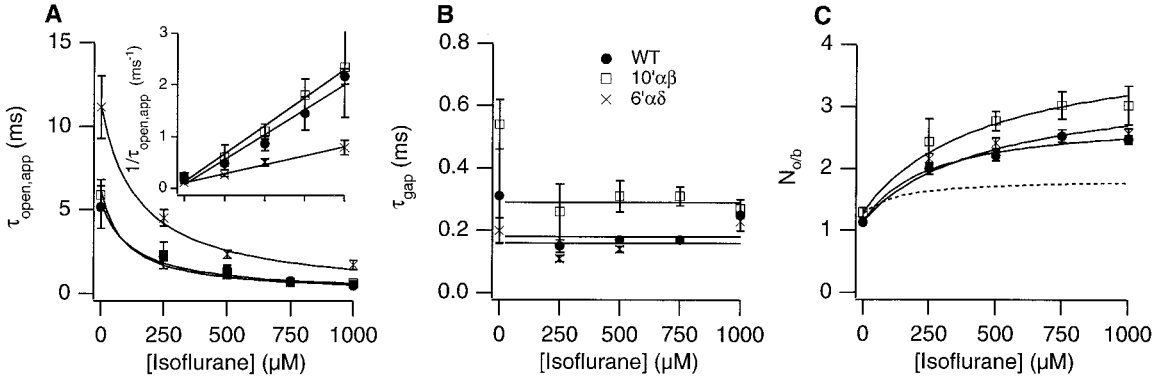


Fig. 8. Summary of the single-channel results for apparent open durations (A), gap durations (B), and openings per burst (C) for WT, 6′$\alpha\delta$, and 10′$\alpha\beta$ AChR. The inset to A shows a reciprocal plot for apparent open durations. Solid lines are the fits of the data to eqs. 3 to 5 as described in the text; the parameters are listed in Table 6. The broken line on the openings per burst versus [isoflurane] graph corresponds to the expected result if isoflurane were to bind to open 10′$\alpha\beta$ AChR channels 4-fold more tightly than to WT receptors.

TABLE 6

Parameters used in fitting the single-channel data

The best fit values for α' and b for WT receptors are about 2-fold higher than those reported for the interaction of isoflurane with single channels in BC_3H1 cells (Dilger et al., 1992). This difference may be the result of an incomplete correction for missed events in the analysis of the latter data.

	BC_3H1	WT	10′$\alpha\beta$	6′$\alpha\delta$
α_{app} (s^{-1})	310	160 ± 80	180 ± 80	90 ± 40
α' (s^{-1})	1100	2100 ± 250	900 ± 100	1900 ± 800
f (M^{-1} s^{-1})	2.1×10^6	$1.7 \pm 0.3 \times 10^6$	$1.5 \pm 0.5 \times 10^6$	$0.6 \pm 0.2 \times 10^6$
b (s^{-1})	2000	3600 ± 1000	2500 ± 700	4400 ± 2400
b/f (mM)	0.95	2.1	1.7	7.3

agonist and isoflurane (−/+ protocol; Fig. 6, top center panel) compared with equilibrium exposure. This suggests that the high affinity for isoflurane exists only in the closed state of the 10′$\alpha\beta$ receptor channel. Once the channels are opened, isoflurane binds to the 10′$\alpha\beta$ receptor just as weakly as it binds to WT receptors.

This view is supported by single-channel results. The bursting activity of channels in the presence of isoflurane reflects the effect of isoflurane on open channels. The binding and dissociation rate constants deduced from single-channel data show little difference between WT and 10′$\alpha\beta$ receptors (Table 6). The bursting behavior of 10′$\alpha\beta$ receptor channels, particularly the number of openings per burst (Fig. 8), does not correspond to the prediction that isoflurane has a high affinity for the open state of the channel. This view is also supported by the macroscopic measurements in that the K_i for the −/+ protocol with 10′$\alpha\beta$ receptors (1.6 mM) is nearly the same as the K_i for the +/+ protocol with WT receptors (1.3 mM).

The differences between channel-blocking parameters in the 6′$\alpha\delta$ receptors compared with the WT receptors are complex and not easily understood. In WT receptors, the ratio of dissociation and association rates (Table 6) is in good agreement with the equilibrium constant (Table 5) obtained from equilibrium macroscopic-current measurements (2.1 mM and 1.4 mM, respectively). In 6′$\alpha\delta$ receptors, the values are 6-fold different (7.3 mM and 1.2 mM, respectively). Presumably, the kinetic scheme does not provide a good description of the interaction of isoflurane with 6′$\alpha\delta$ receptors.

Our conclusion is that the decrease in polarity provided by the αS10′A and βT10′A mutations increases the affinity of the closed state of the channel for isoflurane but does not change the affinity of the open state for isoflurane. Thus, the action of isoflurane on WT channels is state-independent, but the action of isoflurane on 10′$\alpha\beta$ mutant receptors is state-dependent. Although the mutation affects the normal kinetic behavior of the channel, this cannot account for the change in affinity for isoflurane.

Unwin (1995) proposed that channel opening involves a rotation of all five α-helices lining the pore of the channel. In the closed state, the leucines at 9′ level of M2 (Fig. 1) form a ring that obstructs the lumen of the channel. In the open state, the helices rotate to an alternative position to create a patent lumen. If our interpretation of the results is correct, this implies that in the WT receptor, isoflurane associates equally well with the residues that extend into the lumen of the pore in the closed and open states. In the 10′$\alpha\beta$ mutation, the favorable binding environment provided by the polar-to-nonpolar substitutions presumably exists only when then channel gate is closed. In contrast, an open channel block by QX-222 is affected by the 10′$\alpha\beta$ mutations (Charnet et al., 1990). This suggests that isoflurane and the aromatic moiety of QX-222 have different modes of interaction with the 10′ region. This is consistent with the observation that another volatile anesthetic, ether, does not exclude but rather stabilizes the binding of QX-222 (Dilger and Vidal, 1994).

Comparisons with Published Data. In another study of the interaction of isoflurane and alcohols with 10′ mutant receptors (Forman et al., 1995), it was also found that the binding affinity, as judged by inhibition of macroscopic currents, was enhanced by nonpolar substitutions at 10′ on single and multiple subunits. Most of their experiments were done by use of the −/+ protocol, so this suggests that the mutations changed the affinity of open channels for the drugs. The substitutions chosen for that study were larger residues (isoleucine, valine, and phenylalanine) than the alanine substitution used here. One could argue that large residues substantially modify the structure of the pore so as to create an anesthetic binding site where one did not exist in WT receptors. This is also a caveat for our study. However, the substitution of alanine (volume in protein interior = 90 Å^3/residue; Harpaz et al., 1994) for serine (94 Å^3/residue) has little effect on volume, and the substitution of alanine for threonine (120 Å^3/residue) decreases volume by 30%. In contrast, the substitution of valine (139 Å^3/residue), isoleucine (165 Å^3/residue), or phenylalanine (193 Å^3/residue) for the two α-subunit serines increases the volume of the 10′ region by 96, 150, and 210%, respectively. Forman et al. (1995) examined neither the state dependence nor the single-channel kinetics of the interaction between isoflurane and the mutant receptors.

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Appendix

Derivation of Burst Equations for Open/Closed Channel Block

Based on Colquhoun and Hawkes (1982); the equation numbers refer to those in this article. The model is,

$$\begin{array}{ccc} & \beta & \\ C & \rightleftarrows & O \\ & \alpha & \end{array} \qquad b \Updownarrow f[\mathrm{B}]$$

$$\begin{array}{ccc} & \beta' & \\ CB & \rightleftarrows & OB \\ & \alpha' & \end{array}$$

We consider both the C and CB states to be long-lived (low agonist concentration) so that sojourns into either state terminate the burst.

The states are grouped according to the following: A = open (O), B = blocked (OB), C = closed (C and CB). We calculated the equations for a single open state.

The Q matrix has the form (eq. 1.6)

$$\mathrm{Q} = \begin{bmatrix} \mathrm{Q}_{AA} & \mathrm{Q}_{AB} & \mathrm{Q}_{AC} \\ \mathrm{Q}_{BA} & \mathrm{Q}_{BB} & \mathrm{Q}_{BC} \\ \mathrm{Q}_{CA} & \mathrm{Q}_{CB} & \mathrm{Q}_{CC} \end{bmatrix}$$

$$= \begin{bmatrix} -(\alpha + \mathrm{f[B]}) & \mathrm{f[B]} & \alpha \\ \mathrm{b} & -(b + \alpha') & \alpha' \\ \beta & \beta' & -(\beta + \beta') \end{bmatrix}$$

We also need G matrices, which are defined as (eq. 1.25)

$$\mathrm{G}_{AB} = -\mathrm{Q}_{AA}^{-1}\,\mathrm{Q}_{AB}$$

Specifically,

$$G_{AB} = \frac{f[B]}{\alpha + f[B]'}, \quad G_{BA} = \frac{b}{b + \alpha'}, \quad G_{BC} = \frac{\alpha'}{b + \alpha'}$$

The number of openings per burst (eq. 3.12)

$$E(r) = \frac{1}{1 - G_{AB}\,G_{BA}} = \frac{1}{1 - \frac{f[B]}{\alpha + f[B]}\left(\frac{b}{b + \alpha'}\right)}$$

$$= \frac{\alpha + f[B]}{\alpha + f[B]\frac{\alpha'}{b + \alpha'}}$$

The burst duration (eq. 3.20) (u_C is the unity matrix = 1)

$$m = \frac{1 - Q_{AB}\,Q_{BB}^{-1}\,G_{BA}}{Q_{AB}\,G_{BC} + Q_{AB}}\,u_C = \frac{1 + f[B]\frac{1}{b + \alpha'}\frac{b}{b + \alpha'}}{f[B]\frac{\alpha'}{b + \alpha'} + \alpha}$$

$$= \frac{1 + f[B]\frac{b}{(b + \alpha')^2}}{\alpha + f[B]\frac{\alpha'}{b + \alpha'}}$$

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