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Inhibition of Wild-Type and Mutant Neuronal Nicotinic Acetylcholine Receptors by Local Anesthetics

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ABSTRACT

Inhibition of neuronal nicotinic receptors can be regulated by the presence of specific amino acids in the β subunit second transmembrane domain (TM2) domain. We show that the incorporation of a mutant β4 subunit, which contains sequence from the muscle β subunit at the TM2 6′ and 10′ positions of the neuronal β4 subunit, greatly reduces the sensitivity of receptors to the local anesthetic [2-(triethylamino)-*N*-(2,6-dimethylphenyl)acetamide] (QX-314). Although differing in potency, the inhibition of both wild-type α3β4 receptors and α3β4(6′F10′T) receptors by QX-314 is voltage-dependent and noncompetitive. Interestingly, the potency of the local anesthetic tetracaine for the inhibition of α3β4 and α3β4(6′F10′T) receptors seems unchanged when measured at −50 mV. However, whereas the onset of inhibition of wild-type α3β4 receptors is voltage-dependent and noncompetitive, the onset of inhibition of α3β4(6′F10′T) receptors by tetracaine is unaffected by membrane voltage, and at concentrations $\leq$30 μM seems to be competitive with acetylcholine. This may be due to either direct effects of tetracaine at the acetylcholine binding site or preferential block of closed rather than open channels in the mutant receptors. Further analysis of receptors containing the 6′ mutation alone suggests that although the 6′ mutation is adequate to alter the voltage dependence of tetracaine inhibition, both point mutations are required to produce the apparent competitive effects.

The members of the neuronal nicotinic gene family have unique patterns of expression in the nervous system and are related to the nicotinic receptor genes that code for subunits of the muscle-type acetylcholine receptor. In total, 12 different genes have been cloned so far that demonstrably code for neuronal nicotinic receptor subunit proteins, based on their ability to form functional receptors when expressed in *Xenopus laevis* oocytes. Functional combinations of mammalian neuronal-type receptor subunits identified so far contain an α2, α3, α4, α7, α8, α9, or α10 subunit, and those containing α2, α3, or α4 must also contain a β subunit (β2 or β4). Although functional neuronal-type receptors require only one or two different types of subunits, they are believed to have a pentameric configuration similar to that of muscle-type receptors (Cooper et al., 1991). It has been proposed that all of the nicotinic subunits have a similar topology, with the relatively hydrophilic amino-terminal half constituting a major extracellular domain of the protein, followed by three hydrophobic transmembrane domains, a large intracellular loop, and then a fourth hydrophobic transmembrane span. The binding sites for agonist activation of the receptors are in the extracellular domain, and it is generally accepted that channel gating and ion conduction are associated with the second transmembrane domain (TM2).

As the focus of gating-associated conformational change, sequence in TM2 of the nAChR is critical not only for the direct binding of channel blocking agents but also for determining the accessibility/affinity of binding sites in other parts of the receptor that may be linked to TM2 by the conformational dynamics of gating and/or desensitization (Francis et al., 1998). We have recently shown that for neuronal nicotinic β subunit-containing receptors, there can be a reciprocal dependence for the sensitivity to noncompetitive inhibition by mecamylamine and the desensitization or autoinhibition by agonists (Webster et al., 1999). Specifically, changes in the amino acid sequence of the β subunit TM2 domain, which diminish sensitivity to mecamylamine, increase the inhibitory or desensitizing effects of agonists. Complementary changes in the amino acid sequence of the muscle β subunit TM2 domain increase the sensitivity of that receptor to mecamylamine and other noncompetitive antagonists [e.g., BTMPS (Webster et al., 1999)], establishing this β subunit domain as a crucial factor for nicotinic receptor pharmacology.

In the present study, we extend our analysis of noncom-

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ABBREVIATIONS: TM2, second transmembrane domain; nAChR, nicotinic acetylcholine receptor; BTMPS, bis(2,2,6,6-tetramethyl-4-piperidinyl)sebacate; QX-314, 2-(triethylamino)-*N*-(2,6-dimethylphenyl)acetamide; tetracaine, *N*,*N*-dimethylaminoethyl-4-butylaminobenzoate; ACh, acetylcholine; lidocaine, 2-diethylamino-*N*-(2,6-dimethylphenyl)acetamide; QX-222, 2-(trimethylamino)-*N*-(2,6-dimethylphenyl)acetamide; AChR, acetylcholine receptor.

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petitive antagonists to two local anesthetics, QX-314 and tetracaine. Our experiments use mutant forms of the receptor that have been characterized as differing in their sensitivity to the ganglionic blocker mecamylamine. Like mecamylamine, QX-314 and tetracaine are both believed to be voltage-dependent channel blockers of nAChR. However, QX-314 and tetracaine differ from one another in their state dependence for inhibition. Specifically, whereas QX-314 most effectively inhibits channels in the open state (Neher, 1983), tetracaine binds to both the resting and open states of muscle-type receptors (Papke and Oswald, 1989). We hypothesized that substitution of sequence from the muscle β subunit at the 6′ and 10′ positions of the β4 TM2 domain would reduce the sensitivity of receptors containing the mutant subunits to the local anesthetics we examined. We found that the β4 TM2 6′ and 10′ mutations change the potency of QX-314 but have no apparent effect on the mechanism of inhibition. Interestingly, these same mutations had no significant effect on the potency of tetracaine inhibition at our standard holding potential. However, mutant receptors containing the 6′ and 10′ substitutions differ from wild-type receptors in the voltage dependence of tetracaine inhibition and, furthermore, show competition effects between ACh and tetracaine.

Materials and Methods

cDNA Clones. For our experiments, we used the rat cDNA clones for the neuronal receptors (Heinemann et al., 1986). The sequences of the TM2 domains of the relevant subunits are shown below. Adopting the terminology proposed by Miller (1989), the 20 residues in the proposed second transmembrane sequence are identified as 1′ through 20′ (Table 1)

Construction of Site-Directed Mutants. Site-directed mutagenesis was conducted with QuikChange kits (Stratagene, La Jolla, CA). In brief, two complementary oligonucleotides were synthesized that contain the desired mutation flanked by 10 to 15 bases of unmodified nucleotide sequence. Using a thermal cycler, *Pfu* DNA polymerase extended the sequence around the whole vector, generating a plasmid with staggered nicks. Each cycle built only off the parent strands; therefore, there was no amplification of misincorporations. After 12 to 16 cycles, the product was treated with *Dpn*I, which digested the methylated parent DNA into numerous small pieces. The product was then transformed into *Escherichia coli* cells, which repaired the nicks. Mutations were confirmed by DNA sequencing.

Preparation of RNA. After linearization and purification of cloned cDNAs, RNA transcripts were prepared in vitro using the appropriate mMessage mMachine kit from Ambion Inc. (Austin, TX).

Expression in *X. laevis* Oocytes. Mature (>9 cm) female *X. laevis* African toads (Nasco, Ft. Atkinson, WI) were used as a source of oocytes. Before surgery, frogs were anesthetized by placing the animal in a 1.5 g/l solution of 3-aminobenzoic acid ethyl ester (MS222) for 30 min. Oocytes were removed through an incision made in the abdomen.

To remove the follicular cell layer, harvested oocytes were treated with 1.25 mg/ml collagenase from Worthington Biochemicals (Freehold, NJ) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 10 mM HEPES, pH 7.6, 0.33 mM $MgSO_4$, and 0.1 mg/ml gentamicin sulfate). Subsequently, stage 5 oocytes were each isolated and injected with 50 nl (5–20 ng) of a mixture of the appropriate subunit cRNAs. Recordings were made 1 to 7 days after injection, depending on the cRNAs being tested.

TABLE 1
TM2 sequences

	Intracellular	Membrane Spanning II	Extracellular
α3		VTLCISVLLSLTVFLLVITETIPST	
β4		MTLCISVLLALTFFLLLISKIVPPT	
β1		MGLSIFALLTLTVFLLLLADKVPET	
		1′ 6′ 10′ 20′	

Chemicals. QX-314, tetracaine, and all other chemicals for electrophysiology were obtained from Sigma Chemical Co. (St. Louis MO). Fresh acetylcholine stock solutions were made daily in Ringer's solution and diluted.

Electrophysiology. Oocyte recordings were made with a OC-725C oocyte amplifier (Warner Instruments, Hamden, CT) interfaced to either a Macintosh or Gateway personal computer. Data were acquired using LabVIEW software (National Instruments, Austin, TX) or pClamp8 (Axon Instruments, Union City, CA) and filtered at a rate of 6 Hz. Oocytes were placed in a Warner RC-8 recording chamber with a total volume of about 0.6 ml and perfused at room temperature with frog Ringer's solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, pH 7.3, and 1.8 mM $CaCl_2$) containing 1 μM atropine to inhibit potential muscarinic responses. A Mariotte flask filled with Ringer's solution was used to maintain a constant hydrostatic pressure for drug deliveries and washes. Drugs were diluted in perfusion solution and loaded into a 2-ml loop at the terminus of the perfusion line. A bypass of the drug-loading loop allowed bath solution to flow continuously while the drug loop was loaded, and then drug application was synchronized with data acquisition by using a two-way electronic valve. The rate of bath solution exchange and all drug applications was 6 ml/min. Current electrodes were filled with a solution containing 250 mM CsCl, 250 mM CsF, and 100 mM EGTA and had resistances of 0.5 to 2 MΩ. Voltage electrodes were filled with 3 M KCl and had resistances of 1 to 3 MΩ.

Experimental Protocols and Data Analysis. Current responses to drug application were studied under two-electrode voltage clamp at a holding potential of −50 mV unless otherwise noted. Holding currents immediately before agonist application were subtracted from measurements of the peak response to agonist. All drug applications were separated by a wash period of 5 min unless otherwise noted. At the start of recording, all oocytes received two initial control applications of 100 μM ACh. Subsequent drug applications were normalized to the second ACh application to control for the level of channel expression in each oocyte. The second application of control ACh was used to minimize the effect of rundown that occasionally occurred after the initial ACh-evoked response. To measure residual inhibitory effects, the experimental coapplications of ACh and inhibitor were followed, after a 5-min washout, by another application of ACh alone. This subsequent control response was compared with the preapplication control ACh response. Means and S.E. were calculated from the normalized responses of at least four oocytes for each experimental concentration.

For concentration-response relations, data were plotted using Kaleidagraph 3.0.2 (Abelbeck Software, Reading, PA), and curves were generated from the Hill equation: Response = $(I_{max}\ [\text{agonist}]^{n_H})$ / $([\text{agonist}]^{n_H} + (EC_{50})^{n_H})$, where I_{max} denotes the maximal response for a particular agonist/subunit combination, and n_H represents the Hill coefficient. I_{max}, n_H, and the EC_{50} were all unconstrained for the fitting procedures. Negative Hill slopes were applied for the calculation of IC_{50} values.

For experiments assessing voltage dependence of inhibition, oocytes were voltage-clamped at the indicated holding potential for both control applications of ACh alone and test applications of experimental agonists and/or antagonists. After a 5-min wash period, cells were given another control ACh application at the indicated potential so that residual inhibition could be evaluated.

Results

TM2 Mutations Change the Potency of the Local Anesthetic QX-314 but Not the Mechanism of Inhibition. As shown in Fig. 1, the introduction of β1 subunit sequence

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into the 6′ and 10′ positions of the β4 TM2 domain reduces the QX-314 sensitivity of receptors formed by coexpression with α3. Specifically, as shown in Fig. 2A, α3β4(6′F10′T) receptors are about 50-fold less sensitive to inhibition by QX-314 than are wild-type receptors (Table 2). When the inhibition produced by QX-314 was measured over a range of transmembrane voltages, it seemed that the inhibition of both the mutant and wild-type receptors was voltage-dependent (Fig. 2B). Likewise, when the concentration-response functions for ACh were determined in the absence and presence of fixed QX-314 concentrations (1 μM and 100 μM for wild-type and mutant receptors, respectively) for both receptors, QX-314 inhibition seemed to be noncompetitive (Fig. 2, C and D). However, whereas QX-314 coapplication reduced the ACh maximum responses of both the wild-type and mutant receptors, there was no significant effect on the ACh EC_{50} of the wild-type receptors, but there was a shift in apparent ACh potency with the mutant receptors (Table 3).

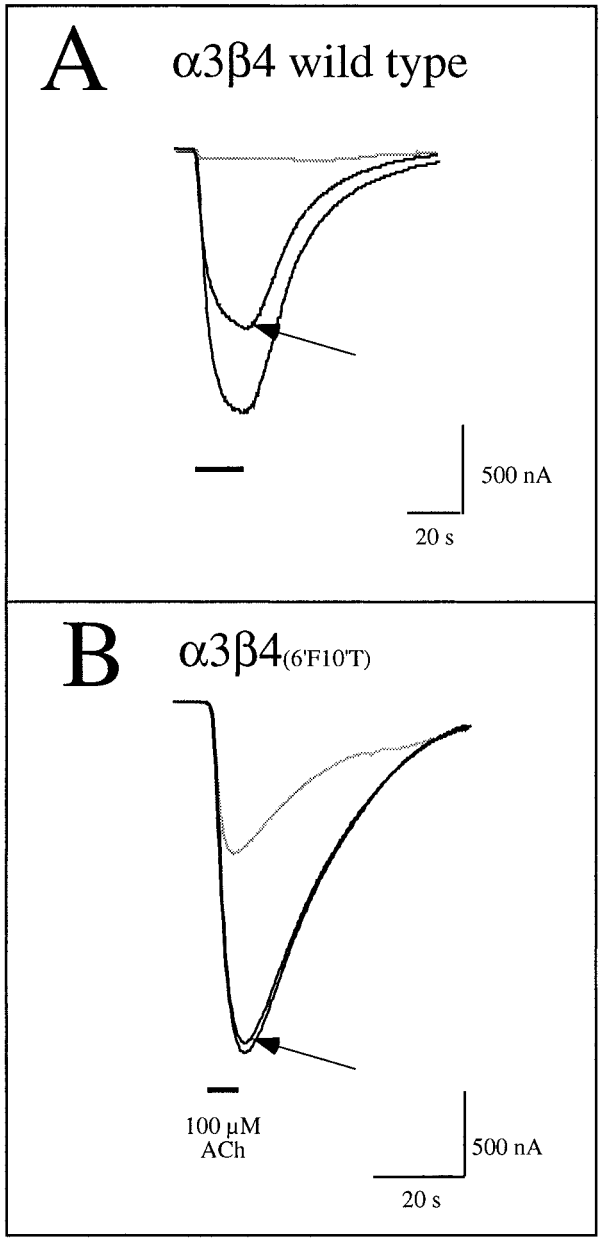

Fig. 1. α3β4(6′F10′T) mutant receptors are less sensitive to the local anesthetic QX-314 than wild-type α3β4 receptors. A, representative traces recorded from oocytes expressing wild-type α3β4 receptors showing the initial control application of 100 μM ACh (dark line), then a response to the coapplication of 100 μM ACh and 100 μM QX-314 (gray line). A final control application of 100 μM ACh response is also shown (arrow), indicating the degree of recovery. Each of these three responses was separated by 5-min washes. B, representative traces recorded from cells expressing the double mutant α3β4(6′F10′T) receptors using the same experimental protocol, indicating that these receptors are less sensitive to blockade by QX-314. The drug application times are indicated by the bars.

TM2 Mutations Do Not Change the Potency of the Local Anesthetic Tetracaine but Do Change the Apparent Mechanism of Inhibition. As shown in Fig. 3, the introduction of the β1 sequence into the 6′ and 10′ positions of the β4 TM2 domain had relatively little effect on the responses of α3-containing receptors to the coapplication of tetracaine and ACh. However, after the application of tetracaine, there was a significant decrease in the control ACh responses of the β4(6′F10′T)-containing receptors. Interestingly, when a concentration-response analysis of tetracaine inhibition was conducted at our standard holding potential of −50 mV, there seemed to be no significant effect of the β4 (6′F10′T) mutations (Fig. 4A). However, when the effect of tetracaine was examined across a range of voltages, it was clear that although the peak responses of wild-type α3β4 receptors showed a significant voltage dependence, the inhibition of α3β4(6′F10′T) receptors during the coapplication of tetracaine and ACh was unaffected by voltage (Fig. 4B). Although there was no significant difference in the inhibition of wild-type and mutant receptors by 10 μM tetracaine when the cells were held at −50 mV, wild-type receptors were far more sensitive ($p < 0.01$) to 10 μM tetracaine than were the mutants when the cell was held at −100 mV. We confirmed this difference in voltage dependence by conducting another concentration-response analysis with a holding potential of −100 mV. As shown in Fig. 5, compared with the data obtained at −50 mV, the concentration-response function measured at the hyperpolarized potential shifted to the left for the wild-type receptors (see Table 2) but not for the α3β4(6′F10′T) receptors.

We further investigated this apparent qualitative difference in tetracaine-induced inhibition by conducting ACh concentration-response analyses in the absence and presence of a fixed (10 μM) concentration of tetracaine. As shown in Fig. 4C, the effect of tetracaine on wild-type receptors fit the predictions for noncompetitive inhibition. Surprisingly, however, the effect of tetracaine on the ACh responses of α3β4(6′F10′T) receptors seems more consistent with a competitive mechanism of inhibition (Fig. 4D). By increasing ACh concentration, the relative amount of inhibition by 10 μM tetracaine was decreased. Although this would be consistent with an inhibitory effect of tetracaine mediated by competition with ACh at the activation site, this interpretation is complicated by the fact that ACh itself may be binding to multiple sites, including sites that intrinsically limit functional response (Webster et al., 1999).

To determine whether the TM2 mutations change the relative affinity of tetracaine for the open and closed states of the receptor, we conducted preapplications of 30 μM tetracaine immediately before the application of 100 μM ACh. We accomplished this by inserting into our perfusion system a second drug application loop that could be filled indepen-

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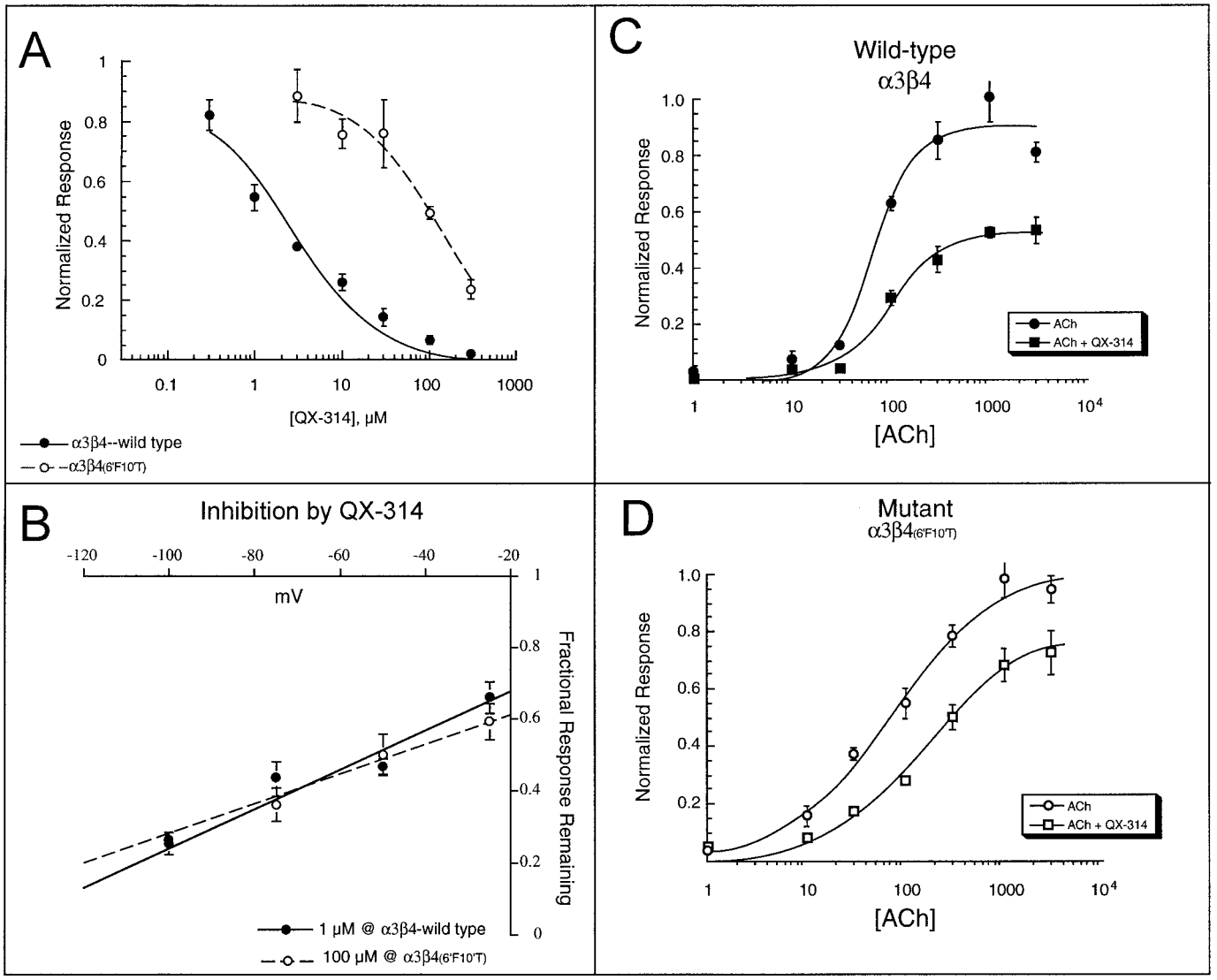

Fig. 2. The effects of QX-314 on $\alpha3\beta4$ and $\alpha3\beta4(6'F10'T)$ receptors. A, concentration-response curves for the effect of QX-314 on the peak currents of $\alpha3\beta2$ and $\alpha3\beta4(6'F10'T)$ receptors when 100 μM ACh was coapplied with QX-314 at the indicated concentrations. Data from each oocyte were normalized to that cell's response to 100 μM ACh alone. B, the voltage dependence of QX-314 inhibition was evaluated by coapplying QX-314 and 100 μM ACh over a range of different voltages and comparing the response obtained with the ACh control response obtained at the same potential. Because of difference in potency for the QX-314 inhibition of wild-type and mutant receptors, 1 μM QX-314 was used with wild-type $\alpha3\beta4$ and 100 μM QX314 was used with $\alpha3\beta4(6'F10'T)$ receptors. C, ACh concentration-response curves for $\alpha3\beta4$ receptors, determined with ACh alone or ACh coapplied with 1 μM QX-314. Data were initially normalized to the 100 μM ACh responses obtained in the same cells and then scaled by the ratio of 100 μM ACh control responses to the maximal ACh responses, obtained with 1 mM ACh. D, ACh concentration-response curves for $\alpha3\beta4(6'F10'T)$ receptors, determined with ACh alone or ACh coapplied with 100 μM QX-314. Data were initially normalized to the 100 μM ACh responses obtained in the same cells and then scaled by the ratio of 100 μM ACh control responses to the maximal ACh responses, obtained with 1 mM ACh. Each point represents the average normalized response of at least four cells.

dently from the ACh application loop. With this method, some mixing does occur between the tetracaine and ACh solutions in the bath; however, most of the tetracaine application is delivered to receptors before ACh-evoked activation. The inhibition of wild-type receptors produced by the preapplication of 30 μM tetracaine was significantly less ($p < 0.05$)

TABLE 2
IC_{50} values

Drug	Receptor	Holding Potential	IC_{50}	Figure
		mV	*μM*	
QX-314	$\alpha3\beta4$	−50	2.9 ± 1.0	2A
QX-314	$\alpha3\beta4_{(6'F10'T)}$	−50	125 ± 25	2A
Tetracaine	$\alpha3\beta4$	−50	9.0 ± 1.6	4A & 5A
Tetracaine	$\alpha3\beta4$	−100	4.4 ± 0.8	5A
Tetracaine	$\alpha3\beta4_{(6'F10'T)}$	−50	23 ± 6.2	4A & 5B
Tetracaine	$\alpha3\beta4_{(6'F10'T)}$	−100	11.4 ± 7.1	5B

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TABLE 3
Curve fits for Hill equations

Receptor	Antagonist	Max Response	n	EC_{50}	Figure
				μM	
$\alpha3\beta4$	None	1[a]	2.1 ± 0.6	68 ± 12	2C & 4C
	10 μM QX-314	0.59 ± 0.02	1.6 ± 0.3	98 ± 12	2
	10 μM tetracaine	0.59 ± 0.03	1.1 ± 0.2	82 ± 15	4
$\alpha3\beta4_{(6'F10'T)}$	None	1[a]	0.8 ± 0.1	72 ± 17	2D, 4D, & 5C
	10 μM QX-314	0.79 ± 0.08	0.8 ± 0.1	183 ± 56	2D
	10 μM tetracaine	1.0 ± 0.2	0.9 ± 0.2	240 ± 62	4D
	100 μM tetracaine	0.64 ± 0.05	0.9 ± 0.14	500 ± 136	5C

[a] By definition.

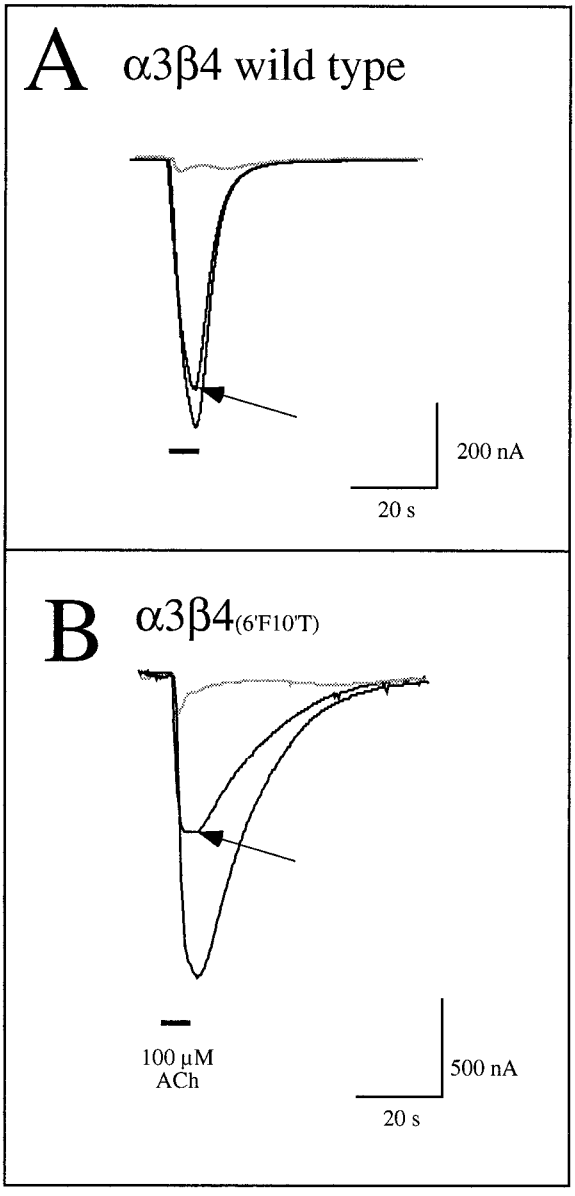

Fig. 3. At the standard holding potential of −50 mV, $\alpha3\beta4(6'F10'T)$ mutant receptors seem at least as sensitive to inhibition by tetracaine as wild-type $\alpha3\beta4$ receptors. A, representative traces recorded from oocytes expressing wild-type $\alpha3\beta4$ receptors showing the initial control application of 100 μM ACh (dark line), then a response to the coapplication of 100 μM ACh and 100 μM tetracaine (gray line). A final control application of 100 μM ACh response is also shown (arrow) indicating the degree of recovery. Each of these three responses was separated by 5-min washes. B, representative traces recorded from cells expressing the double mutant $\alpha3\beta4(6'F10'T)$ receptors using the same experimental protocol, indicating that these receptors are equally sensitive to blockade by tetracaine.

than that produced when tetracaine was coapplied with the ACh. The responses were 45 ± 7% of the ACh controls with preapplication, compared with 20 ± 5% with coapplication (for the coapplication data, see Fig. 4A). In contrast, the mutant receptors showed nearly identical levels of peak current inhibition under these two conditions; responses were reduced to 30 ± 2% of the ACh controls with preapplication, compared with 36 ± 7% with coapplication. These results suggest that for the mutant receptors, inhibition by tetracaine may occur equally well with open and closed receptors.

To determine whether the apparent competitive effects of tetracaine on the mutant receptors were consistent over a wide range of tetracaine concentrations or whether, as previously suggested for muscle-type receptors (Papke and Oswald, 1989), multiple forms of inhibition by tetracaine might exist, additional competition experiments were conducted using 100 μM tetracaine in coapplication with ACh at varying concentrations. As shown in Fig. 5C, this increased concentration of tetracaine both decreased the maximum response and increased the EC_{50} of the ACh concentration-response relationship (Table 3). We evaluated the recovery of $\alpha3\beta4(6'F10'T)$ receptor ACh control responses 5 min after the application of ACh alone or coapplied with either 10 or 100 μM tetracaine (Fig. 5D). Whereas coapplication of 10 μM tetracaine had no significant effect on the recovery of ACh control responses, when 100 μM tetracaine was coapplied with high concentrations of ACh (300 μM–3 mM), there was a greater ($p < 0.01$) depression of subsequent ACh responses. Therefore, whereas the preapplication experiment suggests that lower concentrations of tetracaine may inhibit both open and closed $\alpha3\beta4(6'F10'T)$ receptors, at this high concentration, tetracaine may have additional long-term effects based on a preferential interaction with open channels.

Analysis of Single and Double Mutations: QX-314. We have previously shown that mutations at the 6′ and 10′ positions $\beta1$ or $\beta4$ have additive effects for the reversal of mecamylamine sensitivity and the enhancement of agonist-induced inhibition (Webster et al., 1999). Therefore, we sought to determine whether both mutations contributed to the decrease in QX-314 sensitivity in $\alpha3\beta4(6'F10'T)$ receptors. As shown in Fig. 6A, the 10′ mutation has a sensitivity to QX-314 that is intermediate to the wild-type and $\beta4(6'F10'T)$-containing receptors. When the inhibition measured during the coapplication of QX-314 and ACh is considered, the decrease in sensitivity of the 6′ mutants seems to be nearly the same as that of the 6′/10′ double mutant. However, the 6′ single mutants are, in fact, more sensitive to QX-314 than the 6′/10′ double mutants, as can be seen in an increase in the residual inhibition persisting after a 5-min wash (Fig. 6A, inset). QX-314 inhibition is readily reversible

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in the wild-type receptors (Francis et al., 1998); thus, the appearance of prolonged inhibition in the 6′ mutants was unexpected. It would seem that the 6′ mutation may particularly affect the kinetics of QX-314, making the onset of inhibition slower (and therefore showing less inhibition during the coapplication response, compared with the wild type) but also slower to dissociate. Therefore, it would seem that both the 6′ and 10′ mutations are essential to determine the QX-314 sensitivity.

Analysis of Single and Double Mutations: Tetracaine. In an analysis of inhibition during coapplication of ACh and tetracaine, it seemed that only the 6′ mutation was required to remove the voltage sensitivity of inhibition by tetracaine during the coapplication response (Fig. 6B). However, when residual inhibition of $\alpha3\beta4$(6′F) receptors was measured after a 5-min wash, there was a greater residual effect on cells held at −100 mV than on cells held at −50 mV ($p < 0.05$). Cells held at −50 mV recovered to 88 ± 8% of the pretreatment control and cells held at −100 mV recovered to only 60 ± 6% (Fig. 6B). This was similar to the results obtained with the double mutant (Fig. 6B).

In an attempt to determine whether the 6′ mutation is also sufficient to produce the apparent change in mechanism observed during inhibition of the double mutants by tetracaine (see Fig. 4, C and D), we conducted a competition experiment with a single concentration of ACh and tetracaine in cells expressing $\alpha3$ and the $\beta4$(6′F) mutant. Specifically, 1 mM ACh was applied with or without 10 μM tetracaine, to determine whether this relatively high concentration of ACh would be able to surmount the tetracaine inhibition of the coapplication response, as is the case with the double mutant (Fig. 4D). We found that when 10 μM was coapplied with 1

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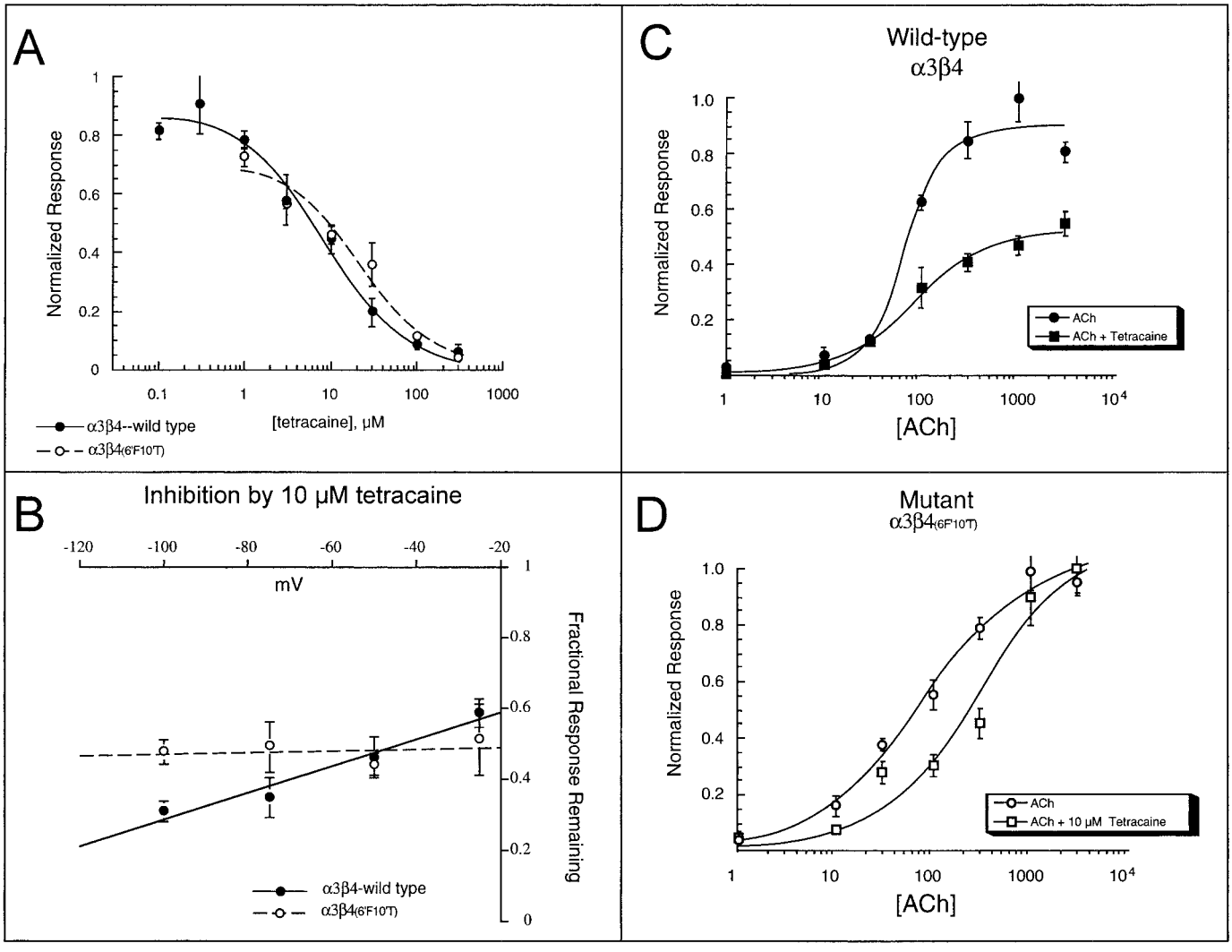

Fig. 4. The effects of tetracaine on $\alpha3\beta4$ and $\alpha3\beta4$(6′F10′T) receptors. A, concentration-response curves for the effect of tetracaine on the peak currents of $\alpha3\beta4$ and $\alpha3\beta4$(6′F10′T) receptors when 100 μM ACh was coapplied with tetracaine at the indicated concentrations. Data from each oocyte were normalized to that cell's response to 100 μM ACh alone. B, the voltage dependence of tetracaine inhibition was evaluated by coapplying 10 μM tetracaine and 100 μM ACh over a range of different voltages and comparing the response obtained to the ACh control response obtained at the same potential. C, ACh concentration-response curves for $\alpha3\beta4$ receptors, determined with ACh alone or ACh coapplied with 10 μM tetracaine. Data were initially normalized to the 100 μM ACh responses obtained in the same cells and then scaled by the ratio of 100 μM ACh control responses to the maximal ACh responses, obtained with 1 mM ACh. D, ACh concentration-response curves for $\alpha3\beta4$(6′F10′T) receptors, determined with ACh alone or ACh coapplied with 10 μM tetracaine. Data were initially normalized to the 100 μM ACh responses obtained in the same cells and then scaled by the ratio of 100 μM ACh control responses to the maximal ACh responses, obtained with 1 mM ACh. Each point represents the average normalized response of at least four cells.

mM ACh to $\alpha3\beta4(6'F)$ receptors, the normalized responses were only 51 ± 6% (n = 4) of the size of the normalized responses to 1 mM ACh alone. This result was similar to the results obtained with the coapplication responses of $\alpha3\beta4$ wild-type receptors (Fig. 4C). Thus, although it seems that the 6′ mutation is sufficient to alter the voltage dependence of the onset of tetracaine inhibition, the presence of this mutation alone is not adequate to produce the apparent change in mechanism of tetracaine inhibition that we observed.

Discussion

We chose to investigate the effects of channel mutations on the inhibition of neuronal-type nAChR by tetracaine and QX-314 because previous studies indicated that these local anesthetics differed in their mechanisms for the inhibition of muscle-type nAChR. Lidocaine and the related compounds QX-314 (lidocaine n-ethyl bromide) and QX-222 have been shown to preferentially block muscle-type nAChR from the extracellular side (Horn et al., 1980), producing inhibition based on an affinity for a site within the open ion channel. Tetracaine, in contrast, has been shown to block muscle-type receptors in both open and closed states (Papke and Oswald, 1989). In our experiments, we sought first to determine whether these compounds also differed in their mechanisms for the inhibition of wild-type $\alpha3\beta4$ receptors, as they do for muscle-type receptors. We then sought to determine whether mutations of the neuronal β subunit TM2 domain to the sequence of the muscle β subunit would make the inhibitory effects of these compounds on neuronal receptors more like their effects on muscle-type receptors.

It has been shown previously that both the 6′ and 10′ residues of the mouse muscle receptor subunits can influence receptor inhibition by lidocaine and the related quaternary compounds QX-314 and QX-222 (Charnet et al., 1990; Pascual and Karlin, 1998). Charnet et al. (1990) proposed that the 6′ residues provide a polar site within the channel that interacts with the charged amine of these local anesthetics,

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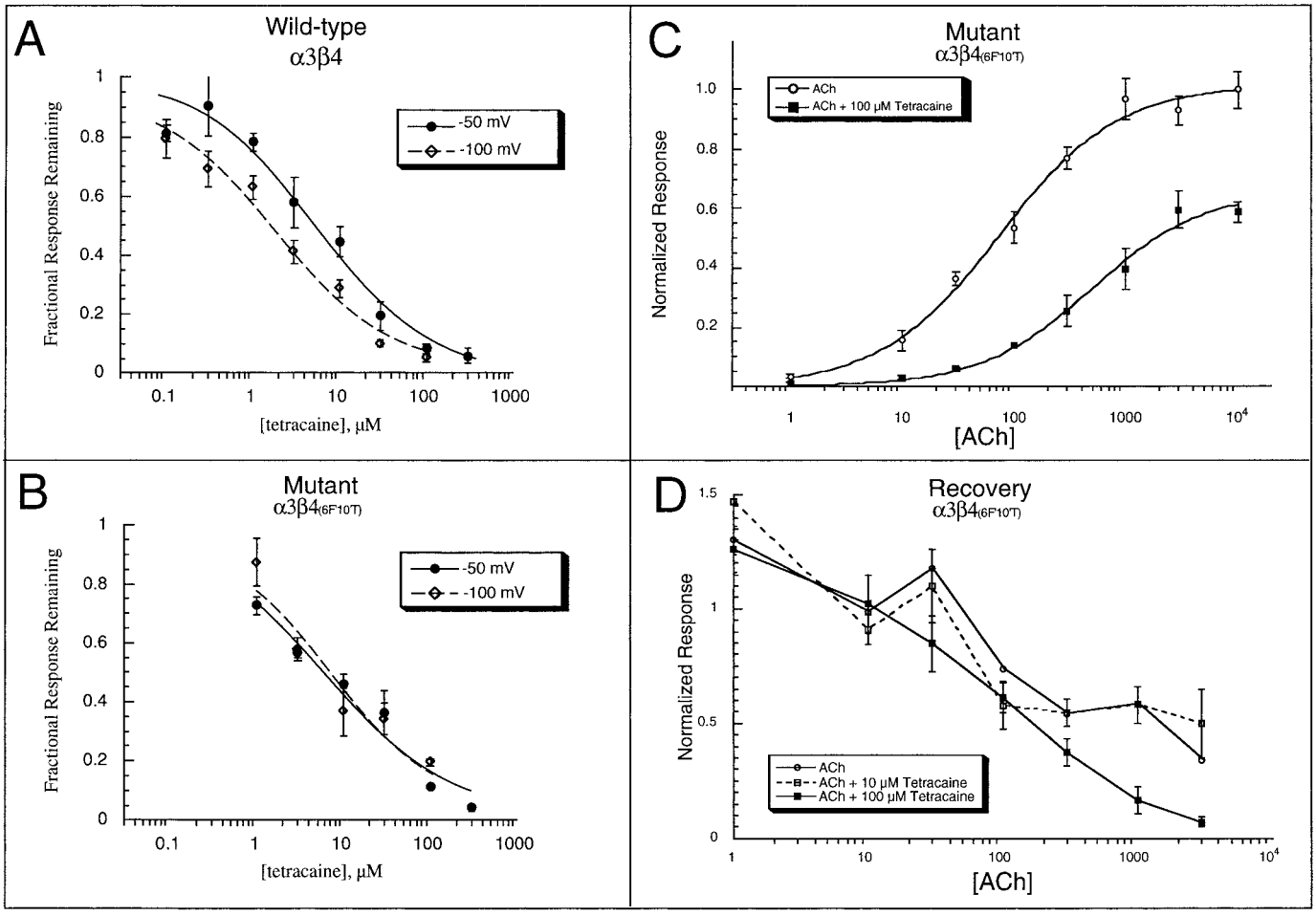

Fig. 5. A and B, the presence of the $\beta4(6'F10'T)$ mutant subunit abolishes the voltage dependence of the onset of tetracaine inhibition. A, concentration-response curves showing the effect of holding potential on the peak currents of cells expressing the wild-type $\alpha3\beta4$ receptor during coapplication of the indicated concentration of tetracaine and 100 μM ACh. B, concentration-response curves for tetracaine and ACh coapplication in cells expressing $\alpha3\beta4$ receptors with the TM2 6′F10′T mutations. The effect of membrane voltage on the degree of inhibition of the coapplication responses can been seen to have been eliminated in the mutant subunit-containing receptors. C, ACh concentration- response curves for $\alpha3\beta4(6'F10'T)$ receptors, determined with ACh alone or ACh coapplied with 100 μM tetracaine. Data were initially normalized to the 100 μM ACh responses obtained in the same cells and then scaled by the ratio of 100 μM ACh control responses to the maximal ACh responses, obtained with 1 mM ACh. The data for responses to ACh alone are the same as those presented in Fig. 4. D, the recovery of the ACh control responses of $\alpha3\beta4(6'F10'T)$ receptors, after the application of ACh alone or ACh coapplied with either 10 μM or 100 μM tetracaine. Each point represents the average normalized response of at least four cells.

whereas the 10′ residues contribute to a hydrophobic binding site. However, in the profile of the muscle receptor, the residues of the β subunit do not fit the model for the ideal QX-314 binding site. The muscle β subunit has a hydrophobic phenylalanine at the 6′ site and a relatively polar threonine at the 10′ site. These residues would not be expected to contribute to the proposed polar and hydrophobic binding sites, and it was shown that appropriate polar and hydrophobic mutations at the 6′ and 10′ sites of the muscle β1 subunit, respectively, had the effect of increasing inhibition by QX-222 (Charnet et al., 1990). In the present experiments, the 6′F and 10′T mutations would both serve to disrupt the QX-314 binding site predicted by the Charnet two-site model, placing hydrophobic residues at the proposed 6′ polar site and more polar residues at the proposed hydrophobic site. Our results are therefore consistent with this two-site model of the QX-314 binding site, with the net effect on the potency of QX-314 being particularly strong, because in the neuronal receptor complexes, each mutation is presumably present in at least two subunits of the pentamer.

Although the effects of the (6′F10′T) mutations on inhibition by QX-314 are consistent with a simple decrease in the affinity of QX-314 for an open-channel-associated site, the effects of the (6′F10′T) mutations on inhibition by tetracaine are more complex. The degree of inhibition during a coapplication of ACh and tetracaine did not seem to differ in the mutant receptors compared with wild-type when measurements were made during the coapplication at the standard holding potential of −50 mV. However, the apparent potency of tetracaine was voltage-dependent in the wild-type receptors but not in the (6′F10′T) mutants. Interestingly, whereas there was no apparent voltage dependence of the inhibition measured during the coapplication of tetracaine and ACh, there was an effect of voltage on the reversibility of inhibition with the (6′F10′T) mutant receptors that was not detectable in the wild-type. Another qualitative difference in the effects of tetracaine on (6′F10′T) mutant receptors was the apparent state dependence for inhibition. In the mutant receptors, inhibition did not depend on the presence of agonist, so inhibition could be obtained if tetracaine was applied before

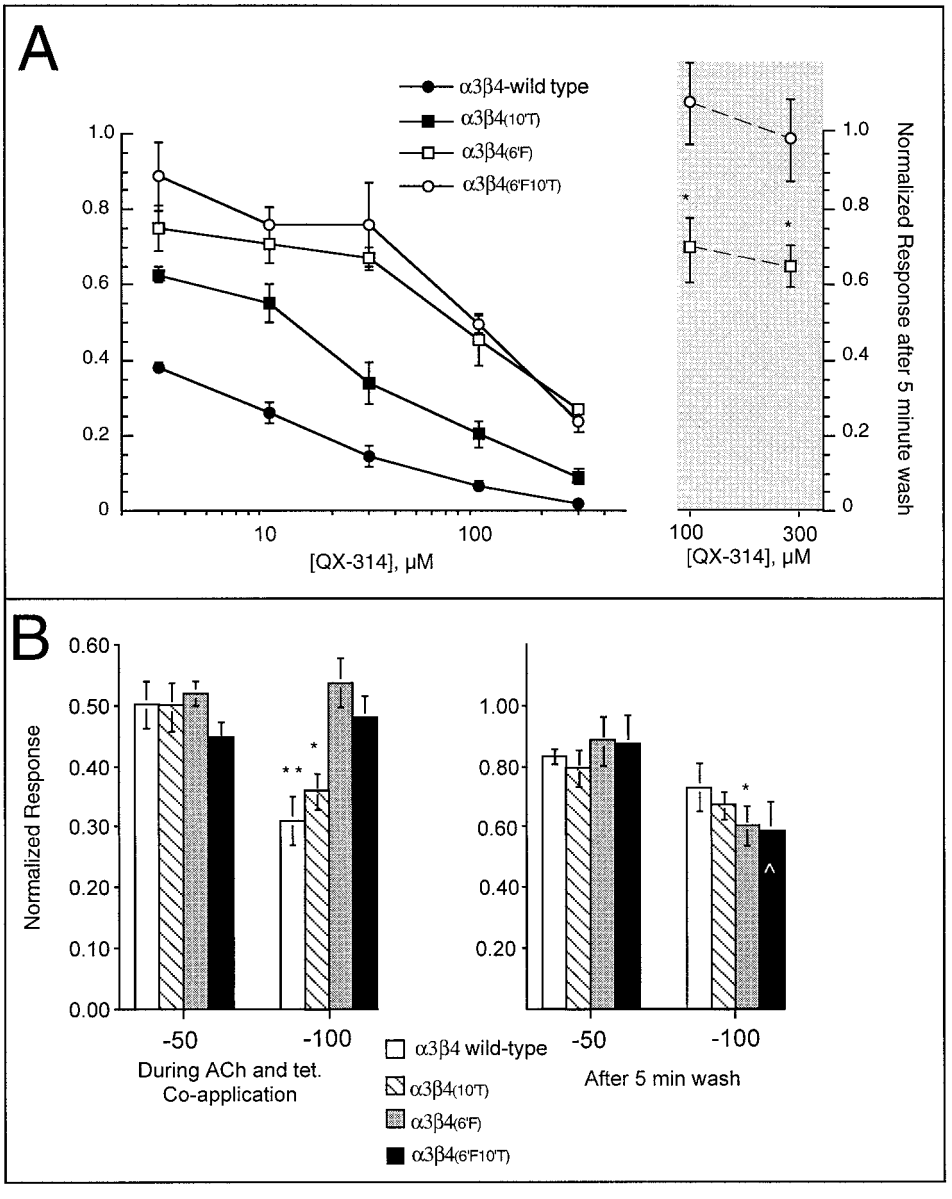

Fig. 6. Effects of point mutations compared with (6′F10′T) double mutants. A, the effects of a range of QX-314 concentrations were measured and compared with the responses of wild-type and $\alpha3\beta4$(6′F10′T) receptors (reproduced from Fig. 2). The main figure shows the effect measured during coapplication of 100 μM ACh and varying concentrations of QX-314. From these data, it would seem that the 6′ mutation was essentially as effective as the 6′10′ double mutation at reducing the potency of QX-314. However, when subsequent ACh control responses were measured after a 5-min wash (shown on right), it was noted that the 6′ mutant showed a residual inhibition that the 6′10′ mutants did not. This observation suggests that the 6′ mutation may be more effective at changing the kinetics of QX-314 inhibition than the actual potency because inhibition of wild-type receptors by QX-314 was fully reversible over the same time period (not shown). B, the effects of single point mutations on the voltage sensitivity of tetracaine-evoked inhibition. Oocytes were treated with 100 μM ACh plus 10 μM tetracaine at a holding potential of either −50 or −100 mV. After a 5-min wash at the test potential, control applications of ACh were measured and expressed relative to initial control responses obtained at the same potential. Although the data measured during the coapplication of ACh and tetracaine might suggest that the 6′ mutation alone was sufficient to eliminate the voltage-dependence of tetracaine-evoked inhibition; in fact, inhibition of the 6′ mutant was more persistent at −100 mV than at −50 mV (see text).

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ACh. Finally, inhibition by 10 μM tetracaine (a concentration close to the apparent IC_{50} values) can be surmounted by increasing agonist concentration in the mutant receptors but not the wild-type receptors. That is, tetracaine shifted the ACh EC_{50} of the mutant receptors but had no effect on the maximum response. However, when the IC_{80} concentration of tetracaine was used, there was both a larger shift in the ACh EC_{50} and a decrease in the maximum response (Table 3).

These data seem to be consistent with multiple interpretations. One hypothesis would be that tetracaine binds to different sites in the wild-type and mutant receptors. Specifically, at least some of the inhibitory effects in the mutant receptor might arise from a direct interaction between tetracaine and ACh at the activation binding site. Alternatively, the binding site for tetracaine might be preferentially exposed or have a higher affinity in the open state of the wild-type receptor, whereas in the mutant receptor tetracaine may bind equally well to both open and closed receptors or even show a preference for binding to closed receptors. If that is the case, then it is possible that preferential binding to closed receptors might appear as a competitive interaction, because increasing agonist concentrations would take the channels away from the state for which the antagonist would have the highest affinity.

Whereas tetracaine does seem to bind primarily to a noncompetitive site in wild-type muscle receptors (Middleton et al., 1999), at high concentrations, it can also compete with agonist (Ryan and Baenziger, 1999). Tetracaine has even been reported to activate mouse muscle-type receptors of BC3H-1 cells (Papke and Oswald, 1989), although tetracaine-evoked channel openings are difficult to detect because of the concomitant channel-blocking activity. Additionally, single-channel data indicated that whereas low concentrations of tetracaine produced inhibition by binding to a site that did not require the channel to be in an open state, higher concentrations of tetracaine seemed to promote increased blockade of open channels. Therefore, data from muscle-type receptors would be consistent with the existence of as many as three different, potentially inhibitory, binding sites for tetracaine. There is evidence for two channel-associated sites that differ in state dependence and might also differ in voltage dependence, based on the disposition of the sites in the membrane's electric field. In addition to producing effects at these channel-associated sites, tetracaine would also inhibit the responses of muscle-type receptors to strong agonists because of its activity as a weak partial agonist. Certainly, analogs of these sites may exist in the wild-type and mutant neuronal AChR used in this study. Mutation of the neuronal subunit to the muscle β subunit sequence may alter the relative affinity of tetracaine for these corresponding sites in the neuronal β subunit mutant.

Although it seems that in the wild-type neuronal receptors, tetracaine produces inhibition in a manner similar to that of QX-314, presumably through open-channel block, it may be that the (6′F10′T) mutations decrease the affinity of tetracaine for the open-channel site. However, because under standard recording conditions, the potency of tetracaine was relatively unaffected in the mutant receptors, it seems likely that if activity is lost at an open-channel site, the effects of tetracaine at alternative sites or for alternative activation states (i.e., closed channels) may be increased. Specifically, in the mutant receptors, tetracaine may be more able to block closed channels. If the binding site in the closed channel were not as deep in the membrane's electric field as the open-channel block site, it would account for the apparent decrease in voltage dependence. Alternatively, tetracaine may bind more effectively to the agonist binding site of mutant receptors. This would be consistent with the apparent competitive effects observed with ACh and 10 μM tetracaine. The EC_{50} for ACh was increased 3-fold in the presence of 10 μM tetracaine. The apparent EC_{50} for ACh was further increased when ACh was applied in the presence of 100 μM tetracaine, also consistent with competitive inhibition. However, the fact that inhibition by 100 μM tetracaine could not be fully surmounted by increasing ACh concentration would argue against inhibition arising solely from competitive interactions at the ACh binding site.

Despite having information about the molecular shapes and hydrophobicity of the drugs, without a better knowledge of the structure of the channel in both the open and closed states, we can only speculate how the mutations in TM2 account for our results. QX-314 is "ellipsoid" with a less polar but bulkier quaternary ammonium group than tetracaine's. Tetracaine is more "rod-like", with a methylammonium group that is smaller and more polar in this part of the molecule. However, it remains difficult to model the drugs in the channel, because we cannot be sure whether the ammonium groups of both drugs bind closest to the cytosol in the wild-type receptor, as has been suggested for the QX-314 orientation in the muscle-type receptor (Charnet et al., 1990). Both tetracaine and QX-314 seem to be binding differently in the mutant compared with the wild-type channels. In the case of QX-314, there seems to be a loss of affinity but not a change in binding site or state dependence. In the case of tetracaine, there may be relative changes in binding to multiple sites or differences in the state dependence of binding to a single site. However, we cannot know whether the point effects of the mutations or more global effects on channel structure cause the effects we observe. Because the tetracaine ammonium group is less polar than that of QX-314, it may be the case that the interaction with the phenylalanine would be stronger with QX-314. We might then speculate that QX-314 would remain more stable with the amine deep in the channel in both the wild-type and mutant but that tetracaine might preferentially change its orientation in the mutant channel, with the amine group more toward the extracellular surface. This could account for the reduced voltage-dependence we observed, if the binding site for the charged group of tetracaine is altered so that it is less deep in the channel.

In conclusion, our data indicate that QX-314 and tetracaine inhibit wild-type $\alpha3\beta4$ receptors through similar mechanisms, such that for both drugs the inhibition is voltage-dependent and noncompetitive, consistent with open-channel blockade. This is in agreement with data for QX-314 inhibition of wild-type muscle nAChR. The TM2 mutations do not seem to alter the mechanism for QX-314 inhibition but do change the inhibition produced by tetracaine. Similar to what has been reported for muscle-type AChR, tetracaine inhibits $\alpha3\beta4$(6′F) receptors in a manner consistent with the block of both open and closed channels and perhaps involving competitive as well as noncompetitive interactions. The findings of the present study are consistent with our previous

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report that β subunit TM2 sequence affects the state-dependent inhibition of muscle-type and neuronal nAChR by the voltage-independent noncompetitive inhibitor BTMPS (Francis et al., 1998). Our results therefore support the hypothesis that sequence in the β subunit TM2 may regulate channel-blocking mechanisms, not only via direct effects at channel-associated sites but also via conformational changes occurring with channel gating and extending beyond the open channel domain.

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