

Reversal of Agonist Selectivity by Mutations of Conserved Amino Acids in the Binding Site of Nicotinic Acetylcholine Receptors*

Received for publication, September 28, 2006, and in revised form, December 21, 2006 Published, JBC Papers in Press, December 21, 2006, DOI 10.1074/jbc.M609202200

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Homomeric $\alpha 7$ and heteromeric $\alpha 4\beta 2$ nicotinic acetylcholine receptors (nAChR) can be distinguished by their pharmacological properties, including agonist specificity. We introduced point mutations of conserved amino acids within the C loop, a region of the receptor critical for agonist binding, and we examined the expression of the mutant receptors in *Xenopus* oocytes. Mutation of either a conserved C loop tyrosine (188) to phenylalanine or a nearby conserved aspartate (197) to alanine resulted in $\alpha 7$ receptors for which the $\alpha 7$ -selective agonist 3-(4-hydroxy,2-methoxybenzylidene)anabaseine (4OH-GTS-21) had roughly the same potency as for wild-type receptors, whereas the physiologic agonist acetylcholine (ACh) showed drastically reduced potency for these mutant receptors. Corresponding mutations in $\alpha 4$ receptors co-expressed with $\beta 2$ resulted in $\alpha 4\beta 2$ receptors for which ACh potency was relatively unchanged, although the efficacy of the $\alpha 7$ -selective agonist 4OH-GTS-21 was increased greatly relative to that of ACh. We also investigated the significance of a conserved lysine (145 in $\alpha 7$), proposed to form a stable salt bridge with Asp-197 in the resting state of the receptor. Mutations of this residue in both $\alpha 7$ and $\alpha 4$ resulted in receptors that were largely unresponsive to both ACh and 4OH-GTS-21. Our results suggest that initiation of gating depends both on specific interactions between residues in the C loop domain and, depending on receptor subtype, the physicochemical properties of the agonist, so that in the altered environment of the $\alpha 4Y190F$ -binding site, large hydrophobic benzylidene anabaseines may close the C loop and initiate channel gating more effectively than the polar agonist ACh.

Nicotinic acetylcholine receptors are homo- or heteropentameric receptors belonging to the Cys loop superfamily of receptor channels. These receptors allow the flow of cations across the membrane in response to agonist binding. The determination of the crystal structure of a soluble acetylcholine-

binding protein (AChBP)³ from *Lymnaea stagnalis* has provided a structural template for the nicotinic ACh receptor extracellular domain (1). This crystal structure revealed that the ligand was bound at the interface between the “plus” and “minus” face of the receptor protomers. In the case of heteromeric receptors (e.g. $\alpha 4\beta 2$), there are three “loops” from the α subunit (referred to as the A, B, and C loops) that form the principal face and interact with three loops (D, E, and F) from the non- α subunit that forms the complementary face. In the case of $\alpha 7$, which forms homomers, each of the five $\alpha 7$ subunits is thought to contribute to the primary face of one binding site and the complementary face of another, so that there may be as many as five ACh-binding sites per receptor (2).

The $\alpha 7$ -type receptors have been proposed as therapeutic targets for both central nervous system pathologies such as Alzheimer and schizophrenia (3, 4) as well as for inflammation (5). Benzylidene anabaseine (BA) compounds, such as 3-(4-hydroxy,2-methoxybenzylidene)anabaseine (4OH-GTS-21), are $\alpha 7$ -selective agonists. 4OH-GTS-21 has been shown to be cytoprotective in both human and rat cells (6). An interesting property of $\alpha 7$ receptors is that they are far more permissive than other nicotinic receptor types in responding to structurally diverse agonists; large hydrophobic agonists such as benzylidene anabaseines and, paradoxically, smaller agonists such as choline are selective for $\alpha 7$ receptors (7). It is thought that a larger, more hydrophobic environment in the $\alpha 7$ ligand-binding domain, compared with other receptor subtypes, is the basis for the selectivity of these receptors for BA-type agonists. In particular, a phenylalanine residue in the C loop has been suggested to make the ligand-binding domain of $\alpha 7$ more accommodating for the binding of large hydrophobic agonists such as GTS-21 (8).

Other groups have made point mutations in the conserved amino acids in the C loop of nicotinic receptors. Although most of these studies have looked at the $\alpha 1$ subunit, sequence conservation of key residues suggests a common activation mechanism for different receptor types (9). Studies conducted before the identification of a structural template for the extracellular domain was available reached various conclusions about the importance of conserved C loop amino acids, particularly regarding mutation of a conserved tyrosine, two amino acids

* This work was supported by National Institutes of Health Grants R01 GM57481, P01 AG10485, and T32 AG00196. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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³ The abbreviations used are: AChBP, acetylcholine-binding protein; nAChR, nicotinic acetylcholine receptor; ACh, agonist acetylcholine; BA, benzylidene anabaseine; 4OH-GTA-21, 3-(4-hydroxy,2-methoxybenzylidene)anabaseine; MLA, methyllycaconitine.

upstream of the vicinal cysteines. Whereas Sine *et al.* (10) concluded that agonist binding affinity was affected by the Tyr-to-Phe mutant, O'Leary and White (11) asserted that this mutant interfered with the coupling of ligand binding and channel opening. With a structural template for nicotinic receptors now available, we can interpret the effect of mutations in the C loop of $\alpha 7$ and $\alpha 4\beta 2$ receptors within the context of their structural topology.

MATERIALS AND METHODS

ACh Receptor Clones—The human nAChR receptor clones were obtained from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia). The $\alpha 7$ gene was subcloned into the pCI-neo vector (Promega, Madison WI) between the NheI and NotI restriction sites.

Site-directed Mutants—The structural orientations of conserved residues in the AChBP and nicotinic ACh receptor model C loops were analyzed to choose functionally important residues. Mutations to $\alpha 7$ and $\alpha 4$ were introduced using the QuikChange kit from Stratagene according to the manufacturer's instructions. Amino acids are numbered as for human $\alpha 7$ (with vicinal cysteines at positions 190 and 191). The mutations were confirmed with automated fluorescent sequencing.

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

Molecular Modeling—Energy-minimized structures of anabaseine and anabasine in their monoprotonated forms were obtained by semi-empirical calculations (MOPAC 2000, PM3 parameter set). Models of the human $\alpha 7$ and $\alpha 4\beta 2$ nAChR extracellular domains were obtained by homology modeling with the AChBP crystal structures with bound nicotine or methyllycaconitine (Protein Data Bank codes 1UW6 and 2BYR). The SwissPDB viewer was used to create alignments between the appropriate AChBP and nAChR, followed by structure generation with the Magic Fit option, and side chain optimization within the Gromos force field, as described by Le Novère (12). Possible binding modes for benzyldine anabaseine were identified with the DOCK program (version 5.3.0).

Expression in *Xenopus laevis* Oocytes—Mature (>9 cm) female *X. laevis* African frogs (Nasco, Ft. Atkinson WI) were used as a source of oocytes. Before surgery, the frogs were anesthetized by placing them in a 1.5 g/liter solution of MS222 for 30 min. Oocytes were removed from an incision made in the abdomen.

Harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM NaHCO₃, 0.82 mM MgSO₄, 15 mM HEPES, pH 7.6, 12 mg/liter tetracycline) to remove the follicular layer. Stage 5 oocytes were isolated and injected with 50 nl (5–20 ng) of each subunit cRNA. Recordings were normally conducted 2–5 days post-injection, although for some mutants longer periods of incubation (up to 8–10 days) were required to obtain measurable currents. Wild-type and mutant $\alpha 7$ receptors were routinely co-injected with the cDNA for

human RIC-3, an accessory protein that improves and accelerates $\alpha 7$ expression (13), without affecting the pharmacological properties of the receptors.

Electrophysiology—Experiments were conducted using OpusXpress6000A (Axon Instruments, Union City, CA). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Both the voltage and current electrodes were filled with 3 M KCl. The oocytes were clamped at a holding potential of –60 mV.

Data were collected at 50 Hz and filtered at 20 Hz for $\alpha 7$ and at 5 Hz for $\alpha 4\beta 2$. The oocytes were bath-perfused with Ringer's solution (115 mM NaCl, 10 mM HEPES, 2.5 mM KCl, and 1.8 mM CaCl₂, pH 7.3) with 1 μ M atropine. Agonist solutions were delivered from a 96-well plate using disposable tips. Flow rates were set at 2 ml/min for $\alpha 7$ and 4 ml/min for $\alpha 4\beta 2$. Drug applications alternated between ACh controls and test solutions of ACh, 4OH-GTS-21, or other experimental agonists at varying concentrations.

Chemicals—4OH-GTS-21 was obtained from Taiho (Tokyo, Japan). Anabaseine and other benzyldine anabaseines were provided by Dr. Bill Kem (University of Florida). TC-1698 and AR-R17779 were provided by Targacept, and tropisetron was provided by Memory Pharmaceuticals. Other chemicals were purchased from Sigma. Fresh acetylcholine stock solutions were made daily in Ringer's solution.

Experimental Protocols and Data Analysis—Responses of $\alpha 7$ wild-type and mutant receptors were calculated as net charge (14), and unless otherwise indicated, responses of $\alpha 4\beta 2$ wild-type and mutant receptors are reported as peak currents. Each oocyte received initial control applications of ACh, then experimental drug applications, and follow-up control applications of ACh. For wild-type $\alpha 7$ receptors, the control ACh concentration was 300 μ M, a concentration that is sufficient to evoke maximal net charge responses (14). For $\alpha 7$ mutants that showed decreased responsiveness to ACh, 3 mM was used for the ACh controls. For both mutant and wild-type $\alpha 4\beta 2$ receptors, ACh controls were 30 μ M unless otherwise indicated. Responses to experimental drug applications were calculated relative to the preceding ACh control responses to normalize the data, compensating for the varying levels of channel expression among the oocytes. Mean values and standard errors were calculated from the normalized responses of at least four oocytes for each experimental concentration. For concentration-response relations, data derived from net charge analyses were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and curves were generated from the Hill Equation 1,

$$\text{Response} = \frac{I_{\max}[\text{agonist}]^n}{[\text{agonist}]^n + (EC_{50})^n} \quad (\text{Eq. 1})$$

where I_{\max} denotes the maximal response for a particular agonist/subunit combination, and n represents the Hill coefficient. I_{\max} , n , and the EC_{50} values were all unconstrained for the fitting procedures, except in the case of the ACh-response curves. Because ACh is our reference full agonist, for the ACh concentration-response curves the data were normalized to the

observed ACh maximum, and the I_{\max} of the curve fits were constrained to equal 1.

Intact Oocyte Binding—MLA binding on intact oocytes was conducted as reported previously (15). In brief, whole *Xenopus* oocytes that were either uninjected or had been injected with mRNAs encoding either wild-type $\alpha 7$ or the $\alpha 7$ K145A or $\alpha 7$ K145D/D190K mutants were placed in single wells of a 96-well plate containing either 20 nM [3 H]methyllycaconitine alone or 20 nM [3 H]methyllycaconitine with 5 mM nicotine. After three 4-s washes in 2.5 ml of Ringer's solution, total radioactivity was measured using an automated liquid scintillation counter and then expressed as femtomoles of MLA binding/cell. The mean values for binding in the presence of nicotine for each treatment ($n = 4-5$ cells) were subtracted from each of the non-nicotine-treated cells for each condition ($n = 4-5$ cells), yielding mean and S.E. values for nicotine displaceable binding. Cells from the same injection sets were also tested for their responses to 1 mM ACh or 100 μ M 4OH-GTS-21.

RESULTS

ACh and 4OH-GTS-21 Concentration-Response Relationships for $\alpha 7$ Wild-type and Mutant Receptors—There are several highly conserved amino acid residues in the C loop of nicotinic receptors. The side chains of two particular amino acids, Tyr-188 (human $\alpha 7$ numbering) and Asp-197, that are conserved in nicotinic receptors and AChBP appeared to be in close proximity in models of the receptor-binding site. We conducted site-directed mutagenesis to determine whether these residues interact with agonists and/or with each other. A tyrosine to phenylalanine mutation was introduced at amino acid position 188. ACh showed a drastic decrease in potency for this mutant receptor (Fig. 1). The EC_{50} in the wild type was 33 μ M, whereas the Y188F mutant had an EC_{50} of 1500 μ M (Table 1). This result is consistent with studies conducted by Galzi *et al.* (16). We hypothesized that this mutation, known to have a large effect on activation by the small, very polar, agonist ACh might have relatively little effect on activation by larger hydrophobic $\alpha 7$ -selective agonists such as 4OH-GTS-21. As shown in Fig. 1, $\alpha 7$ Y188F receptors responded well to 4OH-GTS-21, and there was no significant change in the potency of this agonist (Table 1). The tyrosine at position 188 in $\alpha 7$ has been proposed to be part of an amino acid triad that is essential for initiating the gating for nAChR (9). The other two elements of this triad are the aspartate at 197 and the lysine at 145, which have been proposed to form a salt bridge in the resting state of the receptor. We mutated Asp-197 to alanine, which would have the effect of disrupting the putative salt bridge to Lys-145 and any potential hydrogen bonding to Tyr-188. The D197A mutation in $\alpha 7$ also resulted in receptors for which responses to 300 μ M ACh were severely reduced compared with wild type (Fig. 1). As in the case of the Tyr-188 mutation, this was associated with a reduction in ACh potency (Table 1). The $\alpha 7$ D197A mutant showed an EC_{50} of 430 μ M, in contrast to the wild type, which had an EC_{50} of 33 μ M. As in the case of the Y188F mutation, the D197A mutation had no effect on the potency of 4OH-GTS-21 and produced a

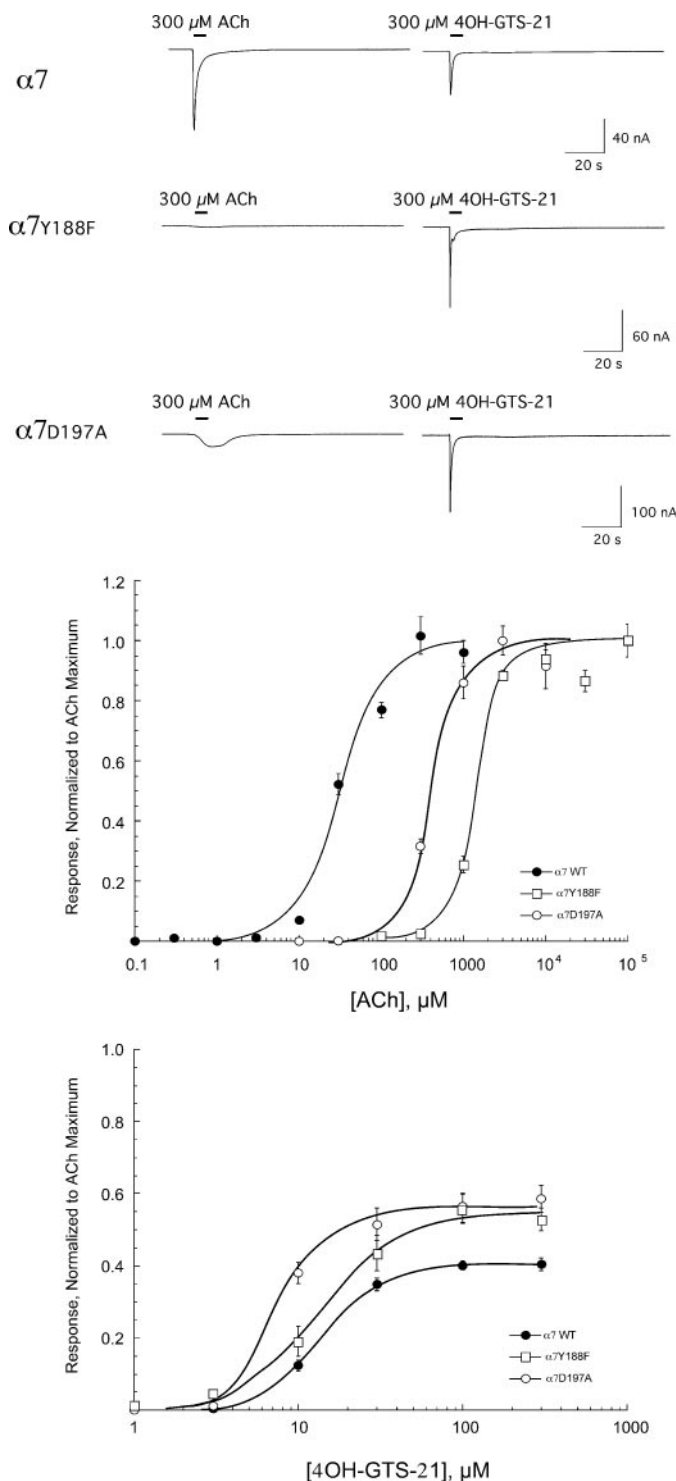


FIGURE 1. Responses of wild-type and mutant $\alpha 7$ Y188F and $\alpha 7$ D197A human $\alpha 7$ receptors to ACh and 4OH-GTS-21. Shown on the top are the responses of oocytes expressing wild-type and mutant $\alpha 7$ receptors to the application of 300 μ M ACh and 300 μ M 4OH-GTS-21. Each pair of responses was obtained from a single cell with 5 min between the two agonist applications. Note that mutations that greatly decrease the responses to 300 μ M ACh have very little effect on the responses to 4OH-GTS-21. Below are shown concentration-response relationships for wild-type and mutant human $\alpha 7$ nAChR expressed in *Xenopus* oocytes. Responses to ACh are shown in the middle section and those to 4OH-GTS-21 in the lower panel. Data were measured relative to ACh control responses and then expressed relative to the ACh maximum for each subtype. The ACh control concentrations used were 300 μ M for the wild-type receptors and 3 mM for the mutants. Each point represents the mean (\pm S.E.) of at least four oocytes.

TABLE 1

Values derived from curve fits

	ACh EC ₅₀	4OH-GTS-21 EC ₅₀	4OH-GTS-21 % ACh maximum	Nicotine EC ₅₀	Nicotine % ACh maximum
α7 receptors					
Wild type	33 ± 4 μM	14 ± 1 μM	40 ± 1%	13 ± 2 μM	81 ± 4%
α7D197A	430 ± 25 μM	8 ± 1 μM	55 ± 2%	73 ± 8 μM	77 ± 3%
α7Y188F	1500 ± 164 μM	14 ± 2 μM	56 ± 2%	230 ± 5 μM	85 ± 2%
α7K145A	1500 ± 260 μM	22 ± 11 μM	29 ± 3%		
α7K145D/D197K	2900 ± 120 μM	37 ± 3 μM	70 ± 2%		
α4-Containing receptors and mutants					
Wild type α4β2	75 ± 20 μM	NA ^a	≤2%	1.6 ± 0.5 μM	20 ± 2%
α4D199Aβ2	115 ± 17 μM	300 ± 100 nM	20 ± 2%	9 ± 2 μM	25 ± 3%
α4Y190Fβ2	76 ± 9 μM	2.2 ± 0.2 μM	450 ± 14%	7 ± 2 μM	32 ± 2%
α4K145Aβ2	NA	NA	NA		
α4K145D/D199Kβ2	NA	2.6 ± 0.3 μM	1240 ± 40%		
α4Y190Fβ2Y114S	60 ± 30 μM	580 ± 140 nM	160 ± 10%		
α4β2Y114S	1.4 ± 0.2 μM	NA	≤2%		
Wild type α4β4	25 ± 2 μM	1.6 ± 0.6 μM	2.1 ± 0.2%		
α4Y190Fβ4	1260 ± 140 μM	2.2 ± 0.1 μM	55 ± 2%		
α4Y190Fβ4S116Y	1040 ± 20 μM	1.0 ± 0.5 μM	30 ± 4%		
α4β4S116Y	4.8 ± 0.8 μM	NA	≤2%		

^a NA indicates the values not available because of the inadequate amplitude of responses.

small but significant increase in the efficacy of the benzyldene anabaseine compared with ACh.

ACh and 4OH-GTS-21 Concentration-Response Relationships for α4β2 Wild-type and Mutant Receptors—To determine whether residues of α4 that are homologous to Tyr-188 and Asp-197 of α7 also regulate the potency of ACh for the high affinity receptors of the brain, we made the corresponding mutations in the human α4 subunit (α4Y190F and α4D199A). As shown in Fig. 2, the ACh concentration-response curves for these mutants were not significantly different from those of wild-type α4β2. 4OH-GTS-21 is highly selective for wild-type α7 receptors compared with α4β2 receptors, normally evoking responses from α4β2 receptors that are no more than 2% of the ACh maximum responses (Fig. 2). Interestingly, although the Y190F and D199A mutations in α4 did not appear to affect ACh potency, they had striking effects on the efficacy of 4OH-GTS-21 for α4β2-type receptors. As shown in Fig. 2, 4OH-GTS-21 is a very efficacious agonist for α4Y190Fβ2 receptors, more so even than ACh. A full concentration-response study showed that the maximum currents stimulated by 4OH-GTS-21 were 4–5-fold larger than the maximal currents that could be stimulated by ACh in the same cells. The α4D199Aβ2 receptors also showed increased responses to 4OH-GTS-21 (Fig. 2) compared with wild-type α4β2 receptors but much less than was seen with the α4Y190Fβ2 receptors. Specifically, for α4Y190Fβ2 receptors there was at least a 200-fold increase in relative efficacy 4OH-GTS-21 compared with wild type, and for α4D199Aβ2 receptors there was only a 10-fold increase in 4OH-GTS-21 efficacy relative to wild type (Table 1).

The α4Y190F mutants were also co-expressed with β4 (Fig. 3 and Table 1). Interestingly, the effects of the mutation were clearly intermediate between those obtained with the Y188F mutation in α7 and the α4Y190F mutation expressed with β2. Specifically, there was a shift in ACh potency, roughly half as large as that seen in the α7 mutants, and there was also a large increase in 4OH-GTS-21 efficacy relative to ACh. The maximal response of wild-type α4β4 receptors to 4OH-GTS-21 was only about 2% of the ACh maximum, and the maximum responses of α4Y190Fβ4 receptors to 4OH-GTS-21 were increased to 55% of the ACh maximum responses obtained.

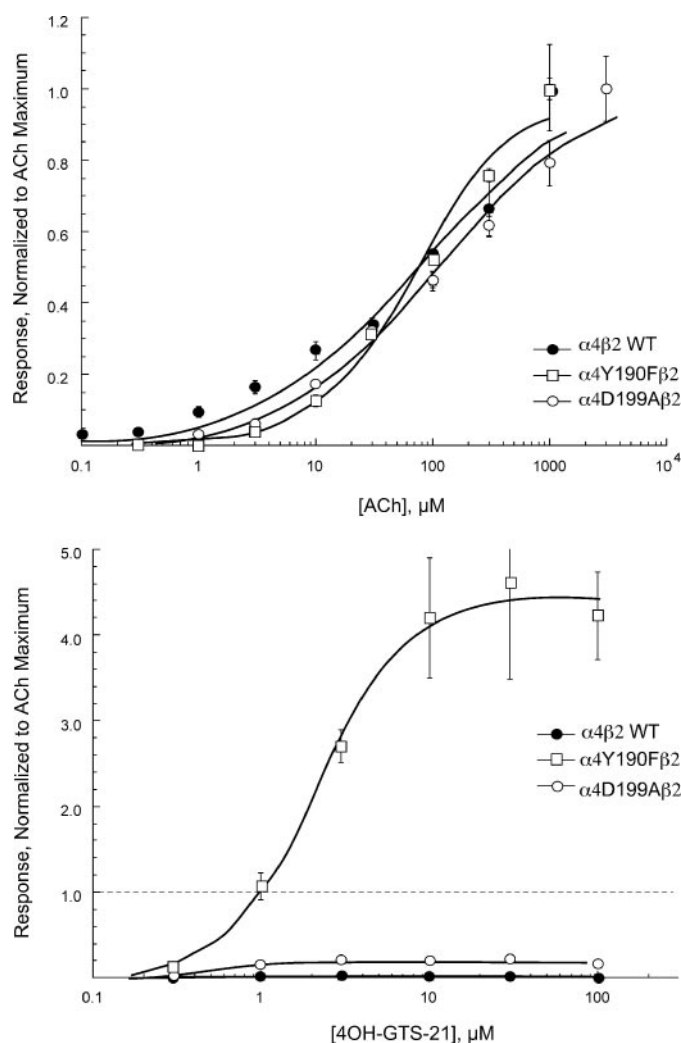


FIGURE 2. Concentration-response relationships for wild-type and mutant human α4β2 nAChR expressed in *Xenopus* oocytes. Data were measured relative to control responses and then expressed relative to the ACh maximum for each subtype. Data for ACh responses are shown in the upper plot, and the concentration-response relationships to 4OH-GTS-21 are shown in the lower plot. Data were measured relative to ACh control responses and then expressed relative to the ACh maximum for each subtype. Each point represents the mean (±S.E.) of at least four oocytes. Note that the maximum responses of the oocytes expressing α4Y190Fβ2 to 4OH-GTS-21 were far larger than their maximal responses to ACh (dashed line).

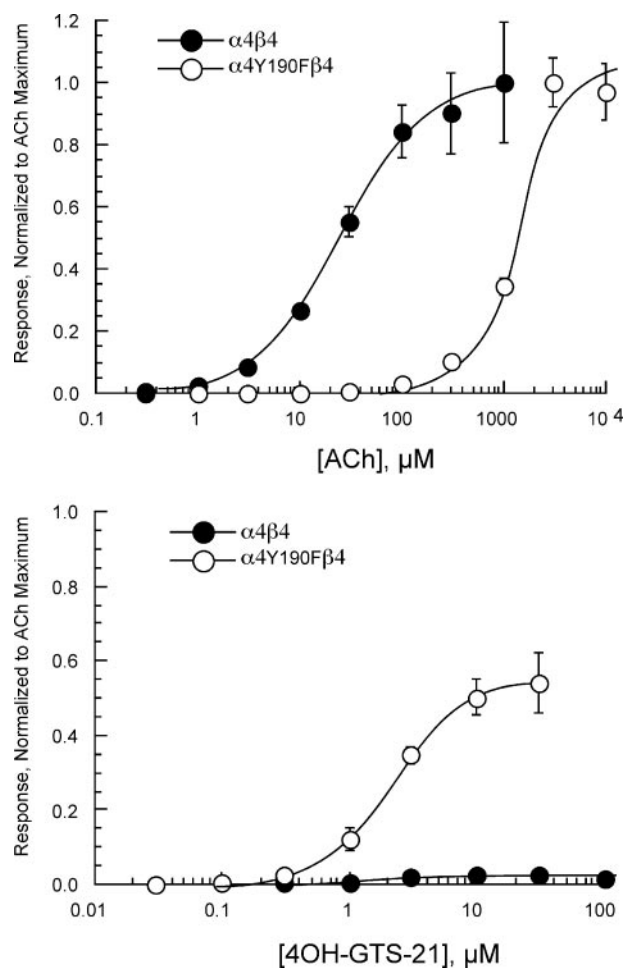


FIGURE 3. Responses of wild-type $\alpha 4\beta 4$ nAChR and mutant $\alpha 4Y190F\beta 4$ receptors to ACh and 4OH-GTS-21. Concentration-response studies for $\alpha 4\beta 4$ nAChR and $\alpha 4Y190F\beta 4$ receptors for ACh (upper) and 4OH-GTS-21 (lower). Each point represents the mean (\pm S.E.) of at least four oocytes.

The specialization of β subunits for agonist binding are thought to focus in regions identified as the D, E, and F loops of the binding site. Differences in the effect of the $\alpha 4Y190F$ mutation when co-expressed with $\beta 2$ or $\beta 4$ on ACh potency may therefore be due to differences between $\beta 2$ and $\beta 4$ in these regions. Because the effect of the Tyr-190 mutant on ACh potency is similar between $\alpha 7$ and $\alpha 4Y190F$ co-expressed with $\beta 4$, it might be due to similarities between $\alpha 7$ and $\beta 4$ in these domains. The sequences of the $\beta 2$, $\beta 4$, and $\alpha 7$ D, E and F loops are shown in Sequence 1,

	---D---	-----E-----	----F----
hbeta2	VWLTQEW	SNAVVSYDGSIFWL	SEVASLDDF
halpha7	IWLQMSW	TNVLVNSSGHCQYL	--EADISGY
hbeta4	VWLKQEW	TNLIVRSNGSVLWL	TPTASMDDF

SEQUENCE 1

As shown above, there are two residues in the E loop where $\beta 4$ diverges from $\beta 2$ and has residues in common with $\alpha 7$. Of these two residues, the tyrosine/serine difference is potentially the more significant difference. It was shown previously that in human $\alpha 7$, this serine residue, along with the glycine residue in the F loop, determined the potency difference of GTS-21

between rat and human $\alpha 7$ (8). However, we created $\beta 2Y114S$ and $\beta 4S116Y$ mutants, and this residue does not appear to be a critical factor for the β subunit-dependent differences in the $\alpha 4Y190F$ effects. Specifically, when co-expressed with wild-type $\alpha 4$, the $\beta 2Y114S$ mutation had the effect of increasing ACh potency, decreasing the EC_{50} from $75 \pm$ to $1.4 \pm 0.2 \mu M$ (Table 1). However, co-expressed with wild-type $\alpha 4$, the $\beta 4S114Y$ mutant also had a similar, albeit smaller, effect of increasing ACh potency, decreasing the EC_{50} from 25 ± 2 to $4.8 \pm 0.8 \mu M$ (Table 1). When co-expressed with $\alpha 4Y190F$, neither β subunit mutation had a significant effect on ACh potency and both produced decreases in the relative efficacy of 4OH-GTS-21 compared with ACh (Table 1).

Noncompetitive Antagonism of Mutant $\alpha 4\beta 2$ Receptors Occurs at High Concentrations of 4OH-GTS-21—It has been reported previously that in addition to being a very weak ($\leq 2\%$) partial agonist for wild-type human $\alpha 4\beta 2$ receptors, 4OH-GTS-21 is also a use-dependent antagonist (6). This sensitivity to channel block by 4OH-GTS-21 at high concentrations was retained in $\alpha 4$ mutants that were strongly activated by 4OH-GTS-21. As shown in Fig. 4, when 4OH-GTS-21 was applied to either $\alpha 4Y190F\beta 2$ or $\alpha 4D199A\beta 2$ receptors at concentrations above $10 \mu M$, there was an initial spike followed by a depression in the response and a subsequent rebound as the 4OH-GTS-21 was washed from the chamber. This is very typical for use-dependent channel block (17). To better characterize the concentration dependence of this antagonist activity, we calculated the ratio of rebound-to-peak currents from $\alpha 4Y190F\beta 2$ receptors across a range of 4OH-GTS-21 concentrations (Fig. 4). From this analysis, the IC_{50} for channel block by 4OH-GTS-21 was estimated to be about $100 \mu M$, whereas the EC_{50} for activation by 4OH-GTS-21 was approximately 2 orders of magnitude lower ($1 \mu M$). The noncompetitive inhibition of wild-type $\alpha 4\beta 2$ receptors also was a relatively low potency effect (6), suggesting that the antagonism seen with the mutants is the retention of a wild-type feature and not the result of the mutations *per se*.

Potency of Nicotine for Wild-type and Mutant $\alpha 7$ and $\alpha 4\beta 2$ Receptors—We investigated the effect of nicotine on $\alpha 7$ and $\alpha 4\beta 2$ mutants. This molecule represents a putative structural intermediate between smaller acyclic agonists like ACh and larger aromatic ringed agonists such as 4OH-GTS-21 and has been crystallized in the AChBP (18). In the AChBP the binding of nicotine appeared to more directly involve the homolog of Tyr-188 than did the binding of ACh. However, we found that the effects of the Y188F and D197A mutations on activation by nicotine were less pronounced than the effects on activation of ACh. The EC_{50} of nicotine for the Y188F $\alpha 7$ mutant increased to $230 \mu M$ from the wild-type value of $13 \mu M$ (Table 1), whereas the EC_{50} for the Asp-to-Ala mutant was $73 \mu M$, so the potency shifts were less than half the magnitude of the changes in the ACh potency. There was also very little change in efficacy of nicotine for these mutants. The percent ACh maximum current of nicotine for the two mutants was approximately the same as for the wild-type value of 81%.

In the $\alpha 4\beta 2$ receptors we observed a modest decrease in potency of nicotine for the mutant receptors (Table 1). The

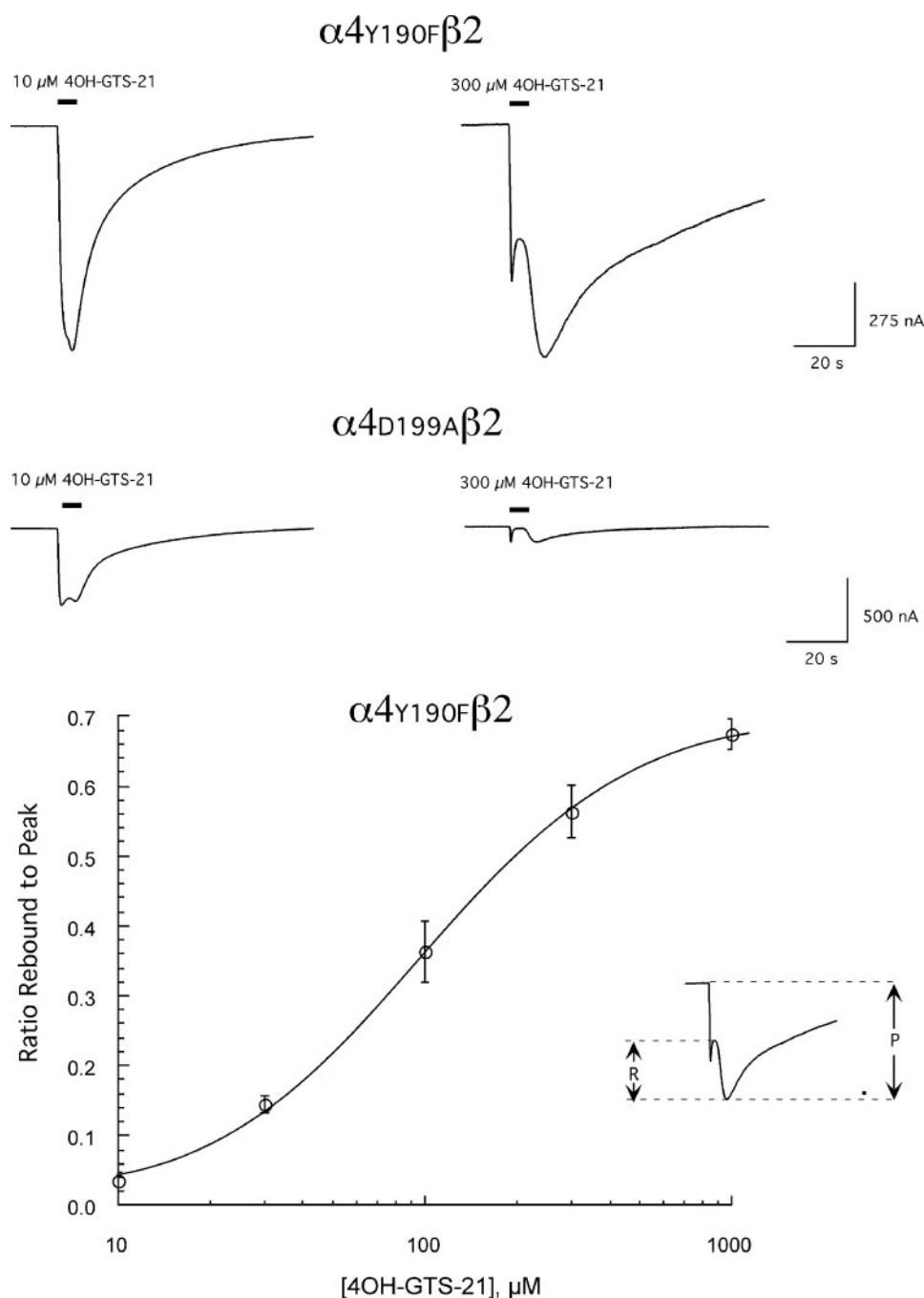


FIGURE 4. Use-dependent inhibition of $\alpha 4\beta 2$ mutant receptors is a limiting factor at high concentrations of 4OH-GTS-21. It has been reported previously that although 4OH-GTS-21 does not produce significant activation of wild-type human $\alpha 4\beta 2$ receptors, it can inhibit $\alpha 4\beta 2$ responses to ACh when it is co-applied at high concentrations. As shown in the upper traces, for the $\alpha 4\beta 2$ mutants that respond to 4OH-GTS-21, when 4OH-GTS-21 is applied at a high concentration ($>10 \mu\text{M}$), there is an initial current that rapidly decays, followed by a rebound as the drug is washed from the chamber. This is typical for use-dependent inhibition. To quantify this effect, peak currents and rebound amplitudes (P and R , respectively, see inset) were measured in the responses of $\alpha 4Y190F\beta 2$ -expressing cells across a range of high 4OH-GTS-21 concentrations. The ratio of the rebound amplitude to the subsequent peak is indicative of the extent of block occurring during the high 4OH-GTS-21 concentration. These values are plotted in the lower panel. The effective concentration for this noncompetitive inhibition was $\sim 100 \mu\text{M}$, 50 times higher than the EC_{50} for channel activation (Fig. 2).

EC_{50} for nicotine was $1.6 \mu\text{M}$ in the wild type, $9 \mu\text{M}$ in the D199A mutant, and $7 \mu\text{M}$ Y190F mutant (Table 1). As seen for $\alpha 7$, the % ACh maximum of nicotine for these mutant receptors did not differ significantly from the wild-type value of 20%.

about 7% of the amplitude of the ACh maximum currents and anabasine producing maximal responses about 2% of the ACh maximum (data not shown). Interestingly, compared with wild-type $\alpha 4\beta 2$, anabasine responses of $\alpha 4Y190F\beta 2$ receptors were

Structural Features $\alpha 7$ -Selective Agonists Showing Enhanced Activation of $\alpha 4$ Mutants—Numerous agonists, varying greatly in structure, have been identified that selectively activate the $\alpha 7$ nAChR. We have previously proposed a general model for key elements common to most of these $\alpha 7$ -selective drugs (19). To resolve the structure-activity determinants for the tyrosine and aspartate mutants in $\alpha 4\beta 2$ receptors, we tested whether $\alpha 4Y190F\beta 2$ mutants responded to other $\alpha 7$ -selective agonists or core structures related to those agonists. As shown in Fig. 5, all of the benzylidene anabaseines tested were capable of activating the $\alpha 4Y190F\beta 2$ receptors, although no others were as efficacious as 4OH-GTS-21 at the test concentration. In contrast, neither the tropane-based agonist tropisetron nor the quinuclidine-based compounds (quinuclidine and AR-R17779) produced significant activation. Likewise, choline did not activate the $\alpha 4Y190F\beta 2$ mutants. Interestingly, TC-1698, which has a structure intermediate to anabaseine and quinuclidine, did show good activation of the mutant $\alpha 4Y190F\beta 2$ receptors at the test concentration. Application of $300 \mu\text{M}$ anabaseine, the core agonist of 4OH-GTS-21, was reasonably effective at activating the $\alpha 4Y190F\beta 2$ receptors (Fig. 5), whereas $300 \mu\text{M}$ anabasine, which lacks the double bond in anabaseine, was relatively ineffective at activating the mutant receptors. A previous study comparing the activity of anabaseine and anabasine on wild-type rat receptors (20) indicated that both compounds were more efficacious for $\alpha 7$ than for $\alpha 4\beta 2$ receptors. Both anabaseine and the reduced analog anabasine are full agonists of wild-type human $\alpha 7$ and weak partial agonists of wild-type human $\alpha 4\beta 2$, with anabaseine producing maximal responses

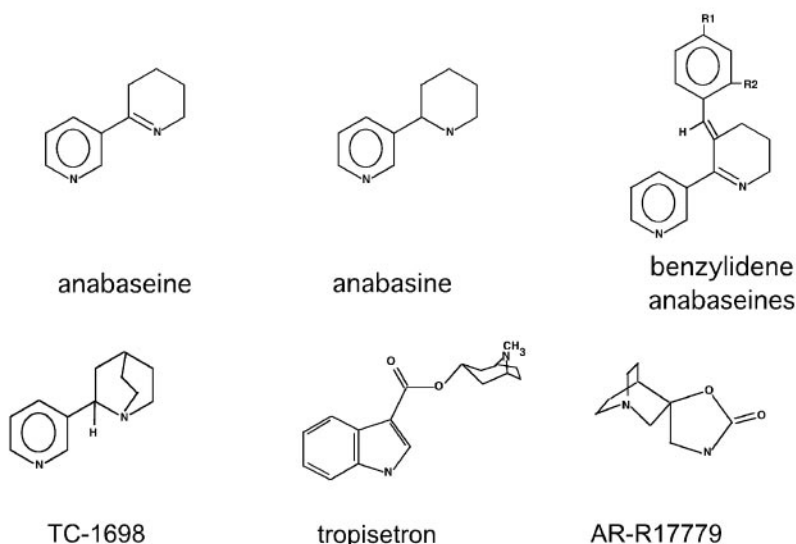
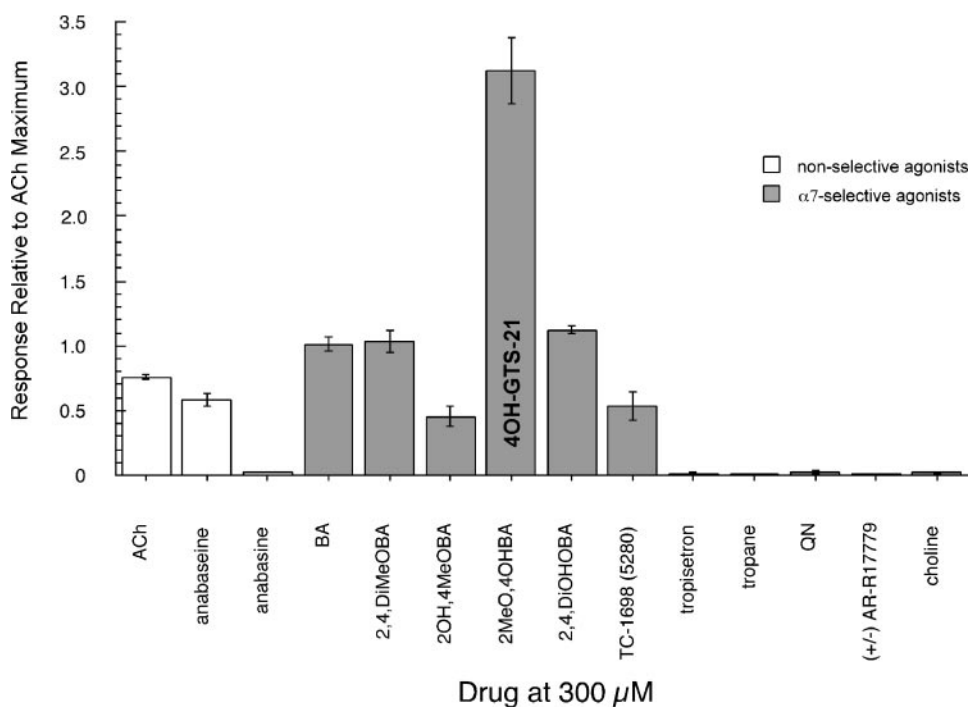
Activation of $\alpha 4Y190F\beta 2$ receptors

FIGURE 5. To determine whether the $\alpha 4Y190F$ mutation produced sensitivity to $\alpha 7$ -selective agonists other than 4OH-GTS-21, $\alpha 4Y190F\beta 2$ -expressing cells were tested for their responses to a variety of alternative agonists, applied at a concentration of 300 μM (top panel). Anabaseine, which activates wild-type $\alpha 4\beta 2$ receptors, also activated this mutant, as did the other benzylidene anabaseines tested. Interestingly, although the somewhat anabaseine-like TC-1698 activated the $\alpha 4Y190F\beta 2$ receptors, other $\alpha 7$ -selective agonists with tropane or quinuclidine-like core structures did not activate $\alpha 4Y190F\beta 2$ receptor (see structure in lower panel).

increased 10-fold to $75 \pm 12\%$ of the ACh maximum, whereas maximum anabaseine responses were less affected by the mutation, increasing only to $6 \pm 1\%$ of the ACh maximum in the full concentration-response analysis (data not shown).

Diminished Activation of $\alpha 7$ and $\alpha 4\beta 2$ Lysine-to-Alanine Mutants and Enhanced Activation of Lysine/Aspartate Double Mutants by 4OH-GTS-21 Compared with ACh—For both $\alpha 7$ and $\alpha 4$ -containing receptors, mutation of the lysine at 145 to alanine greatly reduced the magnitude of current responses compared with wild-type receptors expressed under the same

conditions. The K145A mutation in $\alpha 7$ reduced the potency of ACh to the same extent as did the Y188F mutation and also affected both the potency and efficacy of 4OH-GTS-21 (Fig. 6 and Table 1) compared with ACh. Responses of $\alpha 4K145A\beta 2$ receptors to 3 mM ACh and 300 μM 4OH-GTS-21 were of comparable amplitude but were reduced to near the level of detection (5–10 nA) and so could not readily be characterized. Likewise, double mutants exchanging the tyrosine and aspartate residues in either $\alpha 7$ or $\alpha 4$ gave currents too small to be characterized.

Double mutants were also constructed, which exchanged the location of the aspartate and lysine residues hypothesized to form a salt bridge in the resting state of the receptor. Responses of the $\alpha 7K145D/D197K$ mutant were relatively small but within our limits of reliable measurement (>5 nA) if the cells were kept for 7 days or longer after injection with cRNA. In addition to producing a general reduction in current amplitudes, the reciprocal replacement of the charged residues in the $\alpha 7K145D/D197K$ mutant had an effect on ACh potency similar to that of the Y188F mutation (Fig. 6), whereas 4OH-GTS-21 potency was not affected (Fig. 6). If the cells were kept for 7 days or longer after injection, the ACh responses of $\alpha 4K145D/D199K\beta 2$ mutant receptors were barely at the limits of reliable measurement. However, responses to 4OH-GTS-21 were 12-fold larger than the maximal ACh responses obtainable in the same cells (Fig. 6). Responses to ACh were so small that it was not possible to obtain reliable measurements with ACh concentrations less than 100 μM (Fig. 6).

To improve our ability to interpret the consequences of the K145D/D197K double mutant, we also constructed $\alpha 7K145D$ and $\alpha 7D197K$ single point mutants. When tested 6–10 days post-injection, neither gave detectable responses to either 3 mM ACh or 300 μM 4OH-GTS-21.

Concomitant Binding and Expression Studies—To determine whether the low levels of currents generated by the lysine single and double mutants were primarily because of intrinsically low

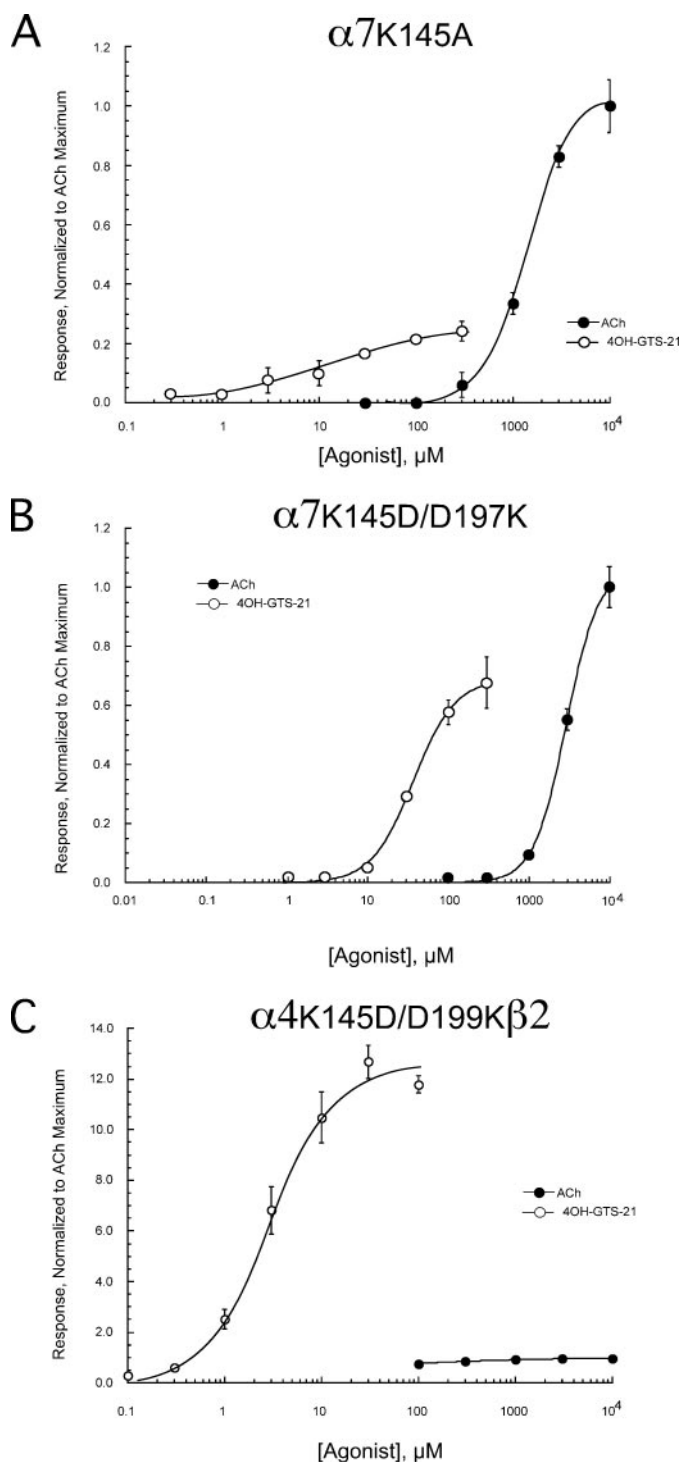


FIGURE 6. Lysine 145 single and double mutants. The $\alpha 7K145A$ mutant nAChR showed reduced response to both ACh and 4OH-GTS-21. The concentration-response relationships for human $\alpha 7K145A$ mutant nAChR expressed in *Xenopus* oocytes to both ACh and to 4OH-GTS-21 are shown in A. Note that to obtain adequately sized responses from these poorly expressing receptors, cells were kept at least 10 days post-injection before being used for recording. Data were measured relative to ACh control responses and then expressed relative to the ACh maximum. The ACh control concentrations used were 3 mM. Each point represents the mean (\pm S.E.) of at least four oocytes. Double mutants were made that reoriented the lysine 145 and aspartate 197 residues in $\alpha 7$. The ACh responses of the $\alpha 7K145D/D197K$ receptors (B) were relatively small, and cells had to be kept for more than a week before ACh responses were above the threshold for detection, and ACh had very low potency for activating this receptor. The effect of the $\alpha 7K145D/D197K$ mutations on the 4OH-GTS-21 responses of $\alpha 7$ receptors was primarily to change the relative

agonist sensitivity or failure of the receptors to express on the surface of the oocytes, we conducted radiolabeled MLA binding experiments on intact oocytes and measured physiological responses on oocytes from the same injection sets 6 days post-injection. Wild-type $\alpha 7$, $\alpha 7K145A$, and $\alpha 7K145D/D197K$ receptors were used for these experiments.

Six days post-injection, responses of wild-type receptors to 3 mM ACh were ~ 80 -fold higher than our threshold for detection (based on measurements from uninjected oocytes from the same oocyte harvest), whereas the cells injected with $\alpha 7K145A$ showed no detectable responses to either 3 mM ACh or 300 μM 4OH-GTS-21. Cells injected with $\alpha 7K145A/D197K$ showed small but detectable responses to 3 mM ACh (~ 3 -fold higher than the detection threshold and less than 5% of the responses of wild-type $\alpha 7$). However, they showed significantly larger responses ($p < 0.001$) to 300 μM 4OH-GTS-21, ~ 8 -fold over our threshold for detection.

MLA binding experiments on cells from the same injection set showed nicotine-displaceable MLA binding for all three receptor subtypes, although the nicotine-displaceable binding in cells injected with the mutant receptors was only about 30–40% that obtained with the wild-type receptors (in femtomoles of MLA bound/cell: wild type, 0.85 ± 0.18 ; $\alpha 7K145A$, 0.36 ± 0.12 ; and $\alpha 7K145D/D197K$, 0.30 ± 0.1). However, although MLA and nicotine have both been well characterized for their effects on wild-type receptors, the lack of similar data for mutant receptors limits the interpretation of MLA binding data. Specifically, the MLA binding may represent a low estimate of the surface expression, because MLA may bind to the mutants with lower affinity than it does to wild-type $\alpha 7$. In the case of the $\alpha 7K145A$ mutant, the MLA binding would suggest that there were $\geq 40\%$ as many receptors in cells expressing wild-type $\alpha 7$, although no functional responses could be detected. In the case of the $\alpha 7K145D/D197K$ mutant, their responses to 4OH-GTS-21 were $\sim 25\%$ that of the wild-type receptors recorded on the same day, relatively consistent with the MLA binding that suggests receptor numbers were $\geq 35\%$ those of controls. These data support the interpretation that although surface expression may be somewhat decreased with the $\alpha 7K145A$ and $\alpha 7K145D/D197K$ mutants, the diminished responses to ACh are largely because of failure of those receptors that are expressed to respond to agonists. The $\alpha 7K145A$ receptors respond poorly to both ACh and 4OH-GTS-21, whereas $\alpha 7K145D/D197K$ receptors respond poorly to ACh and almost as well as wild-type receptors to 4OH-GTS-21.

Modeling of Agonists and the Binding Site—Because the effects of the Y190F mutation in $\alpha 4\beta 2$ receptors on activation by 4OH-GTS-21 may be generalized to other anabaseine-like

efficacy of 4OH-GTS-21 compared with ACh from 40% in the wild type to $\sim 70\%$ in the double mutant. The reorientation of the putative C loop lysine-aspartate residues differentially affects activation by ACh and 4OH-GTS-21 in $\alpha 4\beta 2$ receptors. C shows the concentration-response curves for ACh and 4OH-GTS-21 responses of $\alpha 4K145D/D199K\beta 2$ receptors. Because the ACh-evoked responses were so small, it was not possible to reliably measure responses to ACh at concentrations less than 100 μM . Responses to 100 μM ACh were $76 \pm 3\%$ of the amplitude of the responses to 1 mM ACh. There was, however, a clear effect of the $\alpha 4K145D/D199K\beta 2$ mutations on the responses of $\alpha 4\beta 2$ increasing the relative efficacy of 4OH-GTS-21 compared with ACh from 2% in the wild type to $\sim 1200\%$ in the double mutant.

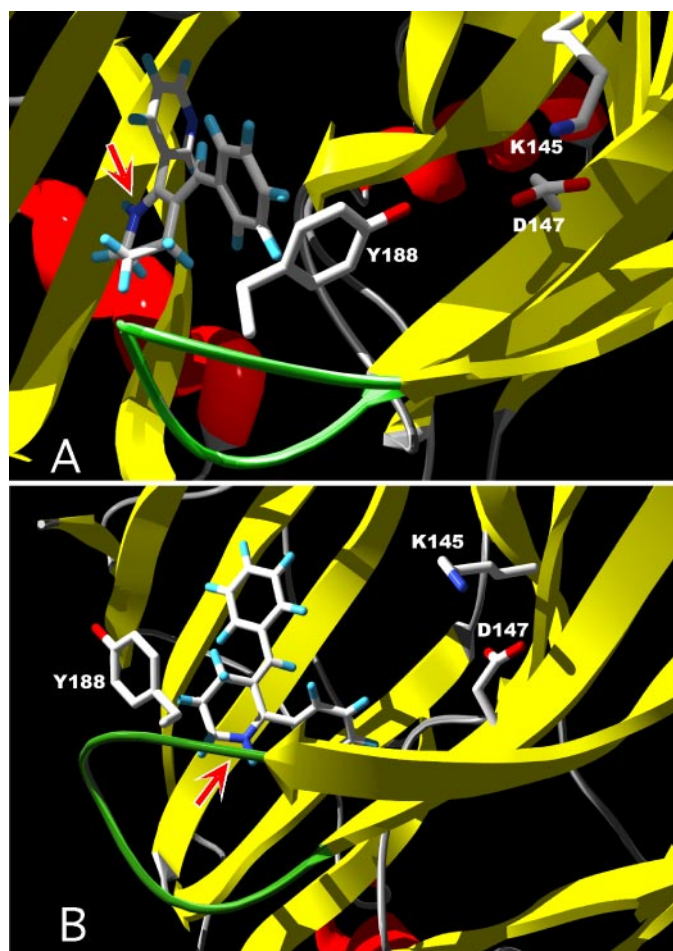


FIGURE 7. **Hypothetical docking of BA into $\alpha 7$ nAChR homology models.** The Tyr-188, Asp-197, and Lys-145 residues are indicated. The first model (A) was derived from the AChBP structure with bound nicotine. Note that favored position for the cationic center of the BA (red arrow) is oriented away from C loop (green). The second model (B) was derived from the AChBP structure with bound methyllycaconitine. In this structure the cationic center of the BA has access to the interior of the C loop.

molecules but not to tropane or quinuclidine-based $\alpha 7$ -selective agonists, or even to anabasine, differences between anabaseine and anabasine may provide a clue to the molecular key that fits the activation lock, as modified by these mutations. These two compounds differ in their overall shape; anabaseine places the pyridine ring in a conformation that is nearly coplanar with the dehydropiperidine ring, whereas in anabasine, the pyridine ring is roughly orthogonal to the plane of the dehydropiperidine ring. It may be the case that anabaseine compounds activate the receptors by using a different sort of intermolecular interaction from ACh to bring down the C loop, perhaps involving more hydrophobic/van der Waals interactions than the cation- π bond effects attributed to ACh.

We prepared homology models for the $\alpha 7$ and $\alpha 4\beta 2$ receptors based on crystal structures of the AChBP. Interestingly, with models based on the structure of nicotine in the binding site, we were unable to dock benzylidene anabaseine into the receptor in a binding mode similar to that of ACh. Receptor-BA complexes were identified, but the BA was found bound at the dimer interface with its charged residue exposed to solvent, rather than interacting with critical C loop residues (Fig. 7A).

Control docking runs with the experimental structure of AChBP, and nicotine or BA correctly reproduced the bound complex of nicotine but did not yield a complex with BA in a similar orientation. However, when we prepared homology models for the $\alpha 7$ and $\alpha 4\beta 2$ receptors based on the crystal structure of AChBP bound to the antagonist MLA, the C loop was swung away from the core residues of the extracellular domain, effectively creating more room for a larger molecule like BA to bind (Fig. 7B). Docking studies based on the MLA-bound structure revealed a family of bound BA-receptor complexes that placed the charged nitrogen in approximately the right place to benefit from the π -charge interactions that agonists like ACh are proposed to enjoy. Due primarily to the limited homology between the AChBP source and the human nAChR target sequences (<30% sequence identity), the $\alpha 7$ and $\alpha 4\beta 2$ homology models alone may not be adequate to identify the specific features of the binding sites that make these subtypes differentially responsive to specific agonists. However, the ligand-induced differences in the AChBP crystal structures (*i.e.* MLA-bound *versus* nicotine-bound) appear to make the models more informative. Considering that nicotine has much higher affinity for $\alpha 4\beta 2$ than for $\alpha 7$, although it is the reverse for MLA, it may be the case that these two ligands act as templates around which the binding site can adapt either a more $\alpha 4\beta 2$ - or $\alpha 7$ -like configuration. If that is the case, then 4OH-GTS-21 may not be an agonist for wild-type $\alpha 4\beta 2$ receptors because it will tend to bind to that receptor in an orientation that does not promote activation.

DISCUSSION

It has been proposed that changes in intramolecular interactions among a triad of amino acids in the ACh-binding site of nAChR initiate the sequence of conformational changes that couple agonist binding to channel gating (9). We confirm that the disposition of this Tyr-188–Asp-197–Lys-145 amino acid triad (human $\alpha 7$ numbering) is important for ACh activation in $\alpha 7$. However, the Tyr-199 and Asp-199 are less important for ACh activation of $\alpha 4\beta 2$ receptors. As reported previously for chick $\alpha 7$, mutation of the tyrosine at position 188 in human $\alpha 7$ produced a decrease in the potency of ACh for activation of the receptor (16). Although potency may be related to binding affinity, it might also be related to other elements in the chain of events coupling binding to conformational change in the receptor, such as the opening rate of the channel once agonist is bound and/or alteration in the concentration dependence of desensitization. For example, if the Y188F mutation slows the opening rate for a bound channel, then that effect might be overcome with higher concentrations of agonist, which would give greater equilibrium occupancy by agonist, providing more bound receptors to open at the slower rate and ultimately produce similar currents to fewer bound channels opening at faster rates. This case would be consistent with the model proposed by Mukhtasimova *et al.* (9), which suggests a direct role for the tyrosine at 188 in perturbing the interaction between lysine 145 and aspartate 197. The observation that mutation of Asp-197 has an effect on ACh activation of $\alpha 7$ similar to the effect of mutating Tyr-188 supports the

hypothesis that there is a functional association between these residues in that process. However, our data indicate that any hypothetical interaction between these residues cannot necessarily be generalized to agonists other than ACh, because activation of human $\alpha 7$ by 4OH-GTS-21 does not appear to be perturbed by either of these mutations.

Although the Y188F and D197A mutations in human $\alpha 7$ appeared to primarily affect only activation by ACh, mutation of Lys-145 to alanine (or aspartate) had an effect on activation by both ACh and 4OH-GTS-21. The single-point mutations of Lys-145 appeared to have a fundamental effect on receptor activation, because the apparent efficacies of both ACh and 4OH-GTS-21 were reduced in this mutant even though binding experiments indicated that substantial numbers of receptors were getting to the surface. Even 7–10 days after RNA injection, ACh-evoked currents in $\alpha 7$ K145D/D197K and $\alpha 4$ K145D/D199K $\beta 2$ receptors remained relatively small compared with wild-type receptors expressed in the same injection set, whereas 4OH-GTS-21-evoked currents were relatively robust. Additionally, the $\alpha 7$ K145D/D197K receptors showed the same selective decrease in ACh potency seen with the Y188F and D197A mutations. These observations suggest that of this amino acid triad the interaction between Tyr-188 and D197 is required most especially for potent activation by ACh and that the lysine (145) is the residue most important for gating by any agonist.

We show that the effects of perturbing the amino acid triad on ACh activation are qualitatively different for $\alpha 7$ and $\alpha 4\beta 2$ receptors. Shifts in ACh potency were seen only in $\alpha 7$, whereas in $\alpha 4\beta 2$ the mutations primarily affected ACh efficacy, relative to the efficacy of 4OH-GTS-21. For the $\alpha 4$ Y190F $\beta 2$ and $\alpha 4$ D199A $\beta 2$ receptors, ACh-evoked responses were essentially indistinguishable from oocytes of the same injection set expressing wild-type $\alpha 4\beta 2$ receptors. This may mean that specialization of the β subunit overcomes the impact of these mutations on ACh potency. Because the Y190F and D199A mutations in $\alpha 4$ create receptors with far larger responses to 4OH-GTS-21 than obtainable with this compound and wild-type receptors, it is clear that they produced an increase in absolute efficacy of 4OH-GTS-21, not just efficacy relative to ACh. Indeed, for $\alpha 4$ Y190F $\beta 2$ receptors, 4OH-GTS-21 appeared to evoke currents larger even than ACh could stimulate from wild-type receptors. This would suggest that an interaction between Tyr-190 and Asp-199 in the wild-type $\alpha 4$ of $\alpha 4\beta 2$ receptors somehow prevents activation by the benzylidene anabaseines. Note that the $\alpha 4$ Y190F and $\alpha 4$ D199A mutations appear to specifically affect the ability of anabaseine compounds to activate the receptors, and it is unclear whether they affect the binding of the compounds *per se*. It has been reported previously that although inactive as agonists, benzylidene anabaseines do typically bind to $\alpha 4\beta 2$ -type receptors with affinity higher (≈ 200 nM (20)) than the potency of 4OH-GTS-21 for activating the mutant receptors. The observation that channel block of $\alpha 4\beta 2$ receptors by high concentrations of 4OH-GTS-21 is a conserved feature in the Y190F and Asp-199 mutations of $\alpha 4\beta 2$ receptors confirms that the limited efficacy of 4OH-GTS-21 with wild-type $\alpha 4\beta 2$ is not because of channel block. Our modeling would suggest that competitive antago-

nism of $\alpha 4\beta 2$ receptors by benzylidene anabaseines might occur because they bind with some affinity but with an incorrect orientation to function as agonists.

Effects of the Y190F and D199A mutations on activation by nicotine were similar to the effects on ACh activation, although somewhat smaller. This was not unexpected because the homolog of Tyr-188 in the AChBP was shown to be an important element for nicotine binding (18). However, it is unclear whether the AChBP structure models anything other than the high affinity binding associated with desensitized receptors, whereas our data focus on the activation process.

The model of Mukhtasimova *et al.* (9) proposes that activation by ACh is associated with movement of the C loop so that Tyr-188 becomes positioned in a way to disrupt a putative salt bridge between the aspartate and lysine. Specifically, positional changes of $\alpha 1$ Lys-145 and $\alpha 1$ Asp-200 in muscle-type nAChR are proposed to initiate the cascade of perturbations that opens the receptor channel; the first perturbation is of β -strand 7, which harbors $\alpha 1$ Lys-145 and is part of the signature Cys loop, and the second is of β -strand 10, which harbors $\alpha 1$ Asp-200 and connects to the M1 domain. However, as discussed above, with neuronal receptors this does not appear to be the case. We show that in $\alpha 7$ receptors gating (even by ACh) still occurs in the Y188F mutant, whereas in $\alpha 4\beta 2$ receptors ACh activation is relatively unaffected by $\alpha 4$ mutations at either Tyr-190 or Asp-199. Our data better support an alternative interpretation that the Y188F mutation is more involved with stabilizing the binding of ACh than disrupting the Asp-Lys salt bridge.

It has been reported that in the AChBP the binding of agonist causes a physical reorientation of the C loop over the binding site, collapsing the ceiling of the binding site on top of the agonist. It is reasonable to speculate that this physical restructuring of the agonist-binding site may account for the increase in affinity for agonist that occurs when nAChRs are activated and subsequently desensitize, a process that is easily measured in heteromeric receptor subtypes, especially those containing the $\beta 2$ subunit. For example, whereas $\alpha 4\beta 2$ receptors in the resting state require concentrations of ACh greater than 10 μ M to effectively activate, subsequent to activation the same receptors bind ACh with ~ 1000 -fold higher affinity. It may be the case that in $\alpha 7$ the Tyr-188 and Asp-197 residues interact to retain a relatively high affinity binding conformation for ACh (compared with the Tyr and Asp mutants) by slowing the return of the C loop to the extended apo-position (21). However, for the β subunit-containing receptors, which show much more profound increases in agonist affinity than $\alpha 7$ receptors following activation, specializations of the β subunits may supercede the role of a Tyr-190 and Asp-199 interaction to retain the stability of the C loop in the activated configuration. This would explain why mutations of these residues did not alter the ACh potency in the $\alpha 4\beta 2$ mutants. The $\beta 4$ subunit may be less specialized in this regard, accounting for why the $\alpha 4$ Y190F $\beta 4$ receptors showed a shift in ACh potency, whereas the $\alpha 4$ Y190F $\beta 2$ receptors did not.

An important question then would be what role does the agonist molecule itself play in initiating the gating cascade? Mukhtasimova *et al.* (9) proposed that the agonist molecules act primarily to stabilize the movement of the C loop. An alter-

native possibility is that the agonist molecules themselves act to perturb other intermolecular interactions that stabilize and destabilize the resting state, interactions very likely involving the Lys-145 residue.

In conclusion, our data indicate that the triad of amino acids proposed to initiate the gating of muscle-type nAChR play important roles in neuronal type nAChR. However, for both $\alpha 7$ and $\alpha 4\beta 2$ receptors, the tyrosine and aspartate residues play a greater role in the specificity of selective agonists than in the activation process *per se*. It appears that in the β subunit-containing receptors, these residues may exclude anabaseine-like $\alpha 7$ -selective agonists from binding in the proper orientation to promote activation. In addition to providing insights into the process of neuronal nAChR activation, our results also identify specific features of an important class of $\alpha 7$ -selective agonists that may be important for future drug development targeting this receptor.

Acknowledgments—We thank Taiho Pharmaceuticals for providing GTS-21 and 4OH-GTS-21 and Targacept for samples of TC-1698 and AR-R17779. Anabaseine and other benzylidene anabaseines were provided by Dr. William Kem (University of Florida), whom we also thank for useful comments on the manuscript. We also thank Dr. Ed Meyer for comments on the manuscript and for help in developing the oocyte MLA binding assay. Technical assistance was provided by Lisa Jacobs, Chris Coverdill, and Dolan Abu-Aouf.

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