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The Structural Basis for GTS-21 Selectivity between Human and Rat Nicotinic α7 Receptors

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ABSTRACT

The α7 nAChR-selective partial agonist 3-(2,4-dimethoxybenzylidene)anabaseine (GTS-21) is more efficacious and potent for rat receptors than for human α7 receptors. Four single amino acid differences exist between human and rat α7 in the agonist binding site, two in the C loop, and one each in the E and F loops. Reciprocal mutations were made in these three domains and evaluated in *Xenopus laevis* oocytes. Mutations in the C and F loops significantly increased the efficacy of GTS-21 for the human receptor mutants but not to the level of the wild-type, and reciprocal mutations in rat α7 did not decrease responses to GTS-21. Whereas mutations in the E loop alone were without effect, the E- and F-loop mutations together increased GTS-21 efficacy and potency for human receptors, but the EF mutations in the rat receptors decreased the GTS-21 potency without changing the efficacy. The only mutants that showed a full reversal of the efficacy differences between human and rat α7 contained complete exchange of all four sites in the C, E, and F loops or just the sites in the C and F loops. However, the reversal of the potency ratio seen with the EF mutants was not evident in the CEF mutants. Our data therefore indicate that the pharmacological differences between rat and human α7 receptors are caused by reciprocal differences in sites within and around the binding site. Specific features in the agonist molecule itself are also identified that interact with these structural features of the receptor agonist binding site.

A crucial assumption for the translation of preclinical research from animal studies to human therapeutics is that receptor pharmacology will be consistent between species. That is, drugs shown to be useful based on their ability to work in animal (rodent) models would also have similar activity on human forms of the receptors. The neuronal α7-type nicotinic acetylcholine receptor (nAChR) has been identified as a potential target for the treatment of Alzheimer's disease (Lindstrom, 1997), and 3-(2,4-dimethoxybenzylidene) anabaseine (GTS-21; also called DMXBA), which selectively targets this receptor, has been shown to improve learning and memory in animal models of cholinergic hypofunction (Kem, 2000). This α7-selective partial agonist has also been shown to prevent the death of differentiated PC-12 cells that occurs after nerve growth factor removal and the death of cultured primary neurons that occurs after high levels of NMDA receptor activation (Martin et al., 1994; Shimohama et al., 1998). It is interesting that although GTS-21 was able to protect PC-12 cells from the cytotoxic effects of amyloid peptide exposure, it was not able to protect human-derived SK-N-SH cells from the same cytotoxic stress, although the GTS-21 4-hydroxy metabolite, 3-(4-hydroxy,2-methoxybenzylidene)anabaseine (4-OH-GTS-21), was cytoprotective in the same assay (Meyer et al., 1998a). A likely explanation for these observed differences in cytoprotective activity came from the observation that GTS-21 was far less efficacious for human α7 receptors than it was for rat α7 receptors (Briggs et al., 1997). Although 4-OH-GTS-21 also activates rat α7 receptors better than human α7 receptors, it is more efficacious than GTS-21 for both receptors, so that at a cytoprotective concentration, it produces activation of human α7 receptors that is comparable with the activation of rat receptors produced by GTS-21 at the same concentration (Papke and Papke, 2002).

Most benzylidene anabaseine (BA) compounds that have been characterized, like GTS-21, are more potent for rat α7

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; GTS-21, 3-(2,4-dimethoxybenzylidene)anabaseine (DMXBA); 4-OH-GTS-21, 3-(4-hydroxy,2-methoxybenzylidene)anabaseine; BA, benzylidene anabaseine; ACh, acetylcholine; MS222, 3-aminobenzoic acid ethyl ester; DMPP, 1-1-dimethyl-4-phenylpiperazinium iodide; 4-OH-BA, 3-(4-hydroxybenzylidene)anabaseine; 4-NH_2-BA, 3-(4-aminobenzylidene)anabaseine; AR-R17779, (−)-spiro[1-azabicyclo[2.2.2]octane-3,5′-oxazolidin-2′-one] 4-propyl-BA, 3-(4-propylbenzylidene)anabaseine; 4-MeO-CA, 3-(4-methoxycinnamylidene)anabaseine.

receptors than for human. Two notable exceptions are 3-(2-hydroxy-4-methoxybenzylidene)anabaseine and 2,4-dihydroxy-benzylidene)anabaseine, which, although less efficacious for human α7 receptors than for rat receptors, have EC_{50} values for human α7 that are less than or equal to their EC_{50} values for rat receptors (Kem et al., 2004). This observation suggests that the location of a hydroxyl group in the 2 position may somehow eliminate the significance of sequence differences between human and rat α7 for the potency of BA compounds. However, it is unclear whether this may be a result of differences in preferred conformations of the various BA compounds (Kem et al., 2004) or specific receptor/ligand interactions in the α7 binding site.

The observation that GTS-21 and related compounds differ in their efficacy for human and rat forms of the receptor means that, at least for these BA compounds, preclinical studies require circumspection before new compounds are proposed for human therapeutics. In the present study, multiple nAChR agonists were examined for their ability to discriminate between human and rat forms of the α7 nAChR expressed in *Xenopus laevis* oocytes. Compared with other classes of α7-selective agonists, the BA compounds were most likely to differ in their activity for human and rat receptors. Specific structural differences between the human and rat receptors that are implicated in the pharmacological differences as well as properties of the BA compounds themselves were investigated. Together, the characterization of binding sites in the receptors with the experimental agonists provide insights into what makes the BA compounds selective for α7 receptors and how new agonists may be designed that will have similar and optimal activity for both human and rat α7 receptors.

Materials and Methods

Sequence Comparisons and Selection of Mutations. Fig. 1 provides a comparison of the human and rat α7 sequences in the extracellular domain, which contains the regions involved with agonist binding. The proposed series of helix and beta sheet domains are based on recent models of the receptor that draw from a large body of data derived from site-directed mutagenesis studies and also, more recently, from the crystallization of a snail ACh binding protein with homology to the receptor's extracellular domain (Corringer et al., 2000; Brejc et al., 2001; Dougherty and Lester, 2001). It is believed that the agonist-binding site of nAChR is located at the interface between adjacent subunits. For receptors that contain both α and non-α subunits, it has been proposed that the α subunit provides the principle binding site, or "plus" face. Other subunits (γ, δ, or ε in the case of muscle receptor, and β2 or β4 in the case of the neuronal ACh receptor, which binds nicotine with high affinity) contain a complementary binding site, the "negative" face. In the case of α7 subunits, there seem to be homologous domains for both the principle binding site and the complementary site, so that each α7 subunit must serve two roles, potentially contributing to two ACh binding sites (Corringer et al., 2000). We investigated the significance of sequence differences between human and rat α7 nAChR, first with an analysis of chimeric receptors and then by focusing on differences in the putative agonist binding domains.

α7 Clones. The rat α7 nAChR clone was obtained from Dr. Jim Boulter (UCLA, Los Angeles, CA), and the human α7 clone was obtained from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). Both of these α7 genes were subcloned into the pCI-neo vector (Promega, Madison WI) between the NheI and NotI restriction sites.

Chimeras. Chimeras HHR and RRH were created by using a unique BclI restriction site within the conserved third transmembrane domain (at residue 280). Because that enzyme is blocked by *dam* methylation, SCS110 competent cells (Stratagene, La Jolla CA) were first transformed with our human and rat α7 constructs. *Dam* methylase-free plasmid DNA preparations were digested with BclI and NotI and then agarose-gel purified (QIAEX II kit; QIAGEN, Valencia CA) to separate the carboxyl-terminal (intracellular domain) portions, which were then exchanged between the human and rat constructs and re-ligated.

Chimeras HRR and RHH were constructed by overlap extension PCR (Horton et al., 1989). Sense and antisense primers correspond-

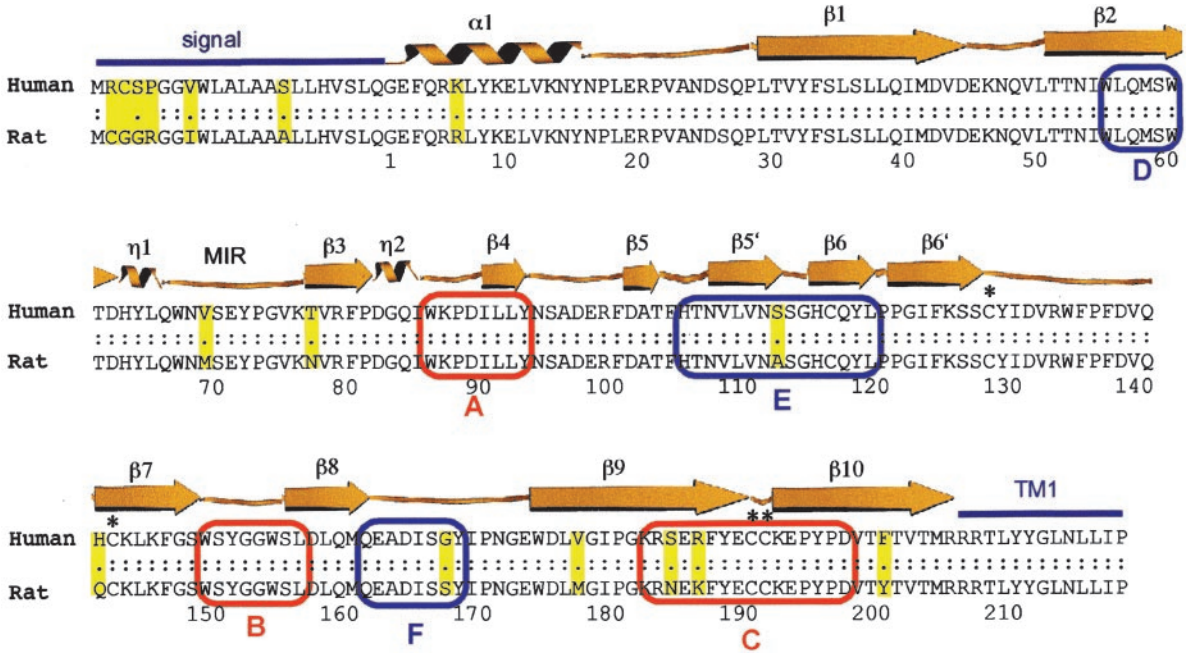

Fig. 1. Sequences of human and rat α7 receptors extracellular domains compared. The proposed arrangement of helical and beta sheet structures (Brejc et al., 2001) is indicated, as well as the putative agonist binding subdomains (Corringer et al., 2000). Sites of sequence difference between human and rat α7 are highlighted.

ing to the conserved sequence at nucleotide bases 425 - 443 (numbered from beginning of start codon) were combined with T7 or T3 promoter primers in each α7/pCI-neo construct to produce the N-terminal sections, approximately 500 base pairs, and the C-terminal sections, approximately 1500 base pairs. The four PCR products were gel-purified. A second PCR was run with the overlapping templates, N-terminal Hα7 plus C-terminal Rα7 or N-terminal Rα7 plus C-terminal Hα7, and the T7 plus T3 promoter primers. The overlapping region provided double-stranded DNA that primed elongation in both directions, and the full-length product was amplified using the T7 and T3 primers. The HRR product was gel-purified and the RHH product was run through the Promega Wizard PCR clean-up kit. These were then digested with NheI and NotI, gel-purified, and ligated into the pCI-neo vector.

Chimeras HRH and RHR were prepared by digesting the purified, full-length products HRR and RHH with BclI and NheI and ligating them with nonmethylated human and rat constructs, which were also digested with these enzymes. All of the chimeras were confirmed by both restriction digests and automated fluorescent sequencing (University of Florida ICBR core facility, Gainesville, FL).

Site-Directed Mutants. Key subdomains in the plus face of the agonist binding site have been identified as regions A, B, and C and, in the negative face, as regions D, E, and F (Fig. 1) (Corringer et al., 2000; Brejc et al., 2001; Dougherty and Lester, 2001). Of the 10 amino acids that differ between the human and rat α7 in the extracellular domain, only four are in these binding site subdomains, one each in the E and F loops, and two in the C loop. Mutants were made that contain reciprocal exchanges in each of these domains, alone or in combination.

Mutant α7 subunits were prepared using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Amino acids are numbered as for human α7 (vicinal cystines at positions 190 and 191). Double and triple mutants were prepared sequentially with the primers shown in Table 1. Mutations were confirmed with automated fluorescent 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, the toads were anesthetized by placing the animal in a 1.5 g/l solution of MS222 for 30 min. Oocytes were removed from an incision made in the abdomen.

To remove the follicular cell layer, harvested oocytes were treated with 1.25 mg/ml collagenase from Worthington Biochemical Corporation (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). Thereafter, stage-5 oocytes were isolated and injected with 50 nl (5–20 ng) of each of the appropriate subunit cRNAs. Recordings were made 5 to 15 days after injection.

Chemicals. Anabaseine, GTS-21, and 4-OH-GTS-21 were obtained from Taiho (Tokyo, Japan). BA (Papke et al., 2004a), 3-(4-methoxycinnamylidene)anabaseine (Meyer et al., 1998b) 3-(4-hydroxybenzylidene)anabaseine (4-OH-BA) (Papke et al., 2004a), as well as the previously unpublished compounds 3-(4-aminobenzylidene)anabaseine (4-NH_2-BA) and 3-(4-propylbenzylidene)anabaseine (4-propyl-BA) were synthesized at the University of Florida. The BA compounds were synthesized according to methods reported previously (Zoltewicz et al., 1993), and their purity was ascertained by NMR, mass spectrometry, and elemental composition measurements. Other chemicals for electrophysiology were obtained from Sigma Chemical Co. (St. Louis, MO). Fresh acetylcholine stock solutions were made daily in Ringer's solution.

Electrophysiology. Experiments were conducted using OpusXpress 6000A (Axon Instruments, Union City, CA). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to 8 oocytes in parallel. Both the voltage and current electrodes were filled with 3 M KCl. Cells were voltage-clamped at a holding potential of −60 mV. Data were collected at 50 Hz and filtered at 20 Hz. Cells were bath-perfused with Ringer's solution, and agonist solutions were delivered from a 96-well plate via disposable tips, which eliminated any possibility of cross-contamination. Flow rates were set at 2 ml/min. Drug applications alternated between ACh controls and experimental agonists. Applications were 12 s in duration followed by 181-s washout periods.

Experimental Protocols and Data Analysis. Responses were calculated as net charge (Papke and Papke, 2002). Each oocyte received two initial control applications of ACh, then an experimental drug application, and then a follow-up control application of 300 μM ACh, a concentration sufficient to evoke a maximal net charge response (Papke and Papke, 2002). 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. Means and S.E.M. were calculated from the normalized responses of at least four oocytes for each experimental concentration. The application of some experimental drugs caused the subsequent ACh control responses to be reduced, suggesting some form of residual inhibition (or prolonged desensitization). Whenever subsequent ACh controls were less than 75% of the preapplication ACh controls, the cells were discarded, and new cells were used to complete the dose response studies. Individual oocytes were used for no more than one dose response study. Because at high concentrations GTS-21 and other benzylidene anabaseines produce long-lived inhibition of control ACh responses, it was not possible to study the entire GTS-21 concentration range on single cells; the data for high concentration effects were therefore obtained on fresh cells that had stable ACh control responses that were used for the internal normalization procedures.

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

TABLE 1
Mutants and primer sequences
Mutations are underlined.

Mutant	Amino Acid(s) Exchanged	Sense Primer
Hα7E	S112A	CTAACGTGTTGGTGAACGCTTCTGGGCATTGCCA
Rα7E	A112S	CAATGTTTTGGTGAATTCATCTGGGCATTGCCA
Hα7F	G167S	CAGGAGGCAGATATCAGTAGCTATATCCCCAATGGAGA
Rα7F	S167G	GAGGCAGATATCAGCGGCTATATCCCCAACGGA
Hα7C	S184N,R186K	TCCCCGGCAAGAGGAATGAAAAGTTCTATGAGTGCTGCAA
Rα7C	N184S,K186R	TCCCTGGCAAAAGGAGTGAGAGGTTCTATGAGTGCTGCAA
Hα7C$_a$	S184N	CGGCAAGAGGAATGAAAGGTTCTATGAGTGCTGCA
Hα7C$_b$	R186K	CGGCAAGAGGAGTGAAAAGTTCTATGAGTGCTGCAAAG
Rα7C$_a$	N184S	CCCTGGCAAAAGGAGTGAGAAGTTCTATGAGTGCTGC
Rα7C$_b$	K186R	CTGGCAAAAGGAATGAGAGGTTCTATGAGTGCTGC
Hα7F(A)	G167A	GCAGATATCAGTGCCTATATCCCCAATGGAGAATGG
Rα7F(A)	S167A	GCAGATATCAGCGCCTATATCCCCAACGGAGAATGG

equation: Response = (I_{max} [agonist]^{nH}) / ([agonist]^{nH} + [EC_{50}]^{nH}), 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 EC_{50} were all unconstrained for the fitting procedures.

Molecular Modeling. We created a structural model for the rat and human α7 dimer based on the structure of acetylcholine binding protein (Protein Data Base entry 1I9B). The BA agonist was drawn in three-dimensional space using a text editor and RASMOL version 2.6, adapted from the PDB structure of nicotine. The superimposed backbones of the rat and human receptor were staggered by a small distance (roughly 0.2 Å) such that regions of both backbones could be seen. The side chains of residues within loops C and F were oriented according to a local energy minimization protocol on SwissPDB. The resulting figures were imported as a BMP files into Canvas 5.0 (Deneba Software, Miami, FL).

Results

The Relative Efficacy of Small Nicotinic Agonists for Human and Rat α7 nAChR. We have previously reported that various benzylidene and cinnamylidene anabaseines discriminate between human and rat α7 receptors in regard to potency and efficacy compared with ACh (Meyer et al., 1998a; Papke and Papke, 2002). We extended our analysis to look at several relatively nonselective nicotinic agonists, as well as a range of α7-selective agonists (Fig. 2).

Figure 3A shows the responses of human and rat α7 receptors to saturating concentrations of four nicotinic agonists that activate multiple receptor subtypes in addition to α7, in each case compared with ACh control responses. The fact that these were saturating concentrations (i.e., concentrations that produced maximal responses) was confirmed with full dose-response studies (data not shown). There were no significant differences in the maximal responses of human and rat α7 responses to nicotine, 1-1-dimethyl-4-phenylpiperazinium iodide (DMPP), or anabaseine, although cytisine was somewhat more efficacious for human α7 receptors than for rat α7 (Papke and Papke, 2002). Regarding whether rat and human receptors differ in their responses to ACh, we have reported previously that EC_{50} values for ACh are 28 ± 8 and 21 ± 3 μM for rat and human α7 receptor, respectively. Therefore, ACh does not seem to discriminate between rat and human α7 receptors with regard to potency (Papke and Papke, 2002). Because ACh is our reference (full) agonist, its efficacy by definition is 1.0 for both human and rat receptors, so direct comparisons of ACh efficacy are therefore not possible.

The Relative Efficacy of Putative α7-Selective Agonists for Human and Rat α7 nAChR. In Fig. 3B, we show the maximal responses of human and rat α7 receptors to saturating concentrations of a variety of α7-selective agonists. Choline is a full agonist (compared with ACh) for both subtypes, whereas AR-R17779 was somewhat more efficacious for human receptors than rat receptors. Tropisetron is a $5HT_3$ receptor antagonist that is also a partial agonist for α7 nAChR but an antagonist for other nAChR subtypes (Macor et al., 2001; Papke et al., 2004b). Like choline and DMPP, tropisetron was equally efficacious for human and rat forms of α7. As shown in Fig. 3, not all agonists discriminate between human and rat α7 receptors in the same way as GTS-21. Relative to ACh, some compounds, such as cytisine (Papke and Papke, 2002) and AR-R17779 (Papke et al., 2004b), are more efficacious for human receptors than for rat. Indeed the core agonist of GTS-21 is equally efficacious for human and rat receptor subtypes, indicating that the discrimination between these two receptors is likely to involve specific interactions with the benzylidene and its substituted side groups. We have examined a substantial number of benzylidene and cinnamylidene anabaseine derivatives; with rare exceptions, those with significant agonist activity were more efficacious for rat receptors than human receptors. Maximal responses of a selection of anabaseine-derived agonists applied at saturating concentrations are also shown in Fig. 3B. For all of the anabaseine derivatives shown, except 4-OH-BA and 4-NH_2-BA, the responses of rat α7 receptors were significantly larger than those of the human α7 nAChR.

Separating Potency and Efficacy Differences with Anabaseine and the Benzylidene Derivatives. GTS-21 is both more potent and more efficacious for rat α7 receptors than for human α7 receptors (Papke and Papke, 2002). As shown in Fig. 3A, however, at saturating concentrations, anabaseine, the core agonist of GTS-21, stimulated similar maximal responses with both human and rat α7 receptors.

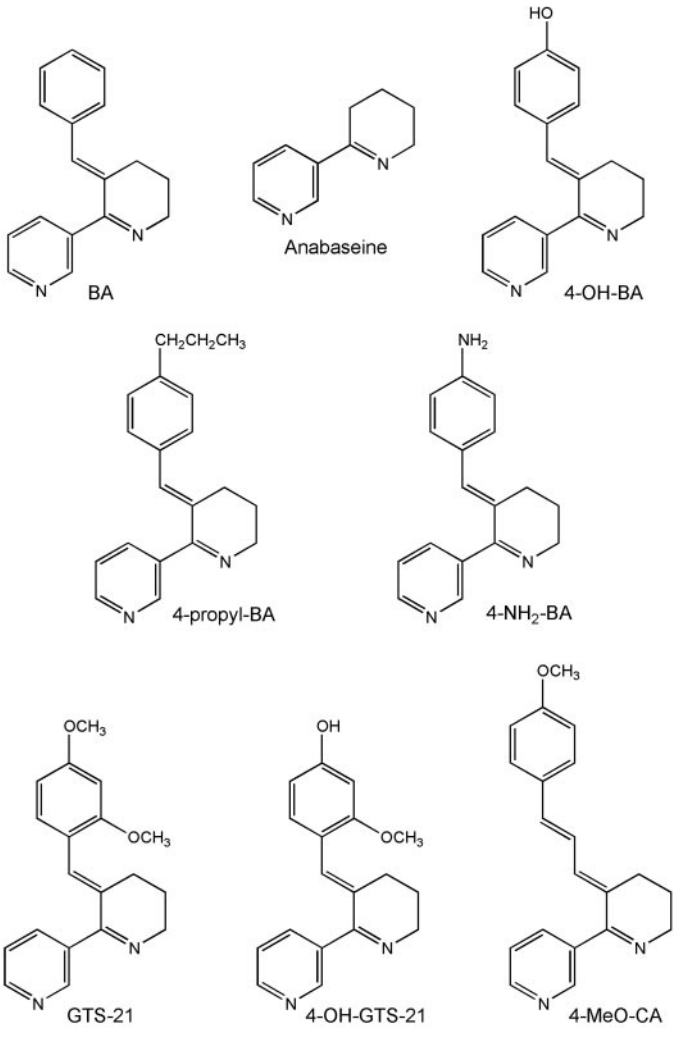

Fig. 2. Anabaseine compounds used to probe functional differences between human and rat α7 receptors. Note that the two-dimensional structures shown are provided to illustrate the various side groups substitutions on the test compounds. The compounds may be stable in one or more three-dimensional structures, and recent data suggest that the benzylidene and tetrahydropyridyl rings are not likely to be coplanar. For more detailed discussion of BA structures, see Kem et al. (2004)

However, the concentration-response curves for human and rat α7 receptors are nonetheless significantly different (Fig. 4A). The activation of rat receptors is best fit with a steeper Hill slope (2.4 versus 1.3 for human) and with a lower EC_{50} (20 versus 40 μM for human), suggesting that anabaseine does discriminate between human and rat α7 receptors in potency.

The full concentration-response curves for 4-OH-BA are shown in Fig. 4B. The efficacy of 4-OH-BA for rat α7 receptors was no more than for human α7 receptors; however, as with both GTS-21 and anabaseine, 4-OH-BA showed a shift to lower potency for human compared with rat. It is interesting that BA and 4-OH-GTS-21 showed efficacy differences but 4-OH-BA did not (Fig. 3B). The difference between 4-OH-GTS-21 and 4-OH-BA is that the 4-OH-GTS-21 has an additional substitution of a methoxy group at the 2 position of the benzylidene. 4-NH_2-BA is another compound with a polar substitution at the 4 position and no side group at the 2 position. As shown in Fig. 4C, this agonist was also equally efficacious for human and rat α7 receptors, consistent with the hypothesis that in the absence of a hydrophobic substitution at the 2 position, a polar substitution at the 4 position may promote increased efficacy for human receptors compared with rat without changing the potency difference present with anabaseine.

Multiple Determinants of GTS-21 Efficacy in the Extracellular Domain of A7. We used GTS-21 as our test agonist to evaluate the importance of the specific sequence

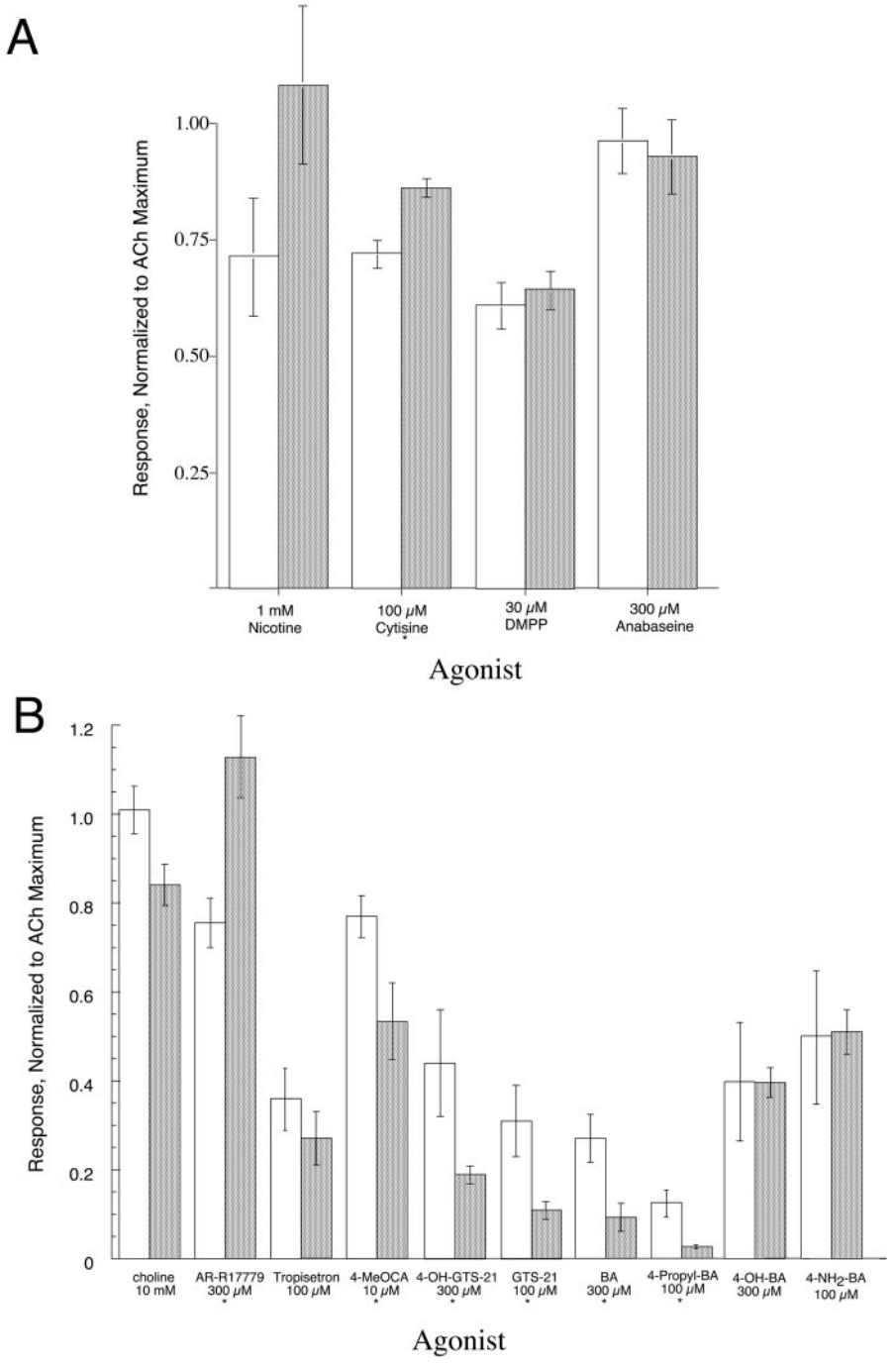

Fig. 3. A, responses of human (filled bars) and rat (open bars) α7 receptors expressed in *X. laevis* oocytes to selected nicotinic agonists at saturating concentrations indicated. A significant difference was observed only in the responses to cytisine ($p < 0.05$). B, responses of human and rat α7 receptors expressed in *X. laevis* oocytes to α7-selective agonists, applied at saturating concentrations, normalized to maximal ACh responses. Human α7 receptors responded better than rat receptors to AR-R17779 ($p < 0.05$). Rat receptors responded better than human receptors to 4-OH-GTS-21, GTS-21, 4 MeO-CA, 4-propyl-BA, and BA ($p < 0.05$, $p < 0.01$, $p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively). Each bar represents the average ± S.E.M. of the responses of at least four oocytes, normalized to 300 μM ACh responses obtained in the same cells.

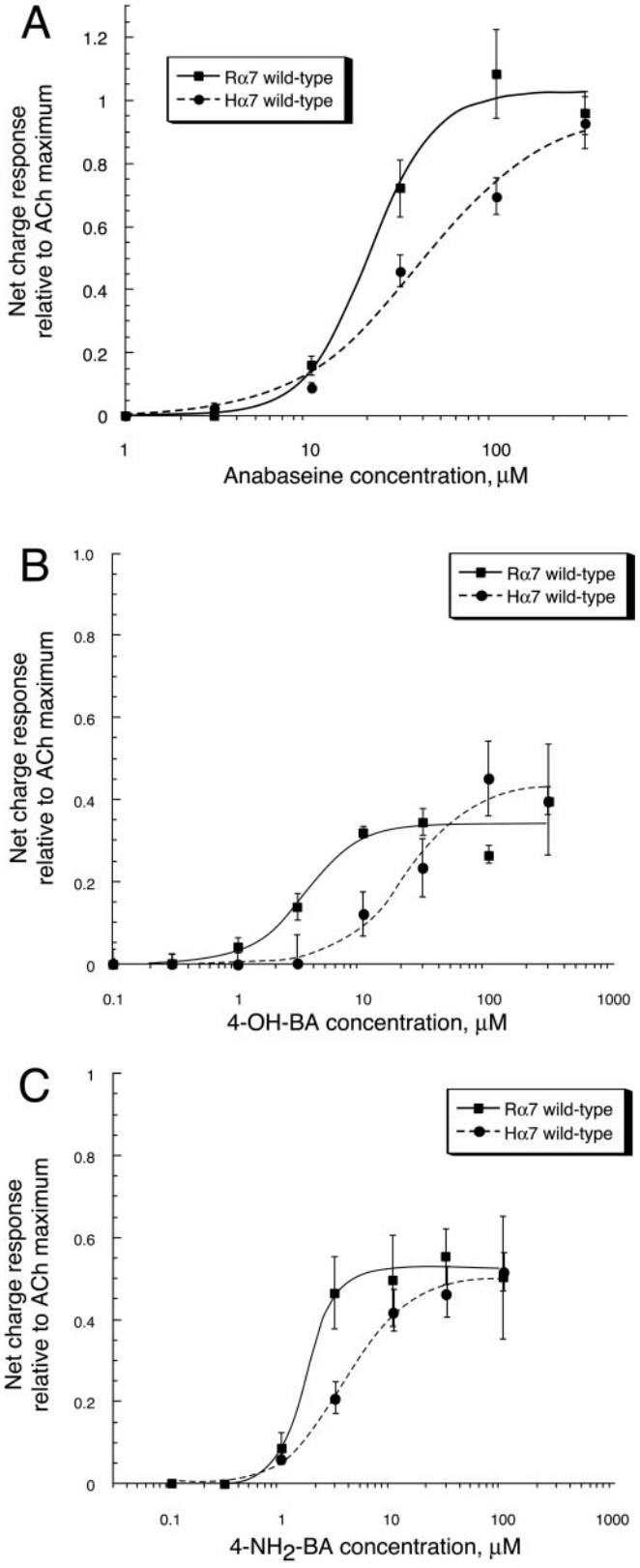

Fig. 4. A, responses of human and rat α7 receptors expressed in *X. laevis* oocytes to anabaseine. B, responses of human and rat α7 receptors expressed in *X. laevis* oocytes to 4-OH-BA. C, responses of human and rat α7 receptors expressed in *X. laevis* oocytes to 4-NH_2-BA. Data were normalized to the net charge of control 300 μM ACh responses obtained 5 min before the experimental agonist-evoked responses. Each point represents the average ± S.E.M. of the normalized responses of at least four oocytes.

differences that exist between these receptors in the domains proposed to contribute to the agonist binding site. We first tested a series of chimeras that exchanged the human and rat sequences. The chimeras either completely exchanged the extracellular domains (HHR and RRH) or exchanged roughly 50% of the sequence in the extracellular domain (HRR, RHH, HRH, and RHR; see *Materials and Methods* for splice sites).

Chimeras that exchanged the entire extracellular domain showed GTS-21 efficacy and potency that was indistinguishable from the wild-type receptor that was the source of the extracellular domain (Table 2). Putting the first half of the rat α7 extracellular domain into the human α7 (RHH) also produced receptors that responded to GTS-21 with an efficacy as high as that for the wild-type rat receptor. Putting the first half of the human α7 sequence into the rat α7 (HRR) had the effect of partially decreasing the efficacy of GTS-21 and also shifting the potency to that of the wild-type human receptor (Table 2).

Chimeras that exchanged the internal half of the extracellular domain (HRH and RHR) showed partial effects on GTS-21 pharmacology. The RHR receptors had fewer GTS-21 responses than the wild-type rat receptor and more than the wild-type human receptor (Table 2). However, the reciprocal chimera, HRH, was not significantly different from the wild-type human receptor (Table 2).

Effects of Mutations in the Agonist Binding Loops. The relative importance of the sequence differences in the putative C, E, and F loops of the ACh binding site were evaluated by testing mutant receptors that exchanged hu-

TABLE 2
GTS-21 curve fit values

I_{max} is expressed relative to the maximum response evoked by ACh. Specifically, because previous studies (Papke and Papke, 2002) have shown that the ACh concentration used for our controls (300 μM) produces maximal net charge responses, I_{max} values for the GTS-21 evoked responses were simply calculated relative the control ACh responses. Error estimates are based on the goodness of fit between the data plotted and the modified Hill equation (see *Materials and Methods*). Values are presented as mean ± S.E.M.

	I_{max}	n	EC_{50}
			μM
Hα7	0.09 ± 0.02	1.6 ± 1.0	11.0±5.7
RHHα7	0.44 ± 0.05	2.2 ± 0.3	12.4±0.7
HRHα7	0.11 ± 0.01	4.8 ± 0.8	7.8±0.4
RRHα7	0.36 ± 0.03	7.7 ± 1.3	7.4±1.5
HHRα7	0.10 ± 0.01	12.6 ± 10.0	6.2±1.3
RHRα7	0.17 ± 0.02	2.3 ± 0.8	4.9±1.0
HRRα7	0.15 ± 0.02	2.0 ± 0.8	20.0±6.0
Rα7	0.32 ± 0.02	2.4 ± 0.5	5.2±0.6
Human			
Wild types	0.09 ± 0.02	1.6 ± 1.0	11±5.7
C	0.24 ± 0.02	1.2 ± 0.2	25±4.3
E	0.09 ± 0.02	2.3 ± 2.1	7.4±3.4
F	0.19 ± 0.01	2.1 ± 0.4	12±0.3
EF	0.22 ± 0.01	2.0 ± 0.4	5.3±0.6
CEF	0.32 ± 0.01	1.1 ± 0.2	15±2.8
CF	0.42 ± 0.04	1.9 ± 0.6	21±8.0
C184 + F	0.14 ± 0.01	1.9 ± 0.5	10±1.5
C186 + F	0.16 ± 0.02	1.6 ± 0.7	4.6±1.4
Rat			
Wild types	0.32 ± 0.02	2.4 ± 0.5	5.2±0.6
C	0.27 ± 0.01	2.3 ± 0.5	8.7±0.8
E	0.27 ± 0.01	2.9 ± 0.4	6.7±0.3
F	0.24 ± 0.01	1.9 ± 0.4	13±0.2
EF	0.35 ± 0.03	1.9 ± 0.4	25±5.1
CEF	0.08 ± 0.02	1.0 ± 0.3	7.2±3.2
CF	0.14 ± 0.02	1.5 ± 0.4	8.5±2.2
C184 + F	0.13 ± 0.02	2.0 ± 4.0	7.0±2.3
C186 + F	0.06 ± 0.01	2.0 ± 2.0	10±3.0

man and rat residues in each single loop. As shown in Fig. 5A, the mutation of rat α7 to the human α7 sequence in the C loop decreased the efficacy of GTS-21 somewhat but not significantly. Mutations of the human α7 to the sequence of the rat α7 in the C loop did increase efficacy of GTS-21 compared with that of the wild-type human α7 ($p < 0.05$ at 100 and 300 μM GTS-21). However, human and rat exchanges in the C loop residues did not have major effects on the potency of GTS-21 for either rat or human α7 (Fig. 5A, Table 2).

Human and rat exchanges in the E loop residues alone had no effects on the potency or efficacy of GTS-21 for either rat or human α7 (Fig. 5B). Whereas the F-loop mutation changed the rat α7 (Rα7F) responses to GTS-21 to be more human-like in potency (Fig. 5C), the maximum responses were not significantly different from wild-type. In contrast, the reciprocal mutation in the human sequence (Hα7F) increased the maximal responses to GTS-21 above those of the wild-type human receptor ($p < 0.05$). Combining the E-loop mutation with the F-loop mutation had one interesting effect that was not observed with the F-loop mutation alone (Fig. 5D); although this combination affected the efficacy of GTS-21 only with the double mutant of the human α7 (Hα7EF), similarly to Hα7F, both the human and rat α7 EF mutants showed shifts in potency toward the potency of the reciprocal wild-types (Table 2).

A pair of mutants, H/Rα7CEF, was constructed that combined all four single point mutations to reverse all of the sequence differences in the proposed agonist binding loops (see Fig. 1). As shown in Fig. 5E, these exchanges were effective at changing the pharmacology of the resulting receptors such that the efficacy of GTS-21 for Rα7CEF was reduced to the level of wild-type human α7 and the efficacy of GTS-21 for Hα7CEF was increased to that of wild-type rat α7. However, although the potency of the Rα7CEF was reduced to the level of wild-type human α7, the potency of the Hα7CEF was not increased compared with that of wild-type human α7 and was less than that with the Hα7EF mutant (Fig. 5D).

Because the combination of mutations in all three binding loops produced a full reversal of GTS-21 efficacy, we next tested the hypothesis that the C and F loops were the specific key to this effect. As shown in Fig. 5F, combining the two mutations in the C loop with the single mutation in F was also effective at reversing the efficacy ratio for GTS-21 (see Table 2). This suggests that although the E-loop site may work in concert with the F-loop site to regulate potency, it does not seem to be key to determining GTS-21 efficacy.

In a further analysis of the interactions between the two residues in the C loop that differ between human and rat α7, and the single amino acid difference in the F loop, we created mutants that exchanged the F loop residue along with one or the other of the two differing C loop residues. As shown in Fig. 6, change at either C loop residue (184 or 186) in conjunction with the F-loop mutant (residue 167), produced a decreased GTS-21 efficacy for the rat, but neither combination produced a significant increase for human. It is noteworthy that the 186 C-loop mutation in combination with the 167 F-loop mutation did seem to reverse the potency differences so that the rat K186R,S167G mutant was like the human wild type with both low GTS-21 efficacy and potency, whereas the human R186K,G167S mutant had a GTS-21 potency similar to that of the rat wild-type receptor.

Evaluation of the Size and Polarity of the F-Loop Site. Because the F-loop mutants (at position 167) produced interesting, albeit intermediate, effects in both the human and rat α7, an additional pair of mutants was constructed to evaluate the importance of this residue in each wild type by replacing the wild-type residues with alanines: Hα7G167A and Rα7S167A. The serine residue of the wild-type rat α7 is both larger and more polar than the glycine residue of the wild-type human α7. Alanine is comparable in size with serine but is nonpolar. If the size of the residue at the F-loop site is the critical factor, then a substitution of alanine for the glycine in human might serve to increase GTS-21 efficacy. If the polarity of serine in the rat sequence is important, then an alanine-for-serine substitution in the rat would be predicted to decrease GTS-21 efficacy. As shown in Fig. 6C, this substitution had pronounced effects on potency for both human and rat α7 receptors and no apparent effects on efficacy. Note that the Hα7G167A data could not be curve-fit with the Hill equation over the range of concentrations tested (the highest concentration of GTS-21 tested was the limiting concentration for aqueous solubility).

Discussion

Our electrophysiological studies define EC_{50} and I_{max} values for GTS-21 activation of each of the wild-type and mutant receptors. These values may represent, respectively, affinity for the activatable states of the receptor and the efficiency with which binding is translated to channel activation (i.e., potency and efficacy). Because we see different structural features of the human and rat receptors selectively affecting EC_{50} differences and I_{max} differences, it is an attractive hypothesis that our mutation studies separate out features that differentially regulate GTS-21 binding and the subsequent activation of human and rat α7 receptors. Nonetheless, it should be noted that for a given receptor, other factors can also affect the measured EC_{50} and I_{max} values. For example, it has been shown (Colquhoun, 1998) that order of magnitude changes in EC_{50} can result from changes in opening and closing rate constants, changes that would typically be considered to impact estimates of efficacy. Likewise, differences in desensitization can affect macroscopic concentration-response relationships. In particular, differences in equilibrium desensitization may manifest as apparent differences in I_{max}. Another factor influencing the concentration response relationships of α7 receptors to various agonists is the phenomenon of residual inhibition, which may represent a particularly long-lived form of desensitization or noncompetitive inhibition (Papke, 2002; Uteshev et al., 2002). However, although some mutations in α7 that affect desensitization rates have been described (Revah et al., 1991), all of the mutants and chimeras in this study showed similar concentration-dependent rapid desensitization and similar sensitivity to the residual inhibition produced by GTS-21. Although in many cases, single-channel recordings can be used to discriminate between effects on binding and activation rate constants, unfortunately, the rapid desensitization of α7 receptors and prolonged inhibitory after-effects of GTS-21 preclude the utility of single-channel experiments for the present study.

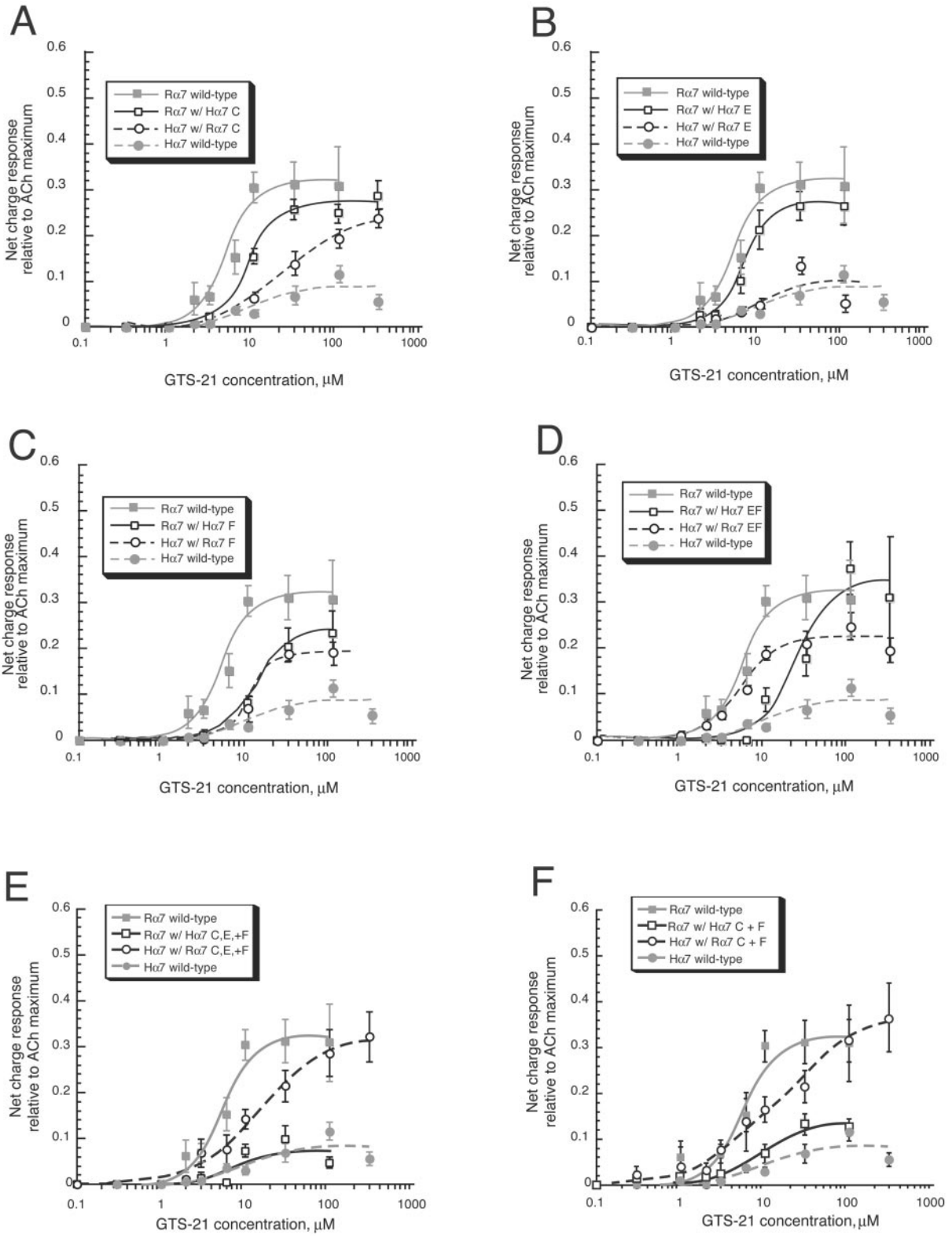

Fig. 5. Responses of mutant human and rat α7 receptors expressed in *X. laevis* oocytes to GTS-21, compared with the results obtained with wild-type human and rat α7. A, data from mutants in which the sequence had been exchanged in the putative C binding loop. B, data from mutants in which the sequence had been exchanged in the putative E binding loop. C, data from mutants in which the sequence had been exchanged in the putative F binding loop. For 30 and 100 μM GTS-21, wild-type human α7 and Hα7 F were significantly different. ($p < 0.05$). D, data from mutants in which the sequence had been exchanged in both the putative E and F binding loops. For 30 and 100 μM GTS-21, wild-type human α7 and Hα7 EF were significantly different. ($p < 0.01$). E, comparison of the responses obtained with human and rat α7 mutants in which the sequence had been exchanged in the putative C, E, and F binding loops with responses obtained with wild-type human and rat α7 receptors expressed in *X. laevis* oocytes to GTS-21. F, comparison of the responses obtained with human and rat α7 mutants in which the sequence had been exchanged in just the putative C and F binding loops with responses obtained with wild-type human and rat α7 receptors expressed in *X. laevis* oocytes to GTS-21. For all concentrations ≥10 μM, GTS-21, wild-type rat α7, and Rα7 CF were significantly different ($p < 0.01$), and wild-type human α7 and Hα7 CF were significantly different ($p < 0.01$). In all cases, data were normalized to the net charge of control 300 μM ACh responses obtained 5 min before the experimental agonist-evoked responses. Each point represents the average ± S.E.M. of the normalized responses of at least four oocytes.

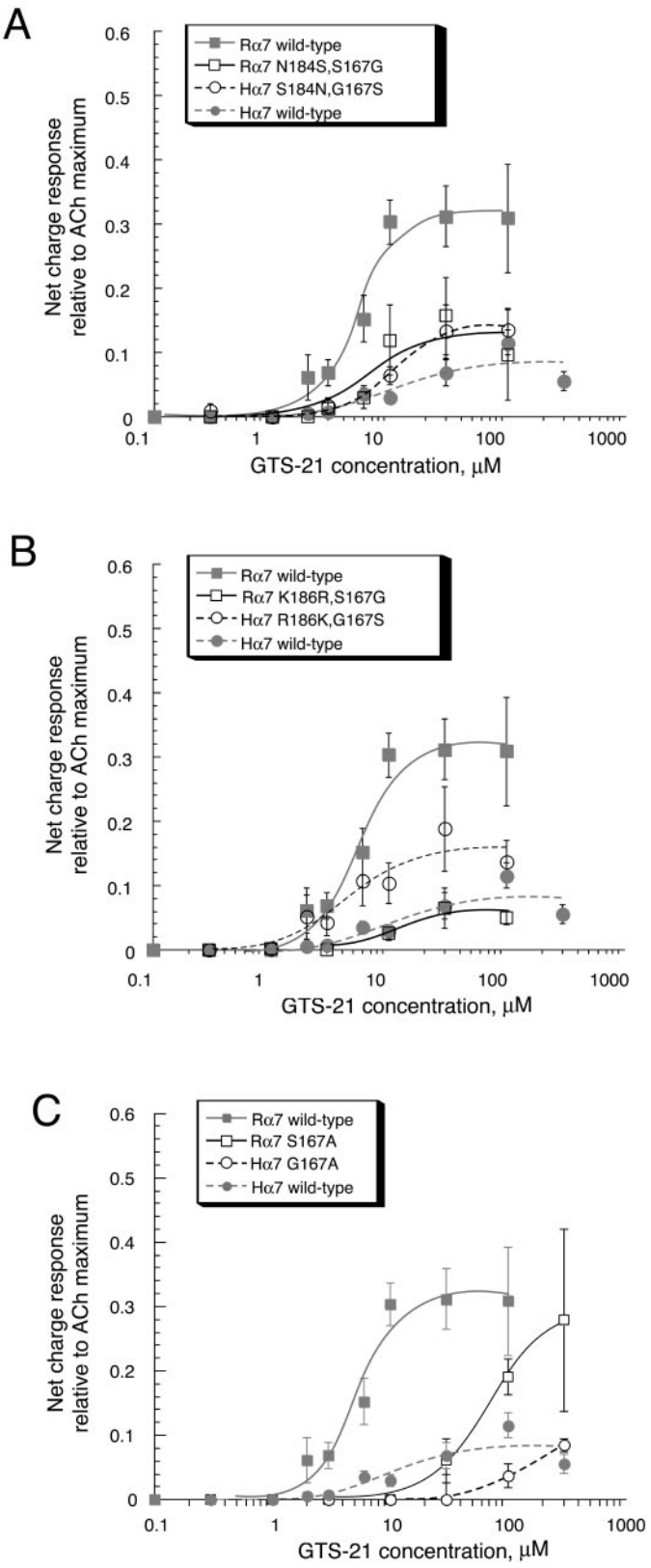

Fig. 6. Comparison of the responses obtained with wild-type human and rat α7 receptors expressed in *X. laevis* oocytes to GTS-21 with the responses obtained with human and rat α7 mutants in which the sequence had been exchanged in the putative F binding loop and one of the two sites within the C binding loop. A, data for mutants in which the sequences were exchanged at residues 167 and 184. B, data for mutants in which the sequences were exchanged at residues 167 and 186. Data were normalized to the net charge of control 300 μM ACh responses obtained 5 min before the experimental agonist-evoked responses. Each point represents the average ± S.E.M. of the normalized responses of at least four oocytes. C, responses of F-loop alanine mutant human and rat α7 receptors expressed in *X. laevis* oocytes to GTS-21, compared with the results obtained with wild-type human and rat α7. Data were normalized to the net charge of control 300 μM ACh responses obtained 5 min before the experimental agonist-evoked responses. Each point represents the average ± S.E.M. of the normalized responses of at least four oocytes.

Benzylidene anabaseine agonists can be considered to have two structural components, the anabaseine core and the benzylidene substituents that are covalently bound to the 3 position on the tetrahydropyridyl ring of anabaseine. Although the anabaseine core agonist can activate multiple nAChR subtypes, the addition of the benzylidene or cinnamylidene ring structure creates a selectivity for α7-type neuronal nAChR (Papke et al., 2004a). Human and rat α7 receptors respond differently to the core agonist in potency, and most benzylidene or cinnamylidene molecules differ in both potency and efficacy for human compared with rat α7 receptors. It is possible that the difference in GTS-21 potency for human and rat receptors may simply reflect the same factors that regulate anabaseine potency.

The combination of the E- and F-loop mutations that reversed the potency difference for GTS-21 between human and rat α7 receptors have the effect of reversing the disposition of serine residues in these two domains. In the human wild type, there is a serine in the E loop but not the F loop and in the rat, vice versa. From the models created by our group and others (Brejc et al., 2001) it seems that there are two separate entry pathways into the agonist binding pocket of α7 receptors. In our models of the α7 extracellular domain, it seems that the E and F loops are located at either end of a channel that might provide access to the binding pocket from two directions (Fig. 7A). Placement of the serine in the F loop, absent the presence of a serine in the E loop, could hypothetically favor easier access of the anabaseine compound to its binding site, perhaps increasing on-rates, or alternatively result in tighter binding through slowing dissociation rates.

Although the addition of both of the C-loop mutations to the E- and F-loop mutations somehow negates some of the effects of the E- and F-loop mutations on potency, they do establish the essential features necessary for regulating the efficacy of GTS-21. Evaluation of the CF mutants shows that the C- and F-loop sequences and how they interact with the agonist are key factors in the efficacy of BA compounds for human and rat α7 receptors. It seems likely that coordination between the C and F loops by the agonist is required during gating and/or activation. As shown in Fig. 7C, the residues 184 to 186 of the C loop lie closest to the lower access pathway to the binding site, which is the pathway located furthest from the synapse. The bulkier, longer arginine residue in human at position 186 (compared with lysine in rat) might result in partial occlusion of this point of entry into the agonist binding domain. On the other hand, the serine in rat loop F might H-bond with the charged side chain of the aspartic acid residue three amino acids upstream. This could stabilize the loop between these residues and thereby open the pathway into the agonist-binding pocket. However, although we hypothesize that the concerted effects of E and F mutations are caused by changes in the access pathway to the binding site and those of the C and F by changes in the binding site itself, it is also possible that the mutations could

be acting at a distance, by changing the orientation of other residues that may directly interact with the reagents, either through regulating access to the binding site or modulating the signal transduction process. Given that rodents and humans diverged approximately 100 million years ago, it is possible that distinct post-translational modifications have also arisen in rats and humans that could affect both potency and efficacy of different agonist types.

It is interesting to note that 4-OH-BA and 4-OH-GTS-21, which are structurally identical except for an *O*-methyl group, have similar relative efficacy compared with ACh for rat; however, whereas the 4-OH-BA is nonselective between rat and human, 4-OH-GTS-21 is selective. Structural differences between 4-OH-BA and 4-OH-GTS-21 may therefore point to specific aspects of the protein-ligand interactions. Two factors may be proposed to account for the selectivity of 4-OH-GTS-21 between rat and human receptors compared with 4-OH-BA. First, the addition of a methoxy group at the 2-position adds a potential hydrogen bond acceptor that may differentially H-bond with rat and human forms of the receptor based on both the preferred conformation of the ligand (see above) and specific residues within the binding site or access pathway. Another possible factor to account for discrimination of the 2-methoxy BAs is that the addition of the OCH_3 group adds steric bulk, which may differentially interact with E- and F-loop serines. The interaction could involve repulsive steric clash and/or favorable van der Waals interactions.

As we have shown in the present study, the differences in sequence that exist in the human and rat extracellular domains are of little consequence for efficacy of agonists with only a polar substitution in the 4 position of the benzylidene (Fig. 4) or for other nonselective and α7-selective agonists (Fig. 3). Our observation that the two C-loop mutations, together with the F-loop mutation in the human α7 receptor, have a greater impact on efficacy than either single C-loop mutation in combination with the F-loop site suggests that the amino acids at both of these positions in the C loop work together with the F loop in the respective organisms either directly at the agonist binding site or through effects at a distance that regulate channel activation. Indeed, our results obtained with the alanine glycine/serine substitutions (Fig. 6) suggest that simple interpretations based on the properties of individual amino acids may neglect important intramolecular effects.

In conclusion, we have identified important domains in the

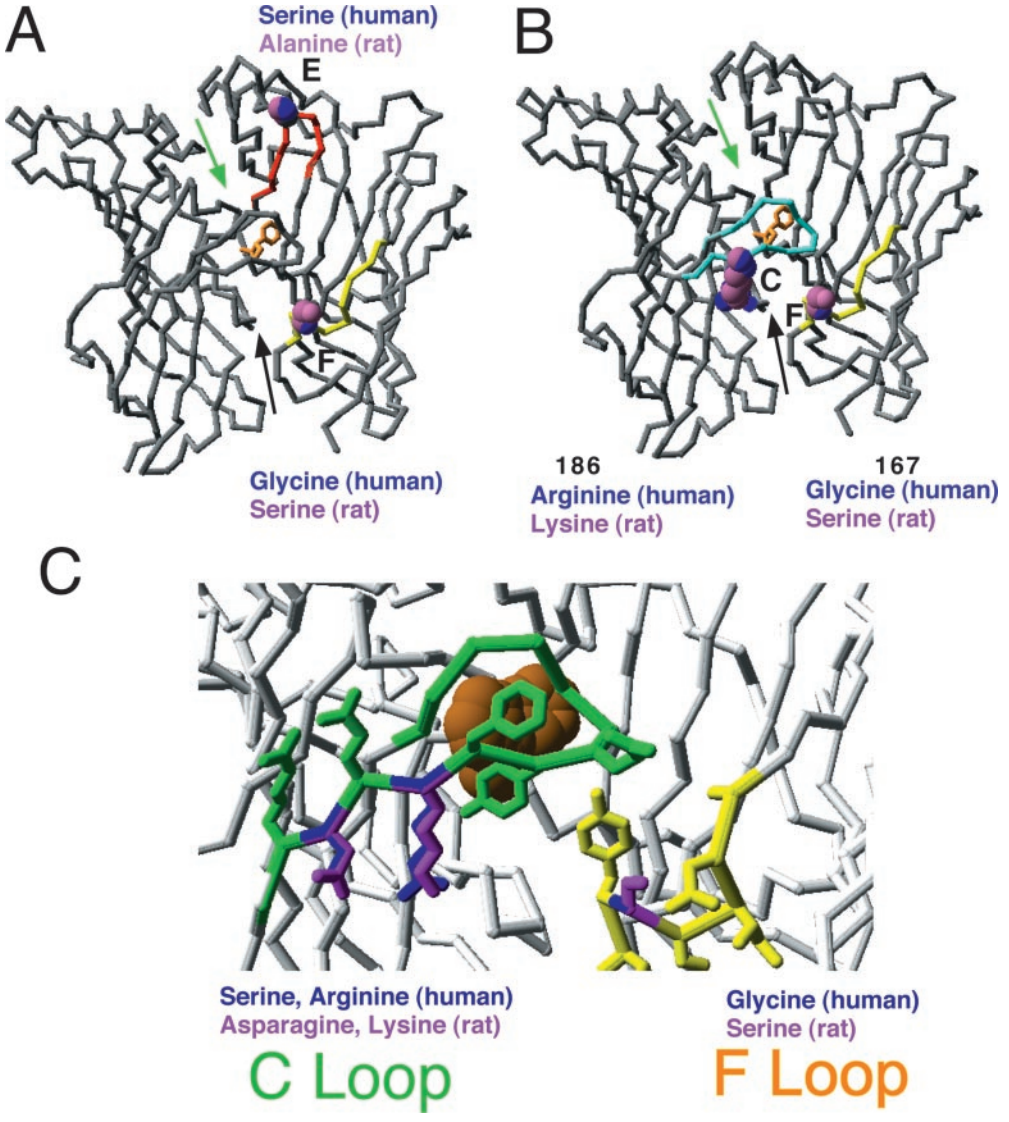

Fig. 7. A, comparison of loops E and F in structural models of rat and human α7 receptors. The two amino acid differences between rat (pink residues) and human (blue residues) in the two loops are shown as space-fill representation. A green arrow is shown near the putative upper entry pathway into the agonist-binding pocket and the black arrow is shown at the lower. Nicotine is shown in orange in its expected orientation within the agonist-binding domain. B, comparison of the residues in the 186 C-loop position and F-loop 167 residues in structural models of rat and human α7 receptors. The two amino acid differences between rat (pink residues) and human (blue residues) in the two loops are shown as space-fill representation. As in A, the green arrow is shown near the putative upper entry pathway into the agonist-binding pocket, whereas the black arrow is shown at the lower. Nicotine is shown in orange in its expected orientation within the agonist-binding domain. C, comparison of loops C and F in models of rat and human α7 receptor. The amino acid differences between rat (pink residues) and human (blue residues) in the two loops are illustrated. Loop C is green and the loop-C amino acid side chains are shown. Loop F is colored yellow and the side chains of residues in this loop are shown as well. All side chains are represented as "sticks". Only the backbone is shown for other regions of the molecule. The BA agonist is colored orange and in this model adopts the same binding orientation as the HEPES molecule from the acetylcholine binding protein structure (Brejc et al., 2001).

α7 receptor that are involved with the pharmacological differences between human and rat forms of the receptor. In addition to providing insights into the α7 binding site, our studies also bring up a number of interesting questions and suggest future experimental directions. For example, how do the specific groups on the benzylidene ring interact with residues in the principle and complementary binding sites? We have reported previously that specific substitutions on the benzylidene ring can have large effects on agonist efficacy and potency (Papke and Papke, 2002). It is important to note that some changes in the benzylidene substitutions have parallel effects on both human and rat α7 receptors. For example, the conversion of GTS-21 to 4-OH-GTS-21 results in a partial agonist with increased efficacy for both human and rat α7 receptors that retains the relative potency differences between human and rat receptors seen with GTS-21 (Papke and Papke, 2002). As our ability to model the extracellular domain of the nAChR is further refined, it is likely that the BA compounds will prove to be valuable research tools to define not only the nature of the binding site but also the conformational changes that occur after agonist binding to produce receptor activation.

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