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European Journal of Pharmacology 524 (2005) 11 – 18
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Rhesus monkey α7 nicotinic acetylcholine receptors: Comparisons to human α7 receptors expressed in *Xenopus* oocytes

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Received 12 May 2005; received in revised form 22 August 2005; accepted 29 August 2005

Abstract

An α7 nicotinic acetylcholine receptor sequence was cloned from Rhesus monkey (*Macaca mulatta*). This clone differs from the mature human α7 nicotinic acetylcholine receptor in only four amino acids, two of which are in the extracellular domain. The monkey α7 nicotinic receptor was characterized in regard to its functional responses to acetylcholine, choline, cytisine, and the experimental α7-selective agonists 4OH-GTS-21, TC-1698, and AR-R17779. For all of these agonists, the EC_{50} for activation of monkey receptors was uniformly higher than for human receptors. In contrast, the potencies of mecamylamine and MLA for inhibiting monkey and human α7 were comparable. Acetylcholine and 4OH-GTS-21 were used to probe the significance of the single point differences in the extracellular domain. Mutants with the two different amino acids in the extracellular domain of the monkey receptor changed to the corresponding sequence of the human receptor had responses to these agonists that were not significantly different in EC_{50} from wild-type human α7 nicotinic receptors. Monkey α7 nicotinic receptors have a serine at residue 171, while the human receptors have an asparagine at this site. Monkey S171N mutants were more like human α7 nicotinic receptors, while mutations at the other site (K186R) had relatively little effect. These experiments point toward the basic utility of the monkey receptor as a model for the human α7 nicotinic receptor, albeit with the caveat that these receptors will vary in their agonist concentration dependency. They also point to the potential importance of a newly identified sequence element for modeling the specific amino acids involved with receptor activation.

Keywords: Voltage-clamp; Alzheimer's disease; Acetylcholine binding protein; α7 nicotinic receptor

1. Introduction

The α-bungarotoxin sensitive α7-type nicotinic acetylcholine receptor is expressed throughout the brain and also in the peripheral nervous system and some peripheral tissues (Sharma and Vijayaraghavan, 2002). In the brain, α7nicotinic acetylcholine receptor are located in high concentrations in the hippocampus, neocortex, and hypothalamus as seen by binding sites (Clarke et al., 1985). The use of nicotinic agonists has improved delay matching in primates (Terry et al., 2002), eye blink memory in rabbits, and spatial-memory related behavior in rats, as well as social memory relationships in rats (Arendash et al., 1995a,b; Meyer et al., 1994; Van Kampen et al., 2004). Some mutations and/or splice variants of the α7 gene have been linked to a decrease in hippocampal auditory gating, which is a symptom of some schizophrenics and approximately 50% of their family members (Freedman et al., 1994, 2000). This may be due to the roles played by α7nicotinic acetylcholine receptor in the activation of GABAergic inhibitory interneurons in hippocampus (Adler et al., 1998; Frazier et al., 2003). The α7 nicotinic receptor may also be involved with the etiology and/or possible treatment of other conditions such as Alzheimer's Disease and Down's Syndrome. Nicotinic receptor agonists have been shown to improve memory and are neuroprotective. Moreover, α7 has been found to coprecipitate with the $A\beta_{1\text{-}42}$ within the histopathological amyloid beta plaques (Wang et al., 2000) and the functional interactions between $A\beta_{1\text{-}42}$ and α7

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doi:10.1016/j.ejphar.2005.08.043

nicotinic acetylcholine receptor (Liu et al., 2001) further support α7 nicotinic acetylcholine receptor as a therapeutic target for Alzheimer's Disease.

The adverse side effects of nicotine or other non-selective cholinergic agonists have promoted the development of more selective α7 agonists for therapeutics. GTS-21 (2,4-dimethoxybenzylidene anabaseine or DMBX), one such selective agonist was tested in phase 1 clinical trials and was found to have no adverse side effects and to increase cognitive functioning in healthy subjects (Kitagawa et al., 2003). With the identification of α7 nicotinic receptors as potential therapeutic targets has also come the need to develop animal models for the testing of novel therapeutic agents. While rodent models are most commonly used, there are numerous pharmacological differences between rat and human α7 nicotinic receptors (Papke and Papke, 2002). Monkey models have the intrinsic advantage of being amenable to more complex behavioral testing than rodents and therefore may have special usefulness for evaluating potential drugs for human therapeutics. We report the cloning and functional characterization of a Rhesus monkey (*Macaca mulatta*) α7 nicotinic acetylcholine receptor (mkα7) in regard to its responses to a series of nicotinic agonists including acetylcholine (acetylcholine), choline, and cytisine, as well as the α7-selective agonists 4OH-GTS-21 (4-hydroxy 2-methoxybenzylidene anabaseine), TC-1698 (2-(3-pyridyl)-1-azabicyclo[3.2.2]nonane) and AR-R17779 ((−)-spiro[1-azabicyclo[2.2.2]octane-3′,5′-oxazolidin-2′-one) (Marrero et al., 2003; Meyer et al., 1998; Papke et al., 2004). These agonists had lower EC_{50}s for activating human α7 (hα7) nicotinic receptors than for monkey α7 (mkα7) nicotinic receptors. Only two amino acids differ between the mkα7 and hα7 sequence in the extracellular domain. Mutations were made of mkα7 sequence to the amino acids present in hα7, and the resulting mutants were tested to determine if changing either or both of the amino acids shifted the concentration–response relationships towards that of hα7.

2. Methods

2.1. Rapid amplification of cDNA ends (RACE) for the 5′- and, 3′-ends of rhesus monkey α7

To identify the 5′- and 3′-ends of rhesus monkey α7, four primers, mkα7-5′R (CTCATCTCCACGCTGGCCAGGTGCAG), mkα7-5′N (CGCACCTTATCCTCTCCCGGCCTCTTCATG), mkα7-3′R (CATGAAGAGGCCGGGAGAGGATAAGGTGCG) and mkα7-3′N (CTGCACCTGGCCAGCGTGGAGATGAG), were designed based on Genbank sequence AJ245976 and a polymerase chain reaction (PCR) was used with a Gene Racer cDNA library generated using rhesus monkey brain mRNA (Biochain). The cDNA fragments from the nested PCR were cloned and sequenced. The 3′ fragment contained a stop codon (TAA) and a polyA+ signal (AATAAA) indicating the end of the transcript. The 5′ fragment extended further upstream of AJ245976, however, failed to reach the starting Met. Additional primers were designed based on new sequence information and used for the second round of 5′ RACE. After the third 5′ RACE, the resulting cDNA fragment contained a Met and an in-frame stop codon, suggesting identification of the starting Met. Additional 5′ RACE primers: mkα7-5′R1 (GACCAGCCTCCATAAGACCAGGATCCAAACTTCAG), mkα7-5′N1 (CGCACGTCGATGTAGCAGGAACTCTTGAATATGC), mkα7-5′R2 (GGTTCTTCTCATCCGCGTCCATGATCTGCAG), and mkα7-5′N2 (GTAGACGGTGAGCGGTTGCGAGTCATTGG).

2.2. Full-length cloning of rhesus monkey α7

A cDNA contig of rhesus monkey α7was built by combining the sequences of the 5′ and 3′ RACE fragments. Two primers spanning the coding sequence of the monkey α7, a 5′ primer CTCAACATGCGCTGCTCGCAGGGAGG and a 3′ primer CCAAGCCAGAGGCCTTGCCCATCTGTGAG, were designed based on the contig and were used to PCR a monkey brain cDNA library. The resulting PCR product was cloned into pcDNA3.1 TOPO vector (Invitrogen) and confirmed by sequencing.

The full length clone we isolated has a predicted amino acid sequence that is 100% identical to that of the unpublished Genbank *M. mulatta* sequence AF486623, although differing by a total of 5 nucleotides in the open reading frame.

2.3. Sequence comparisons and selection of mutations

Two differences were noted within the extracellular region of the rhesus monkey α7 and human α7. Numbering the amino acids as for human α7 (vicinal cysteines at positions 190 and 191), these differing residues were located at positions 171 and 186. Two single point mutants were made in mkα7 (mkα7S171N and mkα7K186R) as well as the double mutant (mkα7S171N,K186R). Mutants were constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions, and were confirmed by automated fluorescent sequencing (University of Florida ICBR core facility, Gainesville, FL, USA).

2.4. Preparation of RNA

The hα7 clone was obtained from Dr. Jon Lindstrom (University of Pennsylvania), and the *M. mulatta* (rhesus) α7 cloned as described above. After linearization and purification of cDNA templates, RNA was prepared using the appropriate mMessage mMachine kit from Ambion, Inc. (Austin, TX, USA), according to the manufacturer's instructions.

2.5. Expression in Xenopus oocytes

The preparation of *Xenopus laevis* oocytes for RNA expression was conducted as previously described (Papke and Papke, 2002). In brief, mature (>9 cm) female *X. laevis* African frogs (Nasco, Ft. Atkinson, WI) were used as a source of oocytes. Prior to surgery, the frogs were anesthetized by placing the animal in a 1.5g/l solution of MS222 (3-aminobenzoic acid

ethyl ester) for 30 min. Oocytes were removed from an incision made in the abdomen.

In order to remove the follicular cell layer, harvested oocytes were treated with 1.25 mg/ml Type 1 collagenase (Worthington Biochemical Corporation, Freehold, NJ) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $MgS0_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.6), 50 mg/l gentamicin sulfate). Subsequently, stage 5 oocytes were isolated and injected with 50 nl (5–20 ng) each of the appropriate subunit cRNAs. Recordings were made 5 to 15 days after injection.

2.6. Chemicals

The source of the 4OH-GTS-21 was Taiho Pharmaceuticals (Tokyo, Japan) and TC-1698 was provided by Targacept (Winston-Salem, NC). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) with the exception of AR-R17779 which was synthesized and supplied by Memory Pharmaceuticals.

2.7. Electrophysiology

Experiments were conducted using OpusXpress 6000A (Axon Instruments, Union City, CA, USA). 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. 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 acetylcholine controls and experimental agonists. Applications were 12 s in duration followed by 181-s washout periods.

2.8. Experimental protocols and data analysis

Responses were calculated as net charge (Papke and Papke, 2002). Except where noted, each oocyte received two initial control applications of 300 μM acetylcholine, then an experimental drug application, and then a follow-up control application of 300 μM acetylcholine, a concentration which is sufficient to evoke a maximal net charge response (Papke and Papke, 2002). Responses to experimental drug applications were calculated relative to the preceding acetylcholine control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. Means and standard errors (S.E.M.) 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

$$\text{Response} = \frac{I_{\max}[\text{agonist}]^n}{[\text{agonist}]^n + (\text{EC}_{50})^n}$$

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} were all unconstrained for the fitting procedures, except in the case of the acetylcholine response curves. Since acetylcholine is our reference full

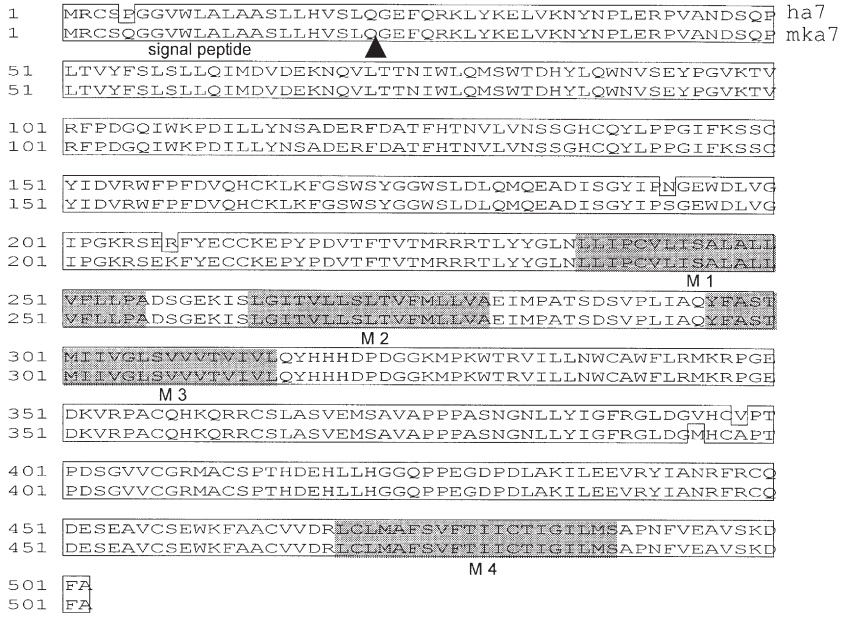

Fig. 1. Protein sequence comparison between human and monkey α7. The sequence alignment was created with Lasergene 6 software using ClustalV. The identical amino acids were boxed. The predicted signal peptide cleavage site was indicated by an arrow head. The predicted transmembrane helices were shaded and labeled as M1, M2, M3 and M4.

agonist, for the acetylcholine concentration response curves the data were normalized to the observed acetylcholine maximum and the I_{max} of the curve fits were constrained to equal one. For the evaluation of antagonists the control acetylcholine concentration used were the EC50 values of 30 μM and 70 μM for human and monkey α7, respectively. Significant differences between responses of the wild-type human and monkey receptors were determined by *t*-tests on the normalized data. Responses of the mutant receptors were compared in separate *t*-tests to the responses of each of the wild-types.

2.9. Molecular modeling

We created a structural model for the monkey and human α7 based on the structure of acetylcholine binding protein (PDB entry 1I9B). The agonist (nicotine) was drawn in 3-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 sidechains 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).

3. Results

3.1. The sequence of monkey α7 acetylcholine receptor

The cloned monkey α7 subunit is 502 amino acids long, with a calculated molecular weight of 56.4 kDa. There are five amino acid changes when compared to human α7 (Fig. 1). Two of the changes are localized in the extracellular N-terminal domain that involves in ligand binding; two more changes in the second intracellular loop between transmembrane domain M3 and M4; one more in the predicted signal peptide which is eliminated in mature receptor.

3.2. Electrophysiological responses of human and monkey α7 acetylcholine receptor

As shown in Fig. 2, both the human and monkey α7 nicotinic receptors responded well to the relatively high acetylcholine control concentrations. However while 30 μM acetylcholine and 300 μM Choline evoked responses from human receptors

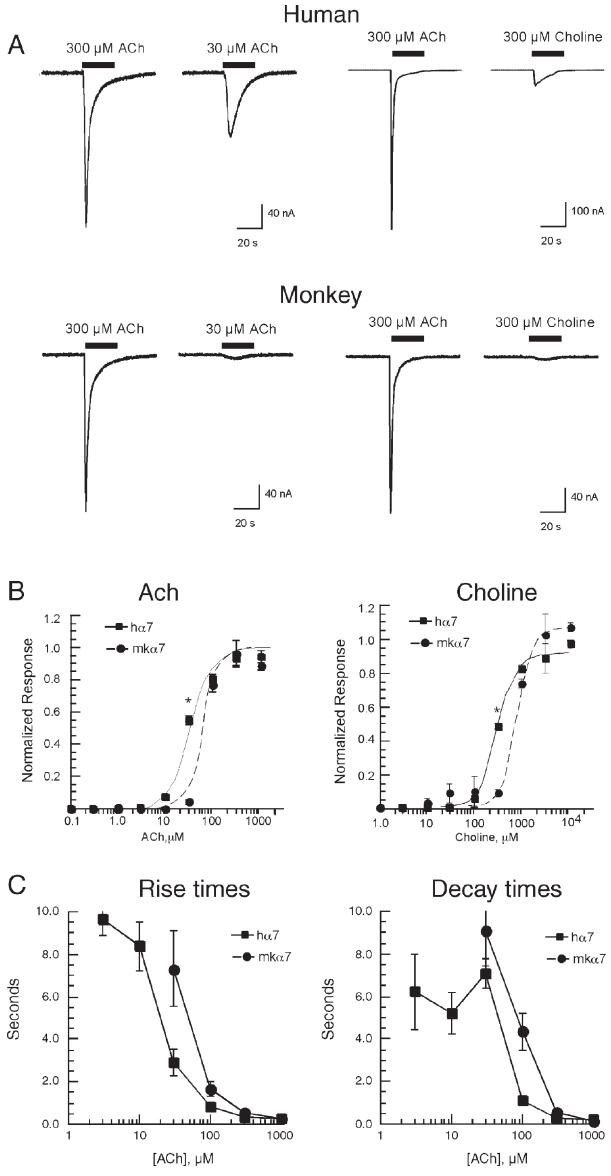

Fig. 2. (A) Representative responses of human (top) and monkey (bottom) α7 receptors expressed in *Xenopus* oocytes to applications of acetylcholine and choline. Both receptors gave similar robust responses to the saturating 300 μM acetylcholine control applications, however the relative responses of the human receptors to 30 μM acetylcholine (top) and 300 μM choline (bottom) were much greater than the responses of the monkey receptors. (B) Concentration response relationships of human and monkey α7 to the endogenous ligands acetylcholine and choline. For both acetylcholine (A) and choline (B), mkα7 is significantly shifted to the right from hα7. Data were normalized to the net charge of control 300μM acetylcholine 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 4 oocytes. Since acetylcholine is our reference full agonist the acetylcholine maximum responses were defined as 1 and the I_{max} values for the curve constrained to equal 1. Concentrations where the responses of the monkey and human α7 receptors to the same concentration of agonist differed significantly ($p<.01$) are indicated (*). (C) Kinetic parameters of human and monkey α7 acetylcholine-evoked responses. As shown on the left, the 10–90% rise time of acetylcholine evoked were several seconds long at relatively low acetylcholine concentrations for both human and monkey α7 responses and decreased to values less that a second at higher concentrations. Consistent with the reduced potency of acetylcholine for monkey rise times of the human responses to 30 μM acetylcholine were significantly faster than those of the monkey receptors ($p<0.05$) to the same acetylcholine concentration. However, the rise times of the monkey responses to 100 μM acetylcholine were not significantly different from the rise times of human responses to 30 μM acetylcholine. Similar results were apparent in the 90–50% fall times (right). Decay times of the human responses to 100 μM acetylcholine were significantly briefer than those of the monkey receptors ($p<0.01$) to the same acetylcholine concentration, while the decay times of the monkey responses to 300 μM acetylcholine were not significantly different from the decay times of human receptor responses to 100 μM acetylcholine.

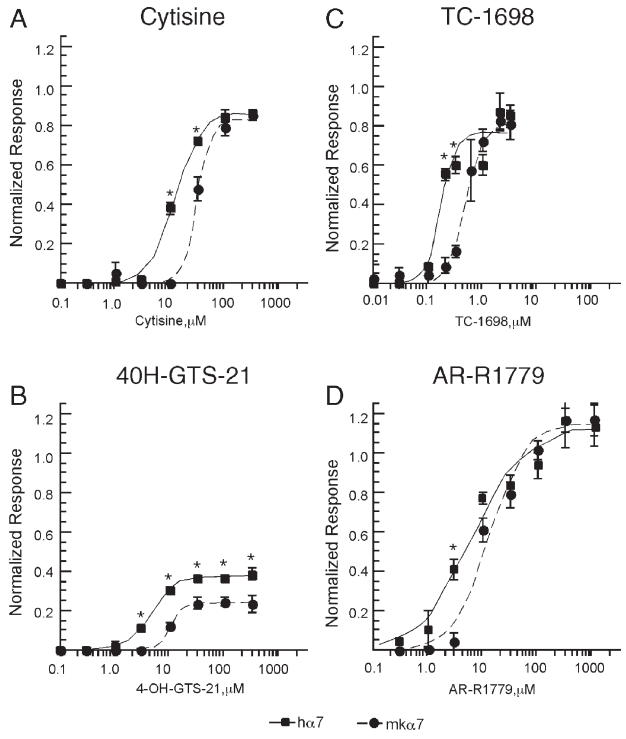

Fig. 3. Concentration response relationships of human and monkey α7 to experimental agonists. For the agonists tested: A) cytisine, B) 4OH-GTS-21, C) TC-1698, and D) AR-R17779, there is a significant potency shift to the right from hα7 to mkα7. Data were normalized to the net charge of control 300μM acetylcholine 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 4 oocytes. Concentrations where the responses of the monkey and human α7 receptors to the same concentration of agonist differed significantly ($p<.01$) are indicated (*).

that had approximately 50% the net charge of the 300 μM acetylcholine controls, the relative responses of monkey receptors were much lower. Fig. 2B shows the net charge concentration response relationships of wild-type human and monkey α7 to the endogenous agonists acetylcholine and choline. The EC_{50} values for these agonists were lower for hα7 than for mkα7 (Fig. 2B) and there were also differences in Hill slope (Table 1). We evaluated a series of experimental agonists; cytisine, 4OH-GTS-21, TC-1698, and AR-R17779, and in each case, the EC_{50} values were significantly higher for monkey α7 than for human α7 (Fig. 3 and Table 1). Specifically, for mkα7 the EC50 values of the agonists tested were on the average 232±13% (S.E.M.) of those for hα7.

The kinetics of macroscopic responses mediated α7 nicotinic acetylcholine receptor are strongly affected by the agonist concentration applied (Papke et al., 2000; Papke and Papke, 2002; Papke and Thinschmidt, 1998; Uteshev et al., 2002). Responses to high effective concentrations of agonist are very brief, showing both rapid rise and rapid decay with the most synchronous channel activation occurring during the rising phase of solution exchange. As shown in Fig. 2C, the rise time and decay times of the responses of monkey α7 nicotinic receptors show similar dependence on the concentration of agonist applied, with an appropriate shift reflecting the reduced potency of acetylcholine for activating monkey α7 nicotinic receptors compared to human α7 nicotinic receptors.

Two antagonists, mecamylamine and MLA (methyllycaconitine) were also evaluated. These antagonists had comparable potency for inhibiting monkey and human α7 (Fig. 4). The Mecamylamine IC_{50} values were 3.3±0.6 μM and 3.0±0.5 μM for monkey and human receptors, respectively and the MLA IC_{50} values were 13.9±1.2 nM and 13. 3±6.5 nM for monkey and human receptors, respectively.

3.3. Human and monkey sequence comparison

The sequences of the human and monkey α7 nicotinic receptor N-terminal extracellular domains are shown in Fig. 5. The proposed arrangement of helical and beta strands (Brejc et al., 2001) is indicated, as well as the putative agonist binding subdomains, including the proposed loops A–F (Corringer et al., 2000). Only two amino acids differ throughout this entire region of the mature protein. One of the point differences (186) is located in the putative C-loop of the positive face of the agonist binding site. The other point difference is near the F-loop of the complementary or negative face of the agonist binding site. This is the portion of the agonist binding site which is associated with the non-alpha subunits in heteromeric receptors.

In addition to the two amino acids which differ in the extracellular domain, there are also two amino acids differences

Table 1
Curve fit values[a] for wild-type human and monkey α7 nicotinic receptors

Agonist	Human receptor			Monkey receptor		
	EC_{50} μM	n	I_{max}	EC_{50} μM	n	I_{max}
Acetylcholine	36±5.1	2.1±0.3	1[b]	74±9.1	3.0±1.0	1
Choline	300±20	2.1±0.3	0.88±0.02	733±65	2.5±0.5	1.0±0.04
Cytisine	11.7±0.6	1.9±0.2	0.85±0.01	27.7± 1.5	3.8±1.8	0.82±0.03
4OH-GTS-21	4.7±0.2	1.9±0.1	0.38±0.01	10.0±0.2	4.2±1.7	0.24±0.01
TC-1698	0.16±0.02	3.2±1.1	0.77±0.05	0.46±0.02	2.85±0.27	0.82±0.02
AR-R17779	6.2±1.8	1.0±0.2	1.1±0.1	12.7±2.9	1.3±0.3	1.1±0.1

[a] Error estimates represent the 95% confidence values from the curve fits.
[b] Since acetylcholine is defined as the reference full agonist, the maximal responses obtained with acetylcholine were defined as 1, and the curve fits for acetylcholine were constrained to have I_{max} equal to 1. For all other agonists, I_{max} is relative to the saturating 300 μM acetylcholine control responses. Note that n represents the Hill coefficients of the fits.

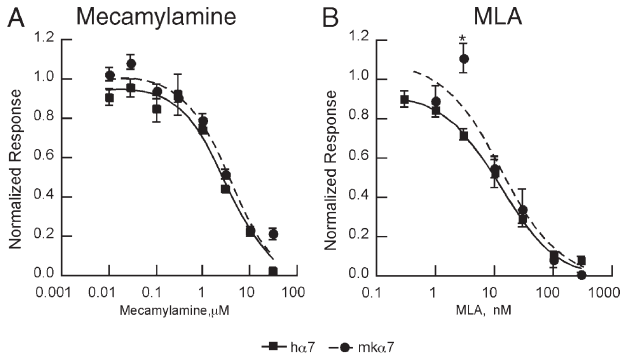

Fig. 4. Concentration response relationships of human and monkey α7 to the antagonists mecamylamine (A) and MLA (B). For these experiments the control acetylcholine concentration used were the EC50 values of 30 μM and 70 μM for human and monkey α7, respectively. Data were normalized to the net charge of the respective control acetylcholine responses obtained 5 min before the co-application of acetylcholine and antagonist at the indicated concentration. Each point represents the average±S.E.M. of the normalized responses of at least 4 oocytes. As indicated (*), monkey receptors responses to 70 μM acetylcholine showed significantly less inhibition ($p<.05$) by 3 μM MLA than did the responses of human α7 receptors to 30 μM acetylcholine.

in the intracellular domain (not shown), but no differences in the putative transmembrane domains.

To test the hypothesis that the sequence differences in the extracellular domain would be most important for the agonist activation differences seen, mutations were made (see methods) in the monkey sequence to switch these residues to those of the human sequence, either singly (mkα7S171N and mkα7K186R) or in tandem (mkα7S171N,K186R).

As shown in Fig. 6A the mkα7S171N,K186R mutant was not significantly different from hα7 in response to acetylcholine application, supporting the hypothesis that the difference in acetylcholine EC_{50} could be attributed to the two single amino acid differences between human and monkey sequences (see also Table 2). Analysis of the single point mutants indicated that the residue at 171 was potentially most important for the effects observed (Fig. 6B), while the mutation at position 186 had no apparent effect on the EC_{50} (Fig. 6C). We also evaluated responses of the monkey mutants to the α7-selective partial agonist 4OH-GTS-21. As shown in Fig. 6D, the double mutant mkα7S171N,K186R was not significantly different from hα7 in its responses to 4OH-GTS-21. However, while the mkα7K186R was essentially like the wild-type monkey receptor in its responses to 4OH-GTS-21 (Fig. 6F), the single point mutant mkα7S171N was apparently more responsive to this partial agonist than even the wild-type human α7 (Fig. 6E). It is interesting to note that while the S171N mutation was effective at shifting the concentration response curves to the left (Figs. 6B and E), the curves were not identical to the wild-type human α7.

4. Discussion

Human and monkey α7 differ significantly in their EC_{50} values for the endogenous agonists acetylcholine and choline, as well as for the experimental agonists, cytisine, 4OH-GTS-21, TC-1698, and AR-R17779. This basic observation should be considered if the rhesus monkey is used as a model system to test α7 agonists for human therapeutics. Specifically, our results suggest that any nicotinic agonist targeting α7 may be less potent in the monkey than it would be in humans, although antagonist activities would be similar.

The observation that the double mutant mkα7S171N,K186R does not differ significantly in EC_{50} for the agonists tested supports the hypothesis that the two amino acid differences in the extracellular domain account for much of the functional differences between the human and monkey receptors. However, it should also be noted that the monkey receptor has significantly higher Hill coefficients for most agonists than the human receptor. While the mutations do reduce the EC_{50}'s they appeared to have less effect on the Hill coefficients. The reason

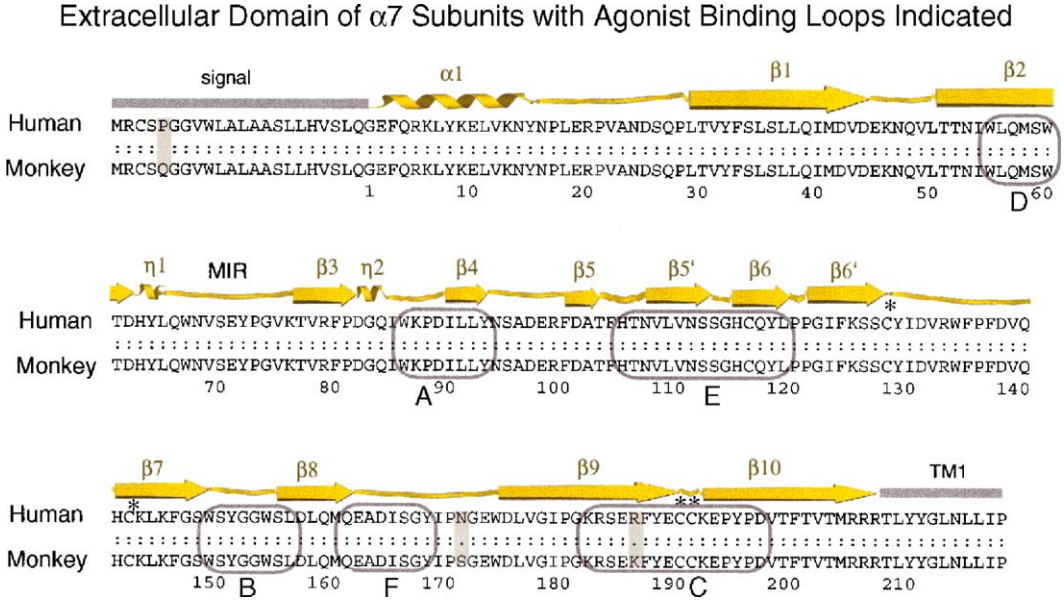

Fig. 5. Sequence alignment of human and monkey α7 sequence in the extracellular domain. The proposed arrangement of helical and beta sheet structures are indicated, as well as the putative agonist binding subdomains. Sites of sequence difference between human and monkey α7 are highlighted.

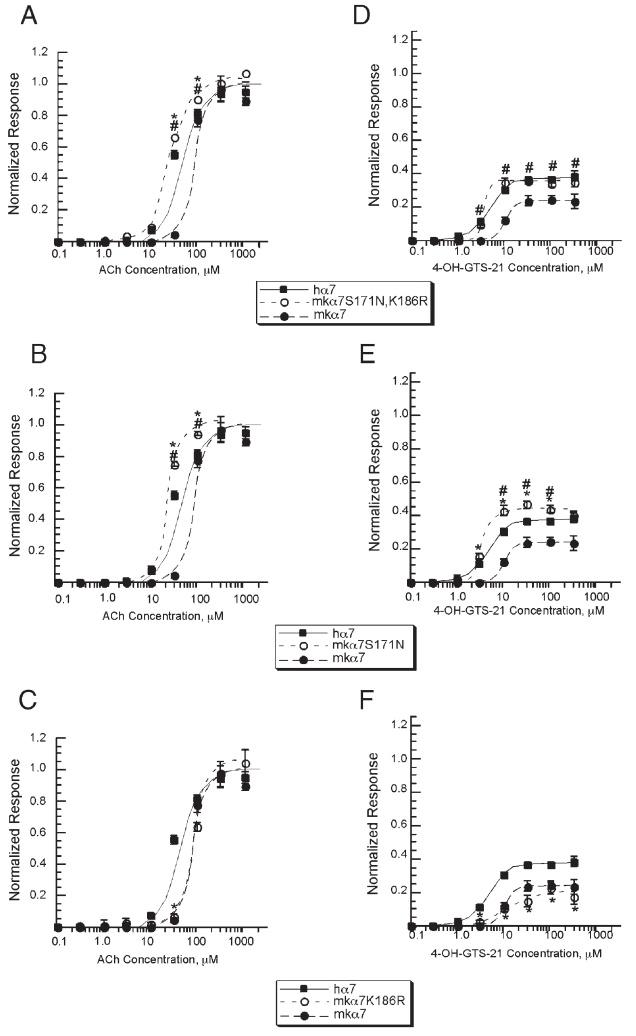

Fig. 6. The effect of mutations on the responses of mkα7 to acetylcholine and 4OH-GTS-21. The responses of the double mutant (mkα7S171N,K186R) were not significantly different from those of wild-type hα7 to either acetylcholine (A) or 4OH-GTS-21 (D). The effects of the single point mutations are shown in the lower panels. See Table 2 for curve fit values. Data were normalized to the net charge of control 300μM acetylcholine responses obtained 5 min before the experimental responses. Each point represents the average±S.E.M. of the normalized responses of at least 4 oocytes. The responses of the mutant receptors were compared to responses of each wild-type receptor. Concentrations at which there were significant ($p<.05$) differences between mutant and human (*) and between mutant and monkey (#) are indicated.

for this is unclear; however, in addition to the two extracellular sites we investigated, there are two amino acids in the intracellular portion of the α7 subunit which differ between human and monkey. Specifically, human has a valine at position 373, where monkey has methionine, and human has valine also at position 376, where monkey has alanine. These residues are all considered to be hydrophobic and so the differences between monkey and human are relatively conservative.

The data obtained with the S171N mutant indicates that this site, which is somewhat outside of the canonical binding site as defined by the hypothetical 6-loop structure (see Fig. 5), is nonetheless important for agonist activation and/or binding. The S171N is a semiconservative difference. However, the 171

Table 2
Curve fit values[a] for mutant monkey α7 nicotinic receptors

Mutant	Acetylcholine			4OH-GTS-21		
	EC_{50} μM	n	I_{max}	EC_{50} μM	n	I_{max}
Human wild-type	36±5.1	2.1±0.3	1[b]	4.7±0.2	1.9±0.1	0.38±0.01
Monkey wild-type	74±9.1	3.0±1.0	1	10.0±0.2	4.2±1.7	0.24±0.01
S171N,K186R	25.5±2.3	1.9±0.3	1	3.5±2.7	7.1±3.7	0.35±0.01
S171N	22.5±2.0	2.8±0.6	1	3.5±0.4	3.8±2.2	0.44±0.1
K186R	85.6±2.9	2.3±0.2	1	10.5±2.8	1.5±0.5	0.20±0.02

[a] Error estimates represent the 95% confidence values from the curve fits.
[b] Since acetylcholine is defined as the reference full agonist, the maximal responses obtained with acetylcholine were defined as 1 and the curve fits for acetylcholine were constrained to have I_{max} equal to 1.

residue is almost invariant among species in the α7 nicotinic receptor, with an asparagine present in the α7 sequence of rat, bovine, mouse, human, chimpanzee and zebra fish, making the serine found in the rhesus monkey sequence rather unusual (see http://www.pasteur.fr/recherche/banques/LGIC/cys-loop.html for α7 subunit sequence accession numbers). Interestingly, although the 171N residue is usually found in α7 subunits, a serine at this site is about equally common in other subunits. Residue 171 is located near the putative F-loop of the complementary face of agonist binding domain. In a structural model of the receptor this residue appears near one of two

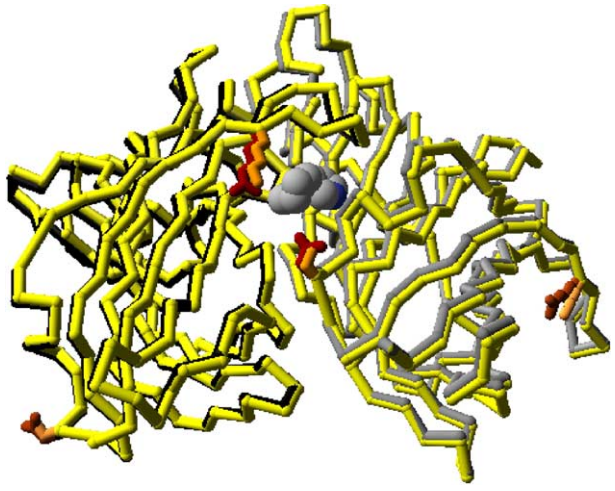

Fig. 7. Structural alignment of human and rhesus α7 nicotinic receptors showing the interface between two contiguous subunits, as they would be arranged in the pentamer. The human receptor backbone is black (positive face) and grey (negative face) while the Rhesus receptor subunits are yellow. An agonist (nicotine) is docked in the putative binding site and shown in spacefill format. The amino acids which differ between human and rhesus α7 are represented as "sticks" on the protein backbone models. The amino acids of the human sequence are colored brown and Rhesus amino acids are colored orange. Backbone structures are staggered by a small distance (0.2 A), so that both protein backbones are visible. The 171 residue appears as part of the negative (complementary) face, below the docked agonist. The 186 residue is in the C-loop of the positive (primary) face and is shown above the nicotine molecule. The human α7 structure was created using Jigsaw3d, and the rhesus α7 was aligned to the human using the SwissModel software package.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

possible entrance to the agonist binding site (Fig. 7) (Stokes et al., 2004).

The K/R substitution at residue 186 is a conservative difference, and although this site is located in the C loop of the agonist binding domain, it had relatively little effect on the activation by acetylcholine or 4OH-GTS-21. This particular residue is rather variable among the different nicotinic acetylcholine receptor subunits, but is most often either arginine (R) or lysine (K) in α7 subunits (Le Novere and Changeux, 1995). It is lysine in the rat, bovine, and mouse α7 sequences, as well as in the rhesus, but arginine in human, chimpanzee, and zebra fish (Genbank: AY247962), although in chicken α7 it is serine.

The observations discussed above suggest that there would have been a common ancestral sequence in early primates with asparagine at residue 171 and lysine at 186. This is consistent with the ancestral sequence as predicted by the PAML software package (Yang, 1997). In this scenario, the two amino acid differences between rhesus and humans in the α7 extracellular domain would have arisen separately as those evolutionary lines diverged. Additionally, the sequence identity present between the chimpanzee and human α7 sequences would suggest that the K to R mutation at 186 occurred prior to the relatively recent human–chimp divergence but after the divergence of the rhesus line.

In conclusion, our results bring to light an important consideration for the use of the rhesus monkey as a model for nicotinic receptor-based therapies related to agonist potency. Additionally, further investigation of the amino acids around 171 in the α7 sequence may delineate the importance of this part of the receptor for agonist activation and/or binding, improving our models for this process. This may ultimately be useful for the development of improved α7 agonists as therapeutics, and to the more complete understanding of the mechanisms of α7-related diseases such as Alzheimer's Disease and Schizophrenia.

Acknowledgements

This work was supported by NIH grant GM57481-01A2 and Memory Pharmaceuticals. We thank Taiho Pharmaceuticals for providing 4-OH-GTS-21 and Targacept for providing TC-1698. We particularly thank Dr. Cathy Smith-Maxwell, and are very grateful to Axon Instruments/Molecular Devices for the use of an OpusXpress 6000A and pClamp9.1.

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