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Cloning and pharmacological characterization of the monkey histamine H_3 receptor

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Abstract

Species differences have been described previously for histamine H_3 receptor pharmacology. Rat selective histamine H_3 receptor ligands such as ciproxifan and A-304121 (2-amino-1-{4-[3-(4-cyclopropanecarbonyl-phenoxy)-propyl]-piperazin-1-yl}-propan-1-one) show over 100-fold selectivity for the rat receptor compared to the human receptor. To date, however, the pharmacology of the cloned monkey histamine H_3 receptor has not been examined. In this study, we cloned the monkey histamine H_3 receptor gene (H_3R) and evaluated the receptor pharmacology in binding and functional assays. The monkey histamine H_3 receptor is highly homologous to the human receptor with 438 identities in their 445 amino acid sequences, but less homologous to the rat receptor. However, unlike the human or rat, we found no evidence for additional splicing for the monkey H_3R. Pharmacological analysis indicated that the monkey receptor exhibited similar pharmacological profiles to those of the human receptor, providing critical information for characterizing histamine H_3 receptor ligands in monkey behavioral models.

Keywords: Histamine H_3 receptor; (Monkey); Cloning; Expression; G-protein coupled receptor; Pharmacology

1. Introduction

Presynaptic histamine H_3 receptors are auto- or hetero-receptors that not only regulate histamine release, but also the release of other neurotransmitters important for cognition, attention and mood (Leurs et al., 1998; Hough, 1988; Lin et al., 1988; Wada et al., 1991; Fox et al., 2002). Recently, the cDNAs of the human and rat histamine H_3 receptor gene (H_3R) have been cloned, each encoding a protein of 445 amino acids (Goodearl, 1999; Lovenberg et al., 1999, 2000; Drutel et al., 2001; Morisset et al., 2000; Morisset et al., 2001). The amino acid sequences of histamine H_3 receptors indicate that they are members of the G-protein coupled receptor superfamily 1, which are characterized by their unique sequence structure, such as the presence of a DRY (or DRF or ERW) motif at the interface of transmembrane domain 3 and intracellular loop 2 (Bockaert and Pin, 1999). Like all G-protein coupled receptors, they contain seven putative transmembrane domains with extracellular N-termini and intracellular C-termini.

Isoforms of the H_3R have also been identified in human, rat and guinea pig (Lovenberg et al., 2000; Tardivel-Lacombe et al., 2000; Drutel et al., 2001; Morisset et al., 2000, 2001; Coge et al., 2001; Wellendorph et al., 2002) that contain deletions of various sizes typically starting at a common position in the third intracellular loop between transmembrane domains 5 and 6, although Liu et al. (2000) had originally found no evidence for the existence of human H_3R isoforms. Where isoforms have been proposed, the nucleotide sequences surrounding the deleted regions resemble those of a splice junction, suggesting that these isoforms may be derived from alternative splicing of the long form of the H_3R. Further, another alternative splicing site at the 3′ end of the gene may result in an 8-amino acid extension at the C-terminal end (Nakamura et al., 2000).

More recently, it has been shown that histamine H_3 receptors from different species exhibit distinct pharmacological profiles. Although the human and rat histamine H_3 receptors are highly conserved, exhibiting 93% identity of their amino acid sequences (Lovenberg et al., 1999, 2000; Tardivel-Lacombe et al., 2000; Drutel et al., 2001; Stark et

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al., 2001; Yao et al., 2003), distinct ligand binding properties have been observed (Arrang et al., 1987; West et al., 1999; Ligneau et al., 2000; Lovenberg et al., 2000; Ireland-Denny et al., 2001, Yao et al., 2003). For example, ciproxifan, thioperamide, imoproxifan and A-304121 bind to the rat histamine H_3 receptor with more than 300-fold higher affinity than to the human receptor (Yao et al., 2003). Using a human/rat chimeric histamine H_3 receptor and site directed mutagenesis in the human histamine H_3 receptors, two amino acids at positions 119 (T for human and A for rat) and 122 (A for human and V for rat) have been identified to be mainly responsible for the pharmacological differences between the two species (Ligneau et al., 2000; Yao et al., 2003). It has been hypothesized that the side chains of these amino acids either directly interact with the ligands or affect the topology of the ligand binding site, which leads to more effective ligand interaction for the rat receptor.

The monkey serves a useful primate model in drug discovery research, particularly in studies of compounds that affect learning and memory (Buccafusco et al., 1995; Decker et al., 1997). Histamine H_3 receptor ligands are currently under investigation for a number of indications including cognition and attention, and understanding the pharmacological characteristics of the monkey histamine H_3 receptor could provide critical information for evaluation of histamine H_3 receptor ligands in monkey behavioral models. Previously, West et al. (1999) published pharmacological characterization of the primate H_3 receptor and found that the binding properties of the non-human primate H_3 receptor are similar to that of the human, but not rodent, in terms of thioperamide affinity. However, we believed additional pharmacological analysis using a broader set of species-selective compounds tested at the cloned monkey receptor plus an analysis of the peptide sequence of the monkey receptor compared to the amino acids previously shown to be important for rat, human pharmacological differences would enhance understanding of the utility of non-human primate studies for predicting human H_3 receptor pharmacology. Therefore, we cloned the monkey H_3R cDNA and compared the receptor pharmacological properties of the expressed monkey histamine H_3 receptor with those of the human and rat histamine H_3 receptors using ligand binding and fluorometric image plate reader (FLIPR) functional assays.

2. Materials and methods

2.1. Materials

Histamine, (*R*)-α-methylhistamine, clobenpropit, thioperamide, benzamidine-HCl, aprotinin, leupeptin and pepstatin and A-23187 were purchased from Sigma (St. Louis, MO). Ciproxifan, imoproxifan, GT-2331 (4-[(*R*,*R*)-2-(5,5-dimethyl-1-hexynyl)cyclopropyl]-1*H*-imidazole), A-304121 (2-amino-1-{4-[3-(4-cyclopropanecarbonyl-phenoxy)-propyl]-piperazin-1-yl}-propan-1-one), A-317920 (furan-2-carboxylic acid (2-{4-[3-(4-cyclopropanecarbonyl-phenoxy)-propyl]-piperazin-1-yl}-1-methyl-2-oxo-ethyl)-amide) and A-320436 (furan-2-carboxylic acid (2-{4-[3-(4′-cyano-biphenyl-4-yloxy)-propyl]-[1,4]diazepan-1-yl}-2-oxo-1-thiazol-4-ylmethyl-ethyl)-amide) were synthesized at Abbott Laboratories. Radiolabeled [^{3}H](*N*)-α-methylhistamine was purchased from Perkin-Elmer Life Sciences (Boston, MA). Fluo-4 AM (acetoxymethyl) dye and pluronic acid were obtained from Molecular Probes (Eugene, OR). Aprotinin, leupeptin and pepstatin were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Standard restriction enzymes, polymerase chain reaction (PCR) cloning vectors including TOPO TA vector, PCR reagents, reverse transcriptase (RT), TRIzol® reagent, Dulbecco's Modified Eagle Medium (D-MEM) cell culture media, hygromycin B and lipofectamine™ 2000 reagent were obtained from Invitrogen/LifeTech (Rockville, MD). The pCI-neo expression vector was purchased from Promega (Madison, WI). The human embryonic kidney (HEK) and rat glioma C6 cell lines were obtained from the American Type Culture Collection (Rockville, MD). The G_{qi5} expression plasmid (pCEP4-G_{qi5}) (Conklin et al., 1993) was obtained from Molecular Devices (Sunnyvale, CA). The HEK-G_{qi5} cell line, stably expressing the G_{qi5} gene was generated at Abbott Laboratories (see below).

2.2. Cloning and isoform detection of the monkey H_3R

Total RNA was extracted using TRIzol® reagent from thalamus tissue of a Rhesus monkey provided by the Alzheimer's Research Center of the Medical College of Georgia. cDNA was synthesized from total RNA using a poly-dT primer and Superscript Reverse Transcriptase (RT).

In order to obtain the monkey H_3R sequence, the initial RT-PCR reaction was performed with monkey thalamus total RNA using a human H_3R primer pair, since the monkey H_3R sequence was not available. These human H_3R primers include the forward primer (5′-$_{450}$GCGGGCAGTGCG-GCGGGCAGTGCGGAAGATGC-3′) in the sense direction and the reverse primer (5′-$_{600}$GATGAGGAAGTAC-GATGAGGAAGTACCAGTTG-3′) in the antisense direction starting at 450 or 600 bp down stream from the start codon as indicated by the base pair number of the first nucleotide in the primer sequences, respectively. The PCR reactions were performed in the presence of GC-Melt™ reagent (Clontech, Palo Alto, CA) using Elongase (Invitrogen) by initial incubation at 96 °C for 3 min followed by 40 cycles of 96 °C for 30 s, 60 °C for 45 s, 72 °C for 3 min and final incubation at 72 °C for 3 min. The monkey H_3R sequence (from 450 to 600 bp) generated by this initial PCR reaction was used to design monkey H_3R-specific primers for subsequent inverse PCR reactions (Pang and Knecht, 1997), in which the double strand monkey thalamus cDNA from the poly-dT primed RT reaction was first circularized or concatenated with ligase, and then served as the PCR template. The monkey H_3R primers used in the

inverse PCR reactions include the forward primer (5′-$_{596}$CATCCCCGAGGGCCACTGCTACGCTGAG-3′) and the reverse primer (5′-$_{584}$GGACAGGTACTCCCAGCTGGACAGGTACTCCCAGCTCAGGATGGC-3′). The PCR reactions were performed in the presence of GC-Melt™ reagent using Elongase by initial incubation at 96 °C for 3 min followed by 40 cycles of 96 °C for 30 s, 55 °C for 45 s, 72 °C for 1.5 min and final incubation at 72 °C for 2 min. The PCR products were separated on an agarose gel, and the products greater than 2 kb were cloned and sequenced in TOPO TA vector. The entire monkey H_3R coding sequence plus the 5′ and 3′ untranslated regions were thus obtained.

To detect isoforms and to clone the monkey H_3R receptor cDNA, monkey H_3R gene-specific primers that cover the 5′ start codon (5′-TTTAAACGCGTACCATG GAGCGCGCGCCGCCCGAC-3′) and the 3′ stop codon (5′-ATATAGTCGACTCA CTTCCAGCACTGCTCCAGG-3′) of the gene were synthesized and used to amplify the monkey H_3R cDNA. In the human control, cDNA was generated in a RT reaction with human thalamus polyA-selected RNA using poly-dT primers and the PCR reaction was performed with human cDNA using a primer pair that covers the 5′ start codon (5′-TTTAAACGCGTATGGAGCGCGCGCCGCCCGAC-3′ and the 3′ stop codon 5′-TATATGTCGACTCA CTTCCAGCAGTGCTCCAGGGAGC-3′). PCR reactions were performed in the presence of GC-Melt™ reagent using Elongase with initial incubation at 96 °C for 3 min followed by 40 cycles of 96 °C for 30 s, 55 °C for 45 s, 72 °C for 1.5 min and final incubation at 72 °C for 2 min. The PCR products were resolved on an agarose gel. The Kozak sequence and restriction sites (*Mlu*I and *Sal*I) were also designed into the monkey H_3R primers and the resulting monkey H_3R PCR fragment was cloned into the pCI-neo expression vector via the *Mlu*I and *Sal*I sites.

2.3. Cell culture, transfection and stable cell line generation

HEK cells were grown in D-MEM with high glucose, containing 10% fetal bovine serum and 20 mM L-glutamine. The HEK-G_{qi5} cell line was generated initially by transient transfection of HEK cells with the pCEP4-G_{qi5} plasmid. The cells were subsequently selected for the ability to grow in the presence of 300 μg/ml hygromycin B 2 days following the initial transfection. Clonal lines were grown and the expression of G_{qi5} was confirmed by Western blot using antibodies against the hemagglutinin tag (Covance, Princeton, NJ) in the G_{qi5} protein. The HEK-G_{qi5} cell line was used as a host for transient expression of the monkey, human and rat H_3R cDNAs. Transient transfection was performed with lipofectamine™ 2000 reagent using protocols provided by the vendor.

2.4. [^{3}H](*N*)-α-methylhistamine binding assay

[^{3}H](*N*)-α-methylhistamine binding assays were conducted as previously described (Esbenshade and Hancock, 2000). Briefly, HEK-G_{qi5} cells transiently transfected with the monkey H_3R were harvested and homogenized in TE buffer (50 mM Tris–HCl, 5 mM EDTA, pH7.4) containing protease inhibitors (benzamidine-HCl, aprotinin, leupeptin and pepstatin) at concentrations recommended by vendor using an ultra-turrax T25 polytron (Janke and Kunkel, Germany) at the maximum setting (24,000 rpm) for 2×10 s, followed by centrifugation at $40{,}000 \times g$. Membrane pellets were further purified by repeating the above homogenization and centrifugation steps. Final membrane preparations were obtained by re-homogenizing the pellets in TE buffer. For binding assays with the human and rat histamine H_3 receptors, membranes were prepared from C6 cells stably expressing the human or the rat histamine H_3 receptors (Esbenshade et al., 2003) using similar methods. Membrane preparations were incubated with [^{3}H](*N*)-α-methylhistamine at various concentrations (from 1×10^{-6} to 1×10^{-10} M) for saturation binding, and $0.5–3 \times 10^{-10}$ M [^{3}H](*N*)-α-methylhistamine for competition binding in the presence or absence of test compounds. The binding reactions were carried out for 30 min at 25 °C and membranes harvested using polyethylenimine (0.3%) pre-soaked Unifilters followed by three brief washes with 4 ml of ice-cold TE buffer. Nonspecific binding was defined with 30 μM thioperamide. K_d values from the saturation binding assays and K_i values from the competition binding assays were determined with one site binding or one site competition curve fitting equations using Prism software (GraphPad, San Diego, CA). Statistical evaluation of pK_d and pK_i values was determined by performing one-way analysis of variance followed by the Bonferroni multiple comparisons test using RS/1 software (BBN Software products, Cambridge, MA), with p values of <0.05 accepted as evidence of statistically significant differences of the means.

2.5. FLIPR assay

HEK cells were co-transfected with H_3Rs and G_{qi5} expression plasmids as described above. The transfected cells were dissociated the next day and seeded in 96-well black wall plates at 75,000 cells per well. The FLIPR assays were performed 2 days following the start of transfection. The cells were incubated with 100 μl per well of 0.5 μg/ml fluo-4 AM in DPBS for 1 to 3 h and washed with DPBS immediately before the assay. Vehicle or test compounds were added to the cells at the first addition and (*R*)-α-methylhistamine (1×10^{-7} M at the final concentration) was added at the second addition. The interval between the first and the second additions was 5 min. The maximum fluorescence units of the wells containing test compounds were compared with those containing vehicle. To obtain the EC_{50} values of (*R*)-α-methylhistamine or the K_B values of antagonists, the data were analyzed from duplicate experiments using the

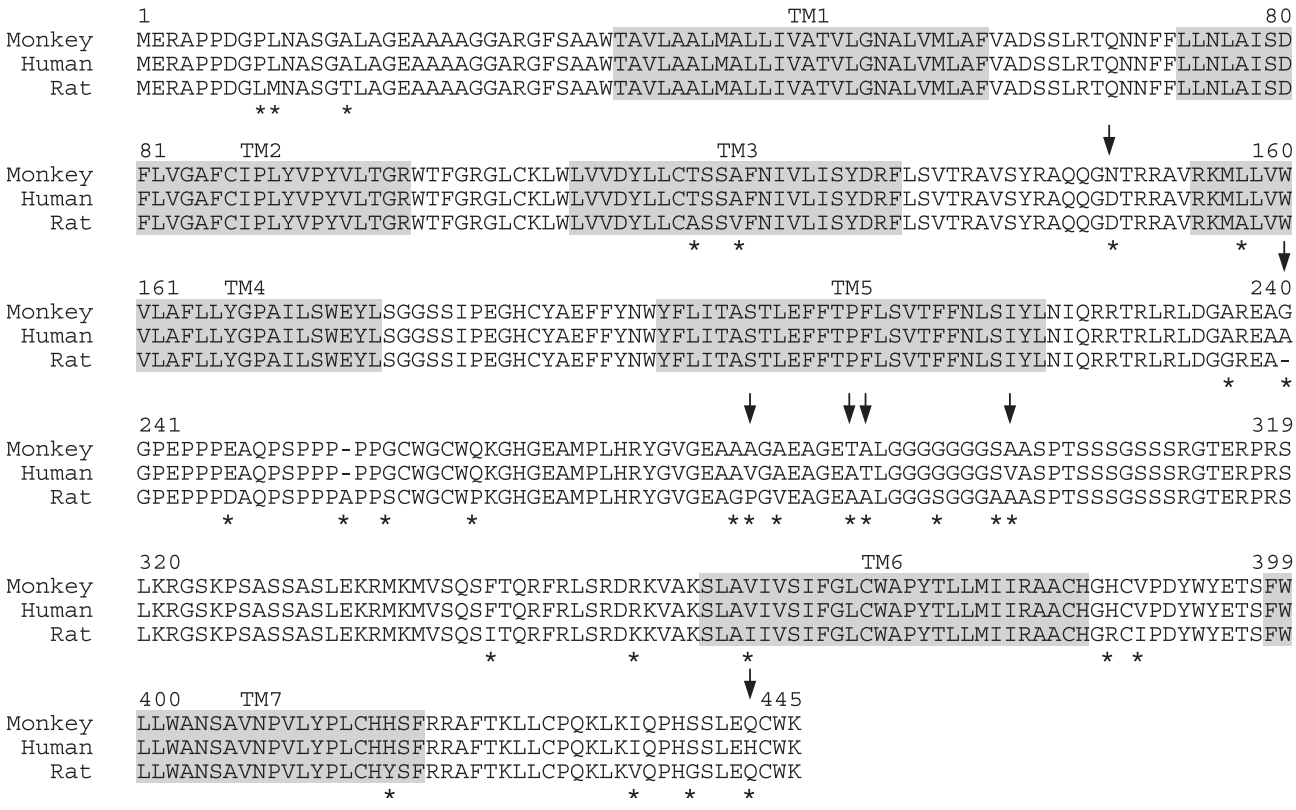


Fig. 1. Alignment of the amino acid sequences of monkey, human and rat histamine H_3 receptors. Putative transmembrane domains are shaded and labeled by the respective transmembrane domain numbers on the top. Amino acids that are distinct in monkey, human and rat histamine H_3 receptors are marked by asterisks. Amino acids that are distinct in monkey and human histamine H_3 receptors are marked by arrows.

sigmoidal dose response with variable slope equation of the nonlinear regression curve fitting analysis provided by Prism. Statistical evaluation of pEC_{50} and pK_B values was determined by performing one-way analysis of variance followed by Bonferroni multiple comparisons test using RS/1 software, with p values of < 0.05 accepted as evidence of statistically significant differences of the means.

3. Results

3.1. Cloning of the monkey H_3R cDNA and sequence comparison with H_3Rs from human and rat

Monkey H_3R cDNA was obtained by PCR reactions from Rhesus monkey thalamus tissue. The monkey hista-

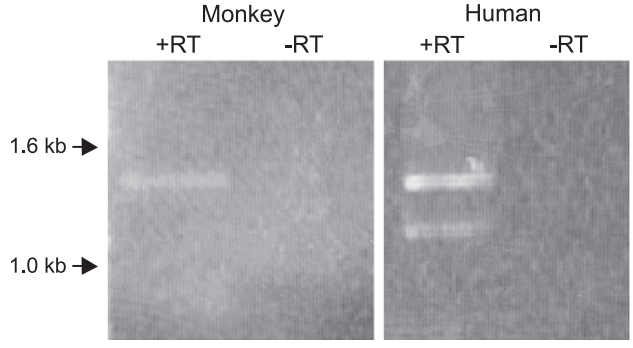


Fig. 2. Detection of H_3R isoforms in monkey and human thalamus using RT-PCR technique. RNA preparations from monkey thalamus or human thalamus were subject to RT-PCR using primer sets that hybridize to the 5′ and 3′ ends of the coding region. The RT reactions were carried out either in the presence (+RT) or in the absence (−RT) of reverse transcriptase prior to the PCR reactions.

mine H_3 receptor protein contains 445 amino acids, like the long form of the human and rat histamine H_3 receptors. The amino acid sequence of the monkey histamine H_3 receptor is 98.4% and 93.7% identical to that of the human and rat histamine H_3 receptors, respectively. There are only 7 amino acid differences between the monkey and human receptors and 28 between the monkey and rat receptors. Further, the amino acids of the monkey histamine H_3 receptor at positions 119 and 122, which have been shown to be important for ligand recognition in human and rat receptors

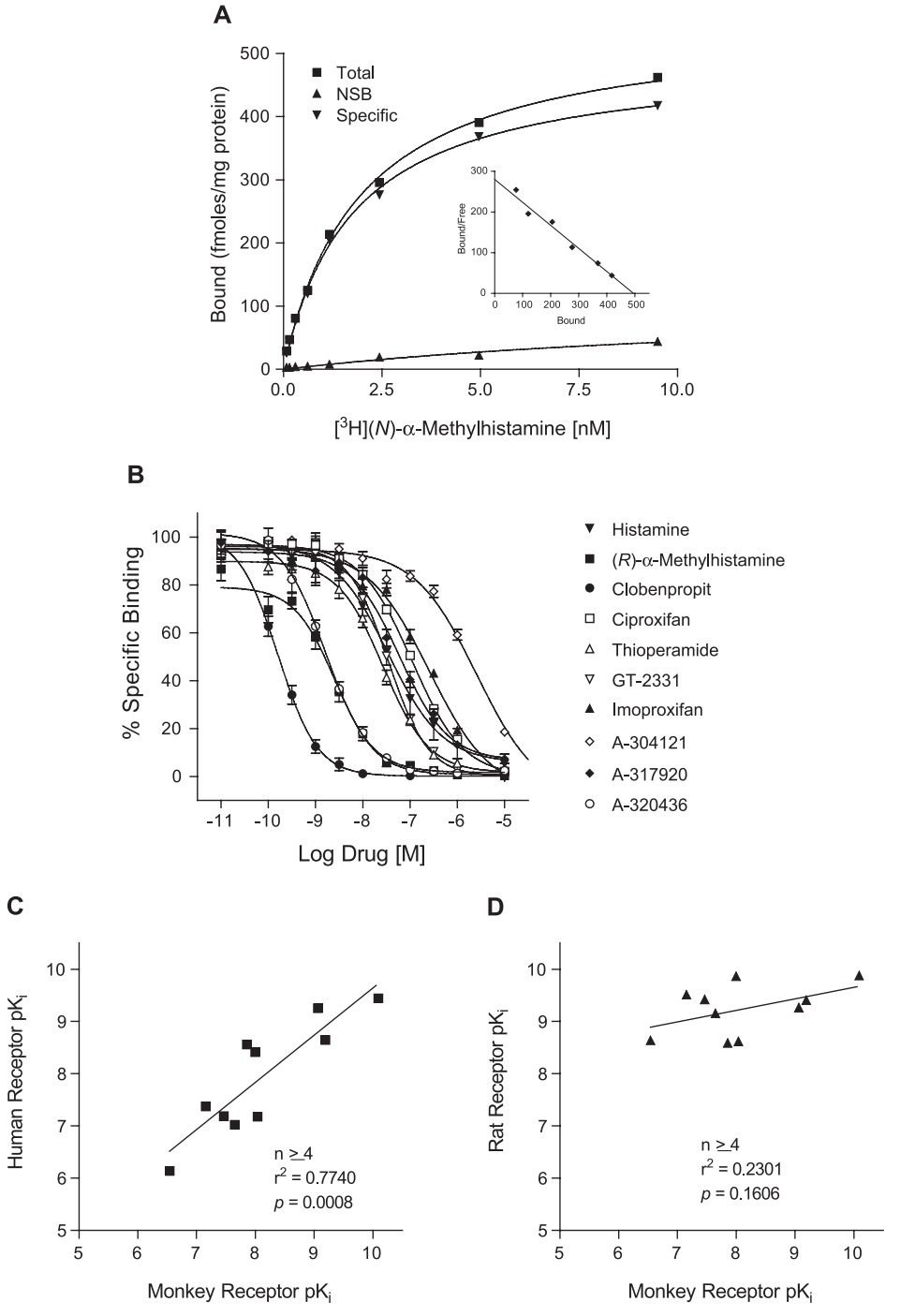


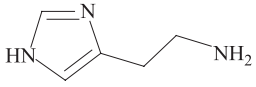
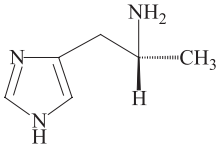
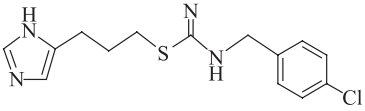
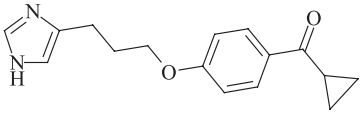
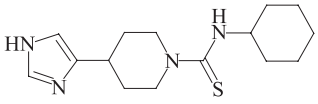
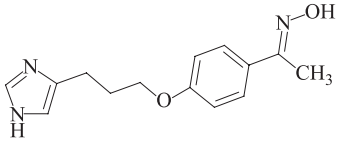
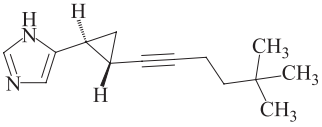
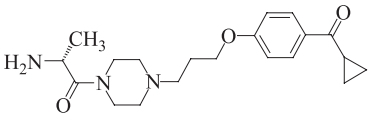
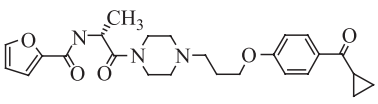
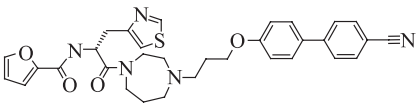
Fig. 3. [^{3}H](*N*)-α-methylhistamine saturation binding assays with the monkey histamine H_3 receptor (A). Total (■), NSB (▲) and specific (▼) represent total, nonspecific and specific bindings, respectively. [^{3}H](*N*)-α-methylhistamine competition binding assays with the monkey histamine H_3 receptor (B). Competition binding assays were performed in the presence of 0.5–3 nM [^{3}H](*N*)-α-methylhistamine. Saturation and competition binding assays were performed with the same membrane preparation made from HEK cells co-expressing the monkey histamine H_3 receptor and G_{qi5}. K_i values were derived using nonlinear regression curve fitting by Prism. Data represent the mean ± S.E.M. of four experiments performed in duplicate. Correlation plots of binding pK_i values of monkey vs. human (B) and monkey vs. rat (C) histamine H_3 receptors are shown with corresponding correlation coefficients (r^2) and p values.

(Ligneau et al., 2000; Yao et al., 2003), are identical to the amino acids in the human receptor. Sequence alignments of the monkey histamine H_3 receptor with the human and rat histamine H_3 receptors are shown in Fig. 1.

3.2. No evidence of short monkey H_3R isoforms

Isoforms of the human and rat H_3R cDNAs containing deletions of various sizes were observed in RT-PCR reactions performed using human and rat RNA preparations (Coge et al., 2001; Drutel et al., 2001; Wellendorph et al., 2002). In order to determine if short isoforms were present for the monkey H_3R, the complete coding region of the monkey H_3R cDNA was obtained with RT-PCR reactions using the monkey thalamus RNA, and a control PCR reaction using the human thalamus RNA was performed simultaneously. Only one unique fragment was observed in the reaction using the monkey RNA, the length of which corresponds to the fragment derived from the long form of the human receptor. In contrast, a short isoform in addition to the long form was observed in the reaction using the human RNA (Fig. 2). Sequencing of these PCR products

Table 1
Comparison of K_i (nM) values of ligands in competition binding assays

Ligand	Structure	K_i (nM)		
		Monkey	Human	Rat
Histamine		12[a] 10–14[b]	2.8 2.5–3.0	2.6 2.1–3.3
(R)-α-methylhistamine		1.03 0.35–3.1	0.56 0.43–0.72	0.54 0.41–0.73
Clobenpropit		0.12 0.04–0.31	0.36 0.30–0.43	0.13 0.10–0.18
Ciproxifan		41 24–70	65 52–79	0.38 0.29–0.50
Thioperamide		13 7.8–23	66 51–86	2.4 1.6–3.7
Imoproxifan		73 48–112	42 35–51	0.31 0.20–0.46
GT-2331		10 7.0–16	3.9 2.9–5.1	0.1 0.08–0.25
A-304121		368 216–628	727 549–962	2.3 1.7–3.2
A-317920		36 16–82	94 78–114	0.70 0.41–1.2
A-320436		1.1 9.05–1.6	2.3 1.7–3.1	0.39 0.26–0.59

[a] $n \geq 4$.
[b] 95% confidence interval.

confirmed that the larger fragments from monkey and human correlated with the long isoform of the H_3 receptor, whereas the sequence of the short fragment from human RNA corresponded to the short isoform that contains the 80-amino acid deletion.

3.3. Pharmacological comparison of monkey, human and rat histamine H_3 receptors

The ligand binding properties of monkey histamine H_3 receptors were characterized using membrane preparations from HEK-G_{qi5} cells transiently transfected with the monkey H_3R (Fig. 3). Competition assays with human and rat histamine H_3 receptors were performed under the same conditions except that membrane preparations were prepared from C6 cells stably expressing the human or rat histamine H_3 receptors. In saturation binding assays performed using [^{3}H](*N*)-α-methylhistamine, the monkey histamine H_3 receptor exhibited a about 5-fold weaker affinity with a K_d value of 1.65 nM ($pK_d = 8.78 \pm 0.051$), whereas the K_d values were 0.340 nM ($pK_d = 9.47 \pm 0.042$) for human and 0.313 nM ($pK_d = 9.50 \pm 0.089$) for rat histamine H_3 receptors. The binding of [^{3}H](*N*)-α-methylhistamine to the monkey histamine H_3 receptor was saturable and best fit by a one-site binding model by Prism. Subsequently, competition-binding assays were performed using 0.5–3 nM [^{3}H](*N*)-α-methylhistamine with membranes containing each of the histamine H_3 receptors. With the exception of histamine and (*R*)-α-methylhistamine, the histamine H_3 receptor ligands used for competition assays with the monkey histamine H_3 receptor were selected based on their differential binding affinities to the human and rat histamine H_3 receptors (Yao et al., 2003) or because of their novel structural and pharmacological properties (i.e. A-317920 and A-320436). The K_i values of these ligands for the monkey, human and rat histamine H_3 receptors are summarized in Table 1. As expected based on similarity of amino acid sequences, the monkey histamine H_3 receptor exhibited similar binding affinities to the human and the rat histamine H_3 receptors for compounds such as clobenpropit and (*R*)-α-methylhistamine that do not differ greatly in their human and rat histamine H_3 receptor affinities. In contrast, lower, human receptor-like affinities were obtained for ciproxifan, imoproxifan and GT-2331 (which are 10- to 200-fold weaker at human than rat histamine H_3 receptors). However, the affinities of the monkey receptor for thioperamide, A-304121, A-317920 and A-320436 were intermediate to those at the human and rat receptors. In contrast, the potency of histamine at the monkey receptor (12 nM) is 5-fold lower than that at the human or the rat receptor (2.8 and 2.6 nM, respectively).

The affinities of the monkey and human histamine H_3 receptors to histamine H_1 and H_2 receptor ligands were tested in [^{3}H](*N*)-α-methylhistamine competition binding assays. These histamine receptor ligands showed limited displacement of the radioligand in these assays. Human and monkey histamine H_3 receptors exhibited similarly low affinities to the H_1 receptor ligand chlorpheniramine ($pK_i = 6.0 \pm 0.02$ for monkey and 6.16 ± 0.11 for human) and the H_2 receptor ligand ranitidine ($pK_i = 5.21 \pm 0.10$ for monkey and 5.81 ± 0.20 for human). Lower affinities were observed for these receptors to histamine H_1 receptor ligands diphenhydramine and mepyramine or histamine H_2 receptor ligands tiotidine and cimetidine.

3.4. Potencies of histamine H_3 receptor antagonists at the monkey receptor are similar to those at the human receptor

In order to investigate if pharmacological similarity of the monkey histamine H_3 receptor and the human histamine H_3 receptor observed in binding assays was also manifested in functional responses, a FLIPR assay was employed to investigate the agonist activation and inhibition by antagonists at the monkey, human and rat histamine H_3 receptors. In this assay, HEK-G_{qi5} cells were transiently transfected with the H_3Rs and the activation by (*R*)-α-methylhistamine was determined by the increase of the intracellular calcium levels as measured by a FLIPR instrument. In antagonist assays, the inhibition of the (*R*)-α-methylhistamine-induced calcium responses was measured and expressed as the percentage of the maximal (*R*)-α-methylhistamine response.

First, in order to determine the potency of (*R*)-α-methylhistamine in activating the monkey, human and rat histamine H_3 receptors, (*R*)-α-methylhistamine concentration-dependent responses were determined and are presented as percentages of the maximal response at 1×10^{-5} M (*R*)-α-methylhistamine concentration (Fig. 4). The peak fluorescence responses for cells transfected with monkey, human and rat H_3Rs were between 6000 and 12,000 units. (*R*)-α-methylhistamine increased intracellular calcium levels in a

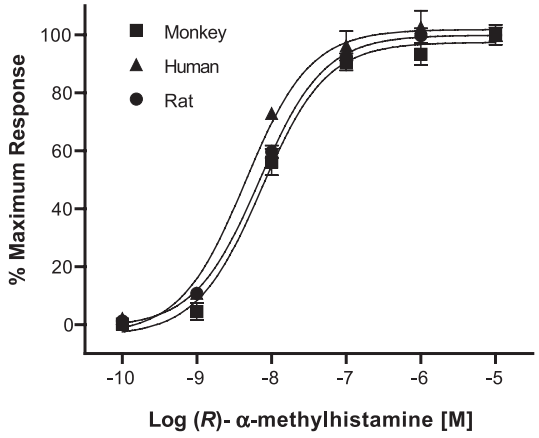


Fig. 4. (*R*)-α-methylhistamine concentration-dependent responses of monkey, human and rat H_3 receptors in FLIPR assays. HEK-G_{qi5} cells were transfected with monkey (■), human (▲) and rat (●) H_3Rs and FLIPR assays were performed 2 days post the initial transfection. (*R*)-α-methylhistamine concentration responses were expressed as percentages of the maximum response at 1×10^{-5} M (*R*)-α-methylhistamine concentration. EC_{50} values were derived using nonlinear regression curve fitting by Prism and are represented by the mean ± S.E.M. of six experiments performed in duplicate.

concentration-dependent manner and the EC_{50} values for (*R*)-α-methylhistamine responses were 7.2, 4.2 and 6.5 nM for the monkey, human and rat histamine H_3 receptors, respectively.

Subsequently, the potencies of histamine H_3 receptor antagonists in blocking the (*R*)-α-methylhistamine-induced calcium responses by histamine H_3 receptors were determined. In this assay, the histamine H_3 receptor antagonists were added to cells at various concentrations, followed by the addition of (*R*)-α-methylhistamine at a final concentration of 1×10^{-7} M. All the histamine H_3 receptor antagonists tested were able to attenuate the response of (*R*)-α-methylhistamine in a concentration-dependent manner (Fig. 5). The K_B values are summarized in Table 2. Ciproxifan, thioperamide, GT-

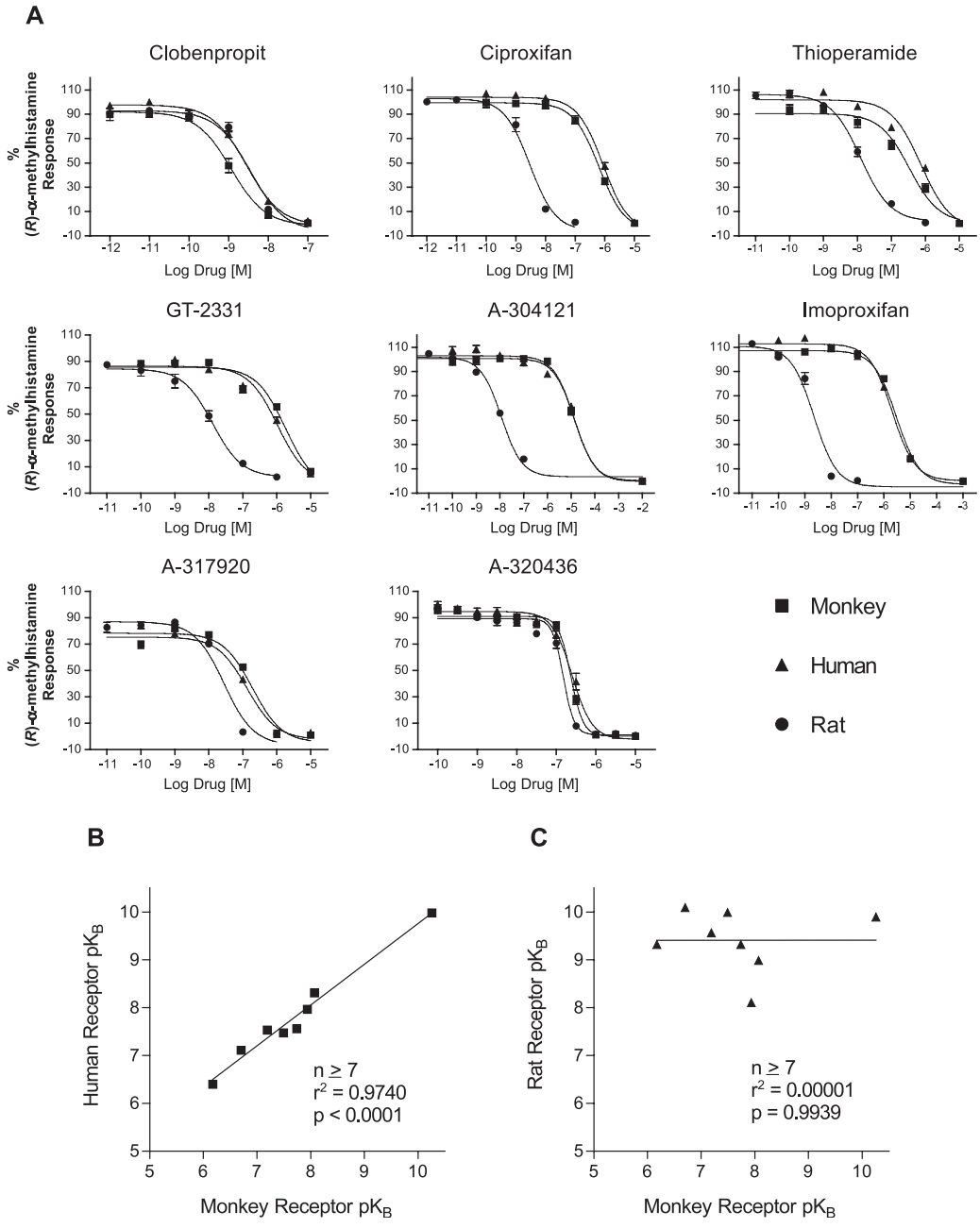


Fig. 5. Antagonist concentration responses of monkey, human and rat histamine H_3 receptors in FLIPR assays (A). HEK-G_{qi5} cells were transfected with monkey (■), human (▲) and rat (●) H_3Rs and FLIPR assays were performed 2 days post the initial transfection. Test ligands were added at various concentrations to the cells followed by the addition of (*R*)-α-methylhistamine (1×10^{-7} M). The interval between the two additions was 5 min. The response at each antagonist ligand concentration was expressed as a percentage of the (*R*)-α-methylhistamine response in the absence of antagonist (vehicle), and plotted against the antagonist concentration. Data were analyzed using nonlinear regression curve fitting by Prism and are represent by the mean ± S.E.M. of six experiments performed in duplicate. Correlation plots of binding pK_B values of monkey vs. human (B) and monkey vs. rat histamine H_3 receptors (C) are shown with corresponding correlation coefficients (r^2) and *p* values.

Table 2
Comparison of K_B (nM) values of antagonist ligands in FLIPR assays

Ligand	K_B (nM)		
	Monkey	Human	Rat
Clobenpropit	0.06[a]	0.11	0.13
	0.03–0.12[b]	0.07–0.15	0.09–0.19
Ciproxifan	32	34	0.10
	24–44	23–48	0.05–0.20
Thioperamide	18	27	0.48
	8.7–38	24–31	0.29–0.77
Imoproxifan	200	78	0.08
	149–267	62–98	0.05–0.12
GT-2331	64	29	0.27
	46–90	19–45	0.05–1.5
A-304121	665	396	0.48
	447–991	204–769	0.40–0.57
A-317920	8.56	4.9	1.0
	6.9–11	3.8–6.3	0.84–1.3
A-320436	12	11	7.9
	8.9–15	9.1–13	5.5–11

[a] $n \geq 7$.
[b] 95% confidence interval.

2331, imoproxifan, A-304121 and A-317920 exhibited similar potencies at the monkey and human histamine H_3 receptors, but their potencies at blocking the rat histamine H_3 receptor were 5–2000 fold higher. Clobenpropit and A-320436 did not differentiate in their potencies for the histamine H_3 receptors from the three species.

4. Discussion

Targeting the H_3 receptor is of potential therapeutic value for cognitive disorders, asthma and obesity (Leurs et al., 1998; Schwartz et al., 2001) and several agonists and antagonists have entered clinical trials (Fozard, 2000; Sansom, 2000). Non-human primates such as the monkey are common species used for preclinical compound testing in drug discovery processes, and studying the pharmacology of target receptors in monkey can provide crucial information towards predicting human clinical efficacy (Buccafusco et al., 1995; Decker et al., 1997). Recently, species selectivity of histamine H_3 receptor ligands has been revealed and key amino acids have been elucidated that directly contributes to the pharmacological differences of histamine H_3 receptors in ligand interaction (Ligneau et al., 2000; Yao et al., 2003). In order to study in vitro pharmacological properties of the monkey histamine H_3 receptor, we have cloned and expressed the monkey H_3R gene and demonstrated that the monkey histamine H_3 receptor exhibits a similar, but not identical pharmacological profile to the human histamine H_3 receptor.

The alignment of the monkey histamine H_3 receptor amino acid sequence with those of the histamine H_3 receptors from other species indicated that the monkey histamine H_3 receptor is most homologous to the human histamine H_3 receptor. However, unlike the human histamine H_3 receptor, only one isoform of the monkey histamine H_3 receptor corresponding to the long form from other species has so far been identified. In contrast, short isoforms of H_3Rs have been identified in rat, guinea pig and human. The sequence of the human H_3R genomic DNA indicates that the human H_3R cDNA is composed of three exons (GenBank accession #NT_030871). Exons 1 and 2 encode amino acids from 1 to 83 and from 84 to 139, respectively. Exon 3 encodes the largest portion of the receptor protein containing amino acids from 140 to 445, which includes transmembrane regions 4, 5, 6 and 7. Several H_3R short isoforms identified in rat, guinea pig and human contain deletions that start at a site in the receptor between transmembrane domains 5 and 6, and are hypothesized to arise from additional splicing in Exon 3 of the long form, generating a fourth exon. It appears that this additional splicing only occurs as a part of a coordinated event when the H_3R precursor RNA is processed to mature mRNA, since the shorter isoform of the human H_3R has not been observed in cell lines that express human H_3R cDNA (data not shown). Alignment of the H_3R nucleotide sequences corresponding to the potential splice junction that gives rise to short isoforms indicated that the monkey H_3R sequence differs from those of human, rat and guinea pig receptors. The H_3Rs from human, rat, and guinea pig contain the highly efficient splicing sequence CAG–GTA at the donor site (Fig. 6), whereas the corresponding monkey sequence contains TCG–GTA instead. The TCG–GTA sequence in

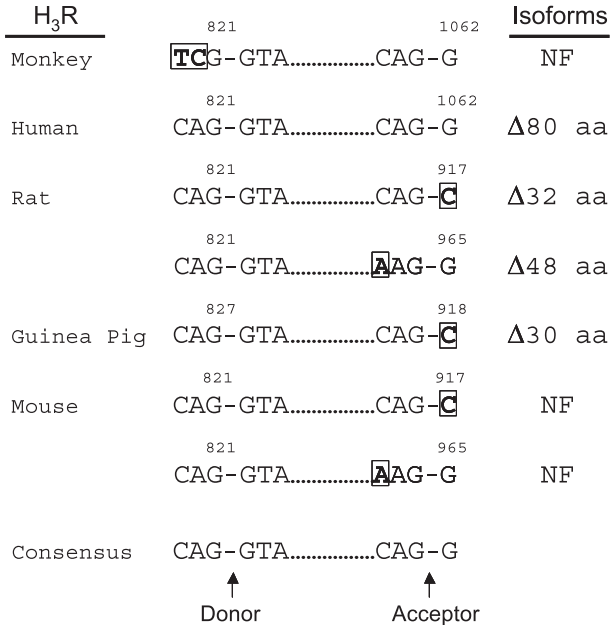


Fig. 6. Nucleotide alignment of the junctions for additional splicing of monkey, human, rat, guinea pig and mouse H_3Rs. The potential splice sites in Exon 3 between transmembrane domains 5 and 6 were aligned with the eukaryotic splicing consensus sequence. Arrows point to the sites of splicing. Nucleotides that are inconsistent with the consensus sequences are boxed and printed in bold. Isoforms arising from the potential splicing are listed. NF represents isoforms not found.

the monkey receptor constitutes a less efficient splicing donor site (Stephens and Schneider, 1992) and may potentially be responsible for the observed absence of short forms. However, controversy exists on the presence of human H_3R isoforms (Liu et al., 2000). Further, in a recent publication (Chen et al., 2003) by the same research group, the absence of mouse H_3 receptor isoforms was also reported, although the mouse H_3 receptor has identical sequences to the human, rat and guinea pig receptors at the potential splice junctions. It is possible that isoforms may exist in lower abundance not readily detected with our PCR techniques. In addition, they may exist in brain regions other than the thalamus since distinct brain tissue distributions have been observed for different isoforms of the rat histamine H_3 receptor (Drutel et al., 2001).

The monkey histamine H_3 receptor exhibited about 5-fold lower affinity for [^{3}H](*N*)-α-methylhistamine binding than the human and rat histamine H_3 receptors in saturation binding assays. However, Scatchard plot indicates that the binding is monophasic and the receptor likely contains one binding site. The differences in affinities may arise from the different cell lines used for expression. Human and rat histamine H_3 receptors were expressed in C6 cells whereas the monkey histamine H_3 receptor was expressed in HEK cells that co-express the receptor and G_{qi5}. This hypothesis is supported by findings that the human histamine H_3 receptor exhibited slightly higher affinity for [^{3}H](*N*)-α-methylhistamine when expressed alone in HEK cells than when co-expressed with G_{qi5} in HEK cells (Sharma and Yao, unpublished data). In general, the monkey histamine H_3 receptor exhibited similar pharmacological properties in competition binding assays to those of the human receptor, but markedly differed from the rat receptor, especially for those compounds that discriminate between the human and rat receptors. This is in general agreement to the findings by West et al. (1999). However, the potency of thioperamide at the cloned monkey histamine H_3 receptor in this study is higher than that obtained with the monkey brain tissue as published by West. The monkey and human histamine H_3 receptors exhibited μM range low affinities to prototype histamine H_1 and H_2 receptor ligands, clearly distinguishing histamine H_3 receptors of these species from histamine H_1 and H_2 receptors.

Recently, it has been elucidated that the amino acid differences at positions 119 and 122 between the human (T119 and A122) and rat (A119 and V122) histamine H_3 receptors lead to significant differences in their affinities for histamine H_3 receptor ligands such as ciproxifan, imoproxifan and GT-2331 (Ligneau et al., 2000; Yao et al., 2003). These rat receptor-selective ligands showed significantly lower potencies at the human receptor. The monkey histamine H_3 receptor contains amino acids identical to those found in the human receptor at these positions and, as expected, the monkey histamine H_3 receptor exhibited similar lower affinities for these histamine H_3 receptor ligands like the human receptor.

Interestingly, some rat-selective ligands such as thioperamide, A-304121 and A-317920 exhibited slightly improved affinity at monkey receptor with K_i values intermediate to those at the human and rat receptors, whereas A-320436 is a potent ligand at monkey, human and rat receptors. Although the monkey and the human receptors are highly homologous, there are seven amino acid differences between their sequences—six of them located in the intracellular loop regions and one in the internally located C-terminus. Unlike amino acids 119 and 122, these amino acids have not been hypothesized to play a direct role in ligand binding. However, these seven amino acids may contribute to ligand binding indirectly by changing the structural conformation of the ligand binding pocket, which may lead to slightly modified ligand affinities of the receptor.

Activation of the monkey H_3 receptor was studied using a FLIPR functional assay measuring (*R*)-α-methylhistamine-induced increases of intracellular calcium levels. Histamine H_3 receptors are normally $G_{i/o}$ coupled G-protein coupled receptors (Endou et al., 1993; Clark and Hill, 1996; Lovenberg et al., 1999) that inhibit adenylyl cyclase upon their activation. However, a FLIPR functional assay was established using a chimeric G_{qi5} protein, which converts $G_{i/o}$-coupling to G_q activation, characterized by an increase of intracellular calcium (Conklin et al., 1993; Selbie and Hill, 1998). (*R*)-α-methylhistamine was able to activate the human, monkey and rat histamine H_3 receptors with similar potencies in FLIPR assays, consistent with the finding that (*R*)-α-methylhistamine has similar binding affinities at these receptors. Subsequently, several diverse antagonist ligands were tested for their ability to attenuate (*R*)-α-methylhistamine-induced calcium responses. These ligands concentration-dependently inhibited the (*R*)-α-methylhistamine-mediated histamine H_3 receptor activation. The potencies of these ligands at the monkey histamine H_3 receptor are comparable to those of the human receptor, even for compounds that showed slight differences at binding potencies, such as thioperamide, A-304121 and A-317920. Although the binding and functional assays were carried out in different systems, the rank order of binding potencies (clobenpropit>A-320436>thioperamide = GT-2331>ciproxifan = A-317920>imoproxifan>A-304121) generally agrees with that of functional potencies (clobenpropit>A-317920>A-320436 = thioperamide>ciproxifan>GT-2331>imoproxifan>A-304121) at the monkey receptor with the exception of A-317920. Interestingly, thioperamide exhibited significantly higher binding potency at the monkey receptor than at the human receptor; however its potency at blocking (*R*)-α-methylhistamine-induced activation of the monkey receptor is comparable to that of the human receptor. Further, GT-2331 exhibited weak agonist properties at the histamine H_3 receptors in FLIPR assays at higher concentrations (1×10^{-6} M for the human and monkey histamine H_3 receptors; and 1×10^{-7} M for the rat histamine H_3 receptor), consistent with the findings by Esbenshade et al. (2001). Several histamine H_3 receptor ligands such as thioperamide and

ciproxifan have been shown to be inverse agonists at the human and rat histamine H_3 receptors (Morisset et al., 2000; Wieland et al., 2001; Pillot et al., 2002; Rouleau et al., 2002). We have also demonstrated that a variety of H_3R antagonists act as inverse agonists using a GTPγS binding assay (Esbenshade et al., 2003). However, these same compounds do not demonstrate inverse agonism in FLIPR assays most likely because of the cellular homestatic mechanisms that regulate intracellular Ca^{2+} levels and permit cell viability. The antagonist added prior to agonist addition did not change basal Ca^{2+} levels. Although it is likely that the monkey histamine H_3 receptor, like the human receptor, is constitutively active, the constitutive activity of histamine H_3 receptors was not readily observed with FLIPR assays and therefore, inverse agonism of these compounds at the monkey H_3 receptor was not assessed.

A-304121, A-317920 and A-320436 are compounds representative of a homologous chemical series (Esbenshade et al., 2003) that all have high potencies at the rat histamine H_3 receptor (0.39 to 2.3 nM). However, significant species selectivity with lower affinities at the human receptor was observed in some early leads from this series, such as A-304121 (>100-fold more selective for the rat receptor). Subsequent medicinal chemistry efforts directed toward improving compound potencies at the human histamine H_3 receptor within this chemical series have been successfully achieved by the incremental decreases in rat–human selectivity by compounds related to A-304121. A-317920 exhibited comparable affinity at the rat histamine H_3 receptor as A-304121, but significantly improved affinity at the human and monkey histamine H_3 receptor. A-320436 showed further improvement in its binding potency at the human and monkey histamine H_3 receptor with K_i values of 2.3 nM and 1.1. Interestingly, A-320436, unlike the other histamine H_3 receptor ligands tested, exhibited consistently higher potencies in binding assays than in FLIPR functional assays for all three species. In contrast, A-317920, although weaker in binding potencies than A-320436, is more potent at receptors in all three species in FLIPR functional assays. Whether these differences are related to actual differential recognition of various conformational states of these receptors across species and assays or are a reflection of the different physicochemical properties of the compounds remains to be determined.

In summary, we have investigated the monkey histamine H_3 receptor with radioligand binding and FLIPR functional assays, and showed that although the pharmacological profile of the monkey histamine H_3 receptor largely resembles that of the human histamine H_3 receptor, some structural and pharmacological differences are present between receptors of the two species. These findings suggest that in order to predict the in vivo potencies of histamine H_3 receptor ligands in human by using monkey behavioral testing, in vitro pharmacological characterization with the monkey H_3 receptor may be necessary to demonstrate the absence of subtle species selectivity between the monkey and human. Further, this information will contribute to the understanding of histamine H_3 receptor ligand properties in monkey behavioral studies, clarify structure and function relationships, and enhance the knowledge necessary for effective drug design.

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