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Molecular and pharmacological characterization of the mouse histamine H_3 receptor

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Abstract

Human, guinea pig and rat histamine H_3 receptors have been investigated at both pharmacological and molecular levels in recent years. Here we report the cloning, molecular, and pharmacological characterization of the mouse histamine H_3 receptor. The amino acid sequence of the mouse histamine H_3 receptor exhibits high homology to rat, guinea pig and human histamine H_3 receptors with 98%, 95%, 94% identities, respectively. The distribution of the mRNA encoding the mouse histamine H_3 receptor was predominant in the brain as detected by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and RNase protection assay. Although several splice forms have been reported for human, guinea pig and rat histamine H_3 receptor mRNAs, we did not detect equivalent isoforms in the mouse in several tissues by either RNase protection assay or robust Polymerase Chain Reaction (PCR) amplifications. Human embryonic kidney (HEK)-293 cells transiently transfected with mouse histamine H_3 receptor cDNA and Gq_{i5} exhibited increases of intracellular Ca^{2+} in response to histamine and several histamine H_3 receptor agonists. COS-7 (African green monkey kidney) cells transfected with mouse histamine H_3 receptor cDNA showed high affinity binding for histamine H_3 receptor ligands in competition binding assays. The pharmacological comparison of human, guinea pig, rat and mouse histamine H_3 receptors indicated that, as expected, the mouse histamine H_3 receptor exhibited a more similar pharmacological profile to the rat histamine H_3 receptor than to either the human or the guinea pig histamine H_3 receptor. Taken together, these findings allow a further appreciation of the histamine H_3 receptor at the molecular level and provide an additional species to assist in the pharmacological assessment of histamine H_3 receptor function.

Keywords: Histamine H_3 receptor; Cloning; Pharmacology; Splice variant

1. Introduction

Histamine plays an important role in a number of physiological functions, including inflammation, gastric acid secretion, neurotransmission and immune regulation through its H_1, H_2, H_3 and H_4 receptors, respectively. Since the first cDNA cloning of the human histamine H_3 receptor (Lovenberg et al., 1999), molecular characterization of histamine H_3 receptors of human, guinea pig and rat have been extensively investigated (Lovenberg et al., 1999, 2000; Tardivel-lacombe et al., 2000; Drutel et al., 2000; Liu et al., 2000). These G_i-coupled presynaptic receptors from different species share high amino acid homology, yet exhibit some differences in pharmacology (Lovenberg et al., 2000). Since the advent of gene-knockout technology, the mouse has become a more important species for the evaluation of gene expressed in the brain. Our interest in the histamine H_3 receptor has led us to explore the histamine H_3 receptor pharmacology in the mouse.

In addition to known pharmacological variations, the existence of splice variants has been reported for rat and guinea pig (Drutel et al., 2000; Tardivel-lacombe et al., 2000). Also, controversial results have also been reported for the existence of the human histamine H_3 receptor splice variants (Liu et al., 2000; Coge et al., 2001;Wiedemann et al., 2002; Wellendorph et al., 2002). These isoforms have been demonstrated to have distinct pharmacological properties and in some cases different tissue distribution from their corresponding "original" or "long" forms.

We have previously identified a fragment of the mouse histamine H_3 receptor cDNA which was used to knockout the histamine H_3 receptor mRNA expression in mice (Toyota et al., 2002). In the present study, we cloned the

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full length of mouse histamine H_3 receptor to characterize it at both molecular and pharmacological levels and investigate whether the alternative splice variants of mouse histamine H_3 receptor exist.

2. Materials and methods

2.1. Cloning of the mouse histamine H_3 receptor cDNA coding region

A 1 kb mouse histamine H_3 receptor cDNA fragment was Polymerase Chain Reaction (PCR)-amplified from mouse brain cDNA (Clontech, Palo Alto, CA) using two primers [forward primer: 5′ GGC AAC GCG CTG GTC ATG CTC GCC TTC GTG 3′ (rat histamine H_3 receptor coding region 151–180) and reverse primer: 5′ GGG CCC AGC AGA GCC CAA AGA TGC TCA CGA TGA 3′ (rat histamine H_3 receptor coding region 1085–1118)] which were designed according to the conserved regions between the human and rat histamine H_3 receptor cDNA sequences (Lovenberg et al., 1999, 2000), using the expand high fidelity Taq DNA polymerase (Roche, Indianapolis, IN). The PCR reaction conditions were: 40 cycles of 94 °C, 40 s; 65 °C, 40 s; 72 °C, 3 min in a Perkin-Elmer GeneAmp System 9600 (Norwalk, CT). The resulting PCR fragment was cloned into PCR2.1-Topo vector (Invitrogen, Carlsbad, CA) and the insert region was sequenced. The resulting sequence appeared to be unique and showed 90% sequence identity to the rat histamine H_3 receptor cDNA. The 5′ end and the 3′ end of the mouse histamine H_3 receptor cDNA were then PCR-amplified from mouse brain Marathon-Ready cDNA (Clontech) by Rapid Amplification of cDNA Ends (RACE) using mouse histamine H_3 receptor gene specific primers and adaptor primers supplied by the manufacturer. The complete coding region of the mouse histamine H_3 receptor cDNA was then PCR-amplified from the mouse brain Marathon-Ready cDNA with two primers, forward primer: 5′ ACG ATA GAA TTC GCC ACC ATG GAG CGC GCG CCG CCC GAC GGG CTG ATG AA 3′ (coding region 1–32) and reverse primer: 5′ ACT AGA GCG GCC GCT CAC TTC CAG CAC TGC TCC AGG GAG CCA TG 3′ (coding region 1309–1338), using the same conditions described above. A single band of 1.4 kb was obtained, and then subcloned into the mammalian expression vector pCIneo (Promega, Madison, WI) and the insert region was sequenced with a Prism 377 DNA sequencer (ABI, Foster City, CA).

2.2. Competition binding assays

COS-7 (African green monkey kidney) cells (CRL-1651, American Type Culture Collection, Manassas, VA) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) with 10% fetal bovine serum and transfected with the mouse histamine H_3 receptor cDNA plasmid using LipofectAMINE (Invitrogen) as described by the manufacturer. Two days after tranfection, the COS-7 cells were detached with phosphate buffered saline plus 10 mM EDTA, washed with phosphate buffered saline, and centrifuged at 1500 rpm for 5 min. The pellets were homogenized in 0.2 × binding buffer (50 mM Tris–HCl, 5 mM EDTA, PH 7.4) for 30 s and centrifuged at 10,000 rpm for 30 min. The membranes were resuspended in the binding buffer, aliquoted into 96-well plates (Greiner Bio-One, Frickenhausen, Germany), incubated with 0.9 nM [^{3}H]-*N*-α-methylhistamine in the presence of various concentrations of test compounds at room temperature for 1 h, and then harvested by filtration through the GF/C (Packard, Meriden, CT) filters pretreated with 0.3% polyethylenimine. The filters were washed four times with ice-cold binding buffer and dried in a 60 °C oven. A 50 μl of Microscint 0 (Packard) was added to each well, and the plates were then counted on Packard TopCount.NXT.

2.3. Ca^{2+} mobilization assays

Human embryonic kidney (HEK)-293 cells (CRL-1573, American Type Culture Collection) were cultured in DMEM with 10% fetal bovine serum in 15 cm tissue culture dishes. Mouse, rat, guinea pig, and human histamine H_3 receptor cDNAs in the mammalian expression vector pCIneo were transiently transfected into HEK-293 cells either with or without a Gq_{i5} cDNA plasmid using LipofectAMINE. Two days after transfection, The cells were detached with phosphate-buffered saline plus 10 mM EDTA, washed with serum free DMEM/Ham's F-12 (Invitrogen) twice, then loaded with 4 μM Ca^{2+} dye Fluo-3 (TEF labs, Austin, TX) in the dye loading buffer (DMEM/Ham's F-12 medium without phenol red plus 2.5 mM probenecid) at room temperature for 1 h. The cells were then washed one time with the dye loading buffer and seeded at 2×10^5 cells/well into polylysine-coated 96-well clear bottom black plates (BD Biosciences, San Jose, CA), and incubated at 37 °C for 1 h. Experimental compounds were diluted in DMEM/Ham's F-12 containing 1% bovine serum albumin. Ligand-induced Ca^{2+} release was measured using a Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices, Sunnyvale, CA).

2.4. PCR detection of mouse histamine H_3 receptor mRNA tissue expression pattern

Two primers: 5′ ACG ATA GAA TTC GCC ACC ATG GAG CGC GCG CCG CCC GAC GGG CTG ATG AA 3′ (coding region 1–32) and 5′ ACT AGA GCG GCC GCT CAC TTC CAG CAC TGC TCC AGG GAG CCA TG 3′ (coding region 1309–1338) were used to amplify the entire coding region of the mouse histamine H_3 receptor cDNA. Nine cDNAs from different mouse tissues (Clontech) were used as templates for PCR amplifications. The PCR conditions were the same as described above. The PCR products were run in a 2% agarose gel, stained with ethidum bromide (10 μg/ml), and then photographed under UV irradiation. The GPDH gene was used as positive control.

The mouse histamine H_3 receptor PCR product was cloned into pBluescript (Stratagene, La Jolla, CA) vector and sequenced with a Prism 377 DNA sequencer (ABI).

2.5. RNase protection assay for the mRNA expression of mouse histamine H_3 receptor in different mouse tissues

The plasmid pBluescript (Stratagene) containing a 429 bp fragment of the putative alternatively spliced region (coding region 768–1196) of the mouse histamine H_3 receptor cDNA was linearized. A ^{32}P-labeled antisense riboprobe was synthesized using T7 RNA polymerase (Promega). A total of 20 μg of total RNA from different mouse tissues were hybridized with mouse histamine H_3 receptor antisense probe overnight as described (Liu et al., 2000). The hybridization mixtures were digested with ribonucleases A and T1 (Sigma, St. Louis, MO), purified, and then run in a 5% polyacrylamide/8 M urea gel with the undigested probe as marker. The gel was dried under vacuum for 1 h at 80 °C and exposed to an X-ray film (MS film, Kodak, Rochester, NY) for 2 h at −80 °C. A mouse β-actin probe was used as control in a parallel experiment.

2.6. PCR detection of the putative alternative splicing variants in the entire mouse histamine H_3 receptor coding region

Three pairs of PCR primers were designed to PCR-amplify three overlapping fragments of the entire mouse histamine H_3 receptor coding region. Primers, P1: 5′ ATG GAG CGC GCG CCG CCC GAC GGG CTG A 3′ and P2: 5′ TGC CGG AGG TGG GAG CTC AAG AAG TGT G 3′, were used to amplify the coding region 1–628. Primers, P3: 5′ CTC TGC AAG CTG TGG CTG GTG GTA GAC T 3′ and P4: 5′ TTC TTG TCC CGC GAC AGC CGA AAG CGC T 3′, were used to amplified coding region 316–1065. Primers, P5: 5′ AGA GGC GCA CTC GTC TTC GGC TGG ATG G 3′ and P6: 5′ TCA CTT CCA GCA CTG CTC CAG GGA GCC A 3′, were used to amplify coding region 677–1338. The mouse brain cDNA was used as the tem-

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     Human:MERAPPDGPLNASGALAGDAAAA-GGARGFSAAWTAVLAALMALLIVATVLGNALVMLAFVADSSLRTQNN
Guinea pig:MERAPPDGLMNASGALAGEAAAAAGGARTFSAAWTAVLAALMALLIVATVLGNALVMLAFVADSSLRTQNN
       Rat:MERAPPDGLMNASGTLAGEAAAA-GGARGFSAAWTAVLAALMALLIVATVLGNALVMLAFVADSSLRTQNN
     Mouse:MERAPPDGLMNASGALAGEAAAA-GGARGFSAAWTAVLAALMALLIVATVLGNALVMLAFVADSSLRTQNN
 Consensus:MERAPPDG--NASG-LAG-AAAA-GGAR-FSAAWTAVLAALMALLIVATVLGNALVMLAFVADSSLRTQNN
                                                  TM1

     Human:FFLLNLAISDFLVGAFCIPLYVPYVLTGRWTFGRGLCKLWLVVDYLLCTSSAFNIVLISYDRFLSVTRAV
Guinea pig:FFLLNLAISDFLVGVFCIPLYVPYVLTGRWTFGRGLCKLWLVVDYLLCTSSVFNIVLISYDRFLSVTRAV
       Rat:FFLLNLAISDFLVGAFCIPLYVPYVLTGRWTFGRGLCKLWLVVDYLLCASSVFNIVLISYDRFLSVTRAV
     Mouse:FFLLNLAISDFLVGAFCIPLYVPYVLTGRWTFGRGLCKLWLVVDYLLCASSVFNIVLISYDRFLSVTRAV
 Consensus:FFLLNLAISDFLVG-FCIPLYVPYVLTGRWTFGRGLCKLWLVVDYLLC-SS-FNIVLISYDRFLSVTRAV
                        TM2                                 TM3

     Human:SYRAQQGDTRRAVRKMLLVWVLAFLLYGPAILSWEYLSGGSSIPEGHCYAEFFYNWYFLITASTLEFFTP
Guinea pig:SYRAQQGDTRRAVRKMVLVWVLAFLLYGPAILSWEYLSGGSSIPEGHCYAEFFYNWYFLITASTLEFFTP
       Rat:SYRAQQGDTRRAVRKMALVWVLAFLLYGPAILSWEYLSGGSSIPEGHCYAEFFYNWYFLITASTLEFFTP
     Mouse:SYRAQQGDTRRAVRKMALVWVLAFLLYGPAILSWEYLSGGSSIPEGHCYAEFFYNWYFLITASTLEFFTP
 Consensus:SYRAQQGDTRRAVRKM-LVWVLAFLLYGPAILSWEYLSGGSSIPEGHCYAEFFYNWYFLITASTLEFFTP
                              TM4                                           TM5

     Human:FLSVTFFNLSIYLNIQRRTRLRLDGAREAAGPEPPPEAQPSPPP-PPGCWGCWQKGHGEAMPLHRYGVGEA
Guinea pig:FLSVTFFNLSIYLNIQRRTRLRLDGGAREAGPDPLPEAQSSPPQPPPGCWGCWPKGQGESMPLHRYGVGEA
       Rat:FLSVTFFNLSIYLNIQRRTRLRLDGG-REAGPEPPPDAQPSPPPAPPSCWGCWPKGHGEAMPLHRYGVGEA
     Mouse:FLSVTFFNLSIYLNIQRRTRLRLDGG-REAGPEPPPDAQPSPPPAPPSCWGCWPKGHGEAMPLHRYGVGEA
 Consensus:FLSVTFFNLSIYLNIQRRTRLRLDG----AGP-P-P-AQ-SPP--PP-CWGCW-KG-GE-MPLHRYGVGEA


     Human:AVGAEAGEATLGGGGGGGSVASPTSSSGSSSRGTERPRSLKRGSKPSASSASLEKRMKMVSQSFTQRFRL
Guinea pig:GPGAEAGEAALGGGS--GAAASPTSSSGSSSRGTERPRSLKRGSKPSASSASLEKRMKMVSQSITQRFRL
       Rat:GPGVEAGEAALGGGSGGGAAASPTSSSGSSSRGTERPRSLKRGSKPSASSASLEKRMKMVSQSITQRFRL
     Mouse:GPGVETGEAGLGGGSGGGAAASPTSSSGSSSRGTERPRSLKRGSKPSASSASLEKRMKMVSQSITQRFRL
 Consensus:--G-E-GEA-LGGG---G--ASPTSSSGSSSRGTERPRSLKRGSKPSASSASLEKRMKMVSQS-TQRFRL


     Human:SRDRKVAKSLAVIVSIFGLCWAPYTLLMIIRAACHGHCVPDYWYETSFWLLWANSAVNPVLYPLCHHSFR
Guinea pig:SRDKKVAKSLAIIVSIFGLCWAPYTLLMIIRAACHGHCVPDYWYETSFWLLWANSAVNPVLYPLCHYSFR
       Rat:SRDKKVAKSLAIIVSIFGLCWAPYTLLMIIRAACHGRCIPDYWYETSFWLLWANSAVNPVLYPLCHYSFR
     Mouse:SRDKKVAKSLAIIVSIFGLCWAPYTLLMIIRAACHGHCVPDYWYETSFWLLWANSAVNPVLYPLCHYSFR
 Consensus:SRD-KVAKSLA-IVSIFGLCWAPYTLLMIIRAACHG-C-PDYWYETSFWLLWANSAVNPVLYPLCH-SFR
                              TM6                                 TM7

     Human:RAFTKLLCPQKLKIQPHGSLEQCWK
Guinea pig:RAFTKLLCPQKLKVQPHGSLEQCWK
       Rat:RAFTKLLCPQKLKVQPHSSLEHCWK
     Mouse:RAFTKLLCPQKLKVQPHSSLEHCWK
 Consensus:RAFTKLLCPQKLK-QPH-SLE-CWK
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Fig. 1. Amino acid sequence comparison of human, guinea pig, rat and mouse histamine H_3 receptors. The consensus sequence is shown in bold. The putative seven transmembrane domains (denoted TM) are indicated with solid lines.

plate for the PCR reactions. The PCR conditions and product detection method were the same as described above.

2.7. Chemicals

All histamine ligands were purchased from Sigma/RBI (Natick, MA). All other reagents were purchased from Sigma.

3. Results

3.1. Comparison of the sequences of human, guinea pig, rat and mouse histamine H_3 receptors

A 1 kb DNA fragment was PCR-amplified from mouse brain cDNA using two primers designed from the conserved region between the human and rat histamine H_3 receptor cDNA sequences. The DNA sequence is unique and shares greater than 90% sequence identity to the human and rat histamine H_3 receptor sequences, suggesting that it is a mouse histamine H_3 receptor cDNA fragment. The 5′ end and 3′ end of the mouse histamine H_3 receptor cDNA were PCR-amplified by RACE using mouse histamine H_3 receptor gene-specific primers and the complete coding region of mouse histamine H_3 receptor was obtained. Our cDNA sequence of the mouse histamine H_3 receptor has been submitted to Genbank (Accession No.: AY142145). A Genbank data base search showed that our mouse histamine H_3 receptor cDNA sequence is identical to an unpublished Genbank submission (Genbank Accession No.: AY044153). The complete coding region of the mouse histamine H_3 receptor

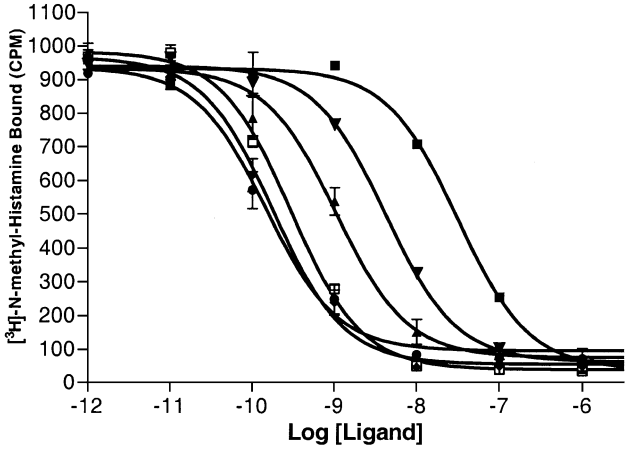

Fig. 2. Characterization of mouse histamine H_3 receptor in the radioligand binding assays. Mouse histamine H_3 receptor cDNA transfected COS-7 cells membranes were added into 96-well plates. [^{3}H]-*N*-α-methylhistamine was added to each well at a final concentration of 0.9 nM. Different concentrations of various unlabeled histamine ligands were added to the assays as the competitors. All assays were performed in triplicates. The results are mean values (± S.E.M.) of the triplicates. ■, Histamine; ▲, *N*-α-methylhistamine; ▼, *R*-α-methylhistamine; ♦, Imetit; ●, Immepip; □, Clobenpropit.

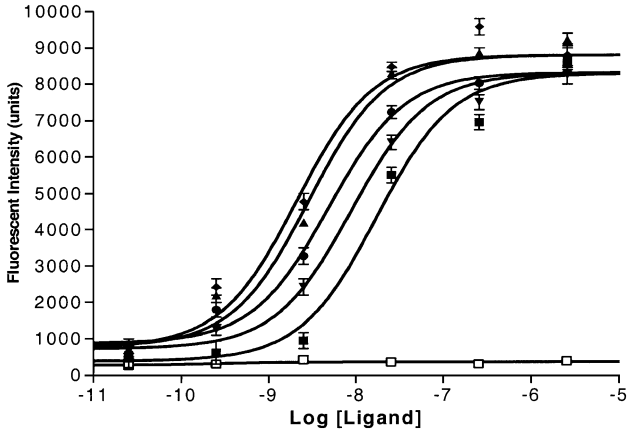

Fig. 3. Histamine ligands stimulate Ca^{2+} mobilization in HEK-293 cells co-expressing mouse histamine H_3 receptor and Gq_{i5}. HEK-293 cells were co-transfected by mouse histamine H_3 receptor and Gq_{i5} cDNAs. The transfected cells were loaded with Ca^{2+} dye Fluo-3 and then stimulated with different concentrations of various histamine ligands. The release of Ca^{2+} was measured with a FLIPR. The results are mean values (± S.E.M.) of triplicate experiments. ■, Histamine; ▲, *N*-α-methylhistamine; ▼, *R*-α-methylhistamine; ♦, Imetit; ●, Immepip; □, Clobenpropit.

cDNA encodes a putative protein of 445 amino acids. Homology comparison among mouse, rat, guinea pig and human histamine H_3 receptors revealed that mouse histamine H_3 receptor shares 98%, 95%, 94% homology with the rat, guinea pig and human histamine H_3 receptors, respectively (Fig. 1). Among the seven transmembrane domains of histamine H_3 receptors from different species, the first and fifth transmembrane domains are completely identical, whereas transmembrane domains 2, 4, 6, 7 have a single amino acid change respectively and there are two amino acid changes in transmembrane domain 3. Rat and mouse are completely identical in all of the transmembrane domains.

3.2. Mouse histamine H_3 receptors have a more similar pharmacological profile to rat histamine H_3 receptors than to human and guinea pig histamine H_3 receptors

A series of six histamine receptor ligands was used to evaluate the pharmacological profile of the mouse histamine H_3 receptor in transfected COS-7 cells. [^{3}H]-*N*-α-methylhistamine bound specifically to the mouse histamine H_3 receptor with high affinity (K_d = 0.9 nM) as determined by a saturation isotherm (data not shown). For competition binding experiments, [^{3}H]-*N*-α-methylhistamine was used at its K_d (0.9 nM). The results showed that the mouse histamine H_3 receptors have high affinity for a number of histamine ligands (Fig. 2). The rank order of potency and mean K_i (nM, ± S.E.M.) values from a triplicate experiments are immepip (0.16 ± 0.2)>imetit (0.2 ± 0.5)>clobenpropit (0.31 ± 0.6)> *N*-α-methylhistamine (1.1 ± 0.3)>*R*-α-methylhistamine (3.9 ± 0.4)>histamine (15 ± 2.7). These ligands were also tested for their ability to activate Ca^{2+} mobilization in HEK-293 cells transiently co-transfected with Gq_{i5} (Conklin et al., 1993) and the mouse histamine H_3 receptor. The results

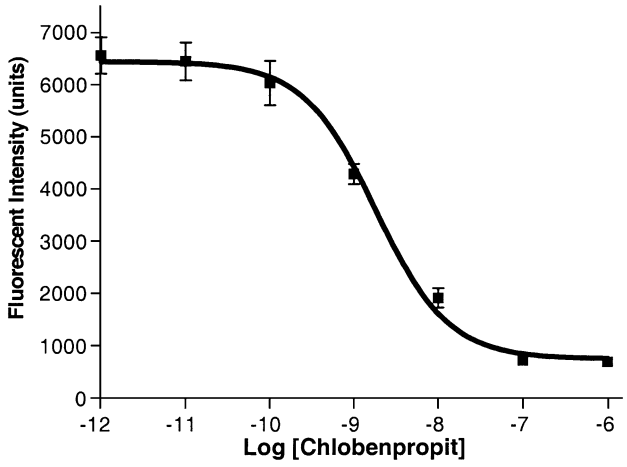

Fig. 4. Clobenpropit is an antagonist for the mouse histamine H_3 receptor. HEK-293 cells cotransfected with mouse histamine H_3 receptor and Gq_{i5} cDNAs were loaded with Fluo-3. Different concentrations of clobenpropit were added to the cells and incubated 20 min at room temperature. *N*-α-methylhistamine was added to cells at a final concentration of 10 nM to stimulate Ca^{2+} mobilization. Release of Ca^{2+} was measured with a FLIPR. The results are mean values (± S.E.M.) of triplicate experiments.

showed similar efficacy, but different potency of the five known histamine H_3 receptor agonists, *N*-α-methylhistamine, *R*-α-methylhistamine, imetit, Immepip and histamine. The histamine H_3 receptor antagonist clobenpropit showed no agonism at the mouse histamine H_3 receptor (Fig. 3).

Clonbenpropit has been shown to be a potent antagonist for the recombinant human and rat histamine H_3 receptors (Lovenberg et al., 1999, 2000). In our results described here for the mouse histamine H_3 receptor, clobenpropit showed that it has high affinity in the binding assay but showed no agonism in the functional assays, suggesting that it may also be an antagonist for the mouse histamine H_3 receptor. To confirm this, we tested the effects of clobenpropit on *N*-α-methylhistamine-induced Ca^{2+} mobilization. Clobenpropit dose-dependently suppressed the effects of *N*-α-methylhistamine thus confirmed that clobenpropit is an antagonist for the mouse histamine H_3 receptor (Fig. 4). The K_B apparent for clonbenpropit was determined to be 1.5 ± 0.5 nM.

To pharmacologically compare the mouse histamine H_3 receptor with histamine H_3 receptors from other species, human, guinea pig, rat and mouse histamine H_3 receptors cDNAs were co-transfected with a Gq_{i5} plasmid into HEK-293 cells. As shown in Fig. 5 and Table 1, imetit, *N*-α-methylhistamine, and histamine were full agonists for all four histamine H_3 receptors, among which imetit was the most potent. Chloroproxyfan behaved as a full agonist only for human histamine H_3 receptor, and displayed partial

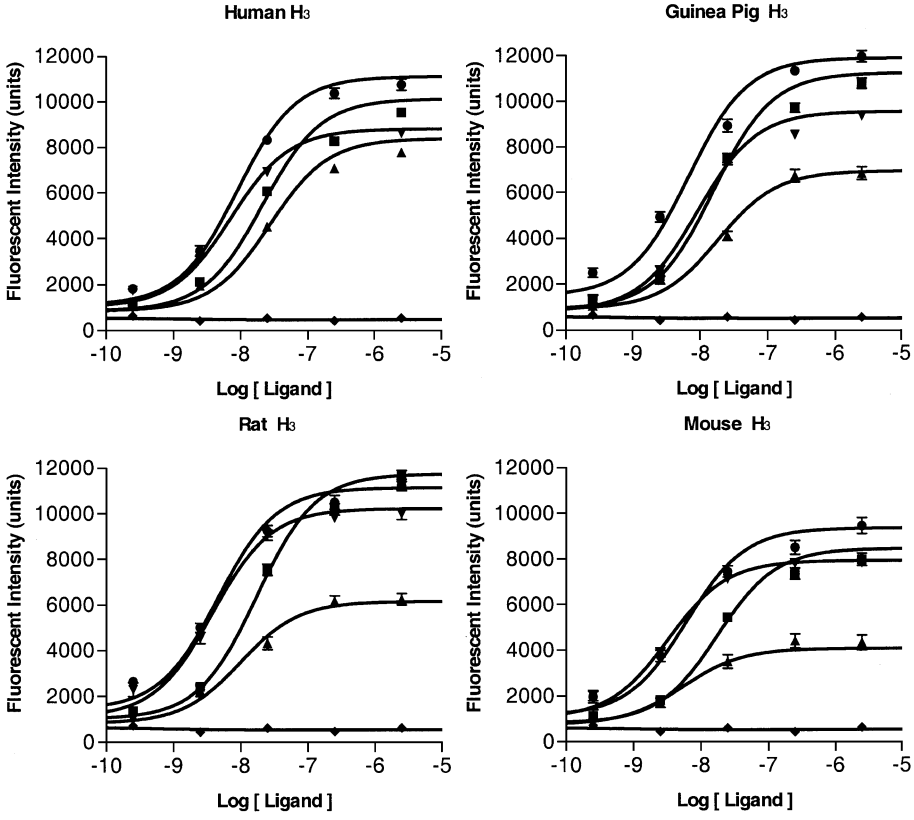

Fig. 5. Pharmacological comparison of histamine H_3 receptors from different species. HEK-293 cells were cotransfected with a Gq_{i5} cDNA and histamine H_3 receptor expression vectors for human, guinea pig, rat and mouse. The transfected cells were loaded with Ca^{2+} dye Fluo-3 and stimulated with different concentrations of various histamine ligands. Release of Ca^{2+} was measured with a FLIPR. The results are mean values (± S.E.M.) of triplicate experiments. ■, Histamine; ▲, Chloroproxyfan; ▼, Imetit; ♦, Clobenpropit; ●, *N*-α-methylhistamine.

Table 1
EC_{50} of histamine ligands for histamine H_3 receptors from different species

Compound	Human H_3	Guinea pig H_3	Rat H_3	Mouse H_3
	EC_{50} (nM ± S.E.M.)			
Histmine	21.8 ± 5.3	15.3 ± 3.7	17.6 ± 3.6	17.0 ± 4.1
Chloroproxyfan	28.3 ± 6.6	18.0 ± 4.9	10.6 ± 3.1	5.3 ± 1.2
Imetit	7.4 ± 0.9	9.2 ± 2.2	3.9 ± 0.7	3.3 ± 0.4
Clobenpropit	>10,000	>10,000	>10,000	>10,000
N-methylhistamine	9.2 ± 2.6	7.0 ± 1.8	4.5 ± 1.1	5.9 ± 0.8

EC_{50} value is determined as the concentration of a ligand to stimulate Ca^{2+} release at its half maximal activation. The data are mean values of triplicate experiments.

agonism for the other three histamine H_3 receptor homologues. Clobenpropit showed no agonist properties for any of the histamine H_3 receptor homologues. In the Ca^{2+} mobilization assays, all ligands had no effect on HEK-293 cells transfected only with the histamine H_3 receptor cDNAs in the absence of Gq_{i5} (data not shown).

3.3. Mouse histamine H_3 receptor is expressed predominantly in the brain

To investigate the mouse histamine H_3 receptor mRNA distribution, we PCR-amplified nine different mouse tissue cDNAs using two primers designed to amplify the entire mouse histamine H_3 receptor coding region. As shown in Fig. 6, a 1400 bp fragment was amplified only from mouse brain cDNA. The PCR amplicon was confirmed by sequencing to be the mouse histamine H_3 receptor coding region.

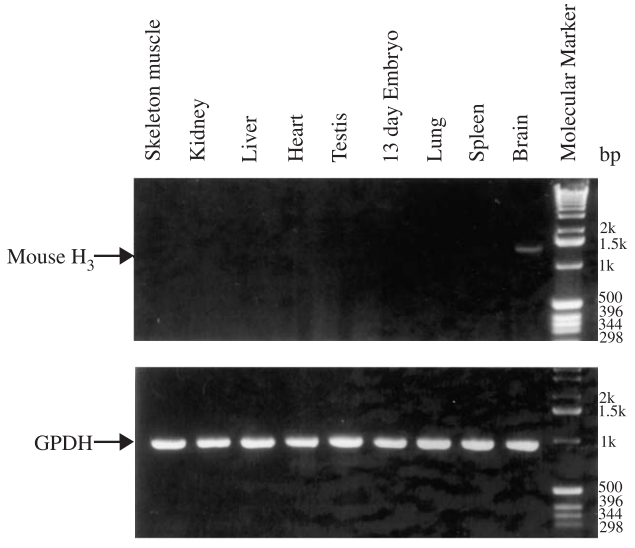

Fig. 6. PCR detection of mouse histamine H_3 receptor mRNA expression profile. cDNAs were synthesized from RNAs of different mouse tissues: brain, spleen, lung, embryo, testis, heart, liver, kidney, and skeleton muscle. Two primers: 5′ ACG ATA GAA TTC GCC ACC ATG GAG CGC GCG CCG CCC GAC GGG CTG ATG AA 3′ and 5′ ACT AGA GCG GCC GCT CAC TTC CAG CAC TGC TCC AGG GAG CCA TG 3′ were used for PCR. PCR-amplification of mouse GPDH was used as an internal control.

3.4. RNase protection assay showed no mouse histamine H_3 receptor isoforms were detected

Alternative splicing of the histamine H_3 receptor gene has been reported in rat and guinea pig (Drutel et al., 2000; Tardivel-lacombe et al., 2000). Contradictory results for the human histamine H_3 receptor alternative splicing have been reported (Liu et al., 2000; Coge et al., 2001; Wiedemann et al., 2002; Wellendorph et al., 2002). To investigate whether mouse histamine H_3 receptor isoform mRNAs are expressed in mouse brain as reported in guinea pig and rat, we used a 429 bp fragment of the mouse histamine H_3 receptor as a probe in RNase protection assay. This fragment corresponds to the region between transmembrane domain 5 and transmembrane domain 7, which has been shown to be a site of putative alternative splicing in the rat and guinea pig histamine H_3 receptors. Six different mouse tissues were evaluated in the assay. The result (Fig. 7) showing that only a single 429 bp protected RNA was detected in mouse brain indicated that the long form of the mRNA is the predominant, if not only, form expressed in the brain.

3.5. PCR results indicated that no alternative splicing occurs in the entire coding region

To confirm the findings of the above-described RNase protection assay, we designed a PCR method to investigate

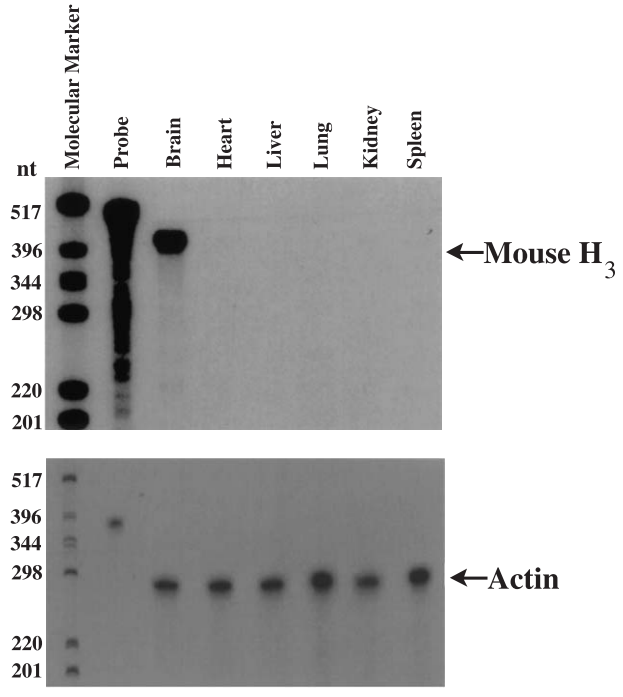

Fig. 7. RNase protection assay detection of mouse histamine H_3 receptor mRNA expression profile. A total of 20 μg of total RNA from mouse brain, heart, liver, lung, kidney and spleen were used in RNase protection assays. Molecular weight standards and undigested probe were run in adjacent lanes as size standards. A mouse β-actin probe was used as an internal control in a parallel experiment.

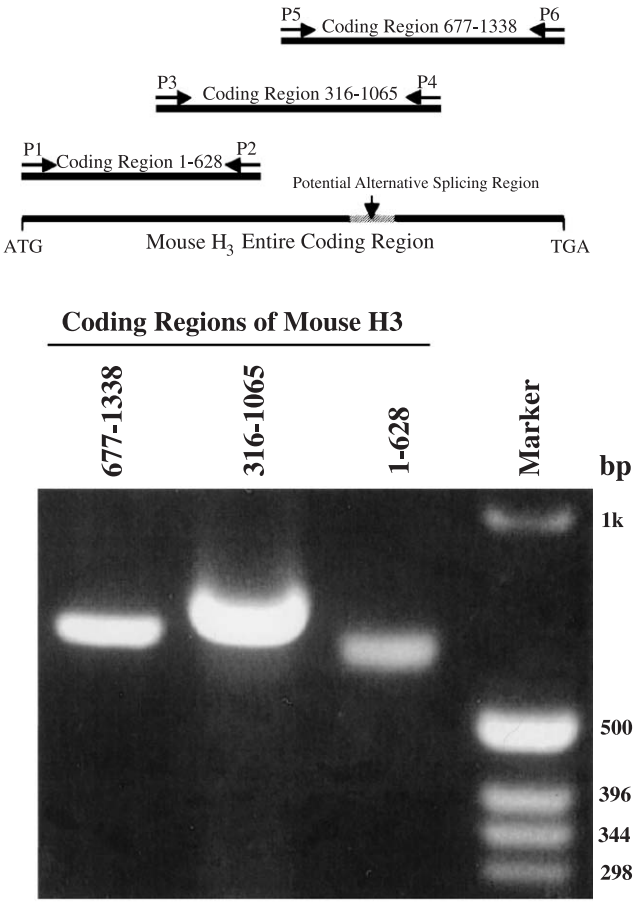

Fig. 8. PCR analysis of mouse histamine H_3 receptor mRNA in the brain. Top panel: PCR diagram. Three pairs of PCR primers that amplify three over lapping fragments of the entire coding region of mouse histamine H_3 receptor were used to PCR amplify the mouse brain cDNA. P1 to P6 indicate the position of the primers used for PCR detection. The hatched box indicates the potential alternative splicing region that has been reported in rat and guinea pig. Bottom panel: The PCR products were run in a 2% agarose gel, stained with ethidium bromide and visualized under UV light.

other splice variants for the mouse histamine H_3 receptor gene. We employed three pairs of PCR primers to amplify three overlapping fragments covering the entire coding region of mouse histamine H_3 receptor mRNA using mouse brain cDNA as the template. Our results clearly demonstrated that a single PCR product was observed in each PCR reaction (Fig. 8). PCR products from three reactions were cloned and sequenced. The results indicated that all products were from the long form of the mouse histamine H_3 receptor mRNA and no isoforms were detected.

4. Discussion

In this study, the cloning and molecular and pharmacological characterization of the mouse histamine H_3 receptor are described. The open reading frame of the mouse histamine H_3 receptor mRNA encodes a 445-amino acid protein that has high homology and similar pharmacology to the rat, guinea pig and human histamine H_3 receptors. The amino acid sequence analysis revealed that the seven transmembrane domains of the mouse histamine H_3 receptor are identical to those of the rat histamine H_3 receptor. Similar to the orthologues of the human, guinea pig and rat receptors, the mouse histamine H_3 receptor contains several conserved amino acid residues such as Asp^{80}, Asp^{114}; Cys^{107}, Cys^{188} and a Asp-Arg-Phe (DRF) motif at the end of transmembrane domain 3 (Leurs et al., 2000). In addition, there are only four amino acid differences between the mouse and rat histamine H_3 receptors (Thr^{286}, Gly^{290}, His^{387} and Val^{389} in the mouse histamine H_3 receptor corresponding to Ala^{286}, Ala^{290}, Arg^{387} and Ile^{389} of the rat histamine H_3 receptor). These minor changes would not be predicted to have influence on the binding pockets (Stark et al., 2001). It is thus not surprising that the pharmacology and ligand binding activity of the mouse histamine H_3 receptor exhibits strong similarity to that of the rat histamine H_3 receptor.

According to the pharmacological profiles of the human, guinea pig and rat histamine H_3 receptors, we selected several typical compounds to characterize the mouse histamine H_3 receptor. As expected, the result is similar to our previous report characterizing the rat histamine H_3 receptor (Lovenberg et al., 1999, 2000). For example, histamine and its derivatives immepip, imetit, *N*-α-methylhistamine, *R*-α-methylhistamine all behave as high affinity, full agonists, whereas clobenpropit is an antagonist. In addition, to confirm whether the recombinant mouse receptor has the same pharmacological characterization to the native counterpart, we examined the binding affinities of the same compounds to homogenized mouse brain tissue. The result obtained from a natural receptor source is similar to that of the recombinant mouse histamine H_3 receptor (data not shown) as well as to previous reports in the literature (Jansen et al., 2000). Comparison of agonism via Ca^{2+} mobilization among the histamine H_3 receptors from the four species also indicated that the mouse histamine H_3 receptor shared a similar pharmacological profile with the other three receptors, particularly with the rat histamine H_3 receptor. We have previously reported that chloroproxyfan behaved as a full agonist of the rat recombinant receptor as measured by inhibition of forskolin-stimulated cAMP accumulation (Lovenberg et al., 2000). In the present study, chloroproxyfan showed agonist properties when evaluated in the Ca^{2+} mobilization assay, although the EC_{50} was shifted substantially to the right compared to its binding affinity.

We have observed that some ligands display discrepancies between the binding K_i's and functional EC_{50}'s. One possible explanation is that the transfected COS-7 cells and HEK-293 cells express different densities of receptors, which may alter ratios and thus lead to discrepant K_d and EC_{50} values. We attempted binding assays using same amount of membranes from both types of transfected cells. In saturation binding assays, although the B_{max} from transfected COS-7 (approximately 11,000 fmol receptor per mg of membrane protein) is routinely five times greater than that

from transfected 293 cells (approximately 2000 fmol receptor per mg of membrane protein), the K_d values derived from the two binding assays are very much comparable.

We chose the transfected COS-7 cells for binding assays, because they gave the large signal to noise ratio, which provided more consistent results. Under similar conditions, transfected HEK-293 cells provided only 1/5 of the signal-to-noise when comparing similar amount of membranes. We chose the HEK-293 cells for the functional assays because in the recombinant receptor expression system we have used, the receptor density is seldom the rate-limiting step for the signal transduction. Instead, the endogenous down stream signal transduction components are rate limiting. Cells with different receptor densities often have the same maximum output for the signal transduction. Because of this, we need a high percentage of cells to be transfected to have a better signal in the functional assays. Since COS-7 cells have higher plasmid copy number upon transfected but have a lower transfection efficiency, they work best for binding assay. However, HEK-293 cells have a higher transfection efficiency, and they produce better results in functional assays.

Another possible reason that ligands display different binding potencies and functional EC_{50} values could be that the binding assay is an equilibration assay while the Ca^{2+} mobilization assay is more close to a kinetic assay. In the Ca^{2+} mobilization assays, we observed the intracellular Ca^{2+} concentration change in a few seconds to 1 min after adding the ligand. Ligands that have slower association rates to the receptor may show lower potencies in the Ca^{2+} mobilization assays. In the binding assays, the K_d is determined at equilibration and thus is more like to reflect a higher affinity. The co-expression of a modified G-protein, in this case Gq_{i5}, could also play a role in causing the potency discrepancy in the two assays. We are currently attempting to address this question using a series of agonist assays (both recombinant and non-recombinant) and a broad assay of agonists. This future study is, however, beyond the scope of this current report.

The tissue distribution of the mouse histamine H_3 receptor mRNA, as best as could be determined, was identical to that of the human, guinea pig and rat histamine H_3 receptors. The mRNA of the mouse histamine H_3 receptor was detected only in mouse brain by either Reverse Transcription (RT)-PCR or RNase protection assay compared to a limited number of peripheral organ tissues. It is possible that there exists the mouse histamine H_3 receptor in other mouse tissues or specialized organ or cell groups as previously reported (Pozzoli et al., 2002).

It has been reported that splice variants of the rat, guinea pig and human histamine H_3 receptors exist, and these isoforms exhibit different patterns of mRNA expression and pharmacological profiles from their corresponding "original" or "long form" receptors (Tardivel-lacombe et al., 2000; Drutel et al., 2000; Coge et al., 2001). In order to detect any isoforms of the mouse histamine H_3 receptor, we employed PCR and RNase protection assay methods, which have been demonstrated to be able to detect the mRNA isoforms (Liu et al., 2000) in rat tissue and artificial human histamine H_3 splice variants. For PCR detection, we designed various primers to amplify the coding region of the mouse histamine H_3 receptor including that corresponding to the alternative splicing intracellular loop 3 by using different annealing temperatures. However, we failed to identify any putative shorter products. One possible reason is that these primers failed to cover the splicing region of the mouse histamine H_3 receptor. To confirm this finding, we employed a RNase protection assay which is very sensitive and has greater fidelity than PCR. A 429 bp fragment within the intracellular loop 3 of the mouse histamine H_3 receptor gene was constructed as a probe. Our finding that no other protected short form was detected suggests that the long form of the receptor mRNA in mouse is the predominant, if not the only, form. This is similar to our finding with the human histamine H_3 receptor (Liu et al., 2000).

In summary, we have identified and characterized the mouse orthologue of the histamine H_3 receptor. In essence, the receptor is pharmacologically undistinguishable from the rat orthologue. With the recent knockout of the histamine H_3 receptor in mice (Toyota et al., 2002), the mouse may become a useful species for histamine H_3 receptor characterization. Thus the availability of the full receptor sequence and pharmacology should help in the advent of the mouse to uncover the function of the histamine H_3 receptor.

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