Comparison of Human, Mouse, Rat, and Guinea Pig Histamine H4 Receptors Reveals Substantial Pharmacological Species Variation

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ABSTRACT
The recently identified histamine H4 receptor has revealed a potential new complexity for the role of histamine in the immune system. To begin to understand the role of this receptor in humans, one must first determine whether homologs exist and can be studied in lower species. To address this, we cloned the human H4 receptor, and there are no clear homologs. The tissue distribution of the rat, mouse, and guinea pig H4 receptors is similar to human in that bone marrow and spleen are the most abundant sources of expression. The human and guinea pig H4 receptors display the highest binding affinity for [3H]histamine (Kd = 5 nM each), whereas the affinities for rat and mouse receptors are substantially lower at 136 and 42 nM, respectively. With respect to the pharmacological profile of known H3/H4 ligands, even greater differences in binding affinities exist among the species homologs. There are also substantial differences in the signal transduction response to each of the four species of H4 receptor. This work demonstrates the existence of histamine H4 receptors in lower species and demonstrates that a clear knowledge of each species pharmacological profile will be essential to elucidate the role of this receptor subtype in vivo.

Histamine is a multifunctional substance exerting influence on many types of cells and physiological processes through a distinct set of G protein-coupled receptors. Generally, histamine modulates inflammatory and allergic responses via H1 receptors (Ash and Schild, 1966), gastrin acid secretion through H2 receptors (Black et al., 1972), and neurotransmitter release in the central nervous system via H3 receptors (Arrang et al., 1983). All of these histamine receptor subtypes are members of the superfamily of G protein-coupled receptors (Gantz et al., 1991; Yamashita et al., 1991; Lovenberg et al., 1999). Recently, we and others have reported cloning and characterization of a new subclass of histamine receptors, H4 (Nakamura et al., 2000; Oda et al., 2000; Liu et al., 2001; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). These reports clearly documented the expression of H4 receptor mRNA on a variety of cell types, including peripheral blood mononuclear cells, neutrophils, eosinophils, mast cells, and resting CD4+ cells. These findings are highly suggestive of a role for the H4 receptor in immune and/or inflammatory modulation. The H4 receptor bears strong sequence and pharmacological similarity to the H3 receptor (Liu et al., 2001) despite the stark contrast in tissue expression patterns. However, given that no ligands have been identified that can clearly pharmacologically distinguish the human H4 receptor, and there are no clear sources of H4 protein identified in animals, it becomes imperative to identify the species homologs of H4.

With the exception of a few reports, the existence of an additional histamine receptor subtype had largely been unpredicted (Raible et al., 1994; Schwoerer et al., 1994). We undertook a retrospective examination of the literature to see whether there were any reports describing histamine responses on bone marrow-derived cells with a pharmacological profile consistent with that of the H4 receptor. Alveolar macrophages have been shown to reduce tumor necrosis factor production and stimulate interleukin-10 production in response to histamine in a manner consistent with either H3 or H4 receptor pharmacology (Sirois et al., 2000). In purified human B cells treated with interleukin-4 and anti-CD58 monoclonal antibody, histamine enhanced IgE and IgG4 production in a thioperoxide-sensitive manner (Kimata et al., 1996). Nakaya and Tasaka (1988) concluded that histamine affects differentiation and proliferation of granulocytic myeloid cells via H2 receptors, and it affects myeloblast and promyelocyte differentiation by a non-H1 non-H2 receptor. While none of these reports provide direct evidence for H4 receptors, they provide evidence of a complex histamine

ABBREVIATIONS: PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; DMEM, Dulbecco’s modified Eagle’s medium; RT-PCR, reverse transcription-polymerase chain reaction; FLIPR, fluorescence imaging plate reader.
pharmacology that cannot be explained with existing pharmacological agents.

To determine what the putative functions of H₄ receptors might be, one must first determine whether they exist and can be studied in animals. Second, one must carefully understand the properties of the pharmacological agents used to ask the biological questions. This is particularly true with respect to histamine, since humans, mice, rats, and guinea pigs respond to histamine with different sensitivity. To begin to address the important biological questions about H₄ receptor function, we cloned and characterized the mouse, rat, and guinea pig H₄ receptors and compared their pharmacological properties with those of the human receptor.

**Experimental Procedures**

**Materials**

SK-N-MC and COS-7 cells were purchased from American Type Culture Collection (Rockville, MD). 293-EBNA cells were purchased from Invitrogen (San Diego, CA). Histamine, imetit, clobenpropit, N-methylhistamine, R-α-methylhistamine, thioperaimide, and clozapine were all purchased from Sigma/RBI (Natick, MA). Human mRNA was purchased from CLONTECH (Palo Alto, CA). cDNA synthesis kits were purchased from Invitrogen (Gaithersburg, MD). Gelzyme was from Invitrogen and pCINeo vector was from Promega (Madison, WI). 418 was purchased from Calbiochem (San Diego, CA). Burimamide was synthesized in-house according to the method of Vollinga et al. (1995). All other reagents were purchased from Sigma (St. Louis, MO).

**Methods**

**Cloning Partial cDNA Fragments of Mouse, Rat, and Guinea Pig H₄ cDNA.** Many different primer sets, designed based on the human H₄ mRNA coding region, were tried to PCR amplify the mouse, rat, and guinea pig H₄ cDNAs without success. One pair of primers: forward primer: 5′-GTC GTG GAC AAC AAT CTT AGA CAT CGA AGT T3′; and reverse primer: 5′-ACT GAG ATG ATC ACG CTT CCA CAG GCT CCA3′ successfully amplified a 500-bp cDNA fragment from mouse and rat spleen and guinea pig bone marrow cDNA. The cDNA fragments were then subcloned into pPCR2 vector (Invitrogen) and the insert sequence (ABL, Prism 377). The resulting DNA sequences from each of the species amplifications were unique and showed 65 to 70% identity to human H₄ sequence. These regions were used as anchoring regions to identify the 5′ end and 3′ end via rapid amplification of cDNA ends (RACE) to obtain the full-length mouse, rat, and guinea pig H₄ coding regions.

**Cloning Full-Length Mouse H₄ cDNA.** The mouse H₄ cDNA 5′ end was PCR-amplified by rapid amplification of cDNA end (RACE) method from mouse spleen Marathon-Ready cDNA (CLONTECH) using the AP: 5′-CCA TCC TAA TAC GAC TCA TAT GAC TGC GAC GCC GCC CCA CAG CAG GCT CCA3′; and mouse gene-specific primer P1: 5′-CAT TGC GTA ACA AAG CCA GGC TCA CAG TC3′. The mouse H₄ cDNA 3′ end was amplified (via RACE) from mouse spleen Marathon-Ready cDNA using the AP and mouse H₄-specific gene primer P2: 5′-TGC ATC TGC TCA CTC AGA AAG TCC TCG TGG AAG 3′. The 5′ end and the 3′ end RACE products of mouse H₄ were sequenced and the complete cDNA sequence assembled. The coding region of mouse H₄ was then PCR amplified from rat spleen cDNA using two primers, forward primer: 5′-ACG TGA ATT CGC CAC CAT GTG GCA GTA GTG TAA CGG CAC TGA 3′; and reverse primer: 5′-ACT GAT GAG GCC GCC GGG AAG CTT GCA ATG AAG CAC ATG CTT CTC 3′. The PCR product was cloned into pCINeo and the insert region sequenced.

**Cloning Full-Length Guinea Pig H₄ cDNA.** Guinea pig bone marrow RNA was purified using an RNA purification kit (Trizol (Invitrogen)) and first-strand cDNA was synthesized using the SMART cDNA synthesis system (CLONTECH). This cDNA was used as a template for guinea pig H₄ 5′ end and 3′ end RACE using guinea pig H₄-specific primers P5: 5′-ATA ATG TAG GGA GAA GAG CAA AGT ACC ACT 3′ and P6: 5′-ACA CTT CTC CAG ACA GGA CCC CGA TTC AAG 3′ together with the adaptor primer provided by the manufacturer. The RACE products were sequenced and the complete cDNA sequence assembled. The guinea pig H₄ complete coding region was PCR-amplified from bone marrow cDNA using two primers: forward primer: 5′-AGC TCT CGA GGC CAC CAT GTG GCA AAA TAA CAC TAC AAT CG 3′ and reverse primer: 5′-ACG ACA GCG GCC GCC GGC TTC AAG TGG ATA TG AGC GGT TTG GTG 3′. The PCR product was then cloned into pCINeo and the insert region sequenced.

**Cell Culture and Transfection.** Human neuroblastoma cell line SK-N-MC cells were cultured in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum. Mouse, rat, and guinea pig H₄ in mammalian expression vector pCINeo were transfected into SK-N-MC cells by electroporation. The transfected cells were cultured under the selection of G418 (600 μg/ml). Colonies surviving the selection were grown and tested for [³H]histamine binding. COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and transiently transfected using LipofectAMINE. Two days after transfection, COS-7 cells were harvested by scraping and membranes prepared as described below.

**Detection of H₄ mRNA Expression in Mouse, Rat, and Guinea Pig by RT-PCR.** Forward primer: 5′-CAG CAC TGT TTA CGT GAA TTT TGG AAT TGG CAT G 3′ and reverse primer: 5′-ACC AAG AAA GCC AGT ATG CAA ACA GCC ACC ATT TGA GC 3′ were designed according to the conserved region between mouse and rat H₄ receptors. cDNAs made from different tissues were used as templates to PCR amplify the mouse and rat fragments. Except for the bone marrow cDNA, all other cDNA templates were purchased from CLONTECH. The PCR reaction conditions (94°C, 40 s; 65°C, 40 s; and 72°C, 2 min) were run for 30 cycles. For guinea pig H₄ mRNA detection, first-strand cDNAs were made from different guinea pig tissues. Forward primer: 5′-TTT ACT AGC TAT TGC TAT AAT CTT AGG CAA TG 3′ and reverse primer: 5′-GAA GCC CAT TTT GGA AGC GAT CAT ATT GCT GT 3′ were used in PCR detection of guinea pig H₄ mRNA. The PCR reactions were performed as described for mouse and rat H₄ mRNA detection. The PCR products were run in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV. Glyceraldehyde-3-phosphate dehydrogenase PCR was used in parallel as a positive control.

**Radioligand Binding Assay.** The H₄ receptor-expressing cells were harvested, washed, and homogenized in 20 mM Tris-HCl, 2 mM EDTA, pH 7.4. Saturation binding was performed by incubating these membranes with different concentrations of [³H]histamine (PerkinElmer Life Science Products, Boston, MA) (0.01–640 nM) plus/minus 50 μM cold histamine in binding buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5). The reaction was incubated at room temperature for 1 h and the membranes were then filtered through the GPC 96-well filters pretreated with 0.01% polyethyleneimine using a 96-well Brandel cell harvester (Gaithersburg, MD). The filter was
washed three times with ice-cold binding buffer and then punched into tubes. Scintiverse E cocktail was added and counts were determined by liquid scintillation counting on a Beckman 3000 counter. All assays at each concentration of [3H]histamine were done in triplicate. Competition binding was performed using 30 nM [3H]histamine as a tracer in the presence of various concentrations of test compounds. The binding assays were incubated at room temperature for 1 h and harvested and counted as described above.

**Calcium Mobilization.** 293-EBNA cells were purchased from Invitrogen and cultured in DMEM plus 10% fetal calf serum. Human, mouse, rat, and guinea pig H4 receptors in the mammalian expression vector pCINeo were cotransfected with Gqi5 (Conklin et al., 1995) plasmid into 293 ENBA cells using LipofectAMINE. Two days after transfection, the cells were detached from dishes with phosphate-buffered saline plus 10 mM EDTA, washed with DMEM F-12 medium and loaded with the calcium dye Fluoro-3 (AM) (TEF Labs, Austin, TX) at a final concentration of 4 μM in dye loading buffer [DMEM F-12 medium without phenol red (Invitrogen) containing 2.5 mM probenecid] at room temperature for 1 h. Cells were then washed one time with dye loading buffer and aliquoted into polylysine-coated black wall 96-well tissue culture plates (BD Biosciences, San Jose, CA). Calcium mobilization in response to different histamine receptor compounds at various concentrations was assayed in a FLIPR 384 Molecular Devices, Sunnyvale, CA).

**Cyclic AMP Accumulation.** Inhibition of cyclic AMP accumulation was measured essentially as previously described (Liu et al., 2001). Briefly, sublines of SK-N-MC cells were created that expressed a reporter gene construct and each of the species H4 receptors. The reporter gene was β-galactosidase under the control of multiple cyclic AMP responsive elements. In 96-well plates, agonists were added directly to the cell media followed 5 min later by an addition of forskolin (5 mM final concentration). After a 6-h incubation at 37°C, the media was aspirated and the cells washed with 200 μl of phosphate-buffered saline followed by a second aspiration. Cells were lysed with 25 μl 0.1% Triton, and 200 μl of the lysate was added to 20 μl of 1 mg/ml substrate solution (chloroformated β-D-galactopyranoside; Roche Molecular Biochemicals, Indianapolis, IN). Color was quantitated on a microplate reader at absorbance 570 nm.

**Results**

**Comparison of Mouse, Rat, Guinea Pig, and Human H4 Receptor Sequences.** Fragments for the mouse, rat, and guinea pig H4 cDNAs were identified via degenerate PCR using primers designed from the human sequence. The 5' and the 3' end of those H4 receptors were identified by RACE and the subsequent full-length cDNAs were PCR-amplified from mouse or rat spleen cDNA pools or guinea pig bone marrow cDNA. The deduced amino acid sequence comparison among the human, mouse, rat, and guinea pig H4 receptors indicated an overall 65 to 70% homology (Fig. 1). Whereas the human H4 receptor is 390 amino acids long, the rat and mouse sequences are longer at 391 amino acids and guinea pig is shorter at 389 amino acids. The degree of homology between the H4 receptors from different species is the lowest among all known histamine receptors. This is quite different from that of the H3 receptor, which shares >92% sequence homology across the species (Fig. 2). The rat and mouse H4 receptors are more related to one another at 84% homology than to either of the other species. Homology between H3 and H4 sequences is consistent from species to species (84–85%). The putative third transmembrane domain is clearly the most conserved among the species, whereas the putative intracellular loop between the fifth and sixth transmembrane domains was clearly the least conserved.

**Mouse, Rat, Guinea Pig, and Human H4 Receptors Are Expressed in Similar Tissues.** Since the sequences for the rat, mouse, and guinea pig H4 receptors were so different from that of the human receptor, we wondered whether we had actually cloned the H4 homologs or whether the sequences represented additional histamine-like receptors. RT-PCR from cDNAs prepared from rat, mouse, and...
guinea pig tissues was used to assess the tissue expression pattern of the various putative homologs that had been isolated. The results (Fig. 3) show that the expression patterns, at least at the whole tissue level, are consistent across all species with the highest expression seen in the spleen and bone marrow. No additional sites of expression were detected with the rat, mouse, and guinea pig clones than had been previously reported for the human receptor.

**Mouse, Rat, Guinea Pig, and Human H$_4$ Receptor Bind [$^3$H]Histamine Differently.** Since [$^3$H]histamine was shown to be the best radioligand for the human H$_4$ receptor in previous studies, we determined its affinity for the mouse, rat, and guinea pig H$_4$ receptors as well. Saturation isotherms for [$^3$H]histamine were generated in membranes from stable cells expressing either the rat, mouse, guinea pig, or human H$_4$ receptors. No [$^3$H]histamine binding was observed in untransfected cells. The results are shown in Table 1. $B_{\text{max}}$ values in the transient and stable cells ranged from 1 to 20 pmol mg/protein depending on culture conditions (data not shown). The human and guinea pig H$_4$ receptors both displayed high affinity for [$^3$H]histamine with $K_D$ values of 5 and 6 nM, respectively. Surprisingly, mouse and rat displayed sub-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Species</th>
<th>[$^3$H]Histamine $K_D$ (nM)</th>
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<tbody>
<tr>
<td>Human</td>
<td>$4.8 \pm 2.5$ (n = 5)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>$6.0 \pm 1.2$ (n = 4)</td>
</tr>
<tr>
<td>Rat</td>
<td>$13.6 \pm 4.1$ (n = 4)</td>
</tr>
<tr>
<td>Mouse</td>
<td>$42 \pm 6$ (n = 4)</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Homology comparison by amino acid of the human (H) and guinea pig (G), mouse (M), and rat (R) H$_1$, H$_2$, H$_3$, and H$_4$ receptors. Percentage of homology was determined using the entire amino acid sequence according to Lipman and Pearson (1985).

**Fig. 3.** Detection of H$_4$ mRNA expression in different tissues by RT-PCR followed by Southern blotting. Agarose gels of positive control glyceraldehyde-3-phosphate dehydrogenase PCR reactions are shown below the blots for each tissue.
stantially reduced affinity for \([3H]\)histamine. The mouse H4 receptor (42 nM) had an almost 10-fold reduction in affinity and the rat H4 (136 nM) had a more than 20-fold reduction compared with human or guinea pig. The \(K_D\) value for the rat H4 may be slightly overestimated because it was difficult to include high enough \([3H]\)histamine concentrations to reach saturation. To ensure that the affinity differences that we observed were not a result of individual variations in the stable clones that were isolated, we tested the binding affinities of the species clones after transient transfection in COS-7 cells. The resulting values were entirely consistent with the values determined using the stable cell lines (data not shown).

**Mouse, Rat, Guinea Pig, and Human H4 Receptor Display Different Binding Profiles.** We tested different H3/H4 histamine receptor ligands as competitors in the radioligand binding assays. All of the assays were run using 30 nM \([3H]\)histamine. IC\(_{50}\) values were converted to \(K_I\) values for each species according to Cheng and Prusoff (1973) using \(K_D\) values as described in Table 1. The results are shown in Fig. 4 and summarized in Table 2. Consistent with the saturation studies using \([3H]\)histamine, the \(K_I\) values for unlabeled histamine were 5.9, 11.8, 43, and 70 for the human, guinea pig, mouse, and rat H4 receptors, respectively. The affinity of histamine in the rat competition assay was approximately 2-fold higher than in the saturation study. As stated above, we believe this is due to the difficulty of saturating the rat binding sites because of the requirement for high \([3H]\)histamine concentrations. Imetit is between 5 and 10 times more potent than histamine at the mouse, rat, and human H4 receptors, but is almost equipotent to histamine at the guinea pig receptor. The opposite is true for clobenpropit, which is almost 10-fold more potent than histamine at the guinea pig receptor, but nearly equipotent to histamine at all other species. \(N\)-Methylhistamine was approximately 10-fold less potent than histamine at all species. As previously shown, \(R\)-\(\alpha\)-methylhistamine is a weak ligand (143 nM) at human H4 receptor (Liu et al., 2001). It is similarly weak at the guinea pig receptor (210 nM), but even weaker at the mouse and rat at 397 and 700 nM, respectively. Thioperamide has roughly the same affinity at all species (23–52 nM). Burimamide, like \(R\)-\(\alpha\)-methylhistamine, shows a rank of human > guinea pig > mouse > rat. Clozapine, which has weak affinity for the human H4 receptor (625 nM), is even weaker at the mouse and rat receptors (>2000 nM), but is surprisingly potent at the guinea pig receptor (80 nM).

**Fig. 4.** \([3H]\)Histamine binding to H4 receptor-transfected cells. Membranes from SK-N-MC cells stably transfected with either human, rat, mouse, or guinea pig receptors were incubated with 30 nM \([3H]\)histamine. Nonspecific binding was determined in the presence of 10 \(\mu\)M histamine: ■, histamine; △, imetit; ◄, clobenpropit; ●, \(N\)-methylhistamine; ●, \(R\)-\(\alpha\)-methylhistamine; □, thioperamide; △, burimamide; ▼, clozapine.
Functional Activity of H₃/H₄ Ligands Varies Greatly between Different Species. To measure functional activity of the H₄ receptors, inhibition of forskolin-stimulated cAMP accumulation was recorded through a reporter gene assay. As shown in Fig. 5, there are dramatic differences in both the potency of histamine, imetit, N-methylhistamine, and R-α-methylhistamine among each of the four species. Histamine was the most potent ligand of all the receptors. Imetit was roughly similar in potency to histamine at the human and guinea pig receptors but was clearly more than histamine at the rat and mouse receptors. All ligands appeared to give full efficacy in this assay. N-Methylhistamine and R-α-methylhistamine were less potent than histamine and imetit at all species with R-α-methylhistamine consistently the weaker of the two. Burimamide was tested at all species but only showed weak agonism at the human receptor and was inactive at the other species (data not shown). Signal-to-noise ratios were consistently higher with the rat and mouse clones. Guinea pig consistently gave barely detectable signals and thus the absolute quantification of the EC₅₀ values was extremely difficult. In all species, thioperamide produced rightward shifts in histamine or imetit dose-response curves, demonstrating that it is an antagonist at this receptor (data not shown).

### TABLE 2
Affinity values (n = 3, mean ± S.D.) of histamine ligands for the H₄ receptors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human H₄ Kᵢ</th>
<th>Guinea Pig H₄ Kᵢ</th>
<th>Rat H₄ Kᵢ</th>
<th>Mouse H₄ Kᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imetit</td>
<td>1.3 ± 0.1</td>
<td>12.9 ± 1.0</td>
<td>6.9 ± 4.1</td>
<td>6.8 ± 3.5</td>
</tr>
<tr>
<td>Histamine</td>
<td>5.9 ± 0.4</td>
<td>11.4 ± 1.3</td>
<td>70 ± 7</td>
<td>43 ± 9</td>
</tr>
<tr>
<td>Clobenpropit</td>
<td>4.9 ± 1.1</td>
<td>1.5 ± 0.1</td>
<td>64 ± 2</td>
<td>15 ± 10</td>
</tr>
<tr>
<td>N-Methylhistamine</td>
<td>48 ± 8</td>
<td>92 ± 3</td>
<td>553 ± 120</td>
<td>316 ± 146</td>
</tr>
<tr>
<td>Thioperamide</td>
<td>52 ± 28</td>
<td>34 ± 7</td>
<td>28 ± 9</td>
<td>23 ± 9</td>
</tr>
<tr>
<td>R-α-Methylhistamine</td>
<td>144 ± 8</td>
<td>203 ± 30</td>
<td>700 ± 274</td>
<td>397 ± 70</td>
</tr>
<tr>
<td>Burimamide</td>
<td>124 ± 19</td>
<td>351 ± 168</td>
<td>960 ± 188</td>
<td>725 ± 121</td>
</tr>
<tr>
<td>Clozapine</td>
<td>625 ± 181</td>
<td>78 ± 16</td>
<td>2197 ± 667</td>
<td>2890 ± 1640</td>
</tr>
</tbody>
</table>

Fig. 5. Inhibition of forskolin-stimulated cAMP-mediated reporter gene (β-galactosidase) activity in SK-N-MC cells stably expressing either human, rat, mouse, or guinea pig H₄ receptor. Shown are average values ± S.E.M. of triplicate determinations. This graph is representative of at least three similar experiments. ■, histamine; ▲, imetit; ●, N-methylhistamine; ○, R-α-methylhistamine.
Calcium Mobilization in Response to H4 Ligands Is Complicated, but Is Not Due to H1 Receptor Activation. For studies with H4 receptors, the cDNAs were cotransfected with Gq5 in 293-EBNA cells and then calcium-mobilizing activity was measured. As shown in Fig. 6, there are dramatic differences in both the potency and efficacy of histamine, imetit, N-methylhistamine, and R-α-methylhistamine among each of the four species. Histamine was clearly the most efficacious ligand at all of the receptor subtypes and was the most potent ligand at both the human and guinea pig receptors. Imetit displayed a more potent EC50 than histamine at the rat and mouse receptors, but it only behaved as a partial agonist. In addition, imetit did not show good agonism at the guinea pig receptor, despite a high binding affinity. N-Methylhistamine was nearly a full agonist at all of the receptors tested. It was roughly 10-fold less potent than histamine at all four receptors. Interestingly, R-α-methylhistamine was roughly equal in efficacy to N-methylhistamine at the human, rat, and mouse receptors, but was less effective at the guinea pig receptor. EC50 values and efficacy estimates are summarized in Table 3. With the exception of imetit at the guinea pig receptor, there were good correlations between KI values and EC50 values that were close to unity for all agonists at all species. The guinea pig data did not correlate with the inclusion of imetit but was well correlated when imetit was not included in the analysis. There was no difference in agonist efficacy or rank order of potency of the agonists when cells were transfected with less cDNA, resulting in lower Bmax values (data not shown).

We have observed in past experiments that HEK-293 cells can up-regulate the expression of histamine H1 receptors in response to transfection of different receptor cDNAs. The calcium response in the guinea pig H4-transfected cells looked similar to what might be expected for H1 receptor activation. To be certain that the agonist responses we currently observed in calcium mobilization assays were due to H4 activation, we compared the pharmacology with that of the transfected human H1 receptor. The H1 receptor responds potently to both histamine and N-methylhistamine, weakly to R-α-methylhistamine, and is unresponsive to imetit (Fig. 7A). The H1 response to histamine could be dose dependently blocked by pyrilamine, where 10 μM pyrilamine could completely abolish the response to every concentration of histamine (Fig. 7B). We therefore tested the effect of 10 μM pyrilamine on H4 activation. Pyrilamine had no effect on histamine or other ligand-stimulated calcium mobilization in the any of the species of H4-transfected cells (data not shown). Likewise, no binding of pyrilamine at concentrations

Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human H4</th>
<th>Guinea Pig H4</th>
<th>Rat H4</th>
<th>Mouse H4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imetit</td>
<td>3, 30%</td>
<td>N.D., 0%</td>
<td>8, 30%</td>
<td>8, 75%</td>
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<tr>
<td>Histamine</td>
<td>8, 100%</td>
<td>8, 100%</td>
<td>80, 100%</td>
<td>30, 100%</td>
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<tr>
<td>N-Methylhistamine</td>
<td>40, 90%</td>
<td>100, 80%</td>
<td>200, 50%</td>
<td>100, 80%</td>
</tr>
<tr>
<td>R-α-Methylhistamine</td>
<td>100, 80%</td>
<td>300, 40%</td>
<td>1000, 40%</td>
<td>250, 80%</td>
</tr>
</tbody>
</table>

N.D., not determined.

Fig. 6. Stimulation of calcium mobilization in Fluo-3-loaded 293-EBNA cells after transient cotransfection of Gq5 and either human, rat, mouse, or guinea pig H4 receptor. Calcium mobilization was determined using FLIPR, and fluorescence intensity was calculated as the maximum minus the minimum fluorescence over a 2-min period. Shown are average values ± S.E.M. of triplicate determinations. This graph is representative of at least three similar experiments. ■, histamine; ▲, imetit; ●, N-methylhistamine; ○, R-α-methylhistamine.
The overall nucleotide and amino acid sequence comparison among human, mouse, rat, and guinea pig $H_4$ receptors showed only 65 to 70% homology, with mouse and rat more similar to one another (84%) than the other species. The $H_4$ receptor homology among species is the lowest among the histamine receptor family, which is in contrast to the $H_3$ receptor, which is approximately 92% conserved among species. We considered the possibility that the receptor homologs we isolated from mouse, rat, and guinea pig could be novel histamine receptor genes other than $H_4$ due to their very low sequence identity to human $H_4$ receptor. We performed additional degenerate PCR with both cDNAs from different tissues and genomic DNAs as templates. No homologs were found except those reported here. Additionally, the tissue expression patterns of $H_4$ mRNA in mouse, rat, and guinea pig are almost identical. In eight tissues tested for each species, $H_4$ mRNA was only found in bone marrow and spleen, similar to what we and others have previously reported for the human $H_4$ mRNA (Liu et al., 2001; Morse et al., 2001; Zhu et al., 2001). This line of evidence further suggests that the cDNAs identified from mouse, rat, and guinea pig are actually species homologs of the human $H_4$ receptor. In addition, the percentage of homology between the $H_3$ and $H_4$ receptors of each species is nearly identical at 34 to 35%. However, given some of the distinct pharmacological distinctions of the guinea pig receptor, as outlined below, one might consider the assignment of this receptor as an $H_4$ homology as tentative.

Histamine has high affinity for the $H_3$ receptor in all species tested to date (Lovenberg et al., 1999, 2000; Tardivel-Lacombe et al., 2000). In this report, we show that histamine is a high-affinity ligand at the human and guinea pig $H_4$ receptors, but appears to have significantly lower affinity for the rat and mouse receptors. In addition to the large variation in the binding affinity of histamine to the $H_4$ species homologs, we report large variations in the binding affinities of other imidazole-based ligands. Clofenpropit had high affinity for the human, guinea pig, and mouse receptors, but had significantly lower affinity for the rat receptor. Imetit had high affinity for all four receptors. N-Methylhistamine had modest affinity for the human and guinea pig receptors, but low affinity for the rat and mouse receptors. R-α-Methylhistamine and burimamide showed a similar pattern, but with slightly reduced affinities. Thioperamide had relatively equal affinity at all four species. Clozapine, which had been shown to bind to the $H_4$ receptor (Oda et al., 2000; Liu et al., 2001), was very weak at the rat and mouse receptors, but substantially more potent at the guinea pig receptor (78 nM). Given the low level of homology between the $H_4$ receptors from the various species in sequence, it is not surprising that we observed large variations in the binding potencies of the various ligands. Indeed, species variations in binding affinity for thioperamide have been observed for the histamine $H_3$ receptor (Lovenberg et al., 2000) and this variation can be attributed to just two amino acid changes (Ligneau et al., 2000).

We reported previously that human $H_4$ receptors could couple to inhibition of adenylate cyclase in SK-N-MC cells (Liu et al., 2001). However, unlike the human $H_3$ receptor (Lovenberg et al., 1999), the EC$_{50}$ values for agonist activation at the $H_4$ receptor are shifted 10-fold to the right relative to their binding $K_I$ values (Liu et al., 2001). In addition, the...
maximal inhibition of cAMP in H4-expressing cells is approximately 50%, whereas H2-expressing cells can inhibit nearly 100% of forskolin-stimulated cAMP accumulation. Rat, mouse, and guinea pig H4 receptors in SK-N-MC cells showed very weak inhibition of adenylyl cyclase, particularly for the guinea pig receptor (Fig. 5). Most of the agonists tested behaved as full agonists relative to histamine. A comparison of the pEC50 values to the pK1 values from binding show that rat and mouse correlate reasonably well. Human and guinea pig correlations are poor and this could be due to the low signal-to-noise ratio, most likely resulting from poor receptor/G protein coupling. Others have shown that the human H4 receptor can couple to calcium mobilization when cotransfected with either Gq15 (Zhu et al., 2001), Gq11,2 (Morse et al., 2001), or G15 (Oda et al., 2000). Taking a similar approach, we cotransfected the H4 receptors with Gq15, resulting in a better signal-to-noise ratio and allowing a direct comparison of the four receptors (Fig. 6). EC50 values for agonist-activated calcium mobilization were well correlated with the binding K1 values. Imetit appeared to maintain good potency at the various species but clearly displayed partial agonism, achieving at best 60% efficacy compared with histamine. This was extreme at the guinea pig receptor where imetit was essentially inactive. R-α-Methylhistamine displayed variable efficacy at the species H4 receptors and was in general weaker than N-methylhistamine.

Since we had observed full agonism in the inhibition of adenylyl cyclase assay, and only what appeared to be partial agonism in the calcium assay, we wondered whether we might be accidentally detecting H4 activation in the calcium assays. To evaluate this possibility, we first determined that 10 μM was the concentration of pyrilamine required to completely block the effects of histamine on the H1 receptor (Fig. 7B). We then tested for H4 agonist responses in the presence of 10 μM pyrilamine. There was no difference in the H4-mediated calcium mobilization in the presence or absence of pyrilamine, suggesting that the effects were due solely to H4 activation. This finding is in contrast to the report of (Nguyen et al., 2001), which claimed that H4 could bind [3H]pyrilamine with modest affinity. We have found no evidence that human or other species of H4 bind pyrilamine, consistent with the report of (Zhu et al., 2001). Hough (2001) noted that the pharmacology reported by Nguyen et al. (2001) was inconsistent with other reports, and we speculate that there may have been contaminating influence of H1 receptors present in their cells. There appear to be two confounding factors that make interpretation of the species pharmacological profiles difficult. The first is the apparent rightward shift in potency of some compounds compared with their binding affinities. The second is the propensity of most of the agonist ligands to exhibit only partial agonism, particularly in the calcium mobilization assay. We have reasoned that the rightward shift could be the result of a continued inappropriate coupling to a non-native G protein or perhaps it could be an inherent feature of the receptor protein. It may also be the result of constitutive activity of the receptor, an effect that has been reported for H1, H2, and H4 receptors (Smit et al., 1996; Bakker et al., 2000; Morisset et al., 2000). We had previously seen a hint of constitutive activity because thioperoxamide was able to dose dependently stimulate forskolin-activated cAMP accumulation (Liu et al., 2001). Also, Morse et al. (2001) reported strong constitutive activity of the H4 receptor as measured by guanosine-5’-O-(3-thio)triphosphate incorporation. The true mechanism of receptor coupling of the H4 receptor will require extensive testing of various G proteins and signal transduction cascades. Isolation of cells natively expressing the H4 receptors will go a long way toward addressing this issue. Because of some of the above-described issues, we would suggest that the K1 and EC50 values reported here may not represent absolute intrinsic values of the ligands for the various receptors. However, it seems clear that the relative comparisons of the ligands among species are reflective of the vast variation in their pharmacology.

In summary, we have cloned and characterized the rat, mouse, and guinea pig cDNAs encoding homologs of the recently described human histamine H4 receptor. The sequences of the species homologs are not well conserved and this is clearly reflected in the variation in both binding and functional activity of numerous H4 agonists and antagonists. These large variations in pharmacological specificity will undoubtedly make interpretation of future in vivo experiments in various species difficult unless selective H4 agents are developed that can maintain relatively equal potency and efficacy across species.

References


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