Molecular Modeling and Site-Specific Mutagenesis of the Histamine-Binding Site of the Histamine H4 Receptor

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ABSTRACT
The histamine H4 receptor is a novel G-protein–coupled receptor with a unique pharmacological profile. The distribution of H4 mRNA suggests that it may play a role in the regulation of immune function, particularly with respect to allergy and asthma. To define the histamine-binding site of this receptor, molecular modeling and site-directed mutagenesis were used to predict and alter amino acids residing in the histamine-binding pocket. The effects of these alterations on histamine binding and receptor activation were then assessed. Our results indicate that Asp94 (3.32) in transmembrane region (TM) 3 and Glu182 (5.46) in TM5 are critically involved in histamine binding. Asp94 probably serves as a counter-anion to the cationic amino group of histamine, whereas Glu182 (5.46) interacts with the N+ nitrogen atom of the histamine imidazole ring via an ion pair. In contrast, Thr178 (5.42) and Ser179 (5.43) in TM5 are not significantly involved in either histamine binding or receptor activation. These results resemble those for the analogous residues in the H1 histamine receptor. Thus far, four pharmacologically distinct histamine receptors have been identified and cloned, all of which are members of the G-protein–coupled receptor family of proteins. Cloning of the first three histamine receptors, the H1, H2, and H3 receptors, was reported previously (Gantz et al., 1991; Yamashita et al., 1991; Lovenberg et al., 1999). Recently, the fourth histamine receptor, the H4 receptor, was cloned independently by several groups (Oda et al., 2000; Liu et al., 2001; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). The H4 receptor is preferentially expressed in tissues of immunological relevance, and its expression seems to be regulated by interleukin-10 or -13 (Morse et al., 2001). Understanding the molecular mechanism for the interaction between histamine and the H4 receptor will probably be useful for the development of selective H4 antagonists and for elucidating and modulating its function in the future.

Site-directed mutagenesis studies were performed previously to investigate the molecular basis for binding of histamine and histaminergic antagonists to H1 and H2 receptors. In transmembrane region (TM) 3, a conserved aspartic acid [amino acid position 107 (3.32) in human H1 and position 98 (3.32) in human H2 receptor] is essential for the binding of both histamine and basic antagonists for both receptors (Gantz et al., 1992; Ohta et al., 1994). In the H1 receptor, antagonists have been shown to vary in the strength of interaction with this aspartate (Nonaka et al., 1998). In the H2 receptor, TM3 residues adjacent to Asp114 (3.32) have been shown to form the basis for species-specific binding of antagonists (Ligneaux et al., 2000; Lovenberg et al., 2000). Residues in TM5 of the guinea pig H1 and human H2 receptors have also been shown to be required for histamine binding (Gantz et al., 1992; Leurs et al., 1994). Asn207 (5.46) in TM5 of the H1 receptor is involved in hydrogen binding with the N+ nitrogen atom of histamine, whereas Asp186 (5.42) in TM5 of the H2 receptor is connected to the same N+ nitrogen atom of histamine by an ion pair (Gantz et al., 1992; Leurs et al., 1994). In addition, Thr190 (5.43) in TM5 of the H2 receptor was shown to be important in establishing the kinetics of histamine binding and action (Gantz et al., 1992). This residue is thought to participate in hydrogen binding to the N+ nitrogen atom of histamine (Gantz et al., 1992). These findings are consistent with prior expectations that all three

Histamine is a biogenic amine that has tremendous influence over a variety of physiological and pathologic processes through different histamine receptors. Thus far, four pharmacologically distinct histamine receptors have been identified and cloned, all of which are members of the G-protein–coupled receptor family of proteins. Cloning of the first three histamine receptors, the H1, H2, and H3 receptors, was reported previously (Gantz et al., 1991; Yamashita et al., 1991; Lovenberg et al., 1999). Recently, the fourth histamine receptor, the H4 receptor, was cloned independently by several groups (Oda et al., 2000; Liu et al., 2001; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). The H4 receptor is preferentially expressed in tissues of immunological relevance, and its expression seems to be regulated by interleukin-10 or -13 (Morse et al., 2001). Understanding the molecular mechanism for the interaction between histamine and the H4 receptor will probably be useful for the development of selective H4 antagonists and for elucidating and modulating its function in the future.

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ABBREVIATIONS: TM, transmembrane region; SFM, serum-free medium-adapted; HEK, human embryonic kidney; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; 5-HT, 5-hydroxytryptamine.
histamine nitrogens participate in binding and receptor activation (Weinstein et al., 1976).

In this study, we carried out computer modeling of the H4 receptor based on its primary sequence and examined the putative histamine-binding pocket. The resulting model suggests three potential interactions between the H4 receptor and histamine. Asp94 (3.32) in TM3 of the H4 receptor, which matches the conserved Asp residues in TM3 of both the H1 and H2 receptor, could interact with cationic amine moieties of histamine. The imidazole ring of histamine would be predicted to interact with the side chains of Glu182 (5.46) and histamine. The imidazole ring of histamine would be predicted to interact with the side chains of Glu182 (5.46) and either Thr179 (5.42) or Ser179 (5.43) in TM5 of the H4 receptor.

In addition, our computer model of the H4 receptor suggested that Asn147 (4.57) in TM4 and Ser230 (6.52) in TM6 could be important for histamine binding. These two residues seem to reside at the opening of the putative binding pocket with their side chains pointing inward, suggesting the potential for a role in guiding histamine into the binding site. Interestingly, corresponding amino acid positions in TM4 of the H1, H2, and H3 receptors are occupied by bulkier residues (Tyr, Trp, and Phe, respectively). A Phe is found in the H1 and H2 receptor in amino acid positions corresponding to Ser230 (6.52) in TM6 of the H4 receptor (Fig. 1). The bulkier side chains at these positions in the H1, H2, and H3 receptors have the potential to impede access of histamine to the binding pocket. The molecular model would predict that the difference in the identities of the residues at these two sites between H1 and the other three described histamine receptors have potential implications for subtype-specific differences in histamine interaction.

To explore the hypotheses regarding the importance of the residues mentioned above for H4 receptor binding and activation by histamine, we mutated these residues individually and in combination. The ability of each mutant and the wild-type receptor to bind and respond to histamine was measured. The results suggest a model for subtype-specific differences in the mechanism of interaction between histamine and its receptor.

Materials and Methods

Modeling of the Histamine H4 Receptor. A molecular model of the human H4 receptor was constructed from the structure of rhodopsin (Palczewski et al., 2000), using a method described previously for modeling the human melanin-concentrating hormone receptor (MacDonald et al., 2000), by using the Look program (Molecular Application Group, Palo Alto, CA), which uses the SEGMED program (Levitt, 1992). A model of histamine was docked to the H4 receptor homology model at a site corresponding to its expected binding pocket in H1 and H2 receptors, based on prior mutagenesis data. The resulting complex model was refined by 1000 steps of molecular mechanics minimization with the Insight II/Discover program (Accelrys, San Diego, CA).

Site-Directed Mutagenesis. The human H4 receptor cDNA (Morse et al., 2001) was subcloned into the Nhe-1 and Not-1 sites of the mammalian expression vector pME18-CD8-Flag, which allows an expressed protein to be epitope-tagged with N-terminal FLAG peptide and includes a signal peptide sequence derived from CD8 that promotes efficient expression. All the point mutations were introduced using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The full-length wild-type and mutant cDNA sequences were verified using the cycle-sequencing method with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA).

Cell Culture, Transfection, and Expression. Serum-free medium-adapted (SFM) HEK-293 F cells (Invitrogen, Carlsbad, CA) were grown at 37°C in a humidified atmosphere with 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum. Cells were transiently transfected with the wild-type or mutant H4 receptor cDNA in pME18-CD8-Flag using LipofectAMINE 2000 reagent (Invitrogen). Receptor expression on the cell surface was determined by flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Briefly, cells were harvested in 5 mM ice-cold EDTA in PBS 24 h after transfection, washed twice with PBS, and stained with biotinylated anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO) on ice for 30 min. After being washed twice with PBS, the cells were then stained with phycoerythrin-conjugated streptavidin (PharMingen, San Diego, CA) for 30 min on ice, followed by two washes with cold PBS before being analyzed.

Membrane Preparation. Twenty-four hours after transfection, the cells were harvested in 50 mM ice-cold Tris-HCl, pH 7.5, and homogenized with a homogenizer (setting 2, 30 s; Polytron; Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged for 5 min at 1,000 rpm to remove nuclei and unbroken cells. The supernatant was centrifuged at 50,000g for 10 min, and the resulting pellet was resuspended in 50 mM ice-cold Tris-HCl, pH 7.5. The protein concentration of the membrane preparation was measured by using BCA Assay Reagent (Pierce Chemical, Rockford, IL).

Histamine H4 Receptor-Binding Studies. For saturation binding, membrane proteins (40–60 μg) were incubated in a total volume of 200 μl of 50 mM Tris-HCl, pH 7.5, with a range of [3H]histamine dihydrochloride (Amersham Biosciences, Piscataway, NJ) concentrations for 1 h at 30°C. Nonspecific binding was determined by inclusion of 1 nM histamine. The bound radioactivity was separated by filtration through polyethylenimine-treated GF/B filters (Packard Biotechnology, Meriden, CT) with a Filtermate 196 harvester (Packard Biotechnology). The filters were washed eight times with 50 mM ice-cold Tris-HCl, pH 7.5, and radioactivity retained on the filters was measured by liquid scintillation counting with a TopCount (Packard Biotechnology) at 34% efficiency. All experiments were performed in triplicate. The binding data were evaluated with Prism (GraphPad Software, San Diego, CA) and analyzed for one- and two-site fits. A single-binding-site model best described all curves.

Ca2+ Mobilization Assay. HEK-293 SFM cells were transiently cotransfected overnight in DMEM and 10% FCS with the wild-type or mutant H4 receptor cDNAs in pME18-CD8-Flag (0.5 μg/cm2) and the chimeric Go11 protein cDNA in pCDNA 3 (0.05 μg/cm2) (Morse et al., 2001) using LipofectAMINE 2000 reagent (1.5 μl/cm2). Twenty-four hours after transfection, the cells were harvested and resseeded at 5 × 10^4 cells/well in DMEM and 10% FCS in the poly(t-lysine)-treated, 96-well, clear-bottomed black plates (BD Biosciences). Forty-eight hours after transfection, the cells were loaded for 1 h with 4 μM Fluo-3AM (Molecular Probes, Eugene, OR) in loading buffer.

Fig. 1. Alignment of amino acid sequences of the third, fourth, fifth, and sixth transmembrane domain in human histamine H1, H2, H3, and H4 receptors. The bold and underlined residues in the H4 receptor are predicted to be involved in the action of histamine. These residues are also numbered using the Ballesteros and Weinstein index system modified by van Rhee and Jacobson (Ballesteros and Weinstein, 1995; van Rhee and Jacobson, 1996).
(10% FCS and 20 mM HEPES in DMEM). After being washed extensively with washing buffer (Hanks’ balanced salt solution and 20 mM HEPES, pH 7.4) to remove excess dye, the cells were evaluated for agonist-induced intracellular mobilization using a Fluorescent Imaging Plate Reader (Molecular Devices, Menlo Park, CA).

**Results**

**Prediction of Histamine H4 Receptor Interaction Based on Receptor Modeling.** The initial model of histamine docked into the hypothetical binding site in the H4 receptor is shown in Fig. 2. Histamine is predicted to bind in a pocket formed by residues in TM3 through TM6, anchored by an ion pair between the side chain of Asp94 (3.32) in TM3 and the cationic amino group of histamine. In TM5, Thr178 (5.42) and/or Ser179 (5.43) could form a hydrogen bond with the imidazole N" nitrogen, whereas Glu182 (5.46) could form an ion pair with the protonated imidazole N" nitrogen. Finally, Asn147 (4.57) in TM4 and Ser230 (6.52) in TM6 point toward the central histamine-binding cavity and may facilitate the binding interaction.

**Investigation of the Histamine-Binding Site by Site-Specific Mutagenesis.** To experimentally explore the interaction of histamine with the amino acids of the H4 receptor that our model predicted would be important, these residues were mutated individually or in combination (see Table 1 for the list of mutants). During the process of subcloning the wild-type H4 receptor cDNA into a mammalian expression vector, we introduced a FLAG epitope at the N terminus of the receptor to facilitate examination of cell-surface expression. This construct was subsequently used to generate the mutant receptors used in this study. Transfection of the FLAG-H4 receptor in HEK-293 SFM cells resulted in the expression of the H4 receptor on the cell surface at a comparable level (Fig. 4).

Molecular modeling also indicated that Glu182 (5.46) in TM5 of the H4 receptor had the potential to bind with the N" nitrogen atom of the histamine imidazole ring and play a role in histamine binding. To examine this possibility, Glu182 (5.46) was mutated to Ala, Gln, or Asp. Cells transfected with either Glu182 (5.46) → Ala or Glu182 (5.46) → Gln mutant H4 receptor exhibited no binding of [3H]histamine (Table 1), although the results of flow cytometric analysis indicated that the two mutant H4 receptors were expressed on cell surface to the same extent as the wild-type H4 receptor (Fig. 4). In contrast, mutation of Glu182 (5.46) to Asp partially preserved [3H]histamine binding, with Bmax and Kd values being reduced by approximately 50% and 10-fold, respectively (Table 1; Fig. 5).

Molecular modeling suggested that Asn147 (4.57) in TM4
and Ser\textsuperscript{320} (6.52) in TM6 of the H\textsubscript{4} receptor were located near the histamine-binding pocket. Increasing the side-chain volume of the residues at these two positions could conceivably affect histamine binding. To investigate whether the amino acids at these two positions play a role in histamine binding to the H\textsubscript{4} receptor, Asn\textsuperscript{147} (4.57) was mutated to Ala or Tyr and Ser\textsuperscript{320} (6.52) was mutated to Ala or Phe. All four resulting mutant receptors were expressed on the cell surface at a level similar to the wild-type receptor (Fig. 4). Replacement of Asn\textsuperscript{147} (4.57) by the larger Tyr or smaller Ala only slightly (two to four times) reduced the affinity of the H\textsubscript{4} receptor for \[^{3}H\]histamine (Table 1; Fig. 5). Similarly, the mutation of Ser\textsuperscript{320} (6.52) to Phe or Ser\textsuperscript{320} (6.52) to Ala resulted in only modest reductions in \[^{3}H\]histamine affinity (two to five times), although the receptor \(B_{\text{max}}\) seemed to be consistently reduced by about 50% compared with the wild type.

**Histamine-Induced Ca\textsuperscript{2+} Flux after Stimulation of Wild-Type and Mutant H\textsubscript{4} Receptors.** The functional capacity of mutant H\textsubscript{4} receptors was examined by measuring histamine-induced Ca\textsuperscript{2+} mobilization in HEK-293 SFM cells transiently cotransfected with constructs expressing the receptors and a chimeric Go\textsubscript{q} protein, as described previously (Morse et al., 2001). In dose-response studies, cells expressing both the wild-type H\textsubscript{4} receptor with the N-terminal FLAG epitope and Go\textsubscript{q} exhibited Ca\textsuperscript{2+} mobilization in response to histamine treatment. The histamine dose for half-maximal response (EC\textsubscript{50}) (21 \(\pm\) 0.6 nM) was similar to those published previously (Oda et al., 2000; Morse et al., 2001) (Table 1; Fig. 6). As expected from the results of the \[^{3}H\]histamine-binding assay, the mutant H\textsubscript{4} receptors that did not demonstrate binding of \[^{3}H\]histamine [Asp\textsuperscript{94} (3.32) \textsuperscript{3}Ala, Asp\textsuperscript{94} (3.32) \textsuperscript{3}Glu, Asp\textsuperscript{94} (3.32) \textsuperscript{3}Asn, Glu\textsuperscript{182} (5.46) \textsuperscript{3}Ala, and Glu\textsuperscript{182} (5.46) \textsuperscript{3}Asp] were not able to mobilize Ca\textsuperscript{2+} in response to histamine treatment.

**TABLE 1**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>(K_{D}) [^{3}H]Histamine nM</th>
<th>(B_{\text{max}}) pmol/mg protein</th>
<th>EC\textsubscript{50} Histamine nM</th>
<th>Efficacy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>15.3 (\pm) 1.7</td>
<td>0.92 (\pm) 0.07</td>
<td>21 (\pm) 6</td>
<td>100</td>
</tr>
<tr>
<td>Asp\textsuperscript{94} \rightarrow Ala</td>
<td>31.4 (\pm) 3.3</td>
<td>1.16 (\pm) 0.12</td>
<td>156 (\pm) 23</td>
<td>49</td>
</tr>
<tr>
<td>Asp\textsuperscript{94} \rightarrow Glu</td>
<td>47.9 (\pm) 6.8</td>
<td>0.63 (\pm) 0.08</td>
<td>91 (\pm) 20</td>
<td>91</td>
</tr>
<tr>
<td>Asp\textsuperscript{94} \rightarrow Asn</td>
<td>64.2 (\pm) 7.1</td>
<td>1.35 (\pm) 0.14</td>
<td>98 (\pm) 22</td>
<td>91</td>
</tr>
<tr>
<td>Asn\textsuperscript{147} \rightarrow Ala</td>
<td>68.3 (\pm) 4.7</td>
<td>0.64 (\pm) 0.02</td>
<td>156 (\pm) 23</td>
<td>49</td>
</tr>
<tr>
<td>Thr\textsuperscript{178} \rightarrow Ala</td>
<td>15.4 (\pm) 1.9</td>
<td>2.51 (\pm) 0.15</td>
<td>95 (\pm) 26</td>
<td>91</td>
</tr>
<tr>
<td>Ser\textsuperscript{179} \rightarrow Ala</td>
<td>34.4 (\pm) 5.5</td>
<td>1.25 (\pm) 0.15</td>
<td>98 (\pm) 22</td>
<td>91</td>
</tr>
<tr>
<td>Glu\textsuperscript{182} \rightarrow Ala</td>
<td>68.3 (\pm) 4.7</td>
<td>1.35 (\pm) 0.14</td>
<td>98 (\pm) 22</td>
<td>91</td>
</tr>
<tr>
<td>Glu\textsuperscript{182} \rightarrow Asp</td>
<td>153.6 (\pm) 15.5</td>
<td>0.54 (\pm) 0.05</td>
<td>571 (\pm) 133</td>
<td>54</td>
</tr>
<tr>
<td>Glu\textsuperscript{182} \rightarrow Gln</td>
<td>61.1 (\pm) 9.5</td>
<td>0.39 (\pm) 0.02</td>
<td>5625 (\pm) 650</td>
<td>199</td>
</tr>
<tr>
<td>Ser\textsuperscript{320} \rightarrow Phe</td>
<td>76.1 (\pm) 9.5</td>
<td>0.54 (\pm) 0.02</td>
<td>93 (\pm) 22</td>
<td>163</td>
</tr>
<tr>
<td>Ser\textsuperscript{320} \rightarrow Ala</td>
<td>32.1 (\pm) 1.9</td>
<td>0.54 (\pm) 0.02</td>
<td>93 (\pm) 22</td>
<td>163</td>
</tr>
</tbody>
</table>

N.D., could not be determined.

\[ K_{D} = 15.3 \pm 1.7 \text{ nM (n=4)} \]
\[ B_{\text{max}} = 0.92 \pm 0.07 \text{ pmol/mg} \]
(5.46) → Gln receptors] also did not stimulate histamine-induced 
Ca2+ mobilization. On the other hand, histamine-induced 
Ca2+ mobilization was observed in all the cells that 
expressed the mutant H4 receptors capable of [3H]histamine 
binding, although the EC50 varied with the different mutant 
receptors. The Thr178 (5.42) → Ala, Ser179 (5.43) → Ala, and 
Thr178 (5.42)/Ser179 (5.43) → Ala/Ala mutant H4 receptors all 
demonstrated EC50 values slightly higher (four times) than 
that of the wild-type receptor. These three mutations did not 
alter the maximal histamine-induced Ca2+ mobilization (Ta-
gle 1; Fig. 6). In contrast, mutation of Glu182 (5.46) 
induced Ca2+ mobilization, although the EC50 varied with the different mutant 
receptors. The Thr178 (5.42)/Ser179 (5.43) 
A1a mutant H4 receptors all 
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that of the wild-type receptor. These three mutations did not 
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gle 1; Fig. 6). In contrast, mutation of Glu182 (5.46) 
induced Ca2+ mobilization, although the EC50 varied with the different mutant 
receptors. In general, the mutant receptors responded simi-
larly to the histamine derivatives (Table 1; Fig. 6).}

**Pharmacological Analysis of Asn147 (4.57) → Tyr and Ser320 → Phe Mutant Receptors.** Because the residues Asn147 (4.57) and Ser320 (6.52) did not seem to contribute strongly to histamine binding but did seem to affect histamine signaling, we investigated the ability of other H4 agonists to activate 
the Asn147 (4.57) → Tyr and Ser320 (6.52) → Phe mutant receptors. In general, the mutant receptors responded simi-
larly to the histamine derivatives (R)-(-)-α-methylhista-
mine, (S)(+)-α-methylhistamine, imetit, and imepip, com-
pared with histamine itself (Table 2; Fig. 7). Thus, the maximum response was reduced for all compounds at the 
Asn147 (4.57) → Tyr mutant compared with the wild type, 
although R-(-)-α-methylhistamine was least affected. The 
EC50 values for the agonists were reduced as well, with the 
exception of S(-)-α-methylhistamine, which exhibited some-
what higher potency at the mutant receptor compared with 
the wild type (Table 2). For the Ser320 (6.52) → Phe mutation, 
all compounds exhibited higher maximal responses, whereas 
the potency of all the compounds was reduced compared with 
the wild type (Table 2). This mutation, however, had the greatest effect on the action of histamine compared with the other compounds.

**Discussion**

Recently, a fourth member of the histamine family of G-
protein–coupled receptors has been identified and character-
ized by several groups. The H4 histamine receptor exhibits 
the highest degree of similarity to the H3 histamine receptor, 
and comparison of the sequences of the four histamine recep-
tors reveals that a number of amino acids that have been 
imPLICATED in histamine binding to the other receptor sub-
types are conserved in the H4 receptor. To begin to delineate 
the histamine-binding site on the H4 receptor, molecular 
modeling and site-directed mutagenesis were carried out to 
determine the involvement of specific amino acid residues in 
histamine binding and receptor activation.

Previous mutagenesis studies on the H1 and H3 receptors 
(Gantz et al., 1992; Ohta et al., 1994), the α and β adrenergic 
receptors (Strader et al., 1987; Strader et al., 1988; Wang et 
al., 1991), and the M1 muscarinic acetylcholine receptor
(Fraser et al., 1989) all argue for a critical role of a conserved aspartic acid residue in TM3 in mediating ligand binding, presumably by providing a negative counter-ion for the protonated amine group of the ligand. In the H₄ receptor, molecular modeling indicated that Asp¹⁸⁴ (3.32) in TM3 might serve such a role in the binding of histamine. In the present study, mutation of Asp³⁴ (3.32) to either Ala or Asn to eliminate the negative charge on the side chain of the amino acid, or altering the position of the carboxylate group in the side chain of Asp³⁴ (3.32) by mutating it to Glu, eliminated [³H]histamine binding and histamine-induced intracellular Ca²⁺ mobilization in cells expressing the mutants. Nevertheless, flow cytometric analysis indicated that each of these mutant receptor proteins was expressed on the cell surface at levels comparable with that of wild-type H₄ receptor. These results indicate that, as for other biogenic amine receptors, the conserved Asp in TM3 plays a critical role in agonist binding and receptor activation.

The fifth transmembrane domain has also been shown to play a critical role in ligand binding to histamine and other biogenic amine receptors. Asn¹⁹⁸ (5.46) in the H₁ histamine receptor or Asp¹⁸⁶ (5.42) in the H₂ histamine receptor have been proposed to interact with the protonated nitrogen atom of histamine (Ganz et al., 1992; Ohta et al., 1994). These two positions are considered to be homologous and form the basis for explaining the relatively flipped position of histamine in the H₁ and H₂ receptor. In TM5 of the human H₄ receptor, position 5.46 is occupied by a Glu residue, Glu¹⁸². Molecular modeling of the H₄ receptor indicated that this residue had the potential to interact with the N⁺ nitrogen atom of the histamine imidazole ring by either a hydrogen bond or an ion pair. Substitution of Glu¹⁸² (5.46) with Ala or Gln resulted in a mutant H₄ receptor that could no longer bind to [³H]histamine and mediate histamine signaling, even when the mutant receptors were expressed on the cell surface at roughly the same level as the wild-type receptor. Mutation of Glu¹⁸² (5.46) to Asp reduced the affinity for [³H]histamine binding and reduced the potency and efficacy of histamine-stimulated Ca²⁺ mobilization. These results indicate that Glu¹⁸² (5.46) in TM5 is another essential element of the H₄ receptor for histamine binding. The mechanism for the interaction between Glu¹⁸² (5.46) and the N⁺ nitrogen atom of histamine is likely to be an ion pair, similar to that proposed between Asp¹⁸⁶ in the TM5 of the H₂ receptor and histamine, because Asn cannot substitute for Asp at this position. The reduced affinity for histamine observed with the Asp¹⁸² mutant H₄ receptor would be consistent with this assignment, with the reduction in affinity arising from the increased distance between the negatively charged oxygen atom and the N⁺ nitrogen atom of histamine.

**Fig. 5.** Binding of [³H]histamine to HEK-293 SFM cell membranes after transient transfection with mutated histamine H₄ receptors. Data points shown are the mean of triplicate determinations and are representative of at least three independent experiments. The data were analyzed by nonlinear regression analysis (Graphpad Prism). A, Asn¹⁴⁷ (4.57) → Tyr and Asn¹⁴⁷ (4.57) → Ala mutants. B, Thr¹⁷⁸ (5.42) → Ala, Ser¹⁷⁹ (5.43) → Ala, and Thr¹⁷⁸ (5.42) Ser¹⁷⁹ (5.43) → Ala Ala mutants. C, Glu¹⁸² (5.46) → Asn mutants. D, Ser³²⁰ (6.52) → Ala and Ser³²⁰ → Phe mutants. The wild-type receptor-binding curve is shown in each figure for comparison.
Other residues in TM5 have also been shown to be involved in the binding of histamine and other biogenic amines to their receptors. In the /H92512 adrenergic receptor, two Ser residues in TM5, corresponding to Ser179 (5.43) and Glu182 (5.46) in the H4 receptor, have been shown to be involved in hydrogen bonding to the meta- and para-hydroxyl groups of the catechol ring of epinephrine (Strader et al., 1989a). In the /H92511-adrenergic receptor, Ser188 (5.42) is critical for binding to the meta-hydroxyl of the endogenous agonists (Hwa and Perez, 1996). Likewise, in the D1 dopamine receptor, mutation of either Ser198 (5.42) or Ser199 (5.43) to Ala disrupts agonist binding (Pollock et al., 1992). In the dopamine D2 receptor, Ser193 (5.42) contributes notably to the binding of dopamine and Ser194 (5.43) is absolutely required for activation of agonists as a result of bonding with the /H9267-hydroxyl group of catecholamines (Cox et al., 1992). For serotonin receptors, Ser5.43 of the human 5-HT4 was proposed to interact with serotonin through a hydrogen bond (Mialet et al., 1992).
al., 2000); and mutation of the analogous Ser 5.43 in the rat 5-HT 2A receptor to alanine caused a 6-fold decrease in 5-HT binding affinity (Shapiro et al., 2000). In the 5-HT 1A receptor, substitution of Ser 5.43 (5.42) or Thr 5.43 (5.43) with alanine resulted in a significant reduction of serotonin binding (Ho et al., 1992). The analogous residue in the rat M 3 muscarinic receptor, Thr 234 (5.42), also affects acetylcholine binding affinity and the ability of the receptor to stimulate agonist-dependent phosphatidylinositol hydrolysis (West et al., 1992). Taken together, these studies clearly demonstrate the conservation of the critical role of TM5 Ser and/or Thr residues in biogenic amine binding. In the case of histamine receptors, however, this role is not as well conserved. Although Thr 5.42 (5.46) in TM5 of the H 2 histamine receptor has been proposed to interact with the N′ nitrogen of the histamine imidazole ring by a hydrogen bond and seems important for establishing the kinetics of histamine binding and activation (Gantz et al., 1992), the homologous TM5 Thr (5.42) of the human and guinea pig H 1 receptors (Thr 174 and Thr 203 , respectively) are not required for histamine binding (Leurs et al., 1994; Ohta et al., 1994). In the H 4 receptor, the corresponding Thr 178 (5.42) and the adjacent Ser 179 (5.43) are predicted by computer modeling to be appropriately positioned to form a hydrogen bond with the N′ nitrogen of histamine. The present study demonstrates, however, that substitution of Ala at these two sites, alone or in combination, does not dramatically alter the affinity of the H 4 receptor for histamine or the ability of the mutant receptors to mediate histamine-induced signaling. Therefore, it seems that in the H 4 receptor, Thr 178 (5.43) and Ser 179 (5.43) do not play an essential role in histamine binding or signaling.

The present results demonstrate that histamine interacts with the H 4 receptor either in different orientations or by different mechanisms compared with the H 1 and H 2 receptors, due to the differences in the chemical nature and location of residues in TM5 that interact with the N′ nitrogen atom of the histamine imidazole ring. Thus, although Asp 186 (5.42) of the H 2 receptor and Glu 182 (5.46) of the H 4 receptor bind the same nitrogen atom by the same mechanism, histamine must adopt a different orientation in these two receptors. In contrast, histamine binds to the H 1 and H 4 receptors in the same orientation, but Asn 198 (5.46) in the H 1 receptor and Glu 182 (5.46) in the H 4 interact with the N′ nitrogen atom by either a hydrogen bond or an ion pair, respectively. Furthermore, although interactions with the potential H-bond donors/acceptors in TM5 are not essential for histamine binding to the H 1 and H 4 receptors, the homologous Thr residue (5.46) in the H 2 receptor was demonstrated to be important in the interaction with the histamine N′ nitrogen atom (Gantz et al., 1992).

![Fig. 7.](attachment:histamine_interactions.png) Fig. 7. Agonist-induced Ca 2+ mobilization in HEK-293 SFM cells coexpressing wild-type or mutant (Ser 320 → Phe or Asn 147 → Tyr) H 4 receptors and Go α i chimeric G-protein. Cells transiently transfected with the indicated receptor constructs were treated with increasing concentrations of R(-)-α-methylhistamine (A), S(+)-α-methylhistamine (B), imetit (C), or immepip (D). Data are presented as relative change of fluorescence. Each data point is the mean of quadruplicate determinations and is typical of three independent experiments. The data were analyzed by nonlinear regression analysis using a sigmoidal dose-response model as described under Materials and Methods.
The computer modeling studies of the H4 receptor also revealed two additional amino acid residues, Asn147 (4.57) and Ser320 (6.52), that were predicted to be in positions that could allow interaction with histamine in the predicted binding pocket. Comparison with the analogous residues in other biogenic amine receptors reveals that Asn147 (4.57) in TM4 of the H4 receptor is unique; this position is occupied by Trp, Phe, or Tyr in the H1, H2, and H3 receptors, respectively. In TM6, the position occupied by Ser320 (6.52) in the H4 receptor is generally found to be Phe in other biogenic amine receptors, except for the H3 receptor, which has Thr at this position. In the β2 adrenergic receptor, the Phe at the corresponding position (6.52) was suggested to be involved in forming an aromatic-aromatic interaction with the catecholamine phenyl ring of norepinephrine and important for the receptor agonist binding (Strader et al., 1989b). The adjacent Phe (6.51) in the α1B adrenergic receptor was found to be necessary not only for agonist binding but also for agonist potency and efficacy (Chen et al., 1999).

Because the affinity of the H4 receptor for [3H]histamine was only slightly affected by mutations, as shown in our [3H]histamine-binding assay, we conclude that Asn147 (4.57) and Ser320 (6.52) of the H4 receptor are not critical for histamine binding. The same conclusion has been made regarding the corresponding serine (4.57) in TM4 of the human 5-HT4 receptor (Mialet et al., 2000). This conclusion contrasts with our model, which suggests that increased side-chain volume at these two sites could impede histamine binding to the H4 receptor. However, both Asn147 (4.57) and Ser320 (6.52) of the H4 receptor seem to be involved in activation of the H4 receptor by histamine. Replacing Asn147 (4.57) of the H4 receptor with Tyr to mimic the H3 receptor seems to be detrimental to histamine signaling through the H4 receptor, as evidenced by a reduction of 50% in the ability of the receptor to respond to histamine with no change in levels of receptor expression. In contrast, changing Ser320 (6.52) of the H4 receptor to Phe to imitate the H1, H2, and other biogenic amine receptors had the unexpected effect of greatly reducing the potency of histamine while at the same time doubling the maximal response of the receptor to histamine treatment. Although the increased efficacy could be due to an increase in receptor expression, paradoxically, this receptor mutant exhibited a reduction in maximal [3H]histamine binding (and a slight change in affinity) that did not seem to be correlated with reduced surface expression as assessed by anti-FLAG antibody staining.

The altered signaling of the Asn147 (4.57) → Tyr and Ser320 (6.52) → Phe receptors was not specific for histamine. Examination of the response to several histamine analogs generally revealed the same pattern of activity at both mutant receptors as seen with histamine, albeit to differing extents. Thus, although the maximal responses for all compounds were reduced at the Asn147 (4.57) → Tyr mutant, this mutation had the smallest effect on R-(−)-α-methylhistamine and the greatest effect on imepip. On the other hand, mutation of Ser320 (6.52) → Phe resulted in decreased potency and increased efficacy for all compounds, although the effects were greatest for histamine.

The observed reduction in maximal [3H]histamine binding and histamine potency at the Ser320 (6.52) mutant might be explained by altered interactions with G-proteins leading to an increase in the proportion of low-affinity histamine-bind-

References


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