0026-895X/02/6201-38–47$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2002 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 62:38–47, 2002

Vol. 62, No. 1
1215/991843
Printed in U.S.A.

Molecular Modeling and Site-Specific Mutagenesis of the Histamine-Binding Site of the Histamine H_4 Receptor

NIU SHIN, ELIZABETH COATES, NICHOLAS J. MURGOLO, KELLEY L. MORSE, MARVIN BAYNE, CATHERINE D. STRADER, and FREDERICK J. MONSMA, JR.

Discovery Technologies Department, Schering-Plough Research Institute, Kenilworth, New Jersey

Received July 11, 2001; accepted April 3, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The histamine H_4 receptor is a novel G-protein–coupled receptor with a unique pharmacological profile. The distribution of H_4 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 Asp^{94} (3.32) in transmembrane region (TM) 3 and Glu^{182} (5.46) in TM5 are critically involved in histamine binding. Asp^{94} probably serves as a counter-anion to the cationic amino group of histamine, whereas Glu^{182} (5.46) interacts with the N^{τ} nitrogen atom of the histamine imidazole ring via an ion pair. In contrast, Thr^{178} (5.42) and Ser^{179} (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 H_1 histamine receptor but contrast with findings regarding the H_2 histamine receptor. Our results also demonstrate that Asn^{147} (4.57) in TM4 and Ser^{320} (6.52) in TM6 play a role in receptor activation but are not involved in histamine binding. Taken together, these data indicate that although histamine seems to bind to the H_4 receptor in a fashion similar to that predicted for the other histamine receptor subtypes, there are also important differences that can probably be exploited for the discovery of novel H_4-selective compounds.

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 H_1, H_2, and H_3 receptors, was reported previously (Gantz et al., 1991; Yamashita et al., 1991; Lovenberg et al., 1999). Recently, the fourth histamine receptor, the H_4 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 H_4 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 H_4 receptor will probably be useful for the development of selective H_4 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 H_1 and H_2 receptors. In transmembrane region (TM) 3, a conserved aspartic acid [amino acid position 107 (3.32) in human H_1 and position 98 (3.32) in human H_2 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 H_1 receptor, antagonists have been shown to vary in the strength of interaction with this aspartate (Nonaka et al., 1998). In the H_3 receptor, TM3 residues adjacent to Asp^{114} (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 H_1 and human H_2 receptors have also been shown to be required for histamine binding (Gantz et al., 1992; Leurs et al., 1994). Asn^{207} (5.46) in TM5 of the H_1 receptor is involved in hydrogen binding with the N^{τ} nitrogen atom of histamine, whereas Asp^{186} (5.42) in TM5 of the H_2 receptor is connected to the same N^{τ} nitrogen atom of histamine by an ion pair (Gantz et al., 1992; Leurs et al., 1994). In addition, Thr^{190} (5.46) in TM5 of the H_2 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^{π} nitrogen atom of histamine (Gantz et al., 1992). These findings are consistent with prior expectations that all three

This project was funded entirely by Schering-Plough Corporation.

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 H_4 receptor based on its primary sequence and examined the putative histamine-binding pocket. The resulting model suggests three potential interactions between the H_4 receptor and histamine. Asp^{94} (3.32) in TM3 of the H_4 receptor, which matches the conserved Asp residues in TM3 of both the H_1 and H_2 receptor, could interact with cationic amine moiety of histamine. The imidazole ring of histamine would be predicted to interact with the side chains of Glu^{182} (5.46) and either Thr^{178} (5.42) or Ser^{179} (5.43) in TM5 of the H_4 receptor. In addition, our computer model of the H_4 receptor suggested that Asn^{147} (4.57) in TM4 and Ser^{320} (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 H_1, H_2, and H_3 receptors are occupied by bulkier residues (Tyr, Trp, and Phe, respectively). A Phe is found in the H_1 and H_2 receptor in amino acid positions corresponding to Ser^{320} (6.52) in TM6 of the H_4 receptor (Fig. 1). The bulkier side chains at these positions in the H_1, H_2, and H_3 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 H_4 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 H_4 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 H_4 Receptor. A molecular model of the human H_4 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). Briefly, the model was built with the Look program (Molecular Application Group, Palo Alto, CA), which uses the SEGMED program (Levitt, 1992). A model of histamine was docked to the H_4 receptor homology model at a site corresponding to its expected binding pocket in H_1 and H_2 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).

```
                TM III                                  TM IV
H4   VFWLTTDYLLCTASVYNIVLISYDRY 113    KIVTLMVAVWVLAFLV-NGPMIL 152
H1   LFWLSMDTVASTASIFSVFILCIDRY 126    RASATILGAWFLSFL--WVIPIL 163
H2   NIYTSLDVMLCTASILNLFMISLDRY 117    RVAISLVLIWVISITLSFLSIHL 166
H3   KLWLVVDYLLCTSSAFNIVLISYDRF 133    RAVRKMLLVWVLAFLL-YGPAIL 172
          3                   3              4       4
          3                   5              5       5
          2                   0              0       7

                TM V                                   TM VI
H4   YILAITSFLEFVIPVILVAYFNMNI 197    LAKSLAILLGVFAVCWAPYSLFTIVLS 327
H1   WFKVMTAIINFYLPTLLMLWFYAKI 213    AAKQLGFIMAAFILCWIPYFIFFMVIA 439
H2   -YGLVDGLVTFYLPLLIMCITYYRI 205    ATVTLAAVMGAFIICWEPYFTAFVYRG 258
H3   YFLITASTLEFFTPFLSVTFFNLSI 221    VAKSLAVIVSIFGLCWAPYTLLMIIRA 382
          55  5   5                                  6 6
          44  4   5                                  5 5
          23  6   0                                  0 2
```

Fig. 1. Alignment of amino acid sequences of the third, fourth, fifth, and sixth transmembrane domain in human histamine H_1, H_2, H_3, and H_4 receptors. The bold and underlined residues in the H_4 receptor are predicated 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).

Site-Directed Mutagenesis. The human H_4 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% CO_2 in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum. Cells were transiently transfected with the wild-type or mutant H_4 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 M_2 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*g* to remove nuclei and unbroken cells. The supernatant was centrifuged at 50,000*g* 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 H_4 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 [^{3}H]histamine dihydrochloride (Amersham Biosciences, Piscataway, NJ) concentrations for 1 h at 30°C. Nonspecific binding was determined by inclusion of 1 mM histamine. The bound radioactivity was separated by filtration through polyethyleneimine-treated GF/B filters (Packard BioScience, Meriden, CT) with a Filtermate 196 harvester (Packard BioScience). 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 BioScience) 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.

Ca^{2+} Mobilization Assay. HEK-293 SFM cells were transiently cotransfected overnight in DMEM and 10% FCS with the wild-type or mutant H_4 receptor cDNAs in pME18-CD8-Flag (0.5 μg/cm^2) and the chimeric $G\alpha_{q/i}$ protein cDNA in pCDNA 3 (0.05 μg/cm^2) (Morse et al., 2001) using LipofectAMINE 2000 reagent (1.5 μl/cm^2). Twenty-four hours after transfection, the cells were harvested and reseeded at 5×10^5 cells/well in DMEM and 10% FCS in the poly(D-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

(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 H_4 Receptor Interaction Based on Receptor Modeling. The initial model of histamine docked into the hypothetical binding site in the H_4 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 Asp^{94} (3.32) in TM3 and the cationic amino group of histamine. In TM5, Thr^{178} (5.42) and/or Ser^{179} (5.43) could form a hydrogen bond with the imidazole N^{π} nitrogen, whereas Glu^{182} (5.46) could form an ion pair with the protonated imidazole N^{τ} nitrogen. Finally, Asn^{147} (4.57) in TM4 and Ser^{320} (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 H_4 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 H_4 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-H_4 receptor in HEK-293 SFM cells resulted in the appearance of cell-surface FLAG staining and high-affinity binding sites for [^{3}H]histamine, with a K_D of 15.3 nM (Fig. 3; Table 1). The K_D of the FLAG-H_4 receptor obtained in this study agrees with that reported previously for the H_4 receptor without the FLAG epitope (Oda et al., 2000; Morse et al., 2001; Zhu et al., 2001). This indicates that a FLAG epitope at the N terminus of the H_4 receptor does not affect histamine binding.

Among the biogenic amine and some peptide G-protein–coupled receptors, a conserved Asp in TM3 has been shown to be critical for interaction with their natural agonists (for discussion see MacDonald et al., 2000). In the H_4 receptor, the corresponding residue is Asp^{94} (3.32). To test its role in histamine binding to the H_4 receptor, Asp^{94} (3.32) was mutated to Ala, Asn, and Glu, respectively. In each case, the mutation resulted in complete loss of [^{3}H]histamine binding to the H_4 receptor (Table 1). This loss of [^{3}H]histamine binding was not caused by a lack of receptor cell-surface expression, because analysis of cell-surface FLAG staining revealed that the wild-type and the three mutant H_4 receptors were expressed on the cell surface at a comparable level (Fig. 4).

Molecular modeling of the H_4 receptor-histamine interaction predicted that Thr^{178} (5.42) and Ser^{179} (5.43) in TM5 were both in a plausible position to hydrogen bond with the N^{π} nitrogen atom of the histamine imidazole ring. To determine the importance of this potential interaction, Thr^{178} (5.42) and Ser^{179} (5.43) were individually mutated to Ala. The affinity of the two resulting mutant receptors for [^{3}H]histamine (K_D) was reduced approximately two to four times; simultaneous substitution of both residues failed to decrease the affinity further (Table 1; Fig. 5). Analysis of histamine binding (B_{max}) and cell-surface FLAG staining (Fig. 4) indicated that neither Ser^{179} (5.43) → Ala nor the Thr^{178} (5.42)/Ser^{179} (5.43) → Ala/Ala mutation had any detrimental effect on the expression of the H_4 receptor on the cell surface (Table 1; Figs. 4 and 5). Introduction of the Thr^{178} (5.42) → Ala mutation alone also did not alter cell-surface FLAG staining intensity (Fig. 4) but reduced the level of [^{3}H]histamine binding to the H_4 receptor by 31% (Table 1; Fig. 5).

Molecular modeling also indicated that Glu^{182} (5.46) in TM5 of the H_4 receptor had the potential to bind with the N^{τ} nitrogen atom of the histamine imidazole ring and play a role in histamine binding. To examine this possibility, Glu^{182} (5.46) was mutated to Ala, Gln, or Asp. Cells transfected with either Glu^{182} (5.46) → Ala or Glu^{182} (5.46) → Gln mutant H_4 receptor exhibited no binding of [^{3}H]histamine (Table 1), although the results of flow cytometric analysis indicated that the two mutant H_4 receptors were expressed on cell surface to the same extent as the wild-type H_4 receptor (Fig. 4). In contrast, mutation of Glu^{182} (5.46) to Asp partially preserved [^{3}H]histamine binding, with B_{max} and K_D values being reduced by approximately 50% and 10-fold, respectively (Table 1; Fig. 5).

Molecular modeling suggested that Asn^{147} (4.57) in TM4

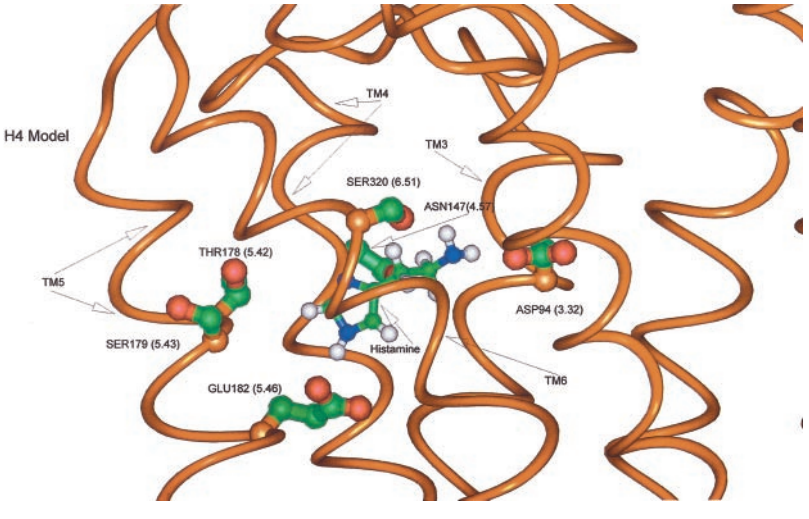

Fig. 2. A molecular model of human H_4 receptor-histamine complex. A molecular model of the human H_4 receptor was constructed from the structure of bovine rhodopsin and refined by 1000 steps of molecular mechanics minimization with the Insight II/Discover program. Asp^{94} (3.32) in TM3 is expected to form an ion pair to the cationic amino group of histamine. Thr^{178} (5.42) and Ser^{179} in TM5 could form a hydrogen bond to the imidazole N^{π} nitrogen. Glu^{182} (5.46) in TM5 could form an ion pair to the protonated imidazole N^{τ} nitrogen. Asn^{147} (4.57) in TM4 and Ser^{320} (6.52) in TM6 point toward the central histamine-binding cavity.

and Ser^{320} (6.52) in TM6 of the H_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_4 receptor, Asn^{147} (4.57) was mutated to Ala or Tyr and Ser^{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^{147} (4.57) by the larger Tyr or smaller Ala only slightly (two to four times) reduced the affinity of the H_4 receptor for [^{3}H]histamine (Table 1; Fig. 5). Similarly, the mutation of Ser^{320} (6.52) to Phe or Ser^{320} (6.52) to Ala resulted in only modest reductions in [^{3}H]histamine affinity (two to five times), although the receptor B_{max} seemed to be consistently reduced by about 50% compared with the wild type.

Histamine-Induced Ca^{2+} Flux after Stimulation of Wild-Type and Mutant H_4 Receptors. The functional capacity of mutant H_4 receptors was examined by measuring histamine-induced Ca^{2+} mobilization in HEK-293 SFM cells transiently cotransfected with constructs expressing the receptors and a chimeric $G\alpha_{q/i}$ protein, as described previously (Morse et al., 2001). In dose-response studies, cells expressing both the wild-type H_4 receptor with the N-terminal FLAG epitope and $G\alpha_{q/i}$ exhibited Ca^{2+} mobilization in response to histamine treatment. The histamine dose for half-maximal response (EC_{50}) (21 ± 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_4 receptors that did not demonstrate binding of [^{3}H]histamine [Asp^{94} (3.32) → Ala, Asp^{94} (3.32) → Glu, Asp^{94} (3.32) → Asn, Glu^{182} (5.46) → Ala, and Glu^{182}

TABLE 1

Effects of point mutations of the histamine H_4 receptor on histamine binding and histamine induced intracellular Ca^{2+} mobilization

Receptors were transiently expressed in HEK-293 SFM cells and used for [^{3}H]histamine binding assays and measurement of histamine-induced intracellular Ca^{2+} mobilization. Data were calculated as the mean ± S.E. of at least three independent experiments. Efficacy is expressed as percentage of the response of the wild type receptor to histamine.

Receptor	K_D [^{3}H]Histamine	H_4 Receptor B_{max}	EC_{50} Histamine	Efficacy
	nM	*pmol/mg protein*	*nM*	*%*
Wild-type	15.3 ± 1.7	0.92 ± 0.07	21 ± 6	100
Asp^{94} → Ala	N.D.	N.D.	N.D.	N.D.
Asp^{94} → Glu	N.D.	N.D.	N.D.	N.D.
Asp^{94} → Asn	N.D.	N.D.	N.D.	N.D.
Asn^{147} →Tyr	31.4 ± 3.3	1.16 ± 0.12	32 ± 6	36
Asn^{147} → Ala	68.3 ± 4.7	0.64 ± 0.02	156 ± 23	49
Thr^{178} → Ala	47.9 ± 6.8	0.63 ± 0.08	91 ± 20	91
Ser^{179} →Ala	34.4 ± 5.5	1.25 ± 0.15	95 ± 26	91
$Thr^{178}Ser^{179}$ → Ala Ala	64.2 ± 7.1	1.35 ± 0.14	98 ± 22	91
Glu^{182} → Ala	N.D.	N.D.	N.D.	N.D.
Glu^{182} → Asp	153.6 ± 15.5	0.54 ± 0.05	571 ± 133	54
Glu^{182} → Gln	N.D.	N.D.	N.D.	N.D.
Ser^{320} → Phe	76.1 ± 9.5	0.39 ± 0.02	5625 ± 650	199
Ser^{320} → Ala	32.1 ± 1.9	0.54 ± 0.02	93 ± 22	163

N.D., could not be determined.

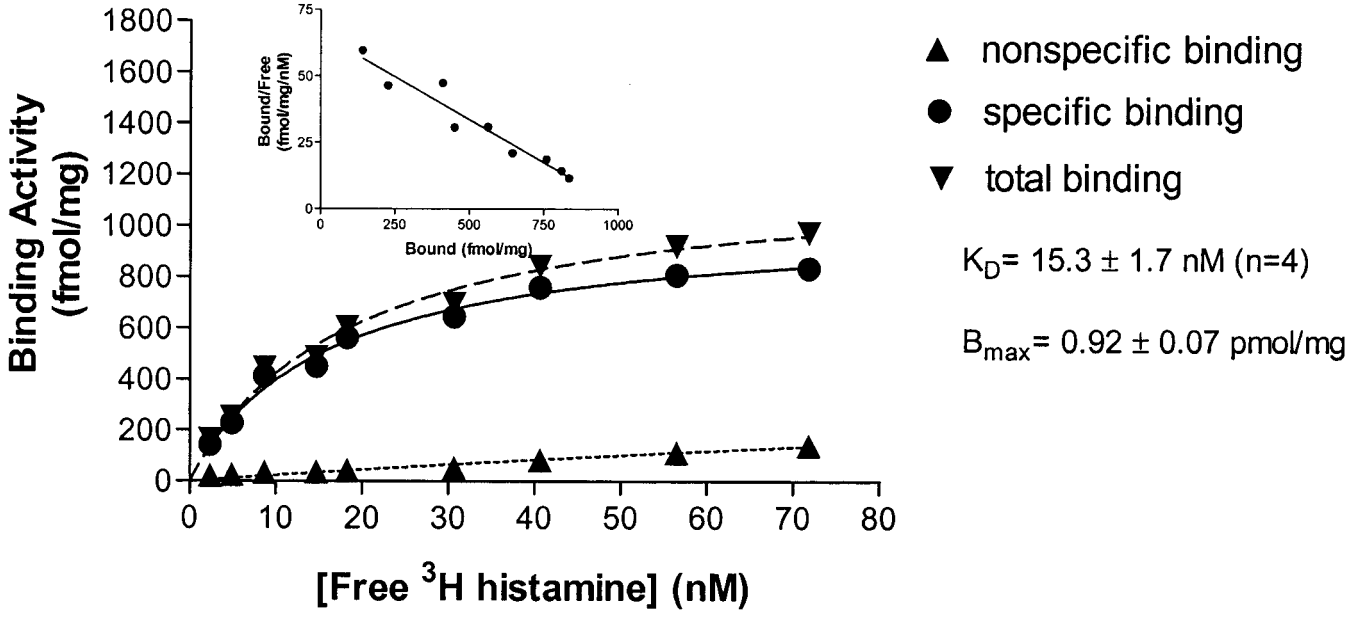

Fig. 3. Saturation binding of [^{3}H]histamine to membranes from HEK-293 SFM cells expressing the N-terminal FLAG-tagged H_4 receptor after transient transfection. Inset, Scatchard transformation of saturation binding data. Each data point is the mean of triplicate determinations and is representative of four independent experiments. The data were analyzed by nonlinear regression analysis using single-site binding models as described under *Materials and Methods*.

(5.46) → Gln receptors] also did not stimulate histamine-induced Ca^{2+} mobilization. On the other hand, histamine-induced Ca^{2+} mobilization was observed in all the cells that expressed the mutant H_4 receptors capable of [^{3}H]histamine binding, although the EC_{50} varied with the different mutant receptors. The Thr^{178} (5.42) → Ala, Ser^{179} (5.43) → Ala, and Thr^{178} (5.42)/Ser^{179} (5.43) → Ala/Ala mutant H_4 receptors all demonstrated EC_{50} values slightly higher (four times) than that of the wild-type receptor. These three mutations did not alter the maximal histamine-induced Ca^{2+} mobilization (Table 1; Fig. 6). In contrast, mutation of Glu^{182} (5.46) → Asp resulted in a 50% reduction in maximal Ca^{2+} mobilization by histamine and a 20-fold increase in the EC_{50} for histamine, compared with the wild-type receptor (Table 1; Fig. 6). For the Asn^{147} (4.57) → Tyr mutant receptor, no change in EC_{50} was observed, although the maximal responses to histamine were reduced by about 60%. Changing the same residue to Ala, however, increased the EC_{50} for histamine by 7-fold and decreased the maximal stimulation by 50% (Table 1; Fig. 6). Interestingly, both mutations of Ser^{320} (6.52) also seemed to affect the signaling of the H_4 receptor. Although mutation of this residue to Ala resulted in some reduction of histamine potency, mutation to Phe resulted in a 250-fold increase in the EC_{50} for histamine. In addition, both mutations consistently exhibited an increase in maximal Ca^{2+} flux of 60 and 100% for both Ser^{320} (6.52) → Ala and Ser^{320} (6.52) → Phe, respectively. The increased efficacy of histamine at these mutant receptors did not seem to correlate with increased [^{3}H]histamine binding; in experiments where binding and functional assays were carried out on the same batch of transfected cells, with the wild-type receptor assayed in parallel, the B_{max} values for [^{3}H]histamine were reduced in the Ser^{320} (6.52) → Phe mutant receptor by 50%, whereas the maximal Ca^{2+} mobilization observed was double that observed with the wild-type receptor.

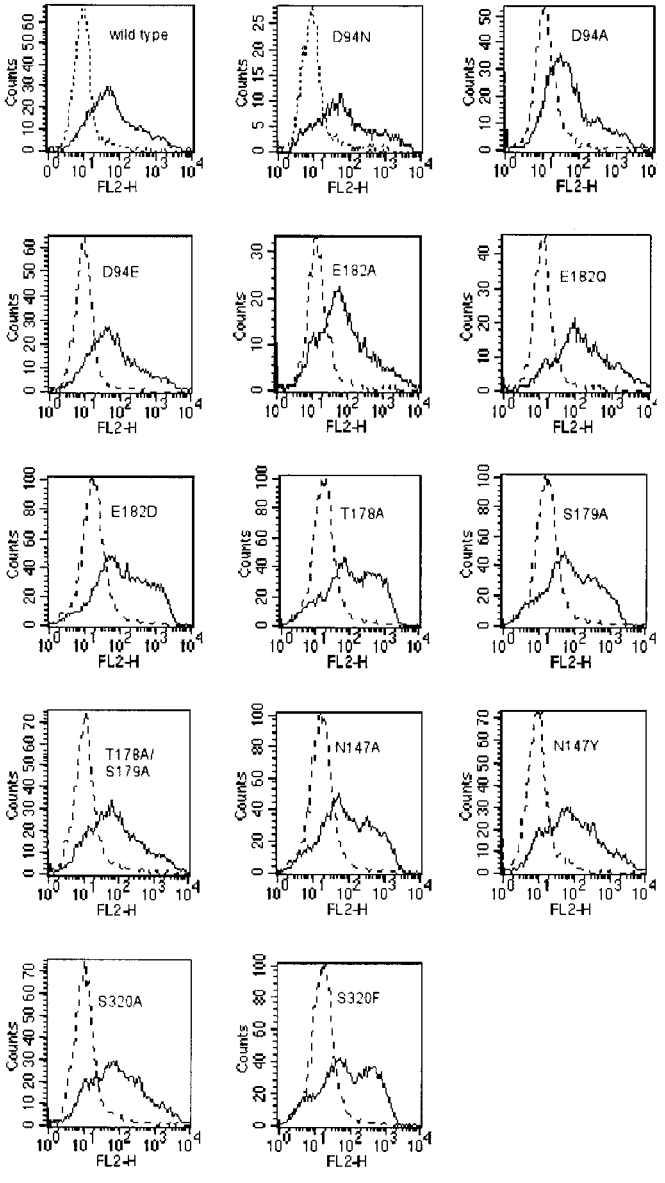

Fig. 4. Surface expression of the FLAG-tagged H_4 receptor and mutants in HEK-293 SFM cell after transient transfection. Cells were transiently transfected with cDNAs of the FLAG-tagged wild-type and mutant H_4 receptors, as indicated. The transfected cells were stained first with either anti-FLAG biotinylated M_2 monoclonal antibody (solid lines) or biotin mouse IgG_1 (dotted lines) and then with phycoerythrin-conjugated streptavidin. The cells were analyzed using a FACSCalibur flow cytometer.

Pharmacological Analysis of Asn^{147} → Tyr and Ser^{320} → Phe Mutant Receptors. Because the residues Asn^{147} (4.57) and Ser^{320} (6.52) did not seem to contribute strongly to histamine binding but did seem to affect histamine signaling, we investigated the ability of other H_4 agonists to activate the Asn^{147} (4.57) → Tyr and Ser^{320} (6.52) → Phe mutant receptors. In general, the mutant receptors responded similarly to the histamine derivatives (*R*)-(−)-α-methylhistamine, (*S*)-(+)-α-methylhistamine, imetit, and imepip, compared with histamine itself (Table 2; Fig. 7). Thus, the maximum response was reduced for all compounds at the Asn^{147} (4.57) → Tyr mutant compared with the wild type, although *R*-(−)-α-methylhistamine was least affected. The EC_{50} values for the agonists were reduced as well, with the exception of *S*-(+)-α-methylhistamine, which exhibited somewhat higher potency at the mutant receptor compared with the wild type (Table 2). For the Ser^{320} (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 characterized by several groups. The H_4 histamine receptor exhibits the highest degree of similarity to the H_3 histamine receptor, and comparison of the sequences of the four histamine receptors reveals that a number of amino acids that have been implicated in histamine binding to the other receptor subtypes are conserved in the H_4 receptor. To begin to delineate the histamine-binding site on the H_4 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 H_1 and H_2 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 H_4 receptor, molecular modeling indicated that Asp^{94} (3.32) in TM3 might serve such a role in the binding of histamine. In the present study, mutation of Asp^{94} (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 Asp^{94} (3.32) by mutating it to Glu, eliminated [^{3}H]histamine binding and histamine-induced intracellular Ca^{2+} 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 H_4 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. Asn^{198} (5.46) in the H_1 histamine receptor or Asp^{186} (5.42) in the H_2 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 H_1 and H_2 receptor. In TM5 of the human H_4 receptor, position 5.46 is occupied by a Glu residue, Glu^{182}. Molecular modeling of the H_4 receptor indicated that this residue had the potential to interact with the N^{τ} nitrogen atom of the histamine imidazole ring by either a hydrogen bond or an ion pair. Substitution of Glu^{182} (5.46) with Ala or Gln resulted in a mutant H_4 receptor that could no longer bind to [^{3}H]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 Glu^{182} (5.46) to Asp reduced the affinity for [^{3}H]histamine binding and reduced the potency and efficacy of histamine-stimulated Ca^{2+} mobilization. These results indicate that Glu^{182} (5.46) in TM5 is another essential element of the H_4 receptor for histamine binding. The mechanism for the interaction between Glu^{182} (5.46) and the N^{τ} nitrogen atom of histamine is likely to be an ion pair, similar to that proposed between Asp^{190} in the TM5 of the H_2 receptor and histamine, because Asn cannot substitute for Asp at this position. The reduced affinity for histamine observed with the Asp^{182} mutant H_4 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^{τ} nitrogen atom of histamine.

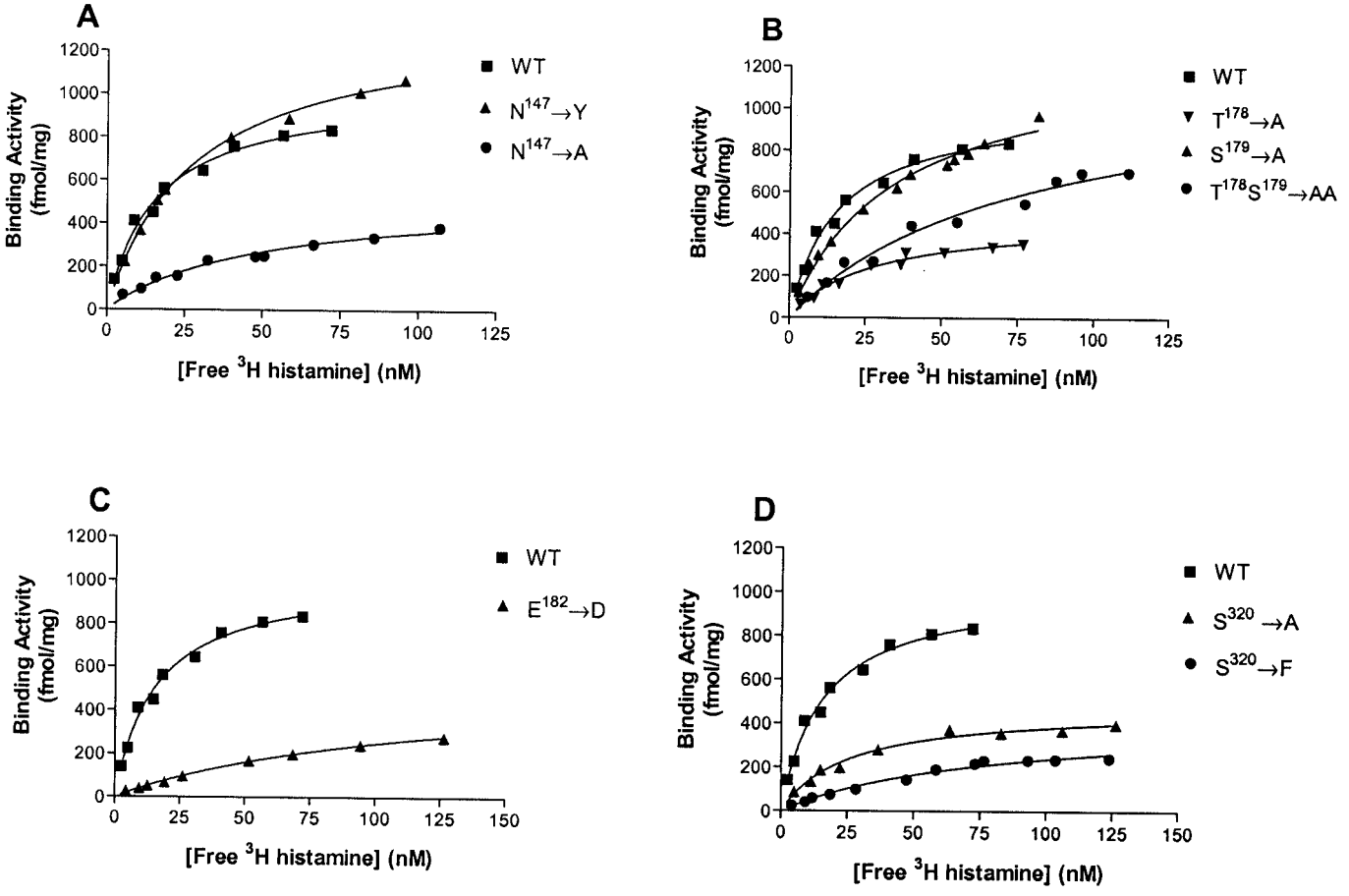

Fig. 5. Binding of [^{3}H]histamine to HEK-293 SFM cell membranes after transient transfection with mutated histamine H_4 receptors. Data points shown are the mean of triplicate determinations and are representative of at least three independent experiments. The data were analyzed by nonlinear regression analysis (Graphpad Prism). A, Asn^{147} (4.57) → Tyr and Asn^{147} (4.57) → Ala mutants. B, Thr^{178} (5.42) →Ala, Ser^{179} (5.43) → Ala, and Thr^{178} (5.42) Ser^{179} (5.43) → Ala Ala mutants. C, Glu^{182} (5.46) → Asn mutants. D, Ser^{320} (6.52) → Ala and Ser^{320} → 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 β_2 adrenergic receptor, two Ser residues in TM5, corresponding to Ser^{179} (5.43) and Glu^{182} (5.46) in the H_4 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 α_1-adrenergic receptor, Ser^{188} (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 Ser^{198} (5.42) or Ser^{199} (5.43) to Ala disrupts agonist binding (Pollock et al., 1992). In the dopamine D2 receptor, Ser^{193} (5.42) contributes notably to the binding of dopamine and Ser^{194} (5.43) is absolutely required for activation of agonists as a result of bonding with the ρ-hydroxyl group of catecholamines (Cox et al., 1992). For serotonin receptors, Ser 5.43 of the human 5-HT_4 was proposed to interact with serotonin through a hydrogen bond (Mialet et

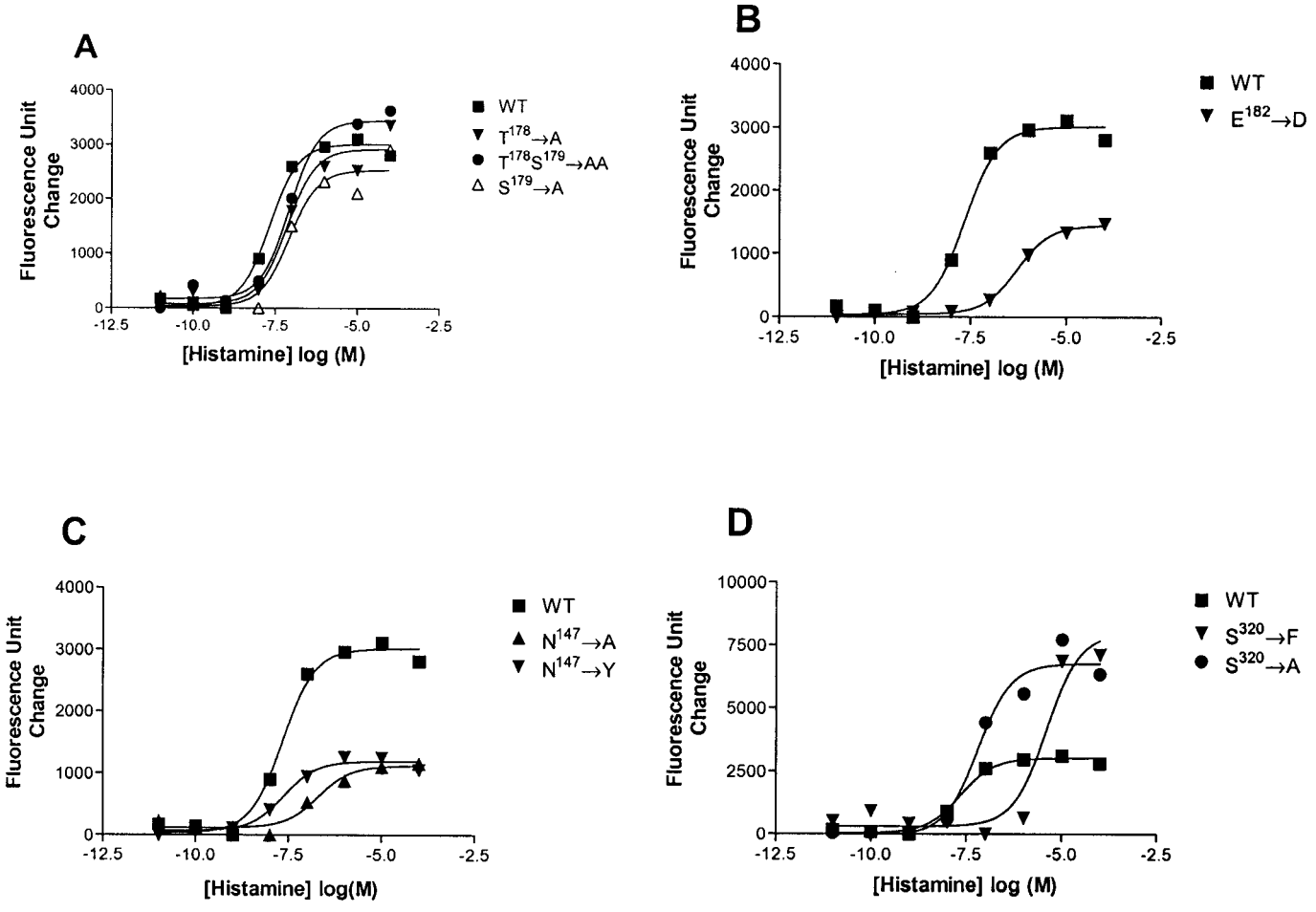

Fig. 6. Histamine-induced Ca^{2+} mobilization in HEK-293 SFM cells coexpressing wild-type or mutant H_4 receptors and $G\alpha_{q/i}$ chimeric G-protein. Data are presented as relative change of fluorescence unit. Each data point is the mean of triplicate determinations and is representative of at least three independent experiments. A, the average maximal fluorescence values for the wild type, Thr^{178} (5.42) → Ala, Thr^{178} (5.42) Ser^{179} (5.43) → Ala Ala, and Ser^{179} (5.43) → Ala mutant H_4 receptors were 3500, 3192, 3192, and 3146 units, respectively. B, the average maximal fluorescence value for the Glu^{182} (5.46) → Asp mutant H_4 receptor was 1886 units. C, the average maximal fluorescence value for the Asn^{147} (4.57) → Ala and Asn^{147} (4.57) → Tyr mutant receptors were 1273 and 1727 units, respectively. D, the average maximal fluorescence value for the Ser^{320} (6.52) → Phe and Ser^{320} (6.52) → Ala mutant receptors were 6941 and 5716 units, respectively.

TABLE 2

Comparison of the agonist induced intracellular Ca^{2+} mobilization in HEK-293 SFM cells expressing the wild-type and mutant histamine H_4 receptors

HEK-293 SFM cells expressing wild type, Ser^{320} → Phe, or Asn^{147} → Tyr mutant histamine H_4 receptors were treated with increasing concentrations of the indicated histamine H_4 receptor agonists. The resulting intracellular Ca^{2+} mobilization was measured by Fluorescent Imaging Plate Reader assay. Data were calculated as the mean ± S.E. of three independent experiments. Efficacy is expressed as percentage of the response of the wild-type receptor to each agonist.

	WT		N^{147} →Y		S^{320} → F	
Agonist	EC_{50}	Efficacy	EC_{50}	Efficacy	EC_{50}	Efficacy
	μM		*μM*		*μM*	
(*R*)-(−)-α-Methylhistamine	0.27 ± 0.03	100	0.46 ± 0.7	76	12.4 ± 1.8	164
(*S*)-(+)-α-Methylhistamine	6.97 ± 0.7	100	0.97 ± 0.3	50	>1000	>100
Imetit	0.09 ± 0.01	100	0.19 ± 0.04	32	1.7 ± 0.14	219
Immepip	0.13 ± 0.03	100	0.32 ± 0.03	31	0.76 ± 0.1	183

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^{198} (5.42) or Thr^{199} (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^{190} (5.46) in TM5 of the H_2 histamine receptor has been proposed to interact with the N^{π} 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^{π} 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^{179} (5.43) and Ser^{178} (5.42) 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^{τ} 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^{τ} 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^{π} nitrogen atom (Gantz et al., 1992).

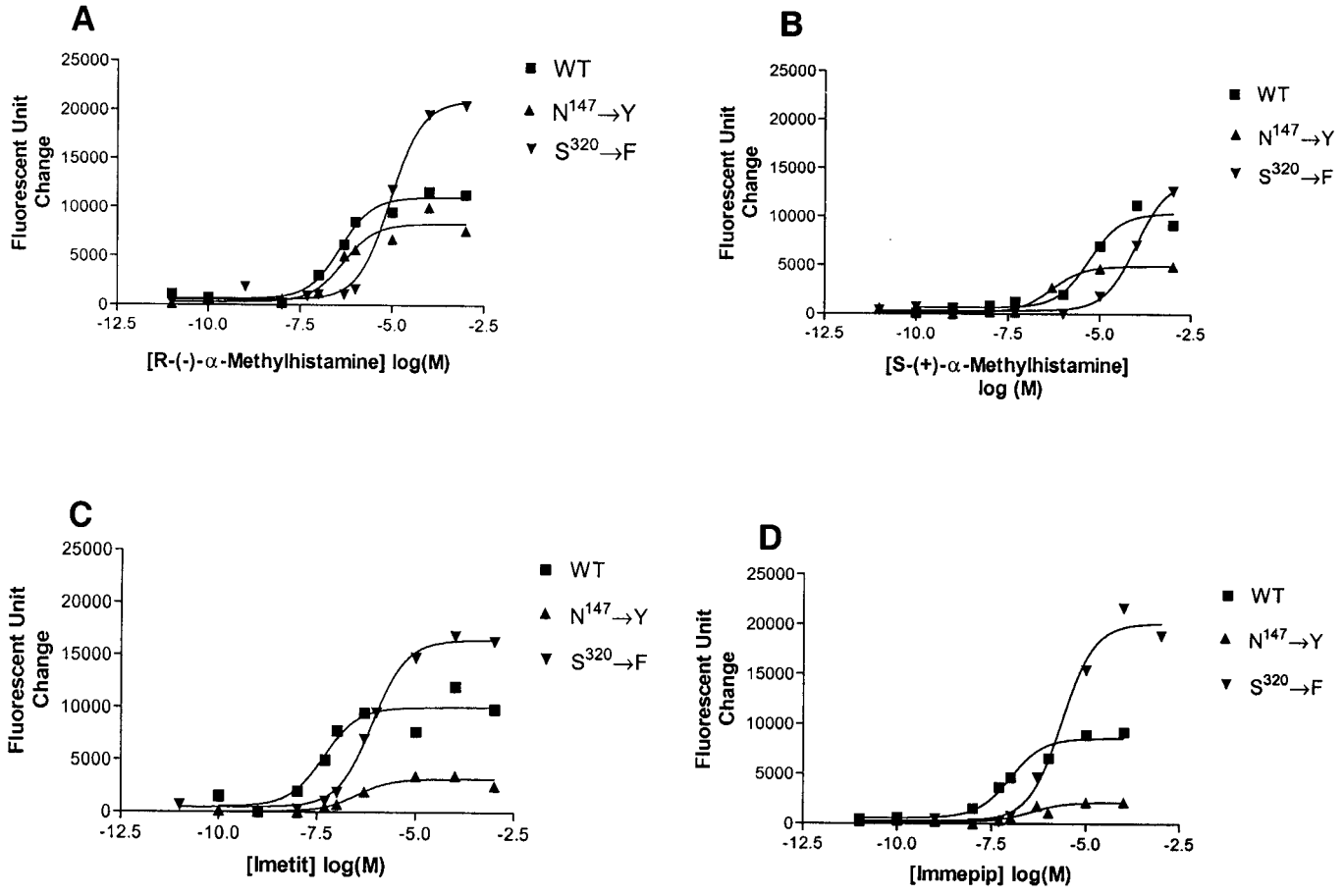

Fig. 7. Agonist-induced Ca^{2+} mobilization in HEK-293 SFM cells coexpressing wild-type or mutant ($Ser^{320} \rightarrow$ Phe or $Asn^{147} \rightarrow$ Tyr) H_4 receptors and $G\alpha_{q/i}$ chimeric G-protein. Cells transiently transfected with the indicated receptor constructs were treated with 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 H_4 receptor also revealed two additional amino acid residues, Asn^{147} (4.57) and Ser^{320} (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 Asn^{147} (4.57) in TM4 of the H_4 receptor is unique; this position is occupied by Trp, Phe, or Tyr in the H_1, H_2, and H_3 receptors, respectively. In TM6, the position occupied by Ser^{320} (6.52) in the H_4 receptor is generally found to be Phe in other biogenic amine receptors, except for the H_3 receptor, which has Thr at this position. In the β_2 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 α_{1B} 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 H_4 receptor for [^{3}H]histamine was only slightly affected by mutations, as shown in our [^{3}H]histamine-binding assay, we conclude that Asn^{147} (4.57) and Ser^{320} (6.52) of the H_4 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-HT_4 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 H_4 receptor. However, both Asn^{147} (4.57) and Ser^{320} (6.52) of the H_4 receptor seem to be involved in activation of the H_4 receptor by histamine. Replacing Asn^{147} (4.57) of the H_4 receptor with Tyr to mimic the H_3 receptor seems to be detrimental to histamine signaling through the H_4 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 Ser^{320} (6.52) of the H_4 receptor to Phe to imitate the H_1, H_2, 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 [^{3}H]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 Asn^{147} (4.57) $\rightarrow$ Tyr and Ser^{320} (6.52) $\rightarrow$ 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 Asn^{147} (4.57) $\rightarrow$ Tyr mutant, this mutation had the smallest effect on *R*-(−)-α−methylhistamine and the greatest effect on imepip. On the other hand, mutation of Ser^{320} (6.52) $\rightarrow$ Phe resulted in decreased potency and increased efficacy for all compounds, although the effects were greatest for histamine.

The observed reduction in maximal [^{3}H]histamine binding and histamine potency at the Ser^{320} (6.52) mutant might be explained by altered interactions with G-proteins leading to an increase in the proportion of low-affinity histamine-binding states, which may not be detected with the ligand and conditions used in the present study. To fully address this issue, however, the development of an H_4 receptor antagonist radioligand will be required. In any case, the H_4 receptor clearly differs from the H_1 and H_2 receptors in the required chemical or physical nature of residue position 6.52.

Previous studies on rhodopsin and β_2 adrenergic receptor have suggested that a critical step in agonist-induced activation of G-protein–coupled receptors is the movement of TM6 and its cytoplasmic extension, which is important for G-protein coupling. For example, photoactivation of rhodopsin involves rotation and tilting of TM6 relative to TM3 (Farrens et al., 1996; Lin and Sakmar, 1996; Dunham and Farrens, 1999). It has been similarly suggested that TM6 of the β_2 adrenergic receptor would move in response to agonist stimulation (Javitch et al., 1997; Jensen et al., 2001). Recently, the movement of TM6 after agonist activation of the β_2 adrenergic receptor was investigated by fluorescence spectroscopy, and the results suggested clockwise rotation of TM6 and/or tilting of the cytoplasmic end of TM6 toward TM5 (Ghanouni et al., 2001).

Specific residues in TM6 have been suggested to be involved in the movement of this TM region upon agonist activation. Grånäs et al. (1998) have suggested that Phe^{331} (6.52) in the $5HT_{1B}$ receptor, analogous to Ser^{320} (6.52) in the H_4 receptor, is involved in the conformational changes associated with signal transduction. In the α_{1B} adrenergic receptor, a highly conserved Phe^{303} (6.44) in TM6 has been postulated to be a key residue in coupling TM6 movement to G-protein activation (Chen et al., 2000, 2002). It remains unclear whether the demonstrated effects of the mutations at the H_4 Ser^{320} (6.52) are due to nonspecific global or local changes in the receptor structure or disruption of particular intramolecular interactions that serve to regulate the ability of histamine to promote H_4 receptor signal transduction. Given the findings mentioned above, which suggest that agonist-induced TM6 movement for G-protein coupling critically depends on the identity of individual residues in TM6, it is possible that Ser^{320} (6.52) in the histamine H_4 receptor is involved TM6 movement and subsequent G-protein coupling on histamine activation. This hypothesis is currently under further investigation.

Taken together, the unique way histamine interacts with residues in the binding pocket of the H4 receptor offers potential for the development of subtype-selective antagonists. These are likely to provide further insights into the physiological role of this newly discovered histamine receptor subtype.

References

Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three dimensional models and computational probing of structure-function relations in G-protein coupled receptors. *Methods Neurosci* **25:**366–428.

Chen S, Lin F, Xu M, and Graham RM (2002) Phe^{303} in TMVI of the α_{1B}-adrenergic receptor is a key residue coupling TM helical movements to G-protein activation. *Biochemistry* **41:**588–596.

Chen S, Lin F, Xu M, Hwa J, and Graham RM (2000) Dominant-negative activity of an α_{1B}-adrenergic receptor signal-inactivating point mutation. *EMBO (Eur Mol Biol Organ) J* **19:**4265–4271.

Chen S, Xu M, Lin F, Lee D, Riek P, and Graham RM (1999) Phe310 in transmembrane VI of the α1B-adrenergic receptor is a key switch residue involved in activation and catecholamine ring aromatic bonding. *J Biol Chem* **274:**16320–16330.

Cox BA, Henningsen RA, Spanoyannis A, Neve RL, and Neve KA (1992) Contribution of conserved serine residues to the interactions of ligands with dopamine D_2 receptors. *J Neurochem* **59:**627–635.

Dunham TD and Farrens DL (1999) Conformational changes in rhodopsin. *J Biol Chem* **274:**1683–1690.

Farrens DL, Altenbach C, Yang K, Hubbell W, and Khorana HG (1996) Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. *Science (Wash DC)* **274:**768–770.
Fraser CA, Wang CD, Robinson DA, Gocayne JD, and Venter JC (1989) Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function. *Mol Pharmacol* **36:**840–847.
Gantz I, DelValle J, Wang L, Tashiro T, Munzert G, Guo Y, Konda Y, and Yamada T (1992) Molecular basis for the interaction of histamine with the histamine H_2 receptor. *J Biol Chem* **267:**20840–20843.
Gantz I, Schaffer M, DelValle J, Logsdon C, Campbell V, Uhler M, and Yamada T (1991) Molecular cloning of a gene encoding the histamine H_2 receptor. *Proc Natl Acad Sci USA* **88:**429–433.
Ghanouni P, Steenhuis JJ, Earrens DL, and Kobika BK (2001) Agonist-induced conformational changes in the G-protein-coupling domain of the β_2 adrenergic receptor. *Proc Natl Acad Sci USA* **98:**5997–6002.
Gråsäs C, Nordvall G, and Larhammar D (1998) Mutagenesis of the human 5-HT_{1B} receptor: differences from the closely related 5-HT_{1A} receptor and the role of residue F331 in signal transduction. *J Recept Signal Transduct Res* **18:**225–241.
Ho BY, Karschin A, Branchek T, Davidson N, and Lester HA (1992) The role of conserved aspartate and serine residues in ligand binding and in function of the 5-HT_{1A} receptor: a site-directed mutation study. *FEBS Lett* **312:**259–262.
Hwa J and Perez DM (1996) The unique nature of the serine interactions for α1-adrenergic receptor agonist binding and activation. *J Biol Chem* **271:**6322–6327.
Javitch JA, Fu D, Liapakis G, and Chen J (1997) Constitutive activation of the β_2 adrenergic receptor alters the orientation of its sixth membrane-spanning segment. *J Biol Chem* **272:**18546–18549.
Jensen AD, Guarnieri F, Rasmussen SGF, Asmar F, Ballesteros JA, and Gether U (2001) Agonist-induced conformational changes at the cytoplasmic side of transmembrane segment 6 in the β_2 adrenergic receptor mapped by site-selective fluorescent labeling. *J Biol Chem* **276:**9279–9290.
Leurs R, Smit MJ, Tensen CP, Ter Laak AM, and Timmerman H (1994) Site-directed mutagenesis of the histamine H_1 receptor reveals a selective interaction of asparagine^{207} with subclasses of H_1-receptor agonists. *Biochem Biophys Res Commun* **201:**295–301.
Levitt M (1992) Accurate modeling of protein conformation by automatic segment matching. *J Mol Biol* **226:**507–533.
Ligneaux X, Morisset S, Tardivel-Lacombe J, Gbahou F, Ganellin CR, Stark H, Schunack W, Schwartz JC, and Arrang JM (2000) Distinct pharmacology of rat and human histamine H_3 receptor: role of two amino acids in the third transmembrane domain. *Br J Pharmacol* **131:**1247–1250.
Lin SW and Sakmar TP (1996) Specific tryptophan UV-absorbance changes are probes of the transition of rhodopsin to its active state. *Biochemistry* **35:**11149–11159.
Liu C, Ma X, Jiang X, Wilson SJ, Hofstra CL, Blevitt J, Pyati J, Li W, Carruthers N, and Lovenberg TW (2001) Cloning and pharmacological characterization of a fourth histamine receptor (H_4) expressed in bone marrow. *Mol Pharmacol* **59:**420–426.
Lovenberg TW, Pyati J, Chang H, Wilson SJ, and Erlander MG (2000) Cloning of rat histamine H_3 receptor reveals distinct species pharmacological profiles. *J Pharmacol Exp Ther* **293:**771–778.
Lovenberg TW, Roland BL, Wilson SJ, Jiang X, Pyati J, Huvar A, Jackson MR, and Erlander MG (1999) Cloning and functional expression of the human histamine H_3 receptor. *Mol Pharmacol* **55:**1101–1107.
MacDonald D, Murgolo N, Zhang R, Durkin JP, Yao X, Strader CD, and Graziano MP (2000) Molecular characterization of the melanin-concentrating hormone/receptor complex: identification of critical residues involved in binding and activation. *Mol Pharmacol* **58:**217–225.
Mialet J, Dahmoune Y, Lezoualc'h F, Berque-Bestel I, Eftekhari P, Hoebeke J, Sicsic S, Langlois M, and Fischmeister R (2000) Exploration of the ligand binding site of the human 5-HT(4) receptor by site-directed mutagenesis and molecular modeling. *Br J Pharmacol* **130:**527–538.
Morse KL, Behan J, Laz TM, West RE, Greenfeder SA, Anthes JC, Umland S, Wan Y, Hipkin RW, Gonsiorek W, et al. (2001) Cloning and characterization of a novel human histamine receptor. *J Pharmacol Exp Ther* **296:**1058–1066.
Nguyen T, Shapiro DS, George SR, Setola V, Lee DK, Cheng R, Rauser L, Lee SP, Lynch KR, Roth BL, et al. (2001) Discovery of a novel member of the histamine receptor family. *Mol Pharmacol* **59:**427–433.
Nonaka H, Otaki S, Ohshima E, Kono M, Kase H, Ohta K, Fukui H, and Ichimura M (1998) Unique binding pocket for KW-4679 in the histamine H_1 receptor. *Eur J Pharmacol* **345:**111–117.
Oda T, Morikawa N, Saito Y, Masuho Y, and Matsumoto S (2000) Molecular cloning and characterization of novel type of histamine receptor preferentially expressed in leukocytes. *J Biol Chem* **275:**36781–36786.
Ohta K, Hayashi H, Mizuguchi H, Kagamiyama H, Fujimoto K, and Fukui H (1994) Site-directed mutagenesis of the histamine H_1 receptor: roles of aspartic acid^{107}, asparagine^{198} and threonine^{194}. *Biochem Biophys Res Commun* **203:**1096–1101.
Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, LeTrong I, Teller DC, Okada T, Stenkamp RE, et al. (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science (Wash DC)* **289:**739–745.
Pollock NJ, Manelli AM, Hutchins CW, Steffey ME, MacKenzie RG, and Frail DE (1992) Serine mutations in transmembrane V of the dopamine D1 receptor affect ligand interactions and receptor activation. *J Biol Chem* **267:**17780–17786.
Shapiro DA, Kristiansen K, Kroeze WK, and Roth B (2000) Differential modes of agonist binding to 5-hydroxytryptamine$_{2a}$ serotonin receptors revealed by mutation and molecular modeling of conserved residues in transmembrane region 5. *Mol Pharmacol* **58:**877–886.
Strader CD, Candelore MR, Hill WS, Sigal IS, and Dixon RA (1989a) Identification of two serine residues involved in agonist activation of the β-adrenergic receptor. *J Biol Chem* **264:**13572–13578.
Strader CD, Sigal IS, and Dixon RA (1989b) Structural basis of β-adrenergic receptor function. *FASEB J* **3:**1825–1831.
Strader CD, Sigal IS, Candelore MR, Rands E, Hill WS, and Dixon RA (1988) Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. *J Biol Chem* **263:**10267–10271.
Strader CD, Sigal IS, Register RB, Candelore MR, Rands E, and Dixon AF (1987) Identification of residues required for ligand binding to the β-adrenergic receptor. *Proc Natl Acad Sci USA* **84:**4383–4388.
van Rhee AM and Jacobson KA (1996) Molecular architecture of G-protein coupled receptors. *Drug Dev Res* **37:**1–38.
Wang CD, Buck MA, and Fraser CM (1991) Site-directed mutagenesis of α2a-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol Pharmacol* **40:**168–179.
Weinstein H, Chou D, Johnson CL, Kang S, and Green JP (1976) Tautomerism and the receptor activation of histamine: a mechanistic model. *Mol Pharmacol* **12:**738–745.
West J, Maggio R, Palmer JR, and Vogel Z (1992) Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation. *J Biol Chem* **267:**19313–19319.
Yamashita M, Fukui H, Sugama K, Horio Y, Ito S, Mizuguchi H, and Wada H (1991) Expression cloning of a cDNA encoding the bovine histamine H_1 receptor. *Proc Natl Acad Sci USA* **88:**11515–11519.
Zhu Y, Michalovich D, Wu H, Tan KB, Dytko GM, Mannan IJ, Boyce R, Alston J, Tierney LA, Li X, et al. (2001) Cloning, expression and pharmacological characterization of a novel human histamine receptor. *Mol Pharmacol* **59:**434–441.

Address correspondence to: Frederick J. Monsma, Jr., Discovery Technologies, Schering-Plough Research Institute, K15-1, 1945; 2015 Galloping Hill Road, Kenilworth, NJ 07033. E-mail: frederick.monsma@spcorp.com