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LYSINE^{200} LOCATED IN THE FIFTH TRANSMEMBRANE DOMAIN OF THE HISTAMINE H_1 RECEPTOR INTERACTS WITH HISTAMINE BUT NOT WITH ALL H_1 AGONISTS

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Previously, we have shown that asparagine^{207} in the fifth transmembrane domain of the histamine H_1 receptor is crucial for the binding of the N^{τ}-nitrogen of the imidazole ring of histamine (Leurs et al., Biochem. Biophys. Res. Commun., 201, 295, 1994). In view of the potential interaction of the imidazole ring of histamine with a binding site, formed by asparagine^{207} and lysine^{200}, we mutated lysine^{200} in the fifth transmembrane domain of the histamine H_1 receptor to a non-functional alanine residue. This mutation did not affect the binding of the tested H_1 receptor antagonists but resulted in a 5-fold lower affinity for histamine. The binding of other H_1 receptor agonists was not affected. In stably transfected CHO cells histamine was 55-fold less effective in activating the H_1Lys^{200}Ala receptor (EC_{50} = 66 μM) compared to the wild type H_1 receptor (EC_{50} = 1.2 μM). Receptor activation by the 2-methyl and the 2-(3-bromophenyl)-analogues however was hardly affected by the mutation, indicating that the 2-substituent probably prevents the interaction with the lysine^{200} residue. Finally, the Lys^{200}Ala mutation reduced the production of [^{3}H]inositol phosphates, stimulated by the non-imidazole H_1 receptor agonist 2-pyridylethylamine. These data indicate that lysine^{200} interacts with the N^{π}-nitrogen of histamine and is important for the activation of the H_1 receptor by histamine and the non-imidazole agonist 2-pyridylethylamine.

Molecular biological approaches have recently been implemented in histamine receptor research. As a result genes or cDNAs encoding for the H_1 and H_2 receptor have been cloned from several species (1-8). The availability of the genetic information encoding for histamine receptor proteins offers a great potential for detailed molecular investigations of ligand receptor interactions. Amino acid sequence alignments of the cloned histamine receptors with amino acid sequences of other aminergic receptors led Birdsall (9) and Timmerman (10) to suggest that histamine binds to the third (TM3) and fifth transmembrane (TM5) domains of the receptor proteins. An aspartic acid residue in TM3, conserved in all aminergic receptors, including the histamine H_1 and H_2 receptors (1-8), has recently been shown to be involved in the binding of histamine and H_1 or H_2 antagonists to the H_1 (11,12) and the H_2 receptor (13). Moreover, Gantz *et al.* (13) showed that an aspartic acid and threonine residue located in TM 5 are probably involved in the interaction of histamine with the H_2 receptor protein. These two residue are located at approximately similar positions as the two serine residues of the β_2 adrenergic receptor that have been shown to be

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implicated in the binding of the catechol moiety of noradrenaline (14). For the H_1 receptor a threonine (Thr^{203}) and asparagine (Asn^{207}) residue are present at homologous positions (fig. 1A). Yet, we recently reported that the Thr^{203}Ala mutation hardly affected the interaction of the receptor protein with histamine (15). In contrast, major changes were noticed for the interaction of histamine with the Asn^{207}Ala receptor mutant (15). A dramatic loss of affinity was accompanied by a loss of agonistic activity as measured by the production of [^{3}H]inositol phosphates in stably transfected CHO cells (15). Similar findings were thereafter reported by other laboratories (11,12). Remarkably, the Asn^{207}Ala mutation did not affect the interaction of the receptor protein with several other H_1 receptor agonists, as e.g. 2-pyridylethylamine (15). Asn^{207} was suggested to interact with the N^{τ}-nitrogen atom of the imidazole ring of histamine (15) as this nitrogen atom is absent in 2-pyridylethylamine.

Based on the results of these mutagenesis studies we assumed that the affinity of histamine for the H_1 receptor is mainly determined by an ionic interaction of the protonated ethylamine sidechain with the aspartic acid residue in TM3 (11,12) and a hydrogen bond between the N^{τ}-nitrogen of the imidazole ring and Asn^{207} in TM5 (15). Nevertheless, structure-activity relationships of H_1 receptor agonists have revealed that the presence of an N^{π}-nitrogen is essential for H_1 agonistic activity (16). Combining these observations we hypothesized that an interaction of the N^{π}-nitrogen of the imidazole ring of histamine with the receptor protein is involved in the H_1 receptor stimulation by this endogenous ligand. Searching for residues capable of hydrogen bond formation with the N^{π}-nitrogen we observed that in the upper part of TM5 of the H_1 receptor a lysine (Lys^{200}) residue is conserved in all species. A specific function of Lys^{200} is suggested by the fact that charged amino acid residues are not present at this position of TM5 in other aminergic G-protein coupled receptors (17,18). These arguments led us to hypothesize that Lys^{200} could be an interesting candidate for an interaction with the imidazole ring of histamine. A molecular modeling study on the interaction between Lys^{200} and Asn^{207} in TM5 of the guinea-pig H_1 receptor and the imidazole ring of histamine has indicated that these two amino acids are indeed likely to form a

A

			*			*				*											
H1 gp	W	F	K	V	M	T	A	I	I	N	F	Y	L	P	T	L	L	M	L	W	F	Y
H2 hum	V	Y	G	L	V	D	G	L	V	T	F	Y	L	P	L	L	I	M	C	T	Y	Y
Beta1 hum	A	Y	A	I	A	S	S	V	V	S	F	Y	V	P	L	C	I	M	A	F	V	Y
Beta2 hum	A	Y	A	I	A	S	S	I	V	S	F	Y	V	P	L	V	I	M	V	F	V	Y
Beta3 hum	P	Y	V	L	L	S	S	S	V	S	F	Y	L	P	L	L	V	M	L	F	V	Y
D2 hum	A	F	V	V	Y	S	S	I	V	S	F	Y	V	P	F	I	V	T	L	L	V	Y
D4 hum	D	Y	V	V	Y	S	S	V	C	S	F	F	L	P	C	P	L	M	L	L	L	Y
5HT1A hum	G	Y	T	I	Y	S	T	F	G	A	F	Y	I	P	L	L	L	M	L	V	L	Y
m1 hum	I	I	T	F	G	T	A	M	A	A	F	Y	L	P	V	T	V	M	C	T	L	Y
m2 hum	A	V	T	F	G	T	A	I	A	A	F	Y	L	P	V	I	I	M	T	V	L	Y
m5 hum	T	I	T	F	G	T	A	I	A	A	F	Y	I	P	V	S	V	M	T	I	L	Y

B

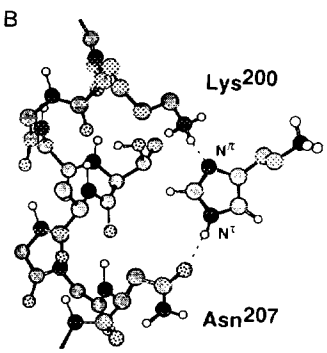


Figure 1.
(A) Alignment of the putative fifth transmembrane domain of the H_1 and H_2 receptor proteins and various other aminergic G-protein coupled receptors. The amino acid sequence alignment was taken from Donnely *et al.* (18) and is shown in single letter notation. The asterisks indicate the position of the mentioned Lys^{200}, Thr^{204} and Asn^{207} residues.
(B) Hypothetical interaction model for the Lys^{200}-Asn^{207} binding site in TM5 and the imidazole ring of histamine. Carbon, hydrogen, nitrogen and oxygen atoms are represented by grey, white, black and dotted circles, respectively. The dotted lines represent potential hydrogen bonds.

binding site for histamine (fig. 1B, Ter Laak *et al.*, in preparation). The side chain of a lysine residue is relatively long and flexible and can easily adopt a conformation that allows the formation of an imidazole binding site with Asn^{207}. In the present study we therefore evaluated the effect of the $Lys^{200}Ala$ mutation of the guinea-pig H_1 receptor on the receptor binding characteristics and receptor activation for various H_1 receptor agonists.

Materials and Methods

Chemicals: Histamine.2HCl was obtained from Sigma Chemical Company (USA). [^{3}H]mepyramine (21 Ci/mmol) and [^{3}H]inositol (18.8 Ci/mmol) were obtained from Amersham. 2-pyridylethylamine.2HCl was taken from laboratory stock. Generous gifts of 2-methylhistamine.2HCl (SmithKline Beecham), 2(3-bromophenyl)histamine dimaleate (Dr. W. Schunack, Berlin), D- and L-chlorpheniramine maleate (Dr. A. Beld, Nijmegen) and (d/l)-mianserine.HCl (Organon) are greatly acknowledged.

Site-directed mutagenesis: The guinea-pig H_1 receptor mutant was constructed by a double Polymerase Chain Reaction (PCR) (19). In the first PCR reaction the synthetic oligonucleotide 5'-GGGAAGCTTGATCAGGTATGTCTGACCTCT, corresponding to nucleotide -33 to -13 and a *Hind*III linker site (1) and the oligonucleotide 5'-GCAGTCATGACCGCGAACCAGGTGAC, corresponding to nucleotides 586-610 with two mutations, were used to amplify a 644 bases DNA fragment of the $H_1Lys^{200}Ala$ receptor mutant. Using 100 ng $pSVgpH_1$ (1) as a template, 0.4 μM of the oligonucleotides, 200 μM of each nucleotide and 2.5 U *Pfu* DNA polymerase (Promega) the desired fragment was amplified in 100 μl using 25 cycles at 94°C for 1 min., 56°C for 1 min. and 72°C for 1 min. and a final extension at 72°C for 10 min. The DNA fragment was gel-purified, and used in a second PCR reaction with 0.4 μM of the oligonucleotide 5'-CGGAGATCTAGGTACCTGTGAGA CAAGGCT, corresponding to nucleotides 1533-1553 of the complementary strand (1) and a *Bgl*II linker site, and 1 μg of $pSVgpH_1$ as template to amplify the complete coding region of the mutant H_1 receptor. Twentyfive cycles at 94°C for 1 min., 56°C for 1 min. and 72°C for 20 min. and a final extension of 10 min at 72°C were used to amplify a 1.6 kb fragment. After gel-purification the fragments were restricted with *Hind*III/*Bgl*II and ligated in the plasmid pSP73 (Promega), which was treated with the same enzymes. The complete nucleotide sequence of the receptor mutant was verified using the dideoxynucleotide chain termination method using Sequenase (USB). Human embryonic kidney cells (HEK-293 cells) were transiently transfected with the eukaryotic expression vectors pRK_5gpH_1WT and $pRK_5gpH_1Lys^{200}Ala$ (20). Chinese Hamster Ovary cells (CHO cells) were stably transfected with the eukaryotic expression vectors $pSVgpH_1WT$ and $pSVgpH_1Lys^{200}Ala$ using Transfectam (Promega) (21).

H_1 receptor binding: [^{3}H]mepyramine binding was performed as described previously (1). In saturation studies increasing concentrations of [^{3}H]mepyramine were incubated with 60 μg membrane protein in the absence or presence of 1 μM mianserine. In displacement studies membranes were incubated with 1.5 nM [^{3}H]mepyramine and increasing concentrations of unlabeled ligands.

[^{3}H]Inositol phosphate production: CHO cells were seeded in 24-well plates and cultured overnight in culture medium. Thereafter cells were labeled overnight in inositol-free culture medium supplemented with 1 μCi/ml [^{3}H]inositol. Cells were washed twice with DMEM, supplemented with 50 mM HEPES (pH = 7.4 at 37°C) and 20 mM LiCl and preincubated for 10 min. at 37°C with 500 μl DMEM/HEPES/LiCl. Incubations were started by the addition of 50 μl of H_1 agonist in DMEM/HEPES/LiCl. After 10 min. incubation at 37°C the reaction was stopped by the addition of 500 μl of cold $CHCl_3$/methanol (1:2, v/v). After extraction with water the [^{3}H]inositol phosphates were isolated by anion exchange chromatography (22).

Results

Fourthyeight hours after the transient transfection of HEK-293 cells with the expression vectors pRK_5H_1WT and $pRK_5H_1Lys^{200}Ala$ a high expression of the respective H_1 receptor

Table 1

Analysis of the binding of [^{3}H]mepyramine and D- and L-chlorpheniramine (ClPhen) to membranes of HEK-293 cells expressing the guinea-pig wild type or $H_1Lys^{200}Ala$ receptor protein. Data shown are mean ± SEM of three independent experiments.

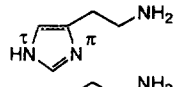
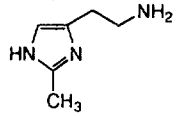
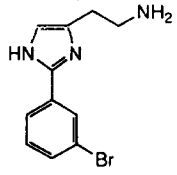
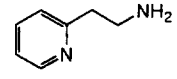
Receptor protein	[^{3}H]mepyramine		D-ClPhen	L-ClPhen
	K_d (nM)	B_{max}(pmol/mg protein)	K_i (nM)	K_i (nM)
wild type	0.86 ± 0.18	7.0 ± 1.6	1.9 ± 0.1	150 ± 40
$Lys^{200}Ala$	0.71 ± 0.25	4.0 ± 1.9	1.4 ± 0.3	140 ± 10

proteins was detected with the H_1 receptor antagonist [^{3}H]mepyramine (Table 1). The $Lys^{200}Ala$ mutation had no effect on the K_d value for the radiolabeled antagonist (Table 1). Moreover, the K_i values for the two stereoisomers of chlorpheniramine did not differ significantly between the two receptor proteins either (Table 1). Displacement of [^{3}H]mepyramine with four selective H_1 receptor agonists did also not reveal dramatic effects of the $Lys^{200}Ala$ mutation (Table 2). The affinity of histamine for the $Lys^{200}Ala$ receptor mutant was 5-fold lower than for the wild type H_1 receptor (Fig. 2A). Yet, for 2(3-bromophenyl)histamine, 2-methylhistamine and 2-pyridylethylamine (Table 2) no differences in the affinities for the two receptor proteins were found.

For studying the effects of the $Lys^{200}Ala$ mutation on the H_1 receptor activation by H_1 receptor agonists the receptor was stably expressed in CHO cells. Transfection of CHO cells deficient in

Table 2

K_i values of various H_1 receptor agonists for the guinea-pig wild type and $H_1Lys^{200}Ala$ receptor protein, transiently expressed in HEK-293 cells. Data shown are mean ± SEM of three independent experiments and were obtained from [^{3}H]mepyramine displacement studies.

H_1-agonist	structure	K_i (μM)	
		wild type	$Lys^{200}Ala$
histamine		30 ± 2	150 ± 10*
2-methylhistamine		98 ± 22	60 ± 11
2-(3-bromophenyl)histamine		0.9 ± 0.1	2.2 ± 0.6
2-pyridylethylamine		69 ± 2	86 ± 14

* Indicates a P-value <0.05 compared to wild type.

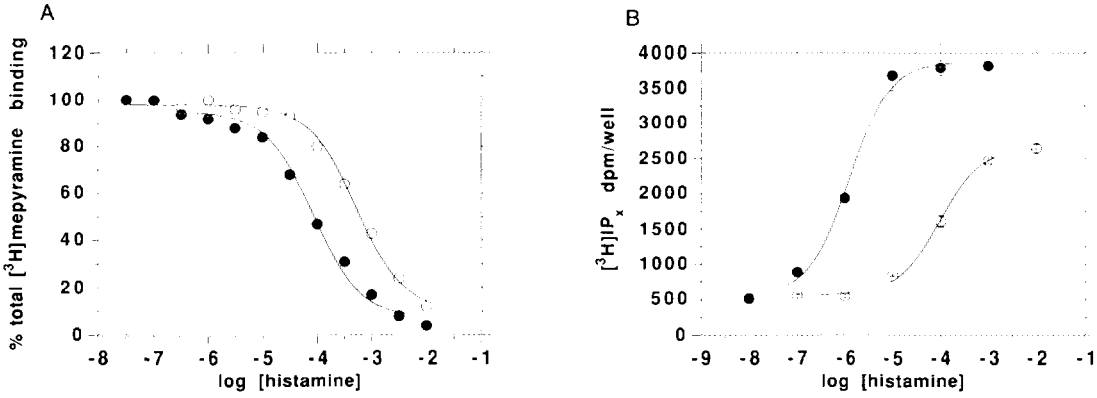


Figure 2.
(A) Displacement by histamine of [^{3}H]mepyramine binding to membranes of HEK-293 cells transiently expressing wild type (filled circles) or $H_1Lys^{200}Ala$ receptors (open circles). A representative experiment out of 3 independent experiments is shown.
(B) Histamine-stimulated accumulation of [^{3}H]inositol phosphates in $CHOgpH_1WT$ (filled circles) and $CHOgpH_1Lys^{200}Ala$ cells (open circles). Data shown are a representative example of 4 independent experiments.

dihydrofolate reductase with the plasmids $pSVgpH_1WT$ and $pSVgpH_1Lys^{200}Ala$ by lipofection resulted, after 10 to 14 days of selection in culture medium deprived of hypoxanthine and thymidine, in the formation of several clonal cell lines. For the wild type H_1 receptor a clone that expressed 450 ± 36 fmol/mg protein [^{3}H]mepyramine binding sites (mean ± SEM, n = 3) was selected. For the $H_1Lys^{200}Ala$ receptor a clone, expressing 256 ± 8 fmol/mg protein [^{3}H]mepyramine binding sites (mean ± SEM, n = 3) was obtained. From the [^{3}H]mepyramine saturation experiments K_d values of 0.69 ± 0.05 nM (mean ± SEM, n = 3) and 0.62 ± 0.08 (mean ± SEM, n = 3) for de radiolabeled antagonist was found for the wild type and $H_1Lys^{200}Ala$ receptor, respectively.

In $CHOH_1WT$ cells histamine stimulated the production of [^{3}H]inositol phosphates 7.5 ± 0.3 fold with an EC_{50} value of 1.2 ± 0.1 μM (mean ± SEM, n = 4). Histamine was clearly less effective in CHO cells expressing the $H_1Lys^{200}Ala$ receptor (Fig. 2B). For the $CHOH_1Lys^{200}Ala$ cells a 55-fold shift in the EC_{50} value for histamine was observed (EC_{50} = 66 ± 6 μM, mean ± SEM, n = 4, P<0.001). Moreover, histamine was significantly less effective in elevating the basal production of [^{3}H]inositol phosphates (Fig. 2B). In $CHOH_1Lys^{200}Ala$ cells histamine stimulated the production of [^{3}H]inositol phosphates 5.2 ± 0.2 fold (mean ± SEM, n = 4, P<0.001). As previously described the selective H_1 receptor agonist 2(3-bromophenyl)histamine acted as a partial agonist in $CHOH_1WT$ cells (Table 3). On the other hand, for 2(3-bromophenyl)histamine neither the potency nor the absolute increase of the [^{3}H]inositol phosphates was affected by the $Lys^{200}Ala$ mutation (Table 3). Also the other 2-substituted histamine analogue, 2-methylhistamine acted as a partial agonist in $CHOH_1WT$ cells. The $Lys^{200}Ala$ mutation only slightly affected the potency of 2-methylhistamine (Table 3). The absolute increase of [^{3}H]inositol phosphates by the partial agonist was not affected by the $Lys^{200}Ala$ mutation. Finally, we observed that the $Lys^{200}Ala$ mutation significantly affected the effectiveness of the non-imidazole H_1 receptor agonist 2-pyridylethylamine (Table 3). In $CHOH_1WT$ cells 2-pyridylethylamine stimulated the basal [^{3}H]inositol phosphate production 6.32 ± 0.58 fold (mean ± SEM, n = 4) with an EC_{50}

Table 3

Characteristics of the agonistic potencies of histamine and three selective H_1 agonists at the guinea-pig wild type or $H_1Lys^{200}Ala$ receptor stably expressed in CHO cells. Agonistic effects were measured as the accumulation of [^{3}H]inositol phosphates. Data shown are mean ± SEM of three to six independent experiments.

	wild type H_1 receptor		$H_1Lys^{200}Ala$ receptor	
H_1 agonist	EC_{50} (μM)	fold stimulation	EC_{50} (μM)	fold stimulation
histamine	1.2 ± 0.1	7.5 ± 0.3	66 ± 6*	5.2 ± 0.2*
2-methylhistamine	8.8 ± 0.9	5.0 ± 0.4	16 ± 2*	4.0 ± 0.4
2-(3-bromophenyl)histamine	0.6 ± 0.2	3.8 ± 0.2	0.6 ± 0.1	4.2 ± 0.5
2-pyridylethylamine	23 ± 4	6.3 ± 0.6	392 ± 101*	4.2 ± 0.6*

* Indicates a P-value <0.05 compared to wild type.

value of 23 ± 4 μM (mean ± SEM, n = 4). Yet, in $CHOH_1Lys^{200}Ala$ cells 2-pyridylethylamine stimulated the basal [^{3}H]inositol phosphate production only 4.2 ± 0.6 fold (mean ± SEM, n = 4, P<0.001) with an EC_{50} value of 392 ± 110 μM (mean ± SEM, n = 4, P<0.001).

Discussion

In recent years the impact of molecular biology on rational drug design has been greatly increased. With the cloning of many genes encoding for G-protein coupled receptors (17,18) mechanistic aspects of neurotransmission and drug action can currently be evaluated in great detail. Site-directed mutagenesis allows the investigation of the role of single amino acid residues in proteins, providing possibilities to study the molecular interactions of small molecules with large receptor protein structures. Using this approach several laboratories have previously reported on the role of TM3 and TM5 of the histamine H_1 receptor in the binding of some H_1 receptor agonists (11,12,15). In TM5 the Asn^{207} residue was suggested to be implicated in the binding of the N^{τ}-nitrogen atom of the imidazole ring of histamine (15). Since previous detailed pharmacochemical studies indicated that the presence of an N^{π}-nitrogen in a heterocyclic ring is necessary for H_1 receptor agonism (16), we evaluated other amino acid residues in TM5 capable of hydrogen bond formation as a putative secondary interaction point with the imidazole ring of histamine. Combining an amino acid alignment of TM5 of several G protein coupled receptors and a computer model of TM5 of the H_1 receptor (Ter Laak *et al.*, in preparation) a binding site for the imidazole ring of histamine was hypothesized to consist of Lys^{200} and Asn^{207} (see Fig. 1). Consequently, site-directed mutagenesis of the Lys^{200} residue to a non-functional alanine residue was applied to challenge the hypothesis of a Lys^{200}-Asn^{207} binding site for histamine. After transient expression of HEK-293 cells high level expression of both the wild type and $H_1Lys^{200}Ala$ receptor was obtained and both receptor proteins showed a similar affinity for the radiolabeled antagonist [^{3}H]-mepyramine (Table 1). Similar findings were observed after stable expression of the wild type and $Lys^{200}Ala$ receptor mutant in CHO cells. Moreover, displacement of the radioligand binding by the stereoisomers of chlorpheniramine showed that the stereoselectivity of the H_1 receptor protein was still observed after the $Lys^{200}Ala$ mutation. These data indicate that the tested antagonists do not interact with Lys^{200}, corroborating previous findings that the binding of these antagonists probably does not occur with TM5 of the H_1 receptor (11,12,15).

Yet, for histamine the Lys^{200}-Asn^{207} couple seems to form a suitable binding site. Mutation of Lys^{200} resulted in a 5-fold lower affinity and severely impaired agonistic potency for histamine. The EC_{50} value of histamine was 55-fold lower for the Lys^{200}Ala mutant and also the maximal effect of histamine was significantly reduced. For the non-imidazole agonist 2-pyridylethylamine an interaction with Lys^{200} was also found. Although the interaction with Lys^{200} does not contribute to agonist affinity, receptor stimulation by this agonist is reduced significantly. These findings indicate that the hypothesis of an interaction of Lys^{200} with the N^{π}-nitrogen of the imidazole ring of histamine is correct. Since Lys^{200} is not crucial for the binding of histamine and 2-pyridylethylamine this residue probably plays a role in the H_1 receptor activation mechanism instead.

The introduction of a 2-methyl substituent on the imidazole ring impairs the interaction of the imidazole ring with the Lys^{200}-Asn^{207} binding site. Our data indicate that the Lys^{200} residue does not contribute to the affinity of 2-methylhistamine, and is only of minor importance for the agonistic potency of this agonist. A further increase of the size of the 2-substituent completely eliminates the interaction of the imidazole ring with Lys^{200}. Neither the binding affinity nor the agonistic potency of 2-(3-bromophenyl)histamine was altered for the H_1Lys^{200}Ala receptor compared to the wild type receptor. Since this agonist does also not interact with the Asn^{207} residue (15) the bulky aromatic substituent probably sterically hinders the interaction of this agonist with the histamine Lys^{200}-Asn^{207} binding site. On the basis of these observations one should conclude that the agonist 2-(3-bromophenyl)histamine uses different amino acids for the interaction with the H_1 receptor than the endogenous agonist histamine.

In conclusion, in the present study we have provided evidence that the Lys^{200} residue of TM5 of the H_1 receptor interacts with the N^{π}-nitrogen atom of histamine and the non-imidazole H_1 receptor agonist 2-pyridylethylamine. A specific interaction of Lys^{200} with these agonists is supported by the observation that the Lys^{200}Ala mutation does not affect H_1 receptor stimulation by the agonist 2(3-bromophenyl)histamine. On the basis of our studies (present study, (15)) we conclude that different histamine H_1 receptor agonists interact in different ways with the receptor proteins. For a good understanding of the molecular basis of H_1 receptor stimulation future site-directed mutagenesis studies should identify amino acid residue(s) involved in the binding and receptor stimulation of 2-phenylhistamines.

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