Vol. 201, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1994 Pages 295-301

SITE-DIRECTED MUTAGENESIS OF THE HISTAMINE H_1-RECEPTOR REVEALS A SELECTIVE INTERACTION OF ASPARAGINE^{207} WITH SUBCLASSES OF H_1-RECEPTOR AGONISTS

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Received March 21, 1994

In this study we investigated the role of the threonine^{203} and the asparagine^{207} residues in the fifth transmembrane domain of the guinea-pig histamine H_1-receptor by site-directed mutagenesis to non-functional alanines. Whereas the threonine^{203} residue is not important for the action of histamine, the asparagine^{207} residue appears to be involved in the binding of the N^{τ}-nitrogen atom of histamine and its 2-methyl-analogue. For the 2-phenyl-analogue and non-imidazole H_1-receptor agonists, this residue is, however, not essential for binding. On the basis of this study we conclude that different histamine H_1-receptor agonists interact in different ways with the H_1-receptor protein. Moreover, we speculate that the interaction with the N^{π}-nitrogen atom is essential for H_1-receptor activation.

The histamine H_1-receptor plays an important role in allergic conditions and has been the therapeutic target for a wide variety of H_1-receptor antagonists (1). From detailed pharmacochemical studies important insights in the interaction of both selective agonists and antagonists have been obtained and used for the development of new, selective drugs (1,2). With the recent cloning of the gene encoding the H_1-receptor (3-6) it became possible to refine these ideas and to study the interaction of subtype specific ligands with the receptor protein.

According to the results of various site-directed mutagenesis studies of receptors of different biogenic amines, the binding of the amines occurs in the transmembrane domains (TM) of the receptor proteins (7). An aspartic acid (Asp) residue in TM3, which is conserved in all receptors of this type, including the histamine H_1- and H_2-receptors (3-6,8-10), interacts with the positively charged amine function (7). Based on the observation that in TM5 of the β_2-adrenergic receptor two serine residues are involved in the binding of the catechol ring of β_2-receptor agonists(11), the binding site for other biogenic amines has also been envisaged in TM5. Recently, Gantz *et al.* (12) showed that an Asp and a threonine (Thr) residue in TM5 are probably involved in the

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0006-291X/94 $5.00

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H1 human	F	K	V	M	T	A	I	I	N	F	Y	L	P	T	L	L	M	L	W	F	Y
H1 guinea-pig	F	K	V	M	T	A	I	I	N	F	Y	L	P	T	L	L	M	L	W	F	Y
H1 bovine	F	K	V	M	T	A	I	I	N	F	Y	L	P	T	L	L	M	L	W	F	Y
H1 rat	F	K	I	M	T	A	I	I	N	F	Y	L	P	T	L	L	M	L	W	F	Y
H2 human	Y	G	L	V	D	G	L	V	T	F	Y	L	P	L	L	I	M	C	I	T	Y
H2 canine	Y	G	L	V	D	G	L	V	T	F	Y	L	P	L	L	V	M	C	I	T	Y
H2 rat	Y	G	L	V	D	G	L	V	T	F	Y	L	P	L	L	I	M	C	I	T	Y

Figure 1.
Alignment of the putative fifth transmembrane domain of the H_1- and H_2-receptor proteins of various species (3-6, 8-10). The asterisks indicate the positions of the amino acids thought to be involved in the action of histamine.

interaction of histamine with the H_2-receptor protein. For the H_1-receptor a Thr and an asparagine (Asn) residue are found at positions homologous to the Asp and Thr residues in the H_2-receptor (Fig. 1). Since it is generally accepted that there are clearly different structural demands for selective histamine H_1- and H_2-receptor agonists (1,2), the different amino acid sequence of TM5 could form the biochemical basis for this paradigm (13). We therefore investigated the role of the Thr^{203} and Asn^{207} residues in TM5 of the guinea-pig H_1-receptor (6) by site-directed mutagenesisof these residues to non-functional alanines (Ala).

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. Gifts of 2-methylhistamine.2HCl, 2-thiazolylethylamine.2HCl (SmithKline Beecham), 2(3-bromophenyl)histamine dimaleate (Dr. W. Schunack, Berlin), D- and L-chlorpheniramine (maleate salts) (Dr. A. Beld, Nijmegen) and mianserin (Organon) are greatly acknowledged.

Site-directed mutagenesis: Guinea-pig H_1-receptor mutants were constructed by a double Polymerase Chain Reaction (PCR) (14). The oligonucleotides S1 (5'-GGGAAGCTTGATCAGG TATGTCTGACCTCT), corresponding to nucleotide -33 to -12 (6) and a *Hind*III linker site (underlined), AS1 (5'-TGATGATGGCAGCCATGACCTTGAA) and AS2 (5'-GGCAGGTAG AAGGCGATGATGGCAGT), corresponding to nucleotides 594-618 and 607-632 of the complementary strand with one or two nucleotide change(s) respectively, and AS3 (5'-CGGAGATCT AGGTACCTGTGAGACAAGGCT), corresponding to nucleotide 1533-1553 of the complementary strand (6) and a *Bgl*II linker site (underlined) were synthesized on an Applied Biosystems DNA synthesizer (model 381A). Using 100 ng $pSVgpH_1$ (6) as a template, 0.4 µM S1, 0.4 µM AS1 or AS2, 200 µM dNTP's and 2.5 U *Pfu* DNA polymerase (Stratagene) a 651 or 665 bases DNA fragment of respectively the Thr^{203}Ala- or the Asn^{207}Ala-H_1-receptor mutant 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 obtained PCR products were gel-purified, and used in a second PCR reaction with 0.4 µM AS3 and 1 µg of $pSVgpH_1$ as template to amplify the complete coding region of the two mutant H_1-receptor genes. 25 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 1.6 kb fragments. After gel-purification the fragments were restricted with *Hind*III/*Bgl*II (Boehringer) and cloned into the plasmid pSP73 (Promega) in order to verify the complete nucleotide sequence using the dideoxy-chain termination method with the Sequenase kit (USB). Subsequently the fragments were used to replace the gene, encoding the wild type H_1-receptor, in $pSVgpH_1$ (6). Chinese Hamster Ovary cells (CHO cells) were grown as described previously (15) and stably transfected with 15 µg of the eukaryotic expression vectors $pSVgpH_1$ (6), $pSVgpH_1Thr^{203}$Ala and $pSVgpH_1Asn^{207}$Ala using Transfectam (Promega).

H_1-receptor binding: [^{3}H]mepyramine binding was performed as described previously (6). 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 competing ligands.

[^{3}H]Inositol phosphate production: CHO cells were seeded in 24-well plates and cultured overnight in culture medium. Thereafter, cells were labelled 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 pre-incubated for 10 min. at 37°C with 500 μl DMEM/HEPES/LiCl. Incubations were started by the addition of 50 μl histamine or H_1 agonist in DMEM/HEPES/LiCl. After 10 min. incubation at 37°C, the medium was removed and the reaction was stopped by the addition of 500 μl of cold $CHCl_3$/methanol (1:2 vol/vol). After extraction with water, the [^{3}H]inositol phosphates were isolated by anion exchange chromatography (16).

Results

Transfection of CHO cells deficient in dihydrofolate reductase with the plasmids pSVgpH_1, pSVgpH_1Thr^{203}Ala and pSVgpH_1Asn^{207}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 each receptor mutant one clone was selected, which expressed 650 fmol/mg protein [^{3}H]mepyramine binding sites approximately (Table 1). Saturation experiments with membranes of these three different cell lines showed in all cases saturable [^{3}H]mepyramine binding. Whereas for the wild type (WT) H_1-receptor a K_D-value of 0.58 ± 0.02 nM (mean ± SEM, n = 3) for [^{3}H]mepyramine was found, slightly different values were obtained for the two mutant receptors (Table 1). The Thr^{203}Ala receptor mutant showed a slightly higher affinity for the radiolabelled H_1-receptor antagonist, whereas the K_D-value of the Asn^{207}Ala mutant was slightly increased (Table 1). These small changes in the affinity for [^{3}H]mepyramine were reflected by the effects of the mutations on the affinity for the stereoisomers of the H_1-receptor antagonist chlorpheniramine. Yet, both mutants still showed the typical H_1-receptor stereospecificity for these stereoisomers (Table 1).

As can be seen in Fig. 2A, important changes were observed for the displacement of [^{3}H]mepyramine by histamine. The Asn^{207}Ala receptor mutant showed a dramatic loss of affinity for histamine. Only at millimolar concentrations of histamine, displacement of [^{3}H]mepyramine was observed (Fig. 2A). In contrast, the Thr^{203}Ala receptor mutant showed only a minor change in the affinity for histamine (Fig. 2A, Table 2). Similar data were obtained for the relatively selective H_1-receptor agonist 2-methylhistamine. For this agonist too, the affinity was dramatically reduced by the Asn^{207}Ala mutation, whereas the Thr^{203}Ala mutation had no effect (Table 2).

For the structurally related agonist 2-(3-bromophenyl)histamine, the Asn^{207}Ala receptor mutant still showed some loss of agonist affinity (Fig. 2B), but the reduction is considerably lower than

Table 1

Analysis of the binding of [^{3}H]mepyramine and D- and L-chlorpheniramine (ClPhen) to membranes of CHO cells expressing the guinea-pig WT, Thr^{203}Ala- and Asn^{207}Ala-H_1-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}(fmol/mg protein)	K_i (nM)	K_i (nM)
WT	0.58 ± 0.02	623 ± 68	0.79 ± 0.14	69 ± 9
Thr^{203}Ala	0.22 ± 0.01*	785 ± 26	0.56 ± 0.10	36 ± 4*
Asn^{207}Ala	0.79 ± 0.09	639 ± 64	5.5 ± 0.7*	321 ± 96*

* indicates a significant difference ($P<0.05$) compared to WT receptor.

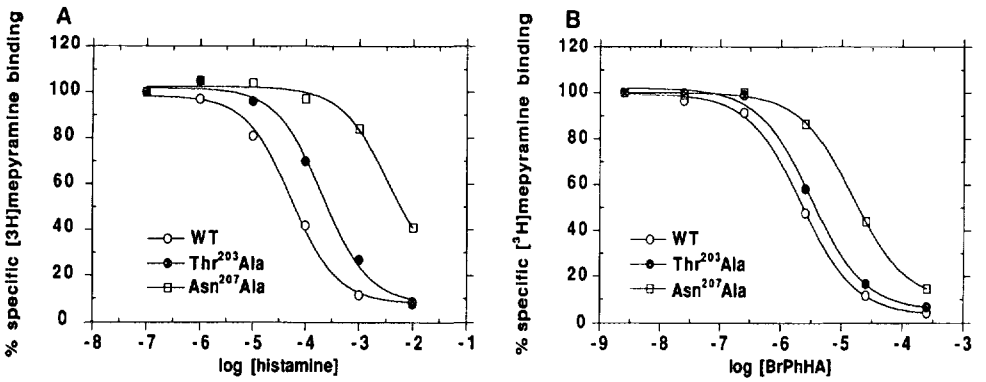
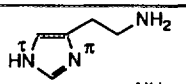
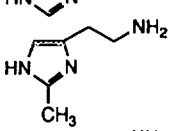
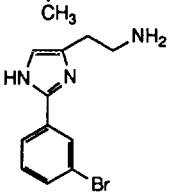
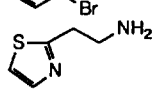
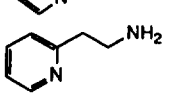


Figure 2.
Displacement of [^{3}H]mepyramine binding to membranes of CHOgpH$_1$WT, CHOgpH$_1$Thr^{203}Ala and CHOgpH$_1$Asn^{207}Ala by histamine (A) and 2-(3-bromophenyl)histamine (BrPhHA) (B). A representative experiment out of 3-4 independent experiments is shown.

for histamine or 2-methylhistamine (Table 2). Also for the selective agonists 2-pyridylethylamine and 2-thiazolylethylamine, only a slight decrease in affinity was found for the Asn^{207}Ala mutation (Table 2). For all agonists no effect of the Thr^{203}Ala mutation was found (Table 2).

The observed effects of the Asn^{207}Ala mutation were reflected in studies with [^{3}H]inositol-prelabelled CHO cells. In CHOH$_1$WT cells, histamine elevated the basal accumulation of

Table 2
K_i-values of various H_1-receptor agonists for the the guinea-pig WT, Thr^{203}Ala- and Asn^{207}Ala-H_1-receptor protein stably expressed in CHO cells. Data shown are mean ± SEM of 3-4 independent experiments.

		K_i-value		
H_1-agonist	structure	wild type	Thr^{203}Ala	Asn^{207}Ala
histamine		12 ± 2 µM (3)	28 ± 4 µM* (3)	> 1000 µM* (4)
2-methylhistamine		42 ± 6 µM (3)	25 ± 5 µM (3)	> 1000 µM* (3)
2-(3-bromophenyl)histamine		0.67 ± 0.15 µM (3)	0.44 ± 0.09 µM (3)	4.3 ± 1.1 µM* (4)
2-thiazolylethylamine		24 ± 3 µM (3)	28 ± 5 µM (3)	147 ± 25 µM* (3)
2-pyridylethylamine		38 ± 3 µM (3)	44 ± 3 µM (3)	129 ± 13 µM* (3)

* indicates a significant difference ($P<0.05$) compared to WT receptor.

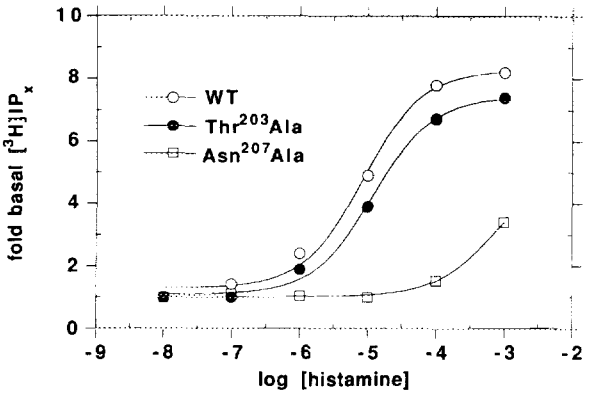


Figure 3.
Histamine-stimulated accumulation of [^{3}H]inositol phosphates in CHOgpH_1WT, CHOgpH_1Thr^{203}Ala and CHOgpH_1Asn^{207}Ala cells. Data shown are a representative example of 5-6 independent experiments.

[^{3}H]inositol phosphates 8.31 ± 0.59 fold (mean ± SEM, n = 5) with an EC_{50}-value of 3.9 ± 2.0 μM (mean ± SEM, n = 5) (Fig. 3). CHO cells expressing the Asn^{207}Ala receptor mutant were clearly refractory to histamine stimulation. Only at very high concentrations of histamine, some stimulation of the [^{3}H]inositol phosphate accumulation could be detected (Fig. 3). For the Thr^{203}Ala receptor mutant no major changes of the EC_{50}-value were noticed (7.5 ± 2.0 μM, mean ± SEM, n = 6), although a slight decrease of the maximal stimulation was observed (5.72 ± 0.50 fold basal accumulation, mean ± SEM, n = 6). The selective H_1-receptor agonist 2-(3-bromophenyl)histamine was found to be a partial agonist for the WT guinea-pig H_1-receptor. Whereas this agonist stimulated the [^{3}H]inositol phosphate accumulation with high potency (EC_{50} = 0.56 ± 0.09 μM, mean ± SEM, n = 3), the intrinsic activity was 0.44 (E_{max} = 3.66 ± 0.36 fold basal values, mean ± SEM, n = 3). As found for histamine, the Thr^{203}Ala mutation did not affect the EC_{50} for this agonist (0.53 ± 0.10 μM, mean ± SEM, n = 4) nor the intrinsic activity (0.36), although the absolute maximal stimulation was somewhat lower (2.08 ± 0.12 fold basal values, mean ± SEM, n = 4). As could be expected on the basis of the radioligand binding studies, the Asn^{207}Ala receptor mutant showed a considerable reduction in potency for this agonist (EC_{50} = 6.6 ± 0.3 μM, mean ± SEM, n = 3). Also in this case the maximal stimulation was reduced (2.06 ± 0.33 fold basal values, mean ± SEM, n = 3). Due to the loss of histamine responsiveness, no intrinsic activity could be determined for the partial agonist.

Discussion

Substitutions of Thr^{203} and Asn^{207} to non-functional Ala residues did not dramatically alter the [^{3}H]mepyramine binding to the mutant H_1-receptors. Only slight changes of antagonist affinity were noticed, whereas both receptor mutants conserved the characteristic stereoselectivity of the histamine H_1-receptor. We assume that these minor differences in antagonist affinity are due to a slight conformational change of the receptor protein and not due to specific interactions of the amino acids with the antagonists.

In contrast, considerable effects were observed on the binding of histamine. The Asn^{207}Ala mutation drastically affected the binding of histamine to the H_1-receptor protein. This effect was also observed in studies, measuring the functionality of the receptor proteins. CHO cells

expressing the Asn^{207}Ala receptor mutant were almost refractory to histamine in producing [^{3}H]inositol phosphates.

Comparable results were obtained for the selective H_1-receptor agonist 2-methylhistamine, but were not evident for the structurally related 2-(3-bromophenyl)histamine and the non-imidazole agonists 2-pyridylethylamine and 2-thiazolylethylamine. As found for histamine, the affinity of these compounds was not markedly reduced by the Thr^{203}Ala replacement. Yet, also the Asn^{207}Ala mutation hardly affected the binding of these selective agonists; only a 3-6 fold reduction in agonist affinity was observed for 2-(3-bromophenyl)histamine, 2-pyridylethylamine and 2-thiazolylethylamine.

For H_1-receptor agonism only the N^{π}-nitrogen atom of the imidazole moiety of histamine is generally accepted to be essential for H_1-receptor activity (1,2). Other heterocyclic ring systems with a nitrogen atom ortho to the ethylamine side chain (e.g. 2-pyridylethylamine and 2-thiazolylethylamine) are known as rather potent and selective H_1-receptor agonists (1,2). None of the amino acid substitutions clearly affected the interaction of the non-imidazole agonists 2-pyridylethylamine and 2-thiazolylethylamine. On the basis of these results we conclude that neither the Thr^{203}, nor the Asn^{207} residue is involved in the interaction with the N^{π}-nitrogen atom of H_1-agonists. Yet, on the basis of our study, we conclude that the Asn^{207} residue is involved in the binding of histamine and 2-methylhistamine. Since no important effects of the Asn^{207}Ala mutation were noticed for agonists lacking N^{τ}-nitrogen atom (2-pyridylethylamine and 2-thiazolylethylamine), Asn^{207} apparently interacts with this nitrogen atom of the imidazole ring of histamine via hydrogen bonding.

Although the N^{τ}-nitrogen atom has bee accepted to be non-essential for H_1-receptor agonism (1,2), we now should refine these ideas. The presence of a N^{τ}-nitrogen atom is essential for a good affinity for histamine, although this structural feature is not essential for receptor activation. These findings are in accordance with the observation that N^{τ}-methylhistamine is almost devoid of H_1-receptor activity (1,2). Compounds that lack the N^{τ}-nitrogen, but are able to bind to the receptor, activate the H_1-receptor if a N^{π}-nitrogen atom is present. For such compounds we should assume a binding mode, different from that of histamine. If we accept that for histamine the interaction of Asn^{207} with the N^{τ}-nitrogen atom is essential for receptor binding of histamine, the amino acid residue(s) that will interact with the N^{π}-nitrogen in the various imidazole and non-imidazole agonist can be postulated to be essential for H_1-receptor activation. Consequently, we propose a discrimination between receptor binding and activation for this receptor type.

The data obtained with 2-(3-bromophenyl)histamine confirm previous ideas about the interaction of the 2-phenylhistamines with the H_1-receptor (1). It is known that 2-methylation results in a subtype-selective H_1-agonist (1,2). Substitution with higher alkyl groups is highly unfavourable for H_1-receptor activity, probably as the result of steric factors (1). It is therefore remarkable that the various 2-phenylhistamine analogues stimulate the H_1-receptor (17,18). In view of their high agonistic activity it was hypothesized that these compounds interact with additional, hydrophobic sites in the receptor protein (1). The results of our site-directed mutagenesis studies indeed reveal a different mode of interaction of histamine and 2-(3-bromophenyl)histamine with the H_1-receptor protein. Whereas for histamine and its 2-methyl-analogue the interaction with the Asn^{207}residue is very important, comparable effects are not seen for its 2-(3-bromophenyl)-analogue. The interaction of this amino acid with the N^{τ}-nitrogen atom is not essential for receptor binding. Aromatic residues, which are probably essential for the high affinity of this agonist, should still be identified.

In conclusion, in the present study we have shown that the Asn^{207} residue of the fifth transmembrane domain of the guinea-pig histamine H_1-receptor, is involved in the binding of the

N^{τ}-nitrogen atom of histamine and its 2-methyl-analogue. For the 2-phenyl-analogues and non-imidazole H_1-receptor agonists this residue does not appear to be essential for binding. On the basis of this study we conclude that different histamine H_1-receptor agonists interact in different ways with the receptor proteins. Moreover, we speculate that the interaction with the N^{π}-nitrogen atom is essential for receptor activation. Our results also suggest that H_1-receptor antagonists do not interact with the two mutated residues in TM5, a finding that corroborates previous reports with other amine receptors (7). Future site-directed mutagenesis studies could resolve these important issues. Amino acid residue(s) involved in the binding of the aromatic rings of 2-phenylhistamines, the non-imidazole agonists and the antagonists should be identified. Moreover, also the identification of the amino acid residue(s) involved in the interaction with the essential N^{π}-nitrogen would be very helpful for future rational drug design.

Acknowledgment

The research of Dr. R. Leurs had been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

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