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Mutational analysis of the histamine H_1-receptor binding pocket of histaprodifens

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Abstract

Histaprodifens constitute a new class of histamine H_1-receptor agonists. These ligands can be regarded as hybrid molecules, consisting of a histamine moiety linked at the two-position of the imidazole ring by a propyl chain to two phenyl rings, one of the characteristic features of several H_1-receptor antagonists. To delineate the binding site of various histaprodifen-like ligands, we generated mutant histamine H_1 receptors, in which various amino acids, involved in the binding of either histamine or H_1-receptor antagonists, were replaced by alanine. Wild-type and mutant H_1 receptors were transiently expressed in African green monkey kidney cells (COS-7) and evaluated for their interaction with histamine and various histaprodifens by [^{3}H]mepyramine radioligand-binding studies and by nuclear factor κB (NF-κB) reporter-gene assays. Our data show that, within the histamine H_1-receptor binding pocket, histaprodifens interact with both agonist and antagonist binding sites, resulting in high affinity histamine H_1-receptor agonists.

Keywords: Histaprodifen; Mutational analysis; Histamine H_1 receptor; Agonist binding site; Antagonist binding site

1. Introduction

The histamine H_1 receptor plays an important role in allergic conditions like rhinitis, asthma, anaphylaxis and uriticaria (Hill et al., 1997). Consequently, histamine H_1-receptor antagonists constitute the medication of choice to alleviate symptoms of allergies. The rationalization of the molecular mechanism of action of these successful therapeutics have for many years been hampered by the lack of detailed knowledge of the histamine H_1 receptor. Using an expression cloning strategy, Yamashita et al. (1991) revealed that the bovine histamine H_1 receptor belongs to the large multigene family of G protein-coupled receptors. Using the bovine histamine H_1-receptor cDNA, the H_1-receptor genes from a variety of species, including man, have been cloned (for review, see Hill et al., 1997). The use of transfected cell lines recently showed that all therapeutically used histamine H_1-receptor antagonists are in fact inverse agonists, i.e. they stabilize the receptor in an inactive conformation (Bakker et al., 2000, 2001).

Moreover, following the cloning of the H_1 receptor, several site-directed mutagenesis studies have been conducted in order to identify the binding pocket of histamine and H_1-receptor antagonists. Asp^{107} in transmembrane domain 3 is a conserved residue among all aminergic receptors and is thought to be responsible for an ionic interaction with the protonated nitrogen of biogenic amines (Shi and Javitch, 2002). In the human histamine H_1 receptor, Asp^{107} is reported to be crucial for the binding of both histamine and histamine H_1-receptor antagonists (Moguilevsky et al., 1998; Nonaka et al., 1998; Ohta et al., 1994). Mutation of a lysine in transmembrane domain 5 in the human (Lys^{191}) and guinea pig (Lys^{200}) histamine H_1 receptor to alanine leads to a decreased affinity for histamine (Gillard et al., 2002; Leurs et al., 1995; Mogui-

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levsky et al., 1998; Wieland et al., 1999). This lysine is suggested to interact with the proximal nitrogen (N^{π}) of the imidazole ring of histamine (Leurs et al., 1995). Asn^{198} is proposed to interact with either the proximal (Moguilevsky et al., 1995, 1998) or the distal (N^{τ}) (Leurs et al., 1994; Ohta et al., 1994) nitrogen of the imidazole ring of histamine, whereas this residue is not involved in the interaction with antagonists. Involvement of the aromatic amino acids Phe^{433} and Phe^{436} in the guinea-pig histamine H_1 receptor (human: Phe^{432} and Phe^{435}, respectively) (transmembrane domain 6) in the binding of histamine and mepyramine has previously also been reported (Wieland et al., 1999). Both phenylalanines were identified as probable interaction points with the trans-aromatic ring of histamine H_1-receptor antagonists. Based on these observations, one can conclude that histamine and the H_1-receptor antagonists have distinct, but partially overlapping binding sites.

Recently, a new class of selective histamine H_1-receptor agonists, the histaprodifens, has been identified and pharmacologically characterized using guinea-pig ileum and rat aortic rings. Interestingly, histaprodifen (2-[2-diphenylpropyl)-1*H*-imidazol-4-yl]ethanamine), the prototype agonist of this family consists of a histamine moiety, representing the endogenous agonist ligand, linked by a propyl chain to two phenyl rings, that are a characteristic feature of the histamine H_1-receptor antagonist pharmacophore (Ter Laak et al., 1995; Zhang et al., 1997). Fig. 1 depicts the structures of histamine, the H_1-receptor antagonist cetirizine and the 'hybrid' agonist histaprodifen. The aromatic rings confer high receptor affinity to these antagonists/inverse agonists. The combination of high affinity, via the diphenyl moiety, with the agonistic properties of histamine is hypothesized to be the rationale behind the reported potent agonism of the 'hybrid' histaprodifens (Elz et al., 2000b).

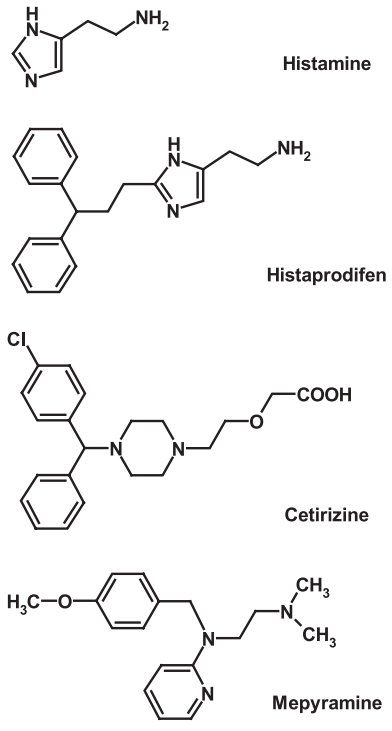


Fig. 1. Structures of histamine, mepyramine, the therapeutically used H_1-receptor antagonist cetirizine, and histaprodifen.

In this study, we have tested this last hypothesis and we have therefore characterized the interaction of histaprodifen and several of its analogues with the human H_1 receptor using [^{3}H]mepyramine binding studies and a H_1-receptor-driven nuclear factor κB (NF-κB) reporter-gene assay. To delineate the histamine H_1-receptor binding sites of these 'hybrid' ligands we studied the interaction with Asp^{107} (transmembrane domain 3), Lys^{191}, Asn^{198} (transmembrane domain 5), Phe^{435} (transmembrane domain 6) (agonist binding site) and Phe^{432} (transmembrane domain 6) (antagonist binding site). These amino acids are conserved among all species.

2. Materials and methods

2.1. Materials

pNF-κB-Luc was obtained from Stratagene (La Jolla, USA). ATP disodium salt, bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine dihydrochloride, mepyramine (pyrilamine maleate) and polyethyl-enimine were purchased from Sigma (USA). D-Luciferin was obtained from Duchefa Biochemie (Haarlem, The Netherlands), glycerol from Riedel-de-Haën (Germany) and Triton X-100 from Fluka (Switzerland). Cell culture media, penicillin and streptomycin were obtained from Invitrogen (Paisley, UK). Fetal bovine serum was obtained from Integro (Dieren, The Netherlands). Cell culture plastics were obtained from Corning Costar (NY, USA). [^{3}H]Mepyramine (20 Ci/mmol) was purchased from ICN Biomedicals (Zoetermeer, The Netherlands).

Gifts of mianserin hydrochloride (Organon, The Netherlands), pcDEF3 (Dr. J. Langer, Goldman et al., 1996) and of the cDNA encoding the human H_1 receptor (Dr. H. Fukui, Fukui et al., 1994) are greatly acknowledged. Histaprodifen dihydrogenmaleate, methylhistaprodifen dihydrogenoxalate, histaprodifen-histaprodifen dimer trihydrogenoxalate and histaprodifen-histamine dimer (suprahistaprodifen) trihydrogenoxalate were prepared at the Institute of Pharmacy Berlin, Germany.

2.2. Cell culture and transfection

COS-7 African green monkey kidney cells were maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) containing 50 IU/ml penicillin, 50 μg/ml streptomycin and 5% (v/v)

fetal bovine serum. COS-7 cells were transiently transfected using the DEAE-dextran method as previously described (Bakker et al., 2001).

2.3. Site-directed mutagenesis

Mutant human histamine H_1 receptors $Asp^{107}Ala$, $Lys^{191}Ala$ and $Asn^{198}Ala$ were previously described by Moguilevsky et al. (1998) and kindly provided by UCB Pharma (Belgium). Mutant receptors $Phe^{432}Ala$ and $Phe^{435}Ala$ were created by Altered Sites® II (Promega) according to manufacturers protocol. All mutant receptors were subcloned into the expression vector pcDEF3.

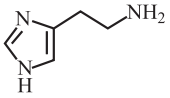
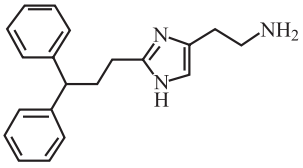
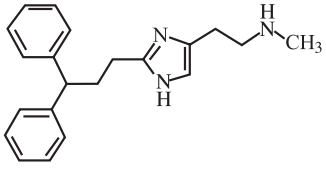
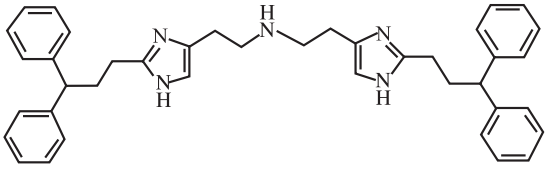
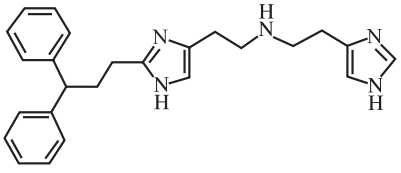
2.4. Reporter-gene assay

Cells transiently co-transfected with pNF-κB-Luc (125 μg/1·10^7 cells) and pcDEF3 containing mutant or wild-type human histamine H_1-receptor cDNA (25 μg/1·10^7 cells) were seeded in 96-well white plates (Costar) in serum free culture medium and incubated with drugs. After 48 h, cells were assayed for luminescence by aspiration of the medium and the addition of 25 μl/well luciferase assay reagent (0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM $MgCl_2$, 0.78 μM $Na_2H_2P_2O_7$, 38.9 mM Tris (pH 7.8), 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100 and 2.6 μM dithiothreitol). After 30 min, luminescence was measured for 3 s/well in a Victor^2 (Wallac).

2.5. Histamine H_1-receptor binding studies

The transfected COS-7 cells used for radioligand binding studies were harvested after 48 h and homogenized in ice-cold 50 mM Na_2/K-phosphate buffer (pH = 7.4) (H_1-binding buffer). The COS-7 cell homogenates were incubated for 30 min at 30 °C in H_1-binding buffer in 200 μl with 3 nM [^{3}H]mepyramine. The non-specific binding was determined in the presence of 1 μM mianserin. The incubations were stopped by rapid dilution with 3-ml ice-cold H_1-binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filters that had been treated with 0.3% polyethyl-enimine. Filters were washed twice with 3-ml H_1-binding buffer and radioactivity retained on the filters was measured by liquid scintillation counting.

Table 1
Affinities and efficacies of H_1-receptor agonists at the WT H_1 receptor

Structure	Name	pK_i	pEC_{50}	α
	histamine (HA)	4.3 ± 0.1	6.7 ± 0.1	1.0
	histaprodifen (HP)	5.6 ± 0.1[c]	5.9 ± 0.1[b]	0.8 ± 0.2
	methylhistaprodifen (MeHP)	6.0 ± 0.1[c]	6.3 ± 0.1	0.9 ± 0.1
	histaprodifen-histaprodifen dimer (HP-HP)	6.2 ± 0.1[c]	6.2 ± 0.1[a]	1.1 ± 0.2
	histaprodifen-histamine dimer (HP-HA)	5.8 ± 0.1[c]	6.4 ± 0.2	0.9 ± 0.2

The binding affinities (pK_i) for the wild-type human histamine H_1 receptor were determined by [^{3}H]mepyramine displacement. Potencies (pEC_{50}) and intrinsic activities (α) of the compounds are determined by an NF-κB-driven reporter-gene assay. All values are calculated as mean ± S.E.M. of at least three experiments, each performed in triplicate.
[a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ vs. histamine.

Table 2
Affinities of [^{3}H]mepyramine and expression levels of mutant human H_1 receptors

	K_d [^{3}H]mepyramine (nM)	H_1 receptor density (pmol/mg protein)
WT	1.3 ± 0.1 (4)	10.1 ± 0.2 (7)
Asp^{107}Ala	>30 (4)^c	ND
Lys^{191}Ala	0.6 ± 0.1 (4)^c	7.1 ± 0.3 (7)^c
Asn^{198}Ala	2.0 ± 0.3 (4)	7.9 ± 0.4 (6)^c
Phe^{432}Ala	>30 (4)^c	ND
Phe^{435}Ala	5.7 ± 0.5 (4)^c	8.6 ± 0.8 (6)^a

The values are determined by saturation radioligand binding assays. Data were calculated as the mean ± S.E.M. In brackets, the number of experiments is shown each performed in triplicate. ND indicates that the value could not be determined.
$^a P<0.05$, $^b P<0.01$, $^c P<0.001$ vs. WT receptor.

2.6. Analytical methods

Protein concentrations were determined according to Bradford (Bradford, 1976), using bovine serum albumine as a standard. Binding and reporter-gene data were evaluated by a non-linear, least squares curve-fitting procedure using GraphPad Prism® (GraphPad Software, San Diego, CA). Herewith obtained pK_i, pEC_{50} and pIC_{50} values are expressed as mean ± S.E.M. Statistical analysis of mean and S.E.M. were carried out by non-paired Student's *t*-test. *P*-values <0.05 were considered to indicate a significant difference ($^a P<0.05$, $^b P<0.01$, $^c P<0.001$).

3. Results

3.1. Characterisation of histaprodifen analogues

Several histaprodifen analogues have been shown to be potent agonists on guinea-pig (ileum, aortic rings, tracheal rings and in vitro), rat (aortic rings, in vivo), bovine (aortic membranes) and human (in vitro) H_1 receptors (Čarman-Kržan et al., 2003; Christophe et al., 2003; Elz et al., 2000a,b; Malinowska et al., 1999; Schlicker et al., 2001; Seifert et al., 2003). In this study, we determined the human histamine H_1-receptor affinity and potency of histaprodifen, methylhistaprodifen, the histaprodifen-histaprodifen dimer and the histaprodifen-histamine dimer. Table 1 shows that the affinities of the histaprodifen analogues for the H_1 receptor are more than ten-fold higher compared to histamine, with the histaprodifen-histaprodifen dimer as most active ligand (pK_i = 6.2). Under our assay conditions, the histaprodifen analogues tested in this study are all full agonists at the human H_1 receptor. However, in contrast to published observations using guinea-pig ileum or rat aortic rings (Elz et al., 2000b), their potencies at the human H_1 receptors are sometimes lower (max. six-fold) then the potency of the endogenous ligand histamine.

3.2. Binding analysis of mutant human H_1 receptors

Mutational analysis of the guinea-pig H_1 receptor has resulted in identification of several amino acids that are involved in the binding of agonists (Leurs et al., 1994, 1995) and antagonists (Wieland et al., 1999). To verify the involvement of corresponding amino acids (Asp^{107}, Lys^{191}, Asn^{198}, Phe^{432} and Phe^{435}) in the human H_1 receptor, these amino acids were individually mutated to alanine. Mutant receptors were initially characterized by [^{3}H]mepyramine saturation binding analysis (Table 2). Mutation of Lys^{191}, Asn^{198} or Phe^{435} into alanine resulted in only slight change in the binding affinity for the inverse agonist radioligand [^{3}H]mepyramine. The expression levels of these mutant receptors did not differ dramatically from the wild-type (WT) H_1 receptor. As expected (Nonaka et al., 1998; Wieland et al., 1999), mutation of Asp^{107} or Phe^{432} to alanine resulted in a loss of [^{3}H]mepyramine binding.

For receptors showing saturable [^{3}H]mepyramine binding (WT, Lys^{191}Ala, Asn^{198}Ala and Phe^{435}Ala), affinities of histamine and histaprodifen-analogues were determined by radioligand displacement studies (Table 3). Fig. 2A shows that mutation of Asn^{198} and Phe^{435} into alanine decreased the affinity of histamine more than 40-fold ($P<0.001$). Furthermore, Table 3 shows that mutation of Lys^{191} into alanine resulted in a 2.5-fold loss in affinity, corresponding to the loss of a hydrogen bond. The data obtained for the binding of histamine to the human H_1 receptor correspond

Table 3
Binding affinities of H_1-receptor agonists for human H_1-receptor mutants

	Affinity agonist (pK_i)				
	Histamine	HP	MeHP	HP-HP	HP-HA
WT	4.3 ± 0.1 (5)	5.6 ± 0.1 (3)	6.0 ± 0.1 (3)	6.2 ± 0.1 (3)	5.8 ± 0.1 (3)
Asp^{107}Ala	ND	ND	ND	ND	ND
Lys^{191}Ala	3.9 ± 0.1 (5)^b	5.9 ± 0.1 (3)^b	6.1 ± 0.1 (3)	6.6 ± 0.1 (3)^b	6.4 ± 0.1 (3)^c
Asn^{198}Ala	2.6 ± 0.1 (5)^c	5.3 ± 0.1 (3)^b	5.4 ± 0.1 (3)^c	6.1 ± 0.1 (3)	5.4 ± 0.1 (3)^b
Phe^{432}Ala	ND	ND	ND	ND	ND
Phe^{435}Ala	2.7 ± 0.1 (4)^c	5.3 ± 0.1 (3)^b	5.5 ± 0.1 (3)^c	5.8 ± 0.1 (3)^b	5.5 ± 0.1 (3)^b

Values are determined by [^{3}H]mepyramine displacement. Data were calculated as the mean ± S.E.M. In brackets, the number of experiments is shown, each performed in triplicate. ND indicates that the value could not be determined.
$^a P<0.05$, $^b P<0.01$, $^c P<0.001$ vs. WT receptor.

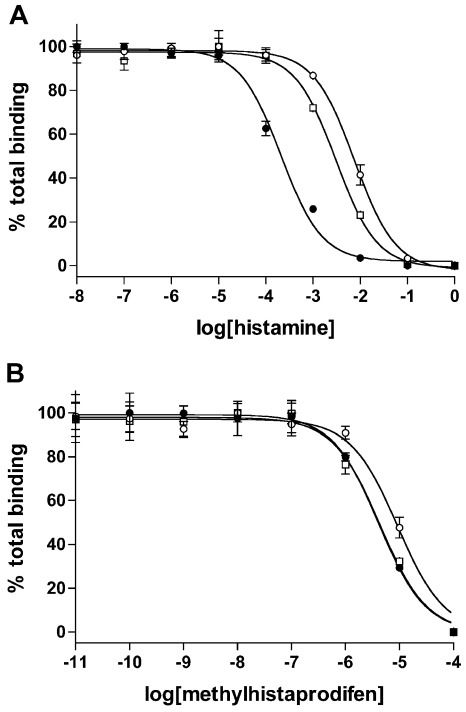


Fig. 2. Displacement of [^{3}H]mepyramine binding to wild-type (●), Asn^{198}Ala (○) or Phe^{435}Ala (□) mutant H_1 receptors by histamine (A) or methylhistaprodifen (B). A representative experiment is shown.

closely to those obtained with the guinea-pig H_1 receptor (Leurs et al., 1994; Wieland et al., 1999). Fig. 2B shows that mutation of Asn^{198} and Phe^{435} to alanine resulted in only minor changes in the affinity for methylhistaprodifen. Table 3 shows that this small decrease is observed for all histaprodifen analogues (one- to four-fold). Mutation of Lys^{191} into alanine resulted in an increase of affinity for most of the histaprodifen analogues. This increase was however absent for methylhistaprodifen but two- to four-fold for histaprodifen, histaprodifen-histaprodifen dimer and the histaprodifen-histamine dimer. Because of the lack of saturable [^{3}H]mepyramine binding of two mutant receptors (Asp^{107}Ala and Phe^{432}Ala), agonist binding could not be studied in these receptors.

3.3. Constitutive activity of WT and mutant H_1 receptors

Recently, constitutive activity of the human H_1 receptor was shown using a NF-κB reporter-gene assay (Bakker et al., 2001). Table 4 shows that all tested mutant H_1 receptors show some degree of constitutive activity. Lys^{191}Ala and Phe^{432}Ala mutant receptors even have a strongly increased basal activity. For Phe^{432}Ala receptor, the basal activity was elevated to such an extent that no (further) agonist-induced NF-κB activation could be detected (data not shown). A 10-fold decrease in the amount of receptor DNA transfected into these cells resulted in a lower basal activity (which is still twice as high as the wild-type level) after which agonist-induced receptor activation could be studied. These

Table 4
Constitutive H_1-receptor activity and inverse agonism by mepyramine

	Basal activity % WT	Potency mepyramine pIC_{50}	Fold histamine stimulation over basal
WT	100 (10)	7.5 ± 0.2 (4)	7.0 ± 1.3 (6)
Asp^{107}Ala	115 ± 13 (8)	5.7 ± 0.1 (4)^c	ND
Lys^{191}Ala	230 ± 36 (10)	7.6 ± 0.2 (3)	3.2 ± 0.3 (6)^a
Asn^{198}Ala	18 ± 2 (10)^c	7.7 ± 0.1 (2)	ND
Phe^{432}Ala	212 ± 111 (4)	5.2 ± 0.2 (4)^c	2.3 ± 0.4 (4)^a
Phe^{435}Ala	39 ± 10 (9)^a	7.1 ± 0.3 (3)	22.1 ± 8.3 (6)

Basal activity of WT histamine H_1 receptors is put at 100%. Values are determined by NF-κB-driven reporter-gene assays. Fold histamine stimulation indicates the window of this reporter-gene assay. Data were calculated as the mean ± S.E.M. In brackets, the number of experiments is shown, each performed in triplicate. ND indicates that the value could not be determined.
$^aP<0.05$, $^bP<0.01$, $^cP<0.001$ vs. WT receptor.

conditions have been used to obtain functional data with this mutant receptor. For the mutant H_1-receptor Asp^{107}Ala, constitutive activity is identical to wild-type human histamine H_1 receptors. For Asn^{198}Ala and Phe^{435}Ala H_1 receptors, the activity is decreased compared to wild-type human histamine H_1 receptors. Table 4 shows that the basal activity of all mutant receptors is still reduced by the H_1-receptor inverse agonist mepyramine. The potency of mepyramine for the Asp^{107}Ala and Phe^{432}Ala mutant receptors however was, respectively, 60- and 200-fold lower compared to the wild-type receptor as is clearly illustrated by Fig. 3. Assuming a linear correlation between the potency and affinity of inverse agonists for the human histamine H_1 receptor (Bakker et al., 2001), this would result in an affinity of mepyramine in the range of, respectively, 70 and 250 nM explaining the loss of [^{3}H]mepyramine binding, observed in our radioligand binding studies. As shown in Fig. 3, mepyramine does not decrease the constitutive activity of the mutant receptors to the same level as the wild-type receptor, indicating that the mutations have most

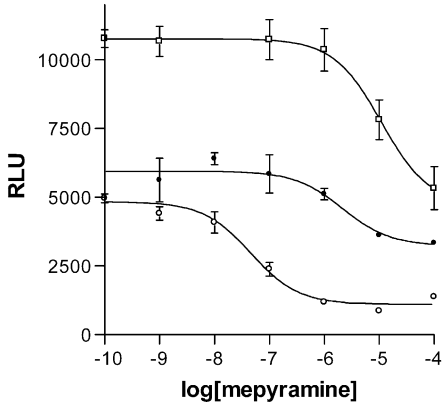


Fig. 3. Mepyramine-mediated inverse agonism on WT (○), Asp^{107}Ala (●) and Phe^{432}Ala (□) mutant H_1 receptors as measured by the inhibition of basal NF-κB activation. A representative experiment is shown.

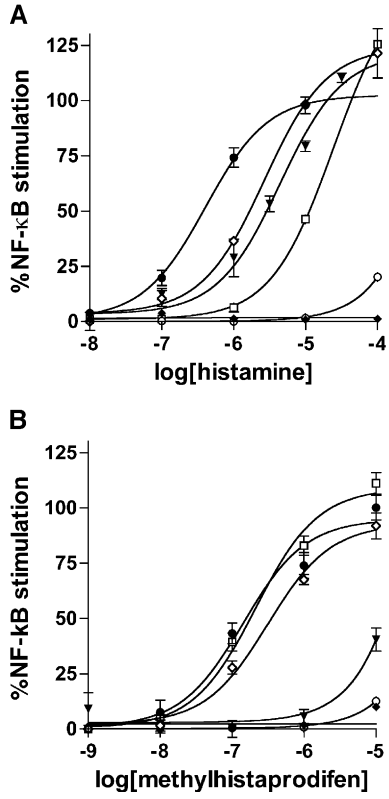


Fig. 4. Representative dose response curves for WT (●), Asp[107]Ala (♦), Lys[191]Ala (◇), Asn[198]Ala (○), Phe[432]Ala (▼) and Phe[435]Ala (□) mutant human histamine H_1 receptors mediated NF-κB activation, induced by histamine (A) or methylhistaprodifen (B). Data are normalized for basal activity of mutant receptors (0%) and maximum histamine stimulation of wild-type receptor (100%).

likely also resulted in a reduction of the negative intrinsic activity of mepyramine.

3.4. Agonist stimulation of mutant human histamine H_1 receptors

Using a NF-κB-driven reporter-gene assay we evaluated the effects of various mutations on the agonist efficacy of the histaprodifens. Fig. 4 clearly shows that potencies of histamine and methylhistaprodifen are affected differently by the various mutations: whereas Lys[191]Ala and Phe[435]Ala mutations greatly affect histamine potency, they hardly affect the potency of methylhistaprodifen. On the other hand, the effect of mutation Phe[432]Ala is substantially larger for methylhistaprodifen then for histamine (≥ 200- and 16-fold, respectively). Mutant receptors Asp[107]Ala and Asn[198]Ala show no agonist-induced NF-κB activation. Remarkably, mutation of Asn[198] to alanine hardly affected methylhistaprodifen binding, but signaling is greatly impaired after stimulation with either histamine or methylhistaprodifen. Similar observations were made for the other histaprodifen analogues; compared to histamine, the potency of these ligands is less sensitive to mutation of either Lys[191] or Phe[435] into alanine and more sensitive to mutation of Phe[432] into alanine (Table 5). One exception is the histaprodifen-histamine dimer. Whereas histamine loses 16-fold in potency and most histaprodifen analogues lose their ability to activate the Phe[432]Ala H_1 receptor, the histaprodifen-histamine dimer shows no significant drop in potency.

4. Discussion

Histaprodifen and its analogues have been reported to be highly potent H_1-receptor agonists with a potency on guinea-pig ileum and rat aortic rings exceeding that of histamine up to 100-fold (Christophe et al., 2003; Elz et al., 2000b). In contrast to these findings using guinea pig and rat models, we find that on the human H_1 receptor, none of the compounds is more potent than histamine. This finding corresponds to a recent report of Seifert and coworkers in which they report that in GTPase assays histaprodifens are generally more potent in membranes expressing guinea pig H_1 receptors than in membranes expressing human H_1 receptors (Seifert et al., 2003). The binding affinities of these compounds, however, exceed that of histamine for the human H_1 receptor 20–80-fold. We note a difference between the relative potencies of histaprodifens and histamine in the binding and functional response. Histamine appears to be a high efficacious agonist whereas histaprodifens are low efficacious agonists, correlating to findings in other experimental systems such as guinea pig ileum, in which most

Table 5
Functional characterization of H_1-receptor agonists on the wild-type (WT) and mutant H_1 receptors

	Agonist potency (pEC_{50})				
	Histamine	HP	MeHP	HP-HP	HP-HA
WT	6.7 ± 0.1 (6)	5.9 ± 0.1 (4)	6.3 ± 0.1 (6)	6.2 ± 0.1 (4)	6.4 ± 0.2 (4)
Asp[107]Ala	< 4[c]	< 4[c]	< 4[c]	< 4[c]	< 4[c]
Lys[191]Ala	5.5 ± 0.1 (6)[c]	5.3 ± 0.2 (4)[a]	6.1 ± 0.2 (6)	5.7 ± 0.3 (4)	6.9 ± 0.1 (4)[a]
Asn[198]Ala	< 4[c]	< 4[c]	< 4[c]	< 4[c]	< 4[c]
Phe[432]Ala	5.5 ± 0.2 (4)[a]	< 4[c]	< 4[c]	< 4[c]	6.1 ± 0.1 (3)
Phe[435]Ala	4.1 ± 0.1 (3)[c]	6.3 ± 0.1 (4)[a]	6.8 ± 0.1 (5)[a]	6.1 ± 0.1 (4)	6.6 ± 0.1 (4)

Potencies (pEC_{50}) of the compounds are determined by an NF-κB-driven reporter-gene assay. Data were calculated as the mean ± S.E.M. In brackets, the number of experiments is shown.
[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ vs. WT receptor.

histaprodifens are partial agonists (Christophe et al., 2003; Elz et al., 2000b). The increased affinity of the histaprodifens combined with their agonistic properties make these compounds interesting for the study of the human H_1-receptor binding pocket. Since the histaprodifens share structural characteristics of both agonists (histamine moiety) and antagonists (diphenyl substituent), we determined the effects of various mutations, that previously have been shown to interact with either H_1-receptor agonists and/or H_1-receptor antagonists (inverse agonists).

Asp^{107} (transmembrane domain 3) is a conserved feature among all aminergic G protein-coupled receptors (Horn et al., 2001), and thought to be the main interaction point of the protonated amine function of aminergic agonists and antagonists with their receptors (for review, see Shi and Javitch, 2002). In line with previous findings, mutation of this residue to alanine leads to a total loss of specific [^{3}H]mepyramine binding.

Some years ago, Nonaka et al. (1998) determined the affinity of mepyramine for the same $Asp^{107}Ala$ receptor mutant using a custom synthesized radioligand [^{3}H]KW-4679. Based on [^{3}H]KW-4679 displacement studies, the authors found that the affinity of mepyramine for this receptor mutant is decreased 280-fold to a K_i value of 1.0 μM. These data clearly explain why [^{3}H]mepyramine is unsuitable as a radioligand for this mutant receptor. Since detection of mutant $Asp^{107}Ala$ receptor expression by [^{3}H]mepyramine binding is not feasible, the mutant receptor was tested for functionality in a NF-κB-driven reporter-gene assay. Previously, we showed that the human H_1 receptor activates NF-κB in both a constitutive and agonist-dependent manner (Bakker et al., 2001). In this study, we show that the constitutive activity of the $Asp^{107}Ala$ mutant H_1 receptor is comparable to that of the wild-type H_1 receptor. Inverse agonist potency of mepyramine was decreased approximately 60-fold upon mutation of Asp^{107} to alanine. This decrease in inverse agonist potencies again demonstrates the involvement of Asp^{107} in inverse agonist binding. Furthermore, constitutive activity and inverse agonism provide indirect evidence for both the presence of the $Asp^{107}Ala$ mutant receptor on the plasma membrane and its 'intrinsic' capability to signal. Despite the presence of constitutive activity of the $Asp^{107}Ala$ mutant receptor, histamine-induced signaling could not be detected. Also none of the histaprodifens was capable of stimulating NF-κB activation. Apparently, also for these compounds the interaction with Asp^{107} is required for high-affinity binding and/or effective activation of the human histamine H_1 receptor.

Phe^{432} (transmembrane domain 6) was previously predicted to accommodate the trans-aromatic ring of classical H_1-receptor antagonists like mepyramine (Wieland et al., 1999). Indeed, mutation of Phe^{432} to alanine leads to a loss of [^{3}H]mepyramine binding, in correspondence with previous work of our group with the guinea-pig H_1 receptor (Wieland et al., 1999). Similar to the $Asp^{107}Ala$ mutation, the $Phe^{432}Ala$ mutation did not abolish receptor expression, as constitutive signaling was easily observed. The level of constitutive signaling of the $Phe^{432}Ala$ mutant receptor is so high that, under standard experimental conditions, no agonist stimulation could be detected. The $Phe^{432}Ala$ H_1 receptor can therefore be considered a constitutively active mutant (CAM) receptor.

Again inverse agonism could be shown for mepyramine, however, with a 200-fold reduced potency. In order to perform agonist studies, the $Phe^{432}Ala$ receptor was expressed at lower levels. Under these conditions, this $Phe^{432}Ala$ receptor can be activated by histamine. Interestingly, agonism could not be shown for most histaprodifen analogues (HP, MeHP and HP-HP). These data implicate that the Phe^{432} is crucial for the binding of these histaprodifen analogues to the H_1 receptor. Consequently, the binding pocket of most histaprodifen analogues appears to overlap more with that of inverse agonists then with that of histamine.

The histaprodifen-histamine dimer is an interesting exception. On the wild-type human histamine H_1 receptor, this ligand acts as a full agonist with a pEC_{50} of 6.4 and a pK_i of 5.8. Like methylhistaprodifen ($pEC_{50}=6.3$, $pK_i=6.0$) the H_1-receptor affinity is higher compared to histamine ($pK_i=4.3$), whereas the potency is lower than histamine ($pEC_{50}=6.7$). Whereas all other histaprodifen analogues lose more then 200-fold in potency upon mutation of Phe^{432}, the histaprodifen-histamine is totally unaffected by the $Phe^{432}Ala$ mutation. This is quite remarkable since the potencies of both smaller (HP, MeHP) and larger compounds (HP-HP dimer) are impaired severely by mutation $Phe^{432}Ala$. These data suggest that the histaprodifen-histamine dimer has an orientation in the H_1-receptor binding pocket that is different from that of the other histaprodifen analogues.

Amino acids Lys^{191}, Asn^{198} and Phe^{435} have been reported to be interaction points for histamine. $Lysine^{191}$ in transmembrane domain 5 has been suggested to uphold a hydrogen bond with the proximal hydrogen of the imidazole ring of histamine (Leurs et al., 1995). For histamine, $Lys^{191}Ala$ mutation results in a decrease in affinity, corresponding to the predicted loss of one hydrogen bond. In contrast, for all histaprodifen analogues, which also contain an imidazole ring, a small increase in affinity was found. This observation suggests that either no hydrogen bond is present, or that the loss of a hydrogen bond caused by the removal of Lys^{191} is compensated for by an increase in space in the binding pocket required for the more bulky and rigid histaprodifen analogues. It is interesting to note that the $Lys^{191}Ala$ mutation also slightly increases the affinity of the classical H_1-receptor antagonist [^{3}H]mepyramine. Comparable findings were previously also reported by Gillard et al. (2002) and Moguilevsky et al. (1998). These data suggest that the binding site of the histaprodifen analogues overlaps in part with the binding site of H_1-receptor antagonists/inverse agonists.

Phe^{435} in transmembrane domain 6 is situated one helical turn above the earlier mentioned Phe^{432}. Whereas the latter is involved in the binding of antagonists, Phe^{435} appears to be involved in histamine binding, probably by stabilizing its imidazole ring (Wieland et al., 1999). The K_d of [^{3}H]mepyramine for the Phe^{435}Ala mutant receptor is only marginally (five-fold) decreased, demonstrating its relative lack of importance in antagonist binding. The decrease in histamine affinity was, as expected, more dramatic (40-fold). In contrast, the effect of this mutation on the affinities of the various histaprodifen analogues is much smaller (max. three-fold). Similarly, Asn^{198} is very important for the binding of histamine, but it is not involved in the binding of either mepyramine or the histaprodifen analogues. Unexpected is the finding that Asn^{198} is essential for histaprodifen signaling and apparently acts as an activation switch in the human H_1 receptor. Clearly, the actual role of Asn^{198} in the activation mechanism of the human histamine H_1 receptor deserves more attention in the future.

Taken together, the binding pocket of histaprodifens consists of several features: like histamine, the interaction with Asp^{107} is obvious, whereas Asn^{198} is required for receptor activation. Lys^{191} and Phe^{435} either are not involved in histaprodifen binding, or, because histaprodifens are larger compounds with more potential interaction points compared to histamine, additional ligand–receptor interactions mask the effect of a single mutation. Phe^{432} is likely to accommodate one of the phenyl rings of HP, MeHP and HP-HP. In contrast to classical H_1-receptor antagonists (Zhang et al., 1997), substitution of the phenyl rings of histaprodifens does not result in a gain of affinity (Elz et al., 2000a). These phenyl rings are therefore probably oriented slightly different in histaprodifens then in most histamine H_1-receptor antagonists.

The fact that HP-HA is not at all affected by the mutation of Phe^{432} into alanine suggests that the phenyl rings of this ligand do not interact with Phe^{432}. It may therefore be speculated that it is not the histaprodifen part, but the histamine part of the HP-HA dimmer that is oriented towards transmembrane domains 5 and 6. The benzhydryl moiety would than possibly be oriented towards transmembrane domains 1 and 2.

Based on the mutagenesis data, we propose that histaprodifen, methyl histaprodifen, and the HP-HP dimer bind to the human histamine H_1 receptor in an 'antagonistic binding mode'; the protonated amine pointing towards Asp^{107}, whereas the phenyl rings point deep into the hydrophilic pocket of the receptor towards Phe^{432}. Since interaction with Asn^{198} is still required, the histamine moiety might be positioned in the binding pocket comparable to histamine. The lack of effect of the mutations Lys^{191} or Phe^{435} into alanine should then be contributed to secondary effects. One of these secondary effects may be that the loss of a relatively minor interaction point for histamine, a small molecule, may be rather large, whereas for histaprodifens, this effect may be nihilated by additional receptor–ligand interactions. Since HP-HP shows the same pattern of mutational sensitivity as HP and MeHP, the binding pocket of one histaprodifen moiety of this histaprodifen-histaprodifen dimer is apparently overlapping with that of histaprodifen. So far, we do not know where the second histaprodifen moiety of the dimer is located.

In conclusion, histaprodifens containing structural features belonging to both H_1-receptor agonists and H_1-receptor antagonists utilize a distinct H_1-receptor binding site. Apparently, the antagonist-like system is stabilized by Phe^{432} and contributes to the high affinity of these compounds. Similar to histamine, the interaction of the protonated amine with Asp^{107} and of the distal nitrogen (N^{τ}) of the imidazole ring with Asn^{198} is required for receptor activation. On the basis of our present results we conclude that the binding site of the hybrid histaprodifens overlaps with that of both H_1-receptor agonists and H_1-receptor antagonists.

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