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Unique binding pocket for KW-4679 in the histamine H_1 receptor

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Abstract

The histamine H_1 receptor has an aspartate (Asp) residue in transmembrane helix 3 (TM3), which is well-conserved among biogenic amine receptors. The Asp residue is one of the most crucial amino acids for ligand binding. The tested histamine H_1 receptor antagonists with tri- and tetracyclic structures were not selective for histamine H_1 receptors and showed affinity for several other biogenic amine receptors. In contrast, KW-4679 ((*Z*)-11-[3-(dimethylamino)propylidene]-6,11-dihydrodibenz[*b*,*e*]oxepin-2-acetic acid hydrochloride), a tricyclic compound, was a selective histamine H_1 receptor antagonist. [^{3}H]KW-4679 had high affinity (K_d value of 2.5 ± 0.12 nM) for wild-type human histamine H_1 receptors. In the [^{3}H]KW-4679 binding assay, replacement of Asp^{107} by alanine by site-directed mutagenesis greatly reduced the affinities (280–2100-fold) of tri- and tetracyclic compounds, whereas this mutation led to a comparatively small reduction (14-fold) in KW-4679 affinity. These results demonstrate that the tested tri- and tetracyclic histamine H_1 receptor antagonists which have a tight interaction with the Asp residue are not selective for the histamine H_1 receptor. Furthermore, the high selectivity of KW-4679 might be explained by a unique binding pocket, which consists of the Asp residue and other acceptor sites, in the histamine H_1 receptor. © 1998 Elsevier Science B.V.

Keywords: Histamine H_1 receptor; KW-4679 ((*Z*)-11-[3-(dimethylamino)propylidene]-6,11-dihydrodibenz[*b*,*e*]oxepin-2-acetic acid; Site-directed mutagenesis; Binding assay

1. Introduction

Histamine mediates a variety of physiologic and pathologic responses in different tissues and cells, and is an important chemical mediator of inflammation in allergic diseases. The histamine H_1 receptor is a member of the G-protein-coupled-receptor superfamily, and studies on the histamine H_1 receptor have identified important interactions in the ligand–receptor complex (Ohta et al., 1994; Leurs et al., 1994; Moguilevsky et al., 1995). Histamine H_1 receptor antagonists are one of the most widely used classes of clinical drugs. Some of these antagonists, such as doxepin, ketotifen, and epinastine, possess a fused ring system, tri- or tetracyclic structures (Fig. 1). These compounds also have affinity for α_1- and α_2-adrenoceptors, 5-HT_{2A} receptors, muscarinic receptors, and so on, indicating that they have low selectivity for the histamine H_1 receptor (Cusack et al., 1994; Kubo et al., 1987; Fügner et al., 1988). Recently we identified a new histamine H_1 receptor-selective, tricyclic antagonist, KW-4679, (*Z*)-11-[3-(dimethylamino)propylidene]-6,11-dihydrodibenz[*b*,*e*]oxepin-2-acetic acid hydrochloride, as an antiallergic drug (Fig. 1) (Ohshima et al., 1992; Ikemura et al., 1996). KW-4679 has antagonistic effects on the contraction of guinea-pig ileum and on the $[Ca^{2+}]_i$ accumulation in guinea-pig tracheal smooth muscle cells induced by histamine (Sasaki et al., 1995).

It is generally assumed that the G-protein-coupled-receptors with a common structural feature of seven transmembrane domains should also have a common mechanism of interaction with their ligands. Since all ligands for catecholamine receptors have a basic amine moiety, this moiety could be a common anchor point of the ligands. Systematic mutagenesis of the negatively charged residues in the transmembrane domain of biogenic amine receptors has led to identification of an Asp residue in the third transmembrane domain (TM3) as the counterion for the basic amine in both agonists and antagonists (Schwartz

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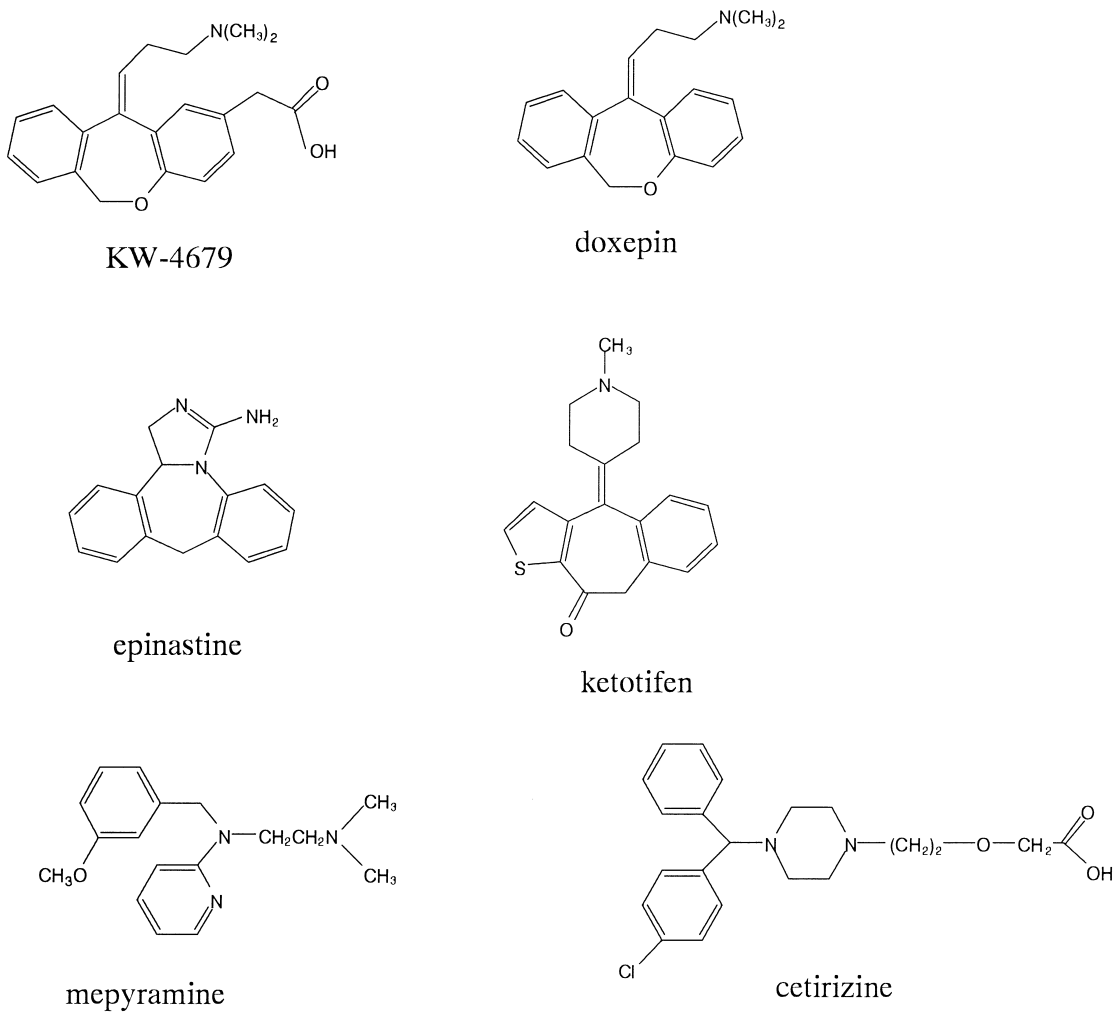

Fig. 1. Structures of histamine H_1 receptor antagonists.

and Rosenkilde, 1996; Strader et al., 1995). It has been suggested that the interaction of ligands with the Asp residue in TM3 is of differential importance for the binding of ligands to cationic amine receptors in each case (Strange, 1996). In the β_2-adrenoceptor, mutagenesis experiments further showed the importance of not only electrostatic interactions (an Asp in TM3) but also hydrogen bonds (two serine residues in TM5) and hydrophobic effects (a phenylalanine residue in TM6) (Strader et al., 1989, 1994). The binding of some antagonists can still be detected at reduced affinity at the Asp-mutated muscarinic acetylcholine receptor, the 5-HT_{1A} receptor, and 5-HT_{2A} receptors (Page et al., 1995; Ho et al., 1992; Wang et al., 1993). Site-directed mutagenesis studies of human histamine H_1 receptors also revealed that the Asp^{107} residue in TM3 was essential for the binding of both the agonist and the antagonist mepyramine, and also for agonist activation such as Ca^{2+} signaling (Ohta et al., 1994). The modeling study also indicated an essential role of the Asp residue for receptor activation (Ter Laak et al., 1995). Moreover, mutations of amino acid residues in TM5 demonstrated that the asparagine^{198} (Asn^{198}) in TM5 is essential for agonist binding and for the production of inositol phosphates but not for antagonist binding (Ohta et al., 1994; Moguilevsky et al., 1995). The same result was obtained for site-directed mutagenesis of guinea-pig histamine H_1 receptors (Leurs et al., 1994). [^{3}H]Mepyramine, the most frequently used radioligand for histamine H_1 receptors, has been reported to have no ability to bind to the mutant receptor that has an Ala residue at position 107 instead of an Asp residue (D107A) (Ohta et al., 1994). Thus, it remains unclear whether the Asp in TM3 is generally important for the binding of histamine H_1 receptor antagonists. The affinities of the ligands for histamine H_1 receptors have been examined by means of the [^{3}H]mepyramine binding assay system in many studies. We have speculated that [^{3}H]mepyramine binding consequently represents the interaction between the Asp in TM3 and the radioligand. Therefore histamine H_1 antagonists, as assessed with binding assays, are not selective owing to an interaction with the Asp conserved in other biogenic amine receptors. Since we found that a new histamine H_1

receptor antagonist, [^{3}H]KW-4679, labels the D107A mutant receptor, the interaction between the Asp in TM3 and each histamine H_1 receptor antagonist was examined. In this report, we demonstrate differences in the contribution of the Asp in TM3 to the histamine H_1 receptor binding of antagonists and suggest that the histamine H_1 receptor binding selectivity of tri- and tetracyclic antagonists might be explained by the strength of the interaction.

2. Materials and methods

2.1. Chemicals

[*N*-methyl-^{3}H]KW-4679 hydrochloride ([^{3}H]KW-4679, 2.70 T Bq/mmol, radiochemical purity of 97%) was tritiated by Amersham International (Buckinghamshire, UK). Mepyramine was from Sigma Chemical (St. Louis, MO). KW-4679 (Ohshima et al., 1992), doxepin, ketotifen, and epinastine were synthesized at the Medicinal Chemistry Department of the Pharmaceutical Research Laboratories of Kyowa Hakko Kogyo. Cetirizine (racemate · 2HCl salt) was kindly provided by UCB (Braine L'Alleud, Belgium). All cell culture supplies were from GIBCO-BRL (Gaithersburg, MD). Other reagents were from standard commercial sources.

2.2. Cells

Previously transfected Chinese Hamster Ovary (CHO) cells (Ohta et al., 1994) that stably expressed the wild-type human histamine H_1 receptor, or stably expressed a given mutant receptor (Asp^{107} in TM3 replaced by alanine (Ala), threonine^{194} (Thr^{194}) in TM5 replaced by Ala and asparagine^{198} (Asn^{198}) in TM5 replaced by Ala), generated by site-directed mutagenesis, were used. Before using these cells for the binding assay, we verified the expression of each mutant receptor by Northern blot analysis. The cells were grown at 37°C in an atmosphere with 5% CO_2 in α-minimum essential medium lacking ribonucleosides and deoxyribonucleosides and supplemented with 10% dialyzed fetal bovine serum.

2.3. [^{3}H]KW-4679 binding

Cultured CHO cells were washed with phosphate-buffered saline twice. Fifty millimolar Na–K phosphate buffer, pH 7.4 (binding buffer), was then added to the cells, and the cells were mechanically scraped off the culture dishes. The obtained cells were pelleted (15 000 rpm, 30 min, 4°C) and washed in fresh binding buffer. The final pellet was kept frozen until the binding assay experiments could be performed. For binding reactions, the assay mixture contained the CHO membranes (100–200 μg of protein), 2 nM [^{3}H]KW-4679 for the D107A receptors or 1 nM [^{3}H]KW-4679 for the wild-type receptors in competition assays and the binding buffer. Saturation assays were carried out using 1–50 nM [^{3}H]KW-4679 for the D107A receptors and 0.2–20 nM for the wild-type and other mutant receptors. Nonspecific binding was defined in the presence of 100 μM KW-4679. Incubations were done at 25°C for 60 min with the D107A receptors and for 360 min with the wild-type and other mutant receptors because these respective times were required for [^{3}H]KW-4679 binding to the D107A mutant receptors, wild-type receptors and other mutant receptors to reach equilibrium (data not shown). Binding reactions were terminated by filtration through Whatman GF/C filters under reduced pressure with a MT-24 cell harvester (Brandel, MD). Filters were washed three times with ice-cold binding buffer (3 ml) and placed in scintillation vials, and bound radioactivity was determined in a liquid scintillation counter, LC6500 (Beckman Instruments, CA). The concentration–response relationship was based on assays in duplicate at 7–10 concentrations for each drug. Protein concentrations were determined by using the Bio-Rad protein assay (Hercules, CA) with bovine serum albumin as the standard.

2.4. Binding of various radioligands

Binding of [^{3}H]thiotidine, [^{3}H]2-([2′,6′-dimethoxy]-phenoxyethylamino)methylbenzodioxan (WB-4101), [^{3}H]clonidine, [^{3}H]spiperone, [^{3}H]quinuclidinylbenzylate, [^{3}H]8-hydroxy-2(di-*n*-propylamino)tetralin, and [^{3}H]ketanserin to histamine H_2 receptors (Gajtkowski et al., 1983), α_1- and α_2-adrenoceptors (Greenberg et al., 1976), dopamine D_2 receptors (Leysen and Gommeren, 1981), muscarinic M_1 receptors (Bloom et al., 1987), 5-HT_{1A} receptors (Gozlan et al., 1983), and 5-HT_{2A} receptors (Leysen et al., 1981), respectively, was measured according to the methods described in the above references.

2.5. Data analysis

Computer analysis (EBDA and LIGAND (Munson and Rodbard, 1980)) was used to evaluate the dissociation constant (K_d value) and receptor density (B_{max} value). IC_{50} values were determined by computerization of logit–log curves. The equation of Cheng and Prusoff (1973) was used to calculate K_i values from IC_{50} values. Data represent means ± S.E.M.

3. Results

3.1. Affinity of KW-4679 for wild-type histamine H_1 receptor

[^{3}H]KW-4679 showed high affinity for the wild-type human histamine H_1 receptor, with a K_d value of 2.5 ± 0.12 nM and a B_{max} value of 1500 ± 250 fmol/mg of protein at a single site (Table 1 and Fig. 2A).

Table 1
Binding parameters of [^{3}H]KW-4679 to the wild-type receptor, or to a H_1 receptor in which Asp^{107} is replaced by Ala (D107A), or Thr^{194} is replaced by Ala (T194A), or Asn^{198} is replaced by Ala (N198A) mutant histamine H_1 receptors

Histamine H_1 receptor	K_d (nM)	B_{max} (fmol/mg of protein)
Wild-type	2.5 ± 0.12	1500 ± 250
D107A	49 ± 4.9	970 ± 64
T194A	3.7 ± 0.28	840 ± 92
N198A	4.2 ± 1.3	2200 ± 940

K_d values and B_{max} values for [^{3}H]KW-4679 were obtained from saturation binding experiments.Values are means ± S.E.M.

[^{3}H]Mepyramine bound to the same receptors with a K_d value of 1.04 ± 0.01 nM and a B_{max} value of 1540 ± 260 fmol/mg of protein (Ohta et al., 1994). The affinities of the histamine H_1 receptor antagonists, as determined by competition experiments using [^{3}H]KW-4679 binding to the wild-type histamine H_1 receptors (Table 2), were in good agreement with those reported for the histamine H_1 receptor using [^{3}H]mepyramine (Cusack et al., 1994; Kubo et al., 1987; Fügner et al., 1988; Snyder and Snowman, 1987; Hill, 1990). These data indicate that [^{3}H]KW-4679 labels the histamine H_1 receptor expressed in CHO cells.

Table 2
Affinities of histamine H_1 antagonists and histamine for the wild-type histamine H_1 receptor or to a receptor in which Asp^{107} is replaced by Ala (D107A)

Ligands	K_i (nM)		K_i ratio
	D107A	Wild-type	
Antagonists			
KW-4679	25 ± 1.5	1.8 ± 0.058	14
Doxepin	190 ± 18	0.69 ± 0.026	280
Epinastine	460 ± 35	1.1 ± 0.16	420
Mepyramine	1000 ± 92	3.6 ± 0.033	280
Ketotifen	1100 ± 33	0.52 ± 0.061	2100
Cetirizine	24000 ± 880	11 ± 0.67	2200
Agonist			
Histamine	> 1000000	7100 ± 350	> 140

K_i values for the histamine H_1 receptor antagonists were obtained from competition binding experiments with [^{3}H]KW-4679, which labeled D107A mutant and wild-type histamine H_1 receptors. K_i ratios are the values of K_i (D107A)/K_i (wild-type). Results are means ± S.E.M. from three experiments.

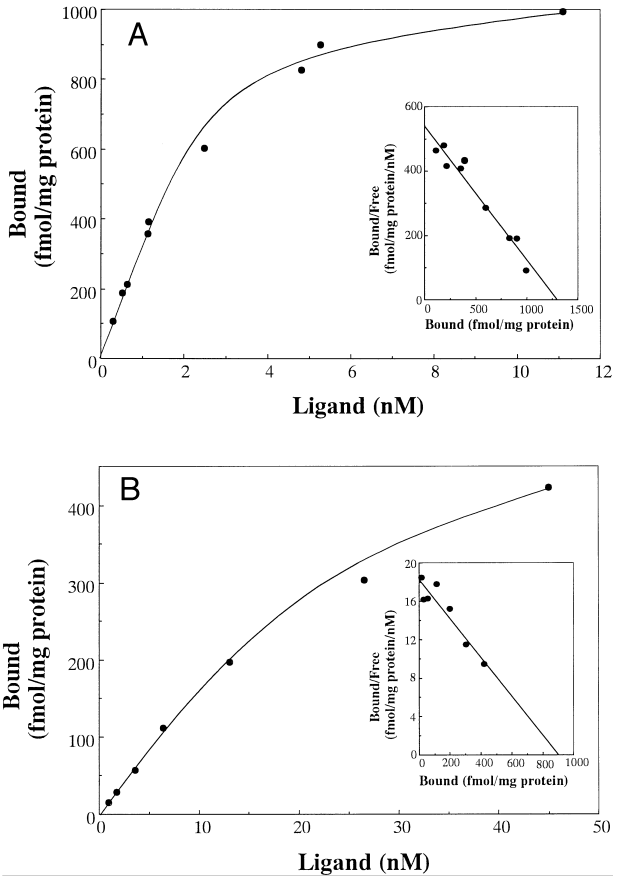

Fig. 2. Representative saturation curves for [^{3}H]KW-4679 binding to CHO cell membranes that stably expressed the wild-type human histamine H_1 receptor (A), or stably expressed the mutant receptor in which Asp^{107} in TM3 is replaced by Ala (D107A) (B). Inset, corresponding Scatchard plot of the data. (A) a K_d value of 2.5 ± 0.12 nM and a B_{max} value of 1500 ± 250 fmol/mg of protein were determined. (B) a K_d value of 49 ± 4.9 nM and a B_{max} value of 970 ± 64 fmol/mg of protein were determined. Values indicate means ± S.E.M. from three separate experiments.

3.2. Affinities of histamine H_1 antagonists and agonist histamine for D107A mutant receptors

The affinity of [^{3}H]KW-4679 was reduced 20-fold by the substitution of alanine for Asp^{107} (Table 1 and Fig. 2B), whereas the affinity of [^{3}H]mepyramine is reported to be substantially lost for the D107A receptors (Ohta et al., 1994). The mutation of Asn^{198} (TM5) to Ala, which reduced the affinity of agonists (Ohta et al., 1994), and that of Thr^{194} (TM5) to Ala did not affect the affinity of KW-4679, indicating that this compound does not interact with these residues (Table 1). This result also accords with the reports that there is no interaction between histamine H_1 receptor antagonists and these residues (Leurs et al., 1994; Moguilevsky et al., 1995). [^{3}H]KW-4679 could label the D107A receptors, and the specific binding was 90% of total binding when 2 nM [^{3}H]KW-4679 was used. The affinities of the representative histamine H_1 receptor antagonists and the agonist, histamine, for the D107A mutant receptors labeled with [^{3}H]KW-4679 were assessed by competition experiments. The affinity of KW-4679 was decreased only 14-fold for the D107A mutant receptors (Table 2 and Fig. 3), whereas the affinities of doxepin, ketotifen, and epinastine, which possess a fused ring system, were dramatically affected, showing a 280-fold, 2100-fold, and 420-fold decrease, respectively (Table 2). Mepyramine and cetirizine, which are nonfused type compounds (Fig. 1), also showed greatly reduced affinity for the D107A receptors, i.e., a 280-fold and 2200-fold de-

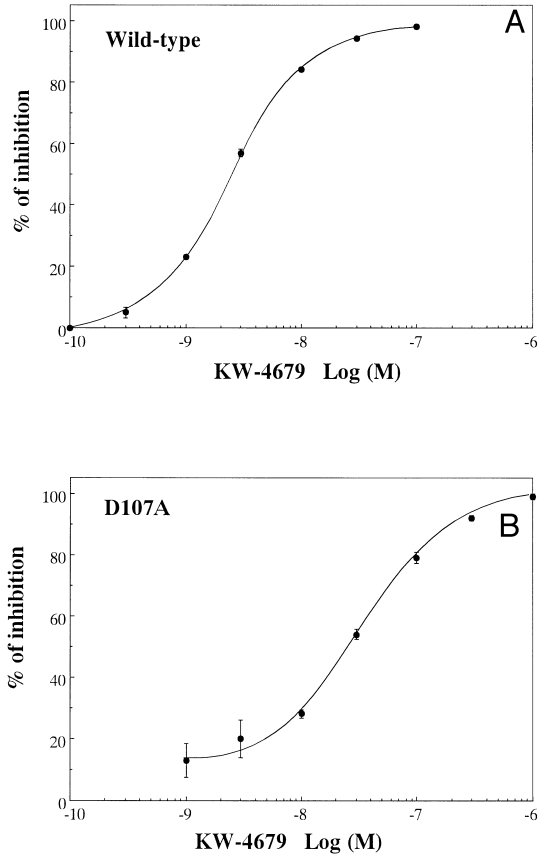

Fig. 3. Inhibition curve of KW-4679 for [^{3}H]KW-4679 binding to CHO cell membranes that stably expressed the wild-type human histamine H_1 receptor (A), or stably expressed the mutant receptor in which Asp^{107} in TM3 was replaced by Ala (D107A) (B). Values are means ± S.E.M. of three separate experiments performed in duplicate.

crease, respectively. The agonist, histamine, showed no affinity for the D107A receptors (Table 2). This result is consistent with the report (Ohta et al., 1994; Moguilevsky et al., 1995; Ter Laak et al., 1995) that the Asp^{107} in TM3 is essential for histamine binding and activation of histamine-induced inositol phosphate formation.

3.3. Receptor profile of histamine H_1 receptor antagonists

Table 3 shows the binding profile of the histamine H_1 receptor antagonists. KW-4679 had intermediate affinity for 5-HT_{2A} receptors only, with a K_i value of 630 nM, whereas doxepin, ketotifen, and epinastine showed high affinity for the receptors, with K_i values of 3.3 nM, 23 nM, and 14 nM, respectively. In addition, these three compounds also exhibited affinity for other receptors, with doxepin showing high affinity for adrenaline α_1, dopamine D_2, and muscarinic M_1 receptors with K_i values of 38 nM, 63 nM, 6.8 nM, respectively. Ketotifen showed high affinity for muscarinic M_1 receptors with a K_i value of 14 nM. Epinastine showed high affinity for adrenaline α_1- and α_2-adrenoceptors with K_i values of 75 nM, 110 nM, respectively. With respect to histamine H_2 and 5-HT_{1A} receptors, doxepin, ketotifen, and epinastine showed affinity, with K_i values of 390–2200 nM. Cetirizine showed high selectivity for histamine H_1 receptors.

4. Discussion

[^{3}H]KW-4679, a tricyclic histamine H_1 antagonist, showed high affinity for the wild-type human histamine H_1 receptor with a K_d value of 2.5 ± 0.12 nM (Table 1 and Fig. 2A) and had an affinity comparable to that of [^{3}H]mepyramine (Ohta et al., 1994). This site-directed mutagenesis study of the histamine H_1 receptor clearly shows that neither Thr^{194} nor Asn^{198} in TM5 is important for the binding of KW-4679 (Table 1). In contrast, Asp^{107} turned out to be involved in KW-4679 binding (Table 1). The affinity of KW-4679 was decreased only 14-fold for the D107A mutant receptors, whereas the affinities of doxepin, ketotifen, and epinastine with tri- or tetracyclic

Table 3
Radioligand binding profiles of histamine H_1 receptor antagonists

Receptor	K_i (nM)				
	KW-4679	Doxepin	Ketotifen	Epinastine	Cetirizine
Histamine H_1	1.8 ± 0.058	0.69 ± 0.026	0.52 ± 0.061	1.1 ± 0.16	11 ± 0.67
Histamine H_2	NE[a]	1100 ± 130	2200 ± 450	640 ± 62	NE[b]
Adrenaline α_1	4900 ± 190	38 ± 2.6	2100 ± 120	75 ± 19	NE[a]
Adrenaline α_2	NE[a]	1800 ± 0	3600 ± 100	110 ± 35	NE[a]
Dopamine D_2	9800 ± 730	63 ± 3.2	1200 ± 280	1100 ± 250	NE[a]
Muscarine M_1	9000 ± 190	6.8 ± 0.28	14 ± 0.15	NE[a]	NE[a]
5-HT_{1A}	NE[a]	390 ± 5.8	1200 ± 60	1500 ± 220	NE[a]
5-HT_{2A}	630 ± 0	3.3 ± 0.10	23 ± 1.5	14 ± 0.32	NE[a]

Assays were run according to standard protocol documented in references. Rat brain membranes were used as receptor sources except for histamine H_1 (CHO cell membranes expressing human recombinant histamine H_1 receptor and [^{3}H]KW-4679 as a receptor source and radioligand, respectively) and H_2 receptors (guinea-pig brain membrane as a receptor source). All assays were validated using appropriate reference standards. Values are means ± S.E.M. of three separate experiments performed in duplicate.
[a] IC_{50} values were more than 100 μM. [b] IC_{50} values were more than 10 μM.

structures were dramatically reduced (Table 2). KW-4679 had an intermediate affinity for 5-HT_{2A} receptors only, whereas doxepin, ketotifen, and epinastine showed high affinity for 5-HT_{2A} receptors and also exhibited affinity for other receptors (Table 3). These compounds, which showed a large decrease in affinity in response to the mutation of Asp^{107}, did not show histamine H_1 receptor selectivity. KW-4679, which was somewhat influenced by the mutation, possessed significant histamine H_1 receptor selectivity (Table 3). The histamine H_1 receptor antagonists with basic amine groups had a tight interaction with Asp^{107}, whereas KW-4679, which also has a basic amino group, had a weaker interaction with the residue. Therefore, KW-4679 might have other acceptor sites that could explain its high affinity binding to histamine H_1 receptors. Since the Asp in TM3 is conserved among G-protein-coupled biogenic amine receptors, it seems that tri- and tetracyclic histamine H_1 receptor antagonists which have a strong interaction with Asp also bind to other biogenic amine receptors.

We could detect the binding of mepyramine in the competition assay even though the tritiated compound showed no binding ability (Ohta et al., 1994), because nonradiolabeled mepyramine added in high concentrations could displace [^{3}H]KW-4679 binding to the D107A receptors (Table 2). Mepyramine and cetirizine, which are nonfused type compounds (Fig. 1), possess high selectivity for histamine H_1 receptors (Table 3) (Snyder and Snowman, 1987; Hill, 1990) in spite of a great decrease in their affinity for the mutant receptor (Table 2). The favorable position of these nonfused type compounds in the histamine H_1 receptor binding pocket could be quite different from that of fused type, tri- and tetracyclic compounds. Thus, they might recognize other crucial residues instead of the Asp^{107} in the wild-type histamine H_1 receptor, and the mutation of Asp^{107} might result in an indirectly altered positioning of these residues in their binding pocket.

The results of our study with mutant receptors imply that among histamine H_1 receptor antagonists KW-4679 interacts in a unique way with the histamine H_1 receptor binding pocket. Doxepin, which lacks the acetic acid moiety of KW-4679 (Fig. 1), showed much lower affinity for the D107A receptor than KW-4679 and no substantial selectivity for the histamine H_1 receptor, suggesting a characteristic role of the acetic acid moiety of KW-4679. The acidic group could provide another electrostatic interaction or a hydrogen bond with a basic or an electropositive group in the receptor.

Our observations provide new insights into the relationship between the Asp residue in TM3 and histamine H_1 receptor antagonists. First, histamine H_1 receptor antagonists which bear a basic nitrogen interact with the Asp in TM3, while the contribution of the Asp to the binding potency of each antagonist is quite different. Second, the histamine H_1 receptor selectivity of the histamine H_1 receptor antagonists with a tri- or tetracyclic structure is suggested to reflect the degree of rigidity in the interaction between a basic amino group on the antagonist and the Asp in TM3 and has an important role in biogenic amine receptors.

This is the first report that describes the functional significance of Asp^{107} in TM3 for the binding of histamine H_1 receptor antagonists on the basis of quantitative data. [^{3}H]KW-4679 was the only radioligand that could label the D107A receptor, and this made it possible to estimate the interaction between histamine H_1 receptor antagonists and the Asp^{107} residue. [^{3}H]KW-4679 is a unique radioligand for histamine H_1 receptors, and additional studies with mutant histamine H_1 receptors and [^{3}H]KW-4679 should provide much data for a better understanding of the nature of the interaction between the histamine H_1 receptor and its ligands at the molecular level.

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