THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 42, Issue of October 15, pp. 29994–30000, 1999

Printed in U.S.A.

Mutational Analysis of the Antagonist-binding Site of the Histamine H_1 Receptor*

(Received for publication, December 31, 1998, and in revised form, June 4, 1999)

Kerstin Wieland‡, Anton M. Ter Laak§, Martine J. Smit‡, Ronald Kühne§, Henk Timmerman‡, and Rob Leurs‡¶

From the ‡Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry, Division of Medicinal Chemistry, Faculty of Chemistry, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, the Netherlands and the §Institute for Molecular Pharmacology, Biocomputing/Molecular Modelling Group, Alfred Kowalke Strasse 4, D-10315 Berlin, Germany

We combined in a previously derived three-dimensional model of the histamine H_1 receptor (Ter Laak, A. M., Timmerman, H., Leurs, H., Nederkoorn, P. H. J., Smit, M. J., and Donne-Op den Kelder, G. M. (1995) *J. Comp. Aid. Mol. Design.* 9, 319–330) a pharmacophore for the H_1 antagonist binding site (Ter Laak, A. M., Venhorst, J., Timmerman, H., and Donné-Op de Kelder, G. M. (1994) *J. Med. Chem.* 38, 3351–3360) with the known interacting amino acid residue Asp^{116} (in transmembrane domain III) of the H_1 receptor and verified the predicted receptor-ligand interactions by site-directed mutagenesis. This resulted in the identification of the aromatic amino acids Trp^{167}, Phe^{433}, and Phe^{436} in transmembrane domains IV and VI of the H_1 receptor as probable interaction points for the *trans*-aromatic ring of the H_1 antagonists. Subsequently, a specific interaction of carboxylate moieties of two therapeutically important, zwitterionic H_1 antagonists with Lys^{200} in transmembrane domain V was predicted. A $Lys^{200} \rightarrow Ala$ mutation results in a 50- (acrivastine) to 8-fold (d-cetirizine) loss of affinity of these zwitterionic antagonists. In contrast, the affinities of structural analogs of acrivastine and cetirizine lacking the carboxylate group, triprolidine and meclozine, respectively, are unaffected by the $Lys^{200} \rightarrow Ala$ mutation. These data strongly suggest that Lys^{200}, unique for the H_1 receptor, acts as a specific anchor point for these "second generation" H_1 antagonists.

Since the initial discovery of the role of histamine in allergic conditions (1) serious efforts have been made to develop drugs that inhibit the actions of histamine. Already in 1933, Fourneau and Bovet (2) reported the first "antihistamine" piperoxan. Following this finding many potent H_1 antagonists that can be considered as variations of diaryl-substituted ethylamines (*e.g.* diphenhydramine and mepyramine) have been developed (for review see Ref. 3). These "first generation" H_1 antagonists are quite effective in humans in allergic rhinitis and urticaria, but because of central nervous system penetration and central H_1 receptor blockade their clinical use is hampered by sedative side effects (3–5). A "second generation" of nonsedative H_1 antagonists (*e.g.* astemizole, acrivastine, cetirizine, loratidine, and terfenadine) has recently been developed (for review see Ref. 3). Their altered pharmacokinetics result in good clinical effectiveness combined with a strongly reduced sedative potential (3–5).

The development of H_1 antagonists has so far been directed by traditional medicinal chemistry (3). With the availability of the genetic information of the histamine H_1 receptor (6), the rationalization of drug-protein interaction has become a major challenge for this therapeutically important class of drugs. Like all aminergic G-protein coupled receptors (GPCR),[1] the H_1 receptor contains an aspartate residue (Asp^{116}) in transmembrane domain (TM) III (6), that is involved in the binding of the protonated amine function found in both agonists and antagonists structures (7, 8). Mutagenesis studies have furthermore shown that the imidazole ring of histamine is accommodated by Lys^{200} and Asn^{207} in TM V (9, 10).

In view of the low sequence similarity between GPCRs and bacteriorhodopsin (BR) much controversy exists on the validity of models derived for GPCRs based on the homology with BR (11–13). Nevertheless, despite the speculative nature of BR-derived GPCR models they have been quite helpful in understanding and predicting drug-receptor interactions for a variety of receptors (see *e.g.* Refs. 14–16). Previously, we also developed a three-dimensional computer model of the histamine H_1 receptor based on the homology with BR, incorporating the results obtained from mutagenesis studies on the agonist binding site (17). In the present study this computer model of the H_1 receptor was combined with a pharmacophoric model of the H_1 antagonistic binding site (18). This ligand-based model for the H_1 antagonistic binding site is based upon an interaction of the protonated amine function of various first generation, semi-rigid H_1 antagonists with an aspartate residue (Asp^{116} in the guinea pig H_1 receptor) (18) and precisely positions the *cis*- and *trans*-aromatic rings of the H_1 antagonists relative to the C_α and C_β carbon atoms of this aspartate residue. Combining the three-dimensional receptor model and the ligand-based pharmacophoric model of the H_1 antagonist binding site resulted in the prediction of interactions of aromatic amino acids in TM IV and VI with the H_1 antagonists. Subsequently, we experimentally confirmed the involvement of these predicted amino acids in the binding of the H_1 antagonist [^{3}H]mepyramine by site-directed mutagenesis. Moreover, on the basis of the three-dimensional model of the antagonist-receptor complex, a specific interaction of carboxylate moieties of therapeutically important, second generation zwitterionic H_1 antagonists (acrivastine and cetirizine) with Lys^{200} in TM V was predicted and experimentally verified.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 31-20-4447579; Fax: 31-20-4447610; E-mail: leurs@chem.vu.nl.

[1] The abbreviations used are: GPCR, G-protein coupled receptor; BR, bacteriorhodopsin; TM, transmembrane domain.

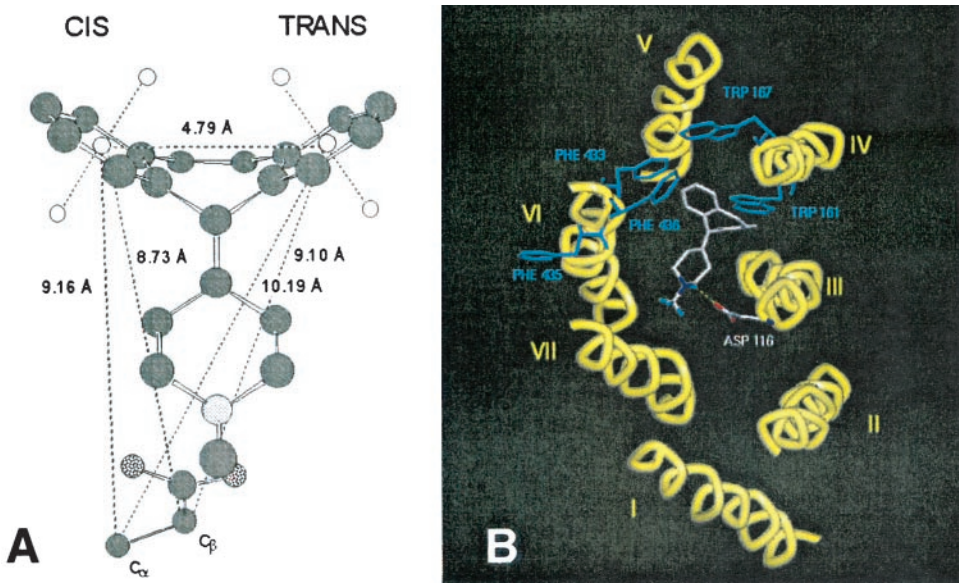


FIG. 1. *A*, the H_1 antagonist pharmacophore of Ter Laak *et al.* (18) describing the position of the *cis*- and *trans*-aromatic rings of H_1 antagonists with respect to the C_α and C_β carbon atoms of a putative aspartate residue. *B*, the H_1 antagonist pharmacophore was docked into the H_1 receptor model based on BR (17). A view from the extracellular side shows the orientation of cyproheptadine between the TMs (*yellow*) and the ionic hydrogen bond interaction with Asp^{116} in TM III. The *trans*-aromatic ring of cyproheptadine is in the proximity of Phe^{433}, Phe^{436} (TM VI), and Trp^{167} (TM IV), and the *cis*-aromatic ring is near Trp^{161} (TM IV). Two other aromatic residues that were mutated in this study (Phe^{435} and Trp^{174}) are not in the proximity of the H_1 antagonist in this model. Phe^{435} points toward the membrane, and Trp^{174} is not shown because in this alignment the residue lies outside the TM region.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, DEAE-dextran, polyethyleneimine, and triprolidine hydrochloride were obtained from Sigma. The mouse anti-FLAG M2 monoclonal antibody was obtained from International Biotechnology Inc. The fluorescein isothiocyanate-conjugated rabbit-anti-mouse secondary antibody was supplied by Dakopatts AB (Stockholm, Sweden). [^{3}H]Mepyramine (28 Ci/mmol) was obtained from Amersham Pharmacia Biotech. Gifts of acrivastine (The Wellcome Foundation Ltd., London, United Kingdom), d-cetirizine hydrochloride, meclozine hydrochloride (UCB, Braine-l'Alleud, Belgium), and mianserin hydrochloride (Organon NV, Oss, the Netherlands) are gratefully acknowledged.

Predicition of Ligand-Receptor Interactions—H_1 antagonists were docked in the previously described three-dimensional receptor model of the guinea pig H_1 receptor (17), using the rigid H_1 antagonist pharmacophoric model of Ter Laak *et al*. (18). This model describes the three-dimensional topology of the *cis*- and *trans*-aromatic rings of cyproheptadine with respect to the positions of the C_α and C_β carbon atoms of an putative Asp residue from the receptor (see Fig. 1*A*). The C_α and C_β carbon atoms of the pharmacophore replaced the corresponding atoms of Asp^{116} in the receptor model. Rotation was carried out along the C_α-C_β bond until cyproheptadine was positioned in the receptor in an energetically favorable orientation. The structure of the zwitterionic compounds acrivastine and d-cetirizine were built and optimized with Chem-X and subsequently docked into the H_1 receptor model onto the cyproheptadine template as described previously (18). Subsequently, all freely rotatable bonds in Lys^{200} and in the side chains of the zwitterionic H_1 antagonist were taken into account in an extensive conformational analysis (MacroModel/AMBER force field (19)).

Site-directed Mutagenesis—The guinea pig H_1 receptor cDNA was subcloned in the pALTER vector (Promega), and point mutations were introduced according to the manufacturer's protocol. The wild type and mutant receptors were epitope-tagged with an N-terminal FLAG peptide (DYKDDDD) after modification of the cDNA sequence with polymerase chain reaction. In our initial binding studies (see Fig. 2) non-tagged receptors were used. The cDNA sequences were verified using the dideoxy chain termination method with the Sequenase kit (U. S. Biochemical Corp.).

Cell Culture and Transfection—COS-7 and HEK-293 cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium, containing 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 5 or 10% (v/v) fetal calf serum, respectively. Cells were transiently transfected with pcDNA3, containing the wild type or mutant H_1 receptor cDNA, using DEAE-dextran (COS-7 cells) or calcium phosphate (HEK-293 cells).

Histamine H_1 Receptor Binding Studies—Transfected cells were harvested after 48 h, homogenized in ice-cold 50 mM Na_2/potassium phosphate buffer (pH 7.4) and used for radioligand binding studies. Cell homogenates (40–50 μg of protein) were incubated for 30 min at 25 °C in 50 mM Na_2/potassium phosphate buffer (pH 7.4) in 400 μl with the indicated concentrations of [^{3}H]mepyramine. The nonspecific binding was defined in the presence of 1 μM mianserin. In displacement studies, cell homogenates were incubated with 1 nM [^{3}H]mepyramine and increasing concentrations of competing ligands. The incubations were stopped by rapid dilution with 3 ml of ice-cold 50 mM Na_2/potassium phosphate buffer (pH 7.4). The bound radioactivity was separated by filtration through Whatman GF/C filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 ml of buffer, and radioactivity retained on the filters was measured by liquid scintillation counting. The binding data were evaluated by a nonlinear, least squares curve fitting procedure. Protein levels were determined according to Bradford (20), using bovine serum albumin as standard.

[^{3}H]Inositol Phosphate Production—HEK-293 cells were seeded in 12-well plates and 24 h after transfection labeled overnight in inositol-free culture medium supplemented with 2 μCi/ml *myo*-[2-^{3}H]inositol. Cells were stimulated for 1 h at 37 °C with Dulbecco's modified Eagle's medium containing 25 mM Hepes (pH 7.4), 20 mM LiCl, and histamine. The incubation was stopped by aspiration of the culture medium and the addition of cold $CHCl_3$/methanol (1:2 v/v). After extraction with water, [^{3}H]inositol phosphates were isolated by anion exchange chromatography (21).

Immunofluorescence—Transfected COS-7 cells were grown on glass coverslips and after 48 h fixed with 4% paraformaldehyde/phosphate-buffered saline for 30 min at room temperature and blocked in phosphate-buffered saline/0.1% bovine serum albumin for 1 h at room temperature. Antigen detection was performed as described (22).

RESULTS

Prediction of Ligand-Receptor Interaction Based on Receptor Modelling—The H_1 antagonist pharmacophoric model of Ter Laak *et al*. (18) represents low energy conformations of several potent and rigid H_1 antagonists (cyproheptadine, phenindamine, triprolidine, epinastine, mequitazine, and mianserine) and is able to discriminate between the stereochemically different *cis*- and *trans*-aromatic rings of these H_1 antagonists (Fig. 1*A*). To predict amino acid residues involved in the ligand-binding site of these H_1 antagonists the cyproheptadine pharmacophore was docked into the previously derived model of the H_1

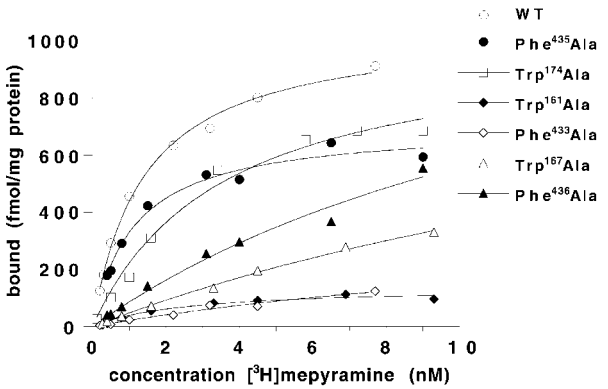


FIG. 2. **Effects of the mutation of aromatic amino acids in TM IV and VI on the binding of the H_1 antagonist [^{3}H]mepyramine to wild type, Phe^{435} → Ala, Trp^{174} → Ala, Trp^{167} → Ala, Phe^{436} → Ala, Trp^{161} → Ala, and Phe^{433} → Ala H_1 receptor after transient expression in COS-7 cells.** The data shown are from a representative example out of at least three independent experiments.

receptor. After rotation over the C_α-C_β bond of Asp^{116} and energy optimizations, a single energetically favorable orientation was found for the H_1 antagonist. In this orientation the aromatic rings of the H_1 antagonist were located in the receptor-binding pocket between the TMs III, IV, V, and VI (Fig. 1*B*). The aromatic rings of cyproheptadine were surrounded by several aromatic amino acids. In the H_1 receptor model, the *cis*-ring of cyproheptadine is located within 5 Å of a tryptophane in TM IV (Trp^{161}), whereas the *trans*-aromatic ring is close to two phenylalanines in TM VI (Phe^{433} and Phe^{436}) and Trp^{167} in TM IV (Fig. 1*B*). All these predicted residues are conserved in all the reported H_1 receptor sequences (23–29).

Verification of the H_1 Antagonist-binding Site by Site-directed Mutagenesis—To verify the interaction of H_1 antagonists with the predicted amino acids these residues were initially mutated to alanines. Moreover, two related amino acids (Trp^{174} in TM IV and Phe^{435} in TM VI) that are also conserved in all the reported H_1 receptor sequences (23–29) but not predicted by the GPCR model were also mutated. In the derived model Phe^{435} points into the phospholipid bilayer (Fig. 1*B*), and Trp^{174} is located just outside TM IV.

The mutant receptors were expressed transiently in COS-7 cells and analyzed by [^{3}H]mepyramine saturation binding studies. Expression of the wild type H_1 receptor in COS-7 cells resulted in a high affinity binding site for [^{3}H]mepyramine (K_D = 0.7 ± 0.1 nM, mean ± S.E., n = 3) (Fig. 2). Mutation of the tryptophane residues Trp^{161}, Trp^{167}, and Trp^{174} in TM IV to alanine residues resulted in distinct effects on the [^{3}H]mepyramine binding to the mutant receptors (Fig. 2). The introduction of the Trp^{174} → Ala mutation did not reduce the affinity of [^{3}H]mepyramine (Fig. 2; K_D = 3.6 ± 0.6 nM, mean ± S.E., n = 3) dramatically. In contrast, for the Trp^{167}Ala receptor the affinity for the H_1 antagonist was reduced more than 10-fold (Fig. 2; K_D > 15 nM, n = 3), whereas cells expressing the Trp^{161} → Ala receptor did not show binding of [^{3}H]mepyramine (Fig. 2) significantly higher than binding to mock-transfected COS-7 cells (15–50 fmol/mg protein; data not shown). Similar findings were obtained with the three Phe-Ala mutations in TM VI. The Phe^{435}Ala receptor mutant still bound [^{3}H]mepyramine with high affinity (Fig. 2; K_D = 1.3 ± 0.2 nM, mean ± S.E., n = 3), whereas for the other two mutants a reduced (Phe^{436} → Ala receptor, K_D > 15 nM, n = 3) or totally impaired (Phe^{433} → Ala receptor) [^{3}H]mepyramine binding was observed (Fig. 2).

To verify protein expression of the two receptor mutants that did not show detectable [^{3}H]mepyramine binding (Trp^{161} → Ala and Phe^{433} → Ala), a FLAG epitope was introduced at the N terminus of the wild type and mutant H_1 receptor proteins. Using confocal laser microscopy we identified specific, anti-FLAG immunofluorescence in the plasma membrane of COS-7 cells expressing the epitope-tagged wild type and the Phe^{433} → Ala H_1 receptor (Fig. 3). For the Trp^{161} → Ala H_1 receptor, the anti-FLAG immunofluorescence was mainly found inside the cell, indicating perturbed receptor expression.

Based on the observed loss of antagonist affinity upon mutation of Trp^{167}, Phe^{433}, and Phe^{436}, we considered these amino acids as likely candidates for the hypothesized interaction with the aromatic rings of the H_1 antagonist. To investigate the role of Trp^{161}, Trp^{167}, Phe^{433}, and Phe^{436} in more detail, we changed the tryptophane residues in TM IV to methionine and phenylalanine (Trp^{161} → Met, Trp^{161} → Phe, Trp^{167} → Met, and Trp^{167} → Phe) and the phenylalanine residues in TM 6 to methionine (Phe^{433} → Met and Phe^{436} → Met). The mutant receptors were epitope-tagged, expressed in COS-7 cells, and evaluated for [^{3}H]mepyramine binding. In contrast to the Trp^{161} → Ala receptor mutant, the Trp^{161} → Met and Trp^{161} → Phe receptor mutants bound [^{3}H]mepyramine with high affinity (Table I). Yet the number of binding sites for the Trp^{161} → Phe receptor mutant was considerably lower compared with the wild type and the Trp^{161} → Met receptor mutant (Table I). As found for the Trp^{167} → Ala receptor mutant, mutation of Trp^{167} to Met or Phe resulted in strongly reduced affinity for [^{3}H]mepyramine (Table I). In contrast, mutating Phe^{433} and Phe^{436} to Met allowed [^{3}H]mepyramine binding with high affinity (Table I).

The affinity of the Trp^{161} → Met and Trp^{161} → Phe mutant receptors for histamine was not changed (Table I), whereas a small to major reduction of the agonist affinity was observed for the Phe^{433} → Met and Phe^{436} → Met receptor mutants (Table I). Because of the lack of saturable [^{3}H]mepyramine binding, the agonist affinity could not be determined for the other mutants.

Histamine-induced [^{3}H]Inositol Phosphate Accumulation after Stimulation of Wild Type and Mutant H_1 Receptors—To test the functionality of the mutant receptors, we initially performed [^{3}H]inositol phosphate accumulation experiments in transfected COS-7 cells. However, in mock-transfected COS-7 cells histamine increased basal [^{3}H]inositol phosphate accumulation, suggesting the presence of an endogenously expressed H_1 receptor.[2] In mock-transfected HEK-293 cells histamine did not stimulate the [^{3}H]inositol phosphate accumulation (Fig. 4), whereas after overexpression of the epitope-tagged wild type H_1 receptor protein (7.1 ± 1.0 pmol/mg protein, mean ± S.E., n = 3) histamine (100 μM) stimulated the [^{3}H]inositol phosphate accumulation 5.9 ± 0.4-fold (mean ± S.E., n = 3) over basal levels (Fig. 4). Evaluation of the various receptor mutants showed that the Trp^{161} → Met, Phe^{433} → Ala, Phe^{433} → Met, Phe^{436} → Ala, and Phe^{436} → Met mutant receptors stimulated the [^{3}H]inositol phosphate accumulation as the wild type H_1 receptor (Fig. 4). Similar EC_{50} values for histamine were observed for the wild type receptor and the Trp^{161} → Met, Trp^{161} → Phe, and Phe^{433} → Ala receptors, whereas the Phe^{433} → Met, Phe^{436} → Ala, and Phe^{436} → Met mutant receptors were stimulated less effectively by histamine (Table I). The expression levels of the mutant receptors in HEK-293 cells ranged from 2.4 (Phe^{436} → Ala) to 10.6 pmol/mg protein (Trp^{161} → Met). For the Phe^{433} → Ala mutant no radioligand binding was found. As expected by the perturbed membrane expression, the Trp^{161} → Ala receptor mutant did not respond to histamine (Fig. 4). As found for the expression in COS-7 cells, the Trp^{161} → Phe was expressed at relatively low levels in HEK-293 cells (0.3 ± 0.1

[2] K. Wieland, H. Timmerman, and R. Leurs, unpublished observations.

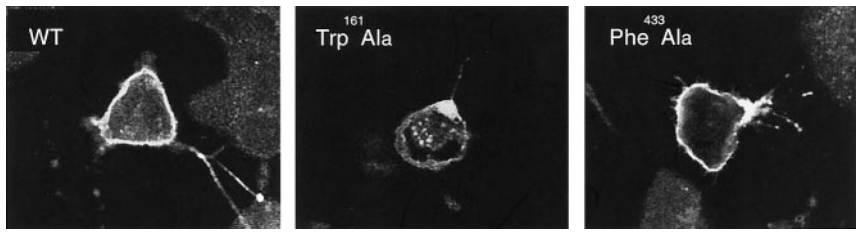


FIG. 3. **Localization of epitope-tagged wild type (*WT*) and mutant H_1 receptors, transiently expressed in COS-7 cells.** Transfected COS-7 cells were grown on glass coverslips and after 48 h fixed with 4% paraformaldehyde. Immunofluorescence was detected with the mouse anti-FLAG M2 antibody and a fluorescein isothiocyanate-conjugated secondary rabbit anti-mouse antibody.

TABLE I

Effects of point mutations in TM 1V and V1 of the histamine H_1 receptor on ligand binding and signal transduction

Receptors were expressed in COS-7 cells or HEK-293 cells and used 48 h after transfection for radioligand binding studies and the accumulation of [^{3}H]inositol phosphates, respectively. Data were calculated as the means ± S.E. from at least three independent experiments. ND indicates that the value was not determined.

Receptor	K_D [^{3}H]mepyramine	H_1 receptor density	K_i histamine	EC_{50} histamine	Basal stimulation
	nM	*pmol/mg protein*	*μM*	*μM*	*fold*
Wild type	0.7 ± 0.1	9.5 ± 1.2	76 ± 19	0.04 ± 0.02	5.9 ± 0.4
$Trp^{161} \rightarrow Ala$		0[a]	ND	ND	0.9 ± 0.1
$Trp^{161} \rightarrow Met$	1.6 ± 0.2	18.6 ± 1.8	124 ± 8	0.08 ± 0.06	6.5 ± 0.5
$Trp^{161} \rightarrow Phe$	1.6 ± 0.1	1.6 ± 0.2	70 ± 6	0.07 ± 0.02	1.5 ± 0.1
$Trp^{167} \rightarrow Ala$	>15	ND	ND	ND	1.2 ± 0.3
$Trp^{167} \rightarrow Met$	>15	ND	ND	ND	1.5 ± 0.2
$Trp^{167} \rightarrow Phe$	>15	ND	ND	217 ± 138	3.0 ± 0.2
$Trp^{174} \rightarrow Ala$	3.6 ± 0.6	1.1 ± 0.4	ND	ND	ND
$Phe^{433} \rightarrow Ala$		0[b]	ND	0.06 ± 0.02	6.0 ± 0.8
$Phe^{433} \rightarrow Met$	5.4 ± 0.4	17.2 ± 0.2	208 ± 13	0.4 ± 0.1	5.6 ± 0.1
$Phe^{435} \rightarrow Ala$	1.3 ± 0.2	0.9 ± 0.3	ND	ND	ND
$Phe^{436} \rightarrow Ala$	>15	ND	ND	210 ± 136	4.7 ± 0.5
$Phe^{436} \rightarrow Met$	3.5 ± 0.3	12.8 ± 4.9	33 ± 36	6.3 ± 2.2	6.8 ± 1.0

[a] Positive anti-FLAG immunofluorescence signals only inside the cell.
[b] Positive anti-FLAG immunofluorescence signals in the membrane.

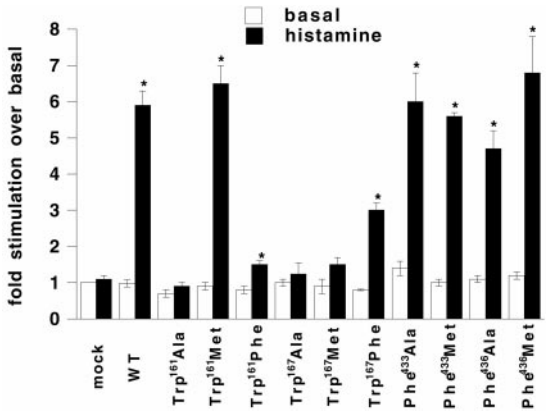


FIG. 4. **Basal (*open bars*) and histamine (100 μM) (*closed bars*) induced production of [^{3}H]inositol phosphates by wild type and mutant H_1 receptors expressed in [^{3}H]inositol prelabeled HEK-293 cells.** The data shown are the means ± S.E. of at least three independent experiments.

pmol/mg protein, means ± S.E., $n = 3$). Despite its low expression level a significant stimulation (Fig. 4) of the mutant receptor by histamine was observed with comparable potency as the wild type receptor (Table I). For the various mutations of Trp^{167} only the $Trp^{167} \rightarrow Phe$ mutation resulted in a mutant receptor that could be activated by histamine, although with a very low efficacy (Fig. 4 and Table I). Because of the lack of saturable binding in the [^{3}H]mepyramine binding studies, the expression levels of these mutant receptors could not be estimated.

Predicted Interaction of the Zwitterionic H_1 Antagonists Acrivastine and Cetirizine with Lys^{200}—To investigate whether the acidic moiety of the nonsedative, zwitterionic H_1 antagonist acrivastine specifically interacts with the H_1 receptor protein, this ligand was docked into the H_1 receptor model on top of the template cyproheptadine. Visual inspection of the resulting ligand-receptor complex suggested a possible interaction with Lys^{200} in TM V. Following this observation a conformational analysis, giving rotational freedom to Lys^{200} and the carboxylate group of the H_1 antagonist, indeed predicted an interaction between the positively charged Lys^{200} and the carboxylate group (Fig. 5*A*). In the case of d-cetirizine, the carboxylate group is attached to the basic nitrogen via a long ether chain. Docking cetirizine in the H_1 receptor model indicated that the carboxylate of d-cetirizine reaches the proximity of Lys^{200}, although the calculated N-O distance of 3.57 Å is somewhat large for a strong (ionic) hydrogen bond interaction (Fig. 5*B*).

Interaction of H_1 Antagonists with Lys^{200} in TM V—To verify the predicted interaction of Lys^{200} with the carboxylate group of acrivastine, we mutated the basic lysine to alanine and methionine to disrupt the potential ionic interaction with the H_1 antagonist. We also mutated Lys^{200} to an arginine residue, because this basic amino acid should be able to interact with the carboxylate group of acrivastine. Previously, we showed that Lys^{200} in TM V specifically interacts with some classes of H_1 agonists (including histamine) but not with the prototypic H_1 antagonists [^{3}H]mepyramine and d- and l-chlorpheniramine (10). Moreover, also the $Lys^{200} \rightarrow Arg$ and the $Lys^{200} \rightarrow Met$ mutations allowed high affinity [^{3}H]mepyramine binding after expression in COS-7 cells and similar to the $Lys^{200} \rightarrow Ala$ mutant (10) showed a slightly altered affinity for histamine (Table II). Functional studies in HEK-293 cells indicated that cells transiently transfected with the mutant receptors all responded to histamine with the accumulation of [^{3}H]inositol phosphates, although the EC_{50} values were higher than found for the wild type receptor (Table II). Displacement of the binding of [^{3}H]mepyramine to the $Lys^{200} \rightarrow Ala$, $Lys^{200} \rightarrow Arg$, and $Lys^{200} \rightarrow Met$ receptor mutants indicated a specific interaction

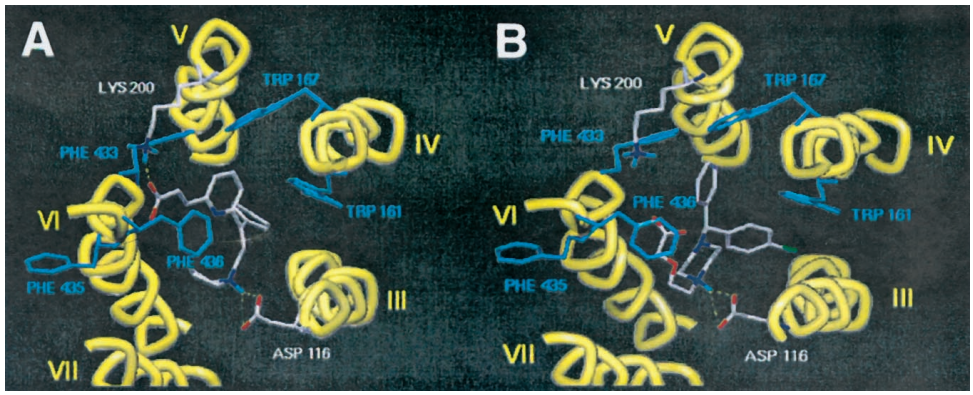


FIG. 5. **Zwitterionic H_1 antagonists acrivastine (*A*) and d-cetirizine (*B*) docked into the H_1 receptor model.** *A*, acrivastine, which fits the cyproheptadine pharmacophore (Fig. 1*A*), makes an additional (ionic) hydrogen bond interaction with Lys^{200} (TM V). *B*, d-cetirizine docked into the same H_1 receptor model and presenting the carboxylate moiety near Lys^{200}.

TABLE II

Effects of mutation of Lys^{200} of the histamine H_1 receptor on ligand binding and signal transduction

Receptors were expressed in COS-7 cells or HEK-293 cells and used 48 h after transfection for radioligand binding studies and the accumulation of [^{3}H]inositol phosphates, respectively. The wild type, $Lys^{200} \rightarrow$ Ala, $Lys^{200} \rightarrow$ Met, and $Lys^{200} \rightarrow$ Arg were expressed in HEK-293 cells at receptor densities of respectively 7.1 ± 1.0, 13.6 ± 1.5, 7.2 ± 1.1, and 7.1 ± 0.4 pmol/mg protein. Data were calculated as the means ± S.E. from at least three independent experiments.

Receptor	K_D [^{3}H]mepyramine	H_1 receptor density	K_i histamine	EC_{50} histamine	Basal stimulation	K_i acrivastine
	nM	*pmol/mg protein*	*μM*	*μM*	*fold*	*nM*
wild type	0.7 ± 0.1	9.5 ± 4.2	76 ± 19	0.04 ± 0.02	5.9 ± 0.4	10 ± 3
$Lys^{200} \rightarrow$ Ala	1.6 ± 0.2	17.1 ± 0.3	320 ± 22	2.3 ± 1.2	5.3 ± 0.5	548 ± 52
$Lys^{200} \rightarrow$ Met	1.9 ± 0.2	17.3 ± 5.1	281 ± 18	1.7 ± 0.6	5.8 ± 0.6	534 ± 97
$Lys^{200} \rightarrow$ Arg	5.7 ± 0.6	13.9 ± 4.7	182 ± 5	1.9 ± 0.8	7.4 ± 0.9	37 ± 11

of Lys^{200} with acrivastine. The K_i value for acrivastine was increased more than 50-fold following the $Lys^{200} \rightarrow$ Ala and $Lys^{200} \rightarrow$ Met mutations (Fig. 6*A* and Table II). In contrast, acrivastine still showed high affinity for the $Lys^{200} \rightarrow$ Arg receptor mutant (Fig. 6*A* and Table II). To further substantiate these findings we tested the affinity of triprolidine, a structural analog of acrivastine lacking the carboxylate moiety (Table III), for the $Lys^{200} \rightarrow$ Ala receptor mutant. As expected, the affinity of this close structural analog was not reduced by the $Lys^{200} \rightarrow$ Ala mutation (Fig. 6*B* and Table III). The Lys^{200} residue is also involved in a specific interaction with the nonsedative, zwitterionic H_1 antagonist d-cetirizine (Fig. 6*C* and Table III). Again, no effect of the $Lys^{200} \rightarrow$ Ala mutation was found on the affinity of the analog meclozine, which does not contain a functional group that can interact with the Lys^{200} residue in TM V (Fig. 6*C* and Table III).

DISCUSSION

More than 25 years after the initial hypothesis of Nauta *et al.* (30) of an interaction of the *trans*-aromatic ring of the H_1 antagonist diphenhydramine with a Phe residue of an hypothetical α-helical structure of the H_1 receptor, we identified the aromatic amino acids Trp^{167}, Phe^{433}, and Phe^{436} in the putative α-helical TMs IV and VI of the H_1 receptor as probable interaction points for the *trans*-aromatic ring of the H_1 antagonists. Moreover, we found Lys^{200} (TM V) to be a specific anchor point for the carboxylate moiety of the nonsedative, zwitterionic second generation H_1 antagonists acrivastine and cetirizine.

The basis for the identification of these amino acids came from the docking of an H_1 antagonistic pharmacophoric model (18) into a previously derived three-dimensional model of the H_1 receptor (17). As a representative example of the first generation H_1 antagonists, the rigid tricyclic cyproheptadine was allowed to interact with its protonated amine function with the highly conserved Asp^{116} in TM III (7, 8). Several aromatic amino acid residues were predicted to interact with the aromatic rings of the H_1 antagonist. Mutation of Trp^{174} and Phe^{435}, which were predicted to not be involved in ligand binding, had indeed no effect on the [^{3}H]mepyramine binding. In contrast, for the mutant $Trp^{167} \rightarrow$ Ala and $Phe^{436} \rightarrow$ Ala receptors a dramatic loss of affinity of [^{3}H]mepyramine was observed. Moreover, the $Phe^{433} \rightarrow$ Ala mutation caused a total loss of H_1 antagonist binding, despite membrane expression of the receptor protein and normal responsiveness toward histamine. Replacing Phe^{433} and Phe^{436} with the hydrophobic but aliphatic Met residue only slightly affected the binding of [^{3}H]mepyramine, indicating that these two Phe residues in TMVI are most likely involved in a hydrophobic interaction with the H_1 antagonist. Furthermore, a large reduction in the affinity and efficacy of histamine was observed for the Phe^{436} mutants. This observation fits well with our recently developed model for the agonist interaction with the H_1 receptor. In this model an interaction of Phe^{436} with the imidazole ring of histamine is predicted.[3] Also for the $Phe^{433} \rightarrow$ Met mutant lower affinity and efficacy are observed. In our model for the agonist-receptor interaction, a direct involvement of Phe^{433} with the agonist binding is not predicted,[3] as substantiated by the full agonist activity at the $Phe^{433} \rightarrow$ Ala mutant. Currently, we cannot explain the reduced agonist responses at the $Phe^{433} \rightarrow$ Met mutant, although we can speculate that the flexible Met side chain prevents optimal agonist-receptor interaction by steric hindrance.

Replacing Trp^{167} with either the aromatic Phe or the aliphatic hydrophobic Met did not allow high affinity [^{3}H]mepyramine binding. Yet, the $Trp^{167} \rightarrow$ Phe receptor mutant was able to functionally interact with histamine. These data suggest that Trp^{167} is important for high affinity [^{3}H]mepyramine binding but that hydrophobicity or aromaticity *per se* is not sufficient for a proper interaction. This could be explained if Trp^{167}would be properly positioned in the binding crevice of the

[3] A. M. Ter Laak and R. Kühne, unpublished observations.

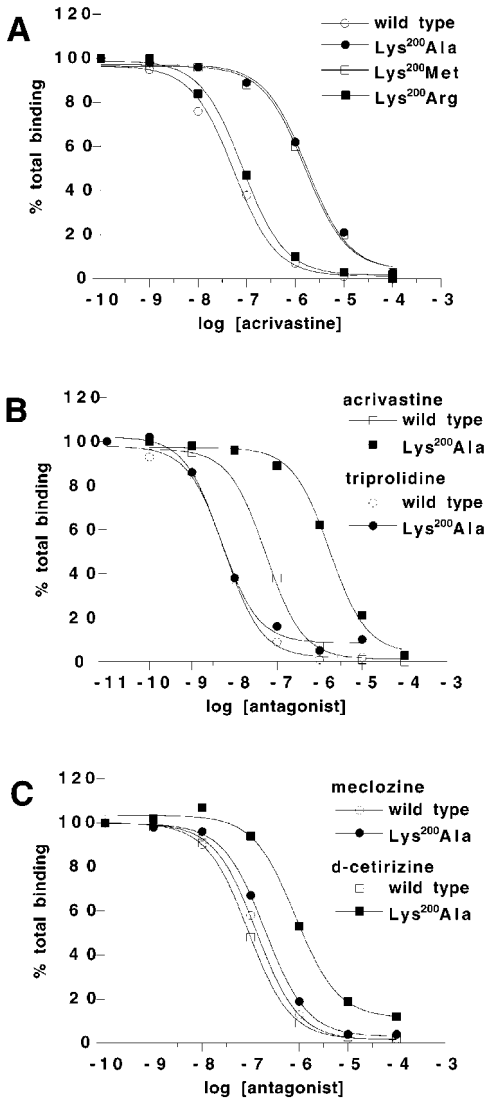


FIG. 6. **Contribution of Lys^{200} in TM V to the binding of the zwitterionic H_1 antagonists acrivastine and cetirizine and their structural analogs triprolidine and meclozine to the H_1 receptor.** *A*, the binding of [^{3}H]mepyramine to the wild type, $Lys^{200} \rightarrow$ Ala, $Lys^{200} \rightarrow$ Met, and $Lys^{200} \rightarrow$ Arg H_1 receptor was displaced by acrivastine. The binding of [^{3}H]mepyramine to the wild type and $Lys^{200} \rightarrow$ Ala H_1 receptor was displaced by acrivastine and triprolidine (*B*) and cetirizine and meclozine (C). The data shown are from a representative example out of at least three independent experiments.

H_1 receptor by an interaction of its indole nitrogen with another (yet unidentified) amino acid of the H_1 receptor.

Searching the GRAP mutant data base (31) for mutated residues at similar positions in other aminergic GPCRs, revealed considerable support for an important role of Trp^{167}, Phe^{433}, and Phe^{436} in the binding of the *trans*-aromatic ring of the H_1 antagonists. A polymorphism of the human β_2 receptor ($Thr^{164} \rightarrow$ Ile) at the position of Trp^{167} in TM IV has been reported to alter ligand binding characteristics (32). Moreover, in the dopamine D_{2L} (33), the $5HT_{2A}$ (34), and the β_2 receptor (35), a Phe residue in homologous position as Phe^{433} has been implicated in hydrophobic or π-π interactions with aromatic rings of receptor ligands. Similarly, in the muscarinic receptors an Asn residue is found at the same position as Phe^{433}, and this Asn^{507} plays a key role in the binding of various muscarinic antagonists to the m3 receptor (36). Finally, in both the dopamine D_{2L} (37) and the α_{1A} receptor (38) residues at a homologous position as Phe^{436} are also involved in ligand binding.

Because of the perturbed protein expression of the $Trp^{161} \rightarrow$ Ala receptor, no conclusive decision could be made regarding its role in the binding of H_1 antagonists. Mutation of Trp^{161} with either Phe or Met is well allowed for high affinity [^{3}H]mepyramine binding or the interaction with histamine. A Trp^{192} residue at homologous position of Trp^{161} in the m3 receptor is important for both agonist and antagonist binding (39), suggesting that Trp^{161} could be involved in an hydrophobic interaction with the *cis*-ring of the H_1 antagonists as well. However, Trp^{161} is 96% conserved throughout the GPCR family, suggesting a fundamental role in GPCR architecture (40). We can therefore not exclude the possibility that a hydrophobic amino acid at position 161 is simply essential to adopt a functional GPCR conformation.

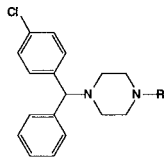
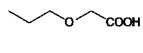
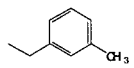
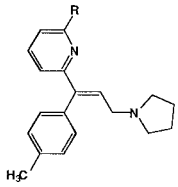
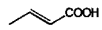
Based on the results of the site-directed mutagenesis studies we concluded that the three-dimensional H_1 receptor model had some predictive value for receptor-ligand interactions. To challenge our three-dimensional model the zwitterionic ligands acrivastine and d-cetirizine were fitted into the H_1 receptor model. Both ligands contain a carboxylate moiety in either the *trans*-ring (acrivastine) or connected with a spacer to the amine-function (d-cetirizine). Modeling studies indicated a possible interaction of the negatively charged group with the amine of Lys^{200} in TM V. The $Lys^{200} \rightarrow$ Ala H_1 receptor mutant shows normal binding of the classical H_1 antagonist [^{3}H] mepyramine (10). Our present findings strongly suggest that Lys^{200} is directly involved in the binding of the carboxylate group of acrivastine and d-cetirizine. The $Lys^{200} \rightarrow$ Ala mutation results in a 30- (acrivastine) to 10-fold (d-cetirizine) loss of affinity of the zwitterionic antagonists, whereas the affinities of acrivastine and cetirizine analogs lacking the negatively charged carboxylate group, triprolidine and meclozine, respectively, are not affected by the $Lys^{200} \rightarrow$ Ala mutation. The Lys^{200} residue was also mutated to Met, which resembles the Lys residue sterically but does not contain a protonated amine function. As predicted, a huge reduction of the affinity of acrivastine was observed for the $Lys^{200} \rightarrow$ Met receptor mutant. Moreover, a basic Arg residue is able to functionally replace $Lys^{200} \rightarrow$ Ala as shown by the high affinity of acrivastine for the $Lys^{200} \rightarrow$ Arg H_1 receptor. These data provide strong evidence for the hypothesized ionic interaction between the carboxylate of acrivastine and the protonated amine function of Lys^{200}. As observed previously for the interaction of histamine with the $Lys^{200} \rightarrow$ Ala mutant receptor (10), histamine was less responsive at the $Lys^{200} \rightarrow$ Met and $Lys^{200} \rightarrow$ Arg H_1 receptor as well. Whereas this is not surprising for the Met mutant (10), the observations with the $Lys^{200} \rightarrow$ Arg H_1 receptor indicate that the longer arginine side chain and the larger guanidinium group cannot optimally accommodate the imidazole ring of histamine. This observation will be important for the refinement of a three-dimensional model for the agonist-binding site.

In view of the emerging cardiotoxicity of several second generation H_1 antagonists (41) and the current interest to combine potent nonsedative H_1 antagonism with other anti-allergic activities (3), the identification of the role of Lys^{200} will be of importance for the design of potent "third generation" H_1 antagonists. The introduction of an carboxylate group in the structure of the H_1 antagonists has by empirical approach been found to be a very effective way to limit central nervous system penetration and to derive nonsedative H_1 antagonists like acrivastine (42) and cetirizine (43). Consequently, this structural element can also been found in other new H_1 antagonists, *e.g.* carebastine, fexofenadine, KF-15766, KW-4679, levocabastine, and pibaxizine (for review see Ref. 3). Besides leading to favorable pharmacokinetics, the carboxylate group also discriminates the receptor binding of second generation H_1 antagonists acrivastine and cetirizine from the first generation H_1 antago-

TABLE III

Structural formulas and affinities of the H_1 antagonists d-cetirizine, meclozine, acrivastine, and triprolidine for the wild type and $Lys^{200} \rightarrow Ala$ H_1 receptor

The affinities of the H_1 antagonists for the wild type and $Lys^{200} \rightarrow Ala$ receptor mutant were determined in [^{3}H]mepyramine displacement studies. Data shown are the means ± S.E. of at least three independent experiments, each performed in triplicate.

general structure	R-group	H_1 antagonist	K_i-value (nM) wild-type	K_i-value (nM) $Lys^{200}Ala$	ratio
		d-cetirizine	60 ± 8	492 ± 45	8.2
		meclozine	170 ± 35	108 ± 1	0.6
		acrivastine	10 ± 3	548± 52	54
	H	triprolidine	4 ± 0.5	2 ± 0.2	0.5

nists via its interaction with Lys^{200}. Because Lys^{200} is an unique residue for the H_1 receptor, interaction of ligands with this residue via a carboxylate group will lead, in addition to limited brain penetration, probably also to good H_1 receptor selectivity. For future design of nonsedative H_1 antagonists the introduction of a carboxylate group capable of interacting with Lys^{200} could therefore be favorable.

In conclusion, our study shows that combining known interacting amino acids of the receptor (Asp^{116}) with a pharmacophore for the H_1 antagonist-binding site and verification by site-directed mutagenesis results in the identification of Trp^{167}, Phe^{433}, and Phe^{436} in TM IV and VI as probable interaction points for the aromatic rings of H_1 antagonists. Moreover, the use of the three-dimensional receptor model allowed the identification of Lys^{200} in TM V as specific anchor point for some nonsedative, zwitterionic H_1 antagonists.

Acknowledgments—We thank Drs. Edwin Roovers and Yvonne van de Vrede for technical assistance.

REFERENCES

1. Dale, H. H., and Laidlaw, P. P. (1910) *J. Physiol.* **41,** 318–344
2. Fourneau, E., and Bovet, D. (1933) *Arch. Int. Pharmacodyn.* **46,** 178–191
3. Zhang, M. Q., Leurs, R., and Timmerman, H. (1997) in *Burger's Medicinal Chemistry and Drug Discovery: Therapeutic Agents* (Wolff, M. E., ed) Vol. 5, 5th Ed., pp. 495- 559, John Wiley & Sons, Inc., New York
4. Janssens, M. M. L., Timmerman, H., and Leurs, R. (1994) *Kirk-Othmer Encycl. Chem. Technol.* **13,** 290–311
5. Du Buske, L. M. (1996) *J. Allerg. Clin. Immunol.* **98,** (suppl.) 307–318
6. Leurs, R., Smit, M. J., and Timmerman, H. (1995) *Pharmacol. Ther.* **66,** 413–463
7. Moguilevsky, N., Varsalona, F., Guillaume, J. P., Noyer, M., Gillard, M., Daliers, J., Henichart, J. P., and Bollen, A. (1995) *J. Receptor Signal Transduc. Res.* **15,** 91–102
8. Ohta, K., Hayashi, H., Mizuguchi, H., Kagamiyama, H., Fujimoto, K., and Fukui, H. (1994) *Biochem. Biophys. Res. Commun.* **203,** 1096–1101
9. Leurs, R., Smit, M. J., Tensen, C. P., Ter Laak, A. M., and Timmerman, H. (1994) *Biochem. Biophys. Res. Commun.* **201,** 295–301
10. Leurs, R., Smit, M. J., Meeder, R., Terlaak, A. M., and Timmerman, H. (1995) *Biochem. Biophys. Res. Commun.* **214,** 110–117
11. Hibert, M. F., Trumpp-Kallmeyer, S., Hoflack, J., and Bruinvels, A. (1993) *Trends Pharmacol. Sci.* **14,** 7–12
12. Pardo, L., Ballesteros, J. A., Osman, R., and Weinstein, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4009–4012
13. Unger, V. M., Hargrave, P. A., Baldwin, J. M., and Schertler, G. F. X. (1997) *Nature* **89,** 203–206
14. Zhao, M. M., Hwa, J., and Perez, D. M. (1996) *Mol. Pharmacol.* **50,** 1118–1126
15. Wieland, K., Zuurmond, H. M., Krasel, C., Ijzerman, A. P., and Lohse, M. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9276–9281
16. Kuipers, W., Link, R., Standaar, P. J., Stoit, A. R., Van Wijngaarden, I., Leurs, R., and Ijzerman, A. P. (1997) *Mol. Pharmacol.* **51,** 889–896
17. Ter Laak, A. M., Timmerman, H., Leurs, H., Nederkoorn, P. H. J., Smit, M. J., and Donne-Op den Kelder, G. M. (1995) *J. Comp. Aid. Mol. Design.* **9,** 319–330
18. Ter Laak, A. M., Venhorst, J., Timmerman, H., and Donné-Op de Kelder, G. M. (1994) *J. Med. Chem.* **38,** 3351–3360
19. Weiner, S. J., Kollman, P. A., Nguyen, D. T., and Case, D. A. (1986) *J. Comp. Chem.* **7,** 230–252
20. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
21. Godfrey, P. (1992) in *Signal Transduction: A Practical Approach* (Milligan, G., ed) pp. 105–121, IRL Press, Oxford
22. Smit, M. J., Timmerman, H., Alewijnse, A. E., Punin, M., van den Nieuwenhof, I., Blauw, J., van Minnen, J., and Leurs, R. (1995) *Biochem. Biophys. Res. Commun.* **214,** 1138–1145
23. Fukui, H., Fujimoto, K., Mizuguchi, H., Sakamoto, K., Horio, Y., Takai, S., Yamada, K., and Ito, S. (1994) *Biochem. Biophys. Res. Commun.* **201,** 894–901
24. Fujimoto, K., Horio, Y., Sugama, K., Ito, S., Liu, Y. Q., and Fukui, H. (1993) *Biochem. Biophys. Res. Commun.* **190,** 294–301
25. Yamashita, M., Fukui, H., Sugama, K., Horio, Y., Ito, S., Mizuguchi, H., and Wada, H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 11515–11519
26. Traiffort, E., Leurs, R., Arrang, J. M., Tardivel-Lacombe, J., Diaz, J., Schwartz, J. C., and Ruat, M. (1994) *J. Neurochem.* **62,** 507–518
27. Moguilevsky, N., Varsalona, F., Noyer, M., Gillard, M., Guillaume, J. P., Garcia, L., Szpirer, C., Bollen, A., and Szpirer, J. (1994) *Eur. J. Biochem.* **224,** 489–495
28. Inoue, I., Taniuchi, I., Kitamura, D., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., and Watanabe, T. (1996) *Genomics* **36,** 178–181
29. De Backer, M. D., Gommeren, W., Moereels, H., Nobels, G., Van Gompel, P., Leysen, J. E., and Luyten, W. H. M. (1993) *Biochem. Biophys. Res. Commun.* **197,** 1601–1608
30. Nauta, W. T., Rekker, R. F., and Harms, A. F. (1968) in *Physico-chemical Aspects of Drug Action* (Ariens, E. J., ed) pp. 305–325, Pergamon, Oxford
31. Kristiansen, K., Dahl, S. G., and Edvardsen, O. (1996) *Proteins* **26,** 81–94
32. Green, S. A., Cole, G., Jacinto, M., Innis, M., and Liggett, S. B. (1993) *J. Biol. Chem.* **268,** 23116–23121
33. Cho, W., Taylor, L. P., Mansour, A., and Akil, H. (1995) *J. Neurochem.* **65,** 2105–2115
34. Choudhary, M. S., Sachs, N., Uluer, A., Glennon, R. A., Westkaemper, R. B., and Roth, B. L. (1995) *Mol. Pharmacol.* **47,** 450–457
35. Dixon, R. A. F., Sigal, I. S., and Strader, C. D. (1988) *Cold Spring Harbor Symp. Quant. Biol.* **53,** 487–497
36. Bluml, K., Mutschler, E., and Wess, J. (1994) *J. Biol. Chem.* **269,** 18870–18876
37. Woodward, R., Daniell, S. J., Strange, P. G., and Naylor, L. H. (1994) *J. Neurochem.* **62,** 1664–1669
38. Hwa, J., Graham, R. M., and Perez, D. M. (1995) *J. Biol. Chem.* **270,** 23189–23195
39. Wess, J., Nanavati, S., Vogel, Z., and Maggio, R. (1993) *EMBO J.* **12,** 331–338
40. Van Rhee, A. M., and Jacobson, K. A. (1996) *Drug Dev. Res.* **37,** 1–38
41. Zhang, M. Q. (1997) *Curr. Med. Chem.* **4,** 187–200
42. Cohen, A. F., Hamilton, M., Philipson, R., and Peck, A. W. (1985) *Clin. Pharmacol. Ther.* **38,** 381–386
43. Rihoux, J. P., and Mariz, S. (1993) *Clin. Rev. Allergy* **11,** 65–88