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A $G_{\alpha_{q/11}}$ -coupled Mutant Histamine H_1 Receptor F435A Activated Solely by Synthetic Ligands (RASSL)*

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Recently, G protein-coupled receptors activated solely by synthetic ligands (RASSLs) have been introduced as new tools to study G_{α_i} signaling *in vivo* (1, 2). Also, G_{α_s} -coupled G protein-coupled receptors have been engineered to generate G_{α_s} -coupled RASSLs (3, 4). In this study, we exploited the differences in binding pockets between different classes of H_1 receptor agonists and identified the first $G_{\alpha_{q/11}}$ -coupled RASSL. The mutant human H_1 receptor F435A (6.55) combines a strongly decreased affinity (25-fold) and potency for the endogenous ligand histamine (200-fold) with improved affinities (54-fold) and potencies (2600-fold) for 2-phenylhistamines, a synthetic class of H_1 receptor agonists. Molecular dynamics simulations provided a mechanism for distinct agonist binding to both wild-type and F435A mutant H_1 receptors.

Receptors activated solely by synthetic ligands (RASSLs)² have recently been developed as tools to control G protein signaling *in vivo* (5). RASSLs are G protein-coupled receptors (GPCRs) that no longer respond to their endogenous ligands but can still be activated by synthetic ligands (5). Given the biological importance of GPCRs, the ability to stimulate a single G protein pathway in a tissue of choice *in vivo* is useful for the understanding of subsequent changes in downstream signaling. The use of RASSLs has already provided valuable insight into the effect of G_{α_i} signaling in the heart. Stimulation of a G_{α_i} -coupled RASSL selectively expressed in the murine heart resulted in a strong (3-fold) and rapid (within 1 min after drug administration) decrease in heart rate (1). In the future, RASSLs may further be applied, *e.g.* to create reversible models of disease states, or combined with microarrays, to result in gene expression fingerprints of specific G protein pathways (for review, see Ref. 6).

There are four major classes of G proteins, named after their respective α -subunit (G_{α_s} , G_{α_i} , $G_{\alpha_{q/11}}$, and $G_{\alpha_{12}}$). Each class couples to specific signal transduction pathways, which are well characterized *in vitro* (for a review, see Ref. 7). The first described RASSL couples through G_{α_i} proteins (5) and thus far is the only RASSL being applied *in vivo* (1, 2, 8). Also, more recently, several G_{α_s} -coupled RASSLs have been developed (3, 4). Thus far, there have been no reports about either $G_{\alpha_{q/11}}$ - or $G_{\alpha_{12}}$ -coupled RASSLs.

The human histamine H_1 receptor (H_1R) couples through $G_{\alpha_{q/11}}$ proteins and thereby activates phospholipase C, resulting in, for example, inositol phosphate hydrolysis and increased concentrations of intracellular Ca^{2+} (9, 10). We previously demonstrated that the H_1 receptor also activates NF- κ B, both in a ligand-induced as well as in a constitutive manner, via $G_{\alpha_{q/11}}$ proteins (11). Although several G_{α_q} -coupled GPCRs also activate $G_{\alpha_{12}}$ (for a review, see Ref. 12), the H_1R does not seem to couple to $G_{\alpha_{12}}$ (10, 11, 13).

By mutational analysis, we previously characterized several amino acids that are involved in the binding of histamine and several synthetic agonists, such as the histaprodifens (a new class of specific H_1R agonists) (14). Mutation of Phe⁴³⁵ (6.55) to Ala in TM6 (transmembrane domain 6) resulted in a strong decrease in affinity and potency of histamine, whereas only marginally affecting affinities and potencies of histaprodifens. In this study, we showed that for another class of H_1R agonists, the substituted 2-phenyl-histamines (PheHAs, Ref. 15), mutation of F435A results in strongly increased affinities and potencies, thereby identifying the first $G_{\alpha_{q/11}}$ -coupled RASSL.

EXPERIMENTAL PROCEDURES

Materials—pNF- κ B-Luc was obtained from Stratagene (La Jolla, CA). ATP disodium salt, bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine dihydrochloride, mepyramine (pyrilamine maleate), glycerol, Triton X-100, and polyethyleneimine were purchased from Sigma. D-Luciferin was obtained from Duchefa Biochemie BV (Haarlem, The Netherlands). Cell culture media, penicillin, and streptomycin were obtained from Invitrogen. Fetal bovine serum was obtained from Integro B. V. (Dieren, The Netherlands). Cell culture plastics were obtained from Corning Costar (Corning, NY). [³H]Mepyramine (20 Ci/mmol) was purchased from ICN Biomedicals B. V. (Zoetermeer, The Netherlands). 2-(3-Chlorophenyl)histamine, 2-(3,4-dichlorophenyl)histamine, 2-(cyclohexyl)-histamine, 2-(methylcyclohexyl)-histamine, and 2-(ethylcyclohexyl)-histamine were synthesized at the Vrije Universiteit Amsterdam, The Netherlands. Gifts of 2-(3-trifluoromethyl)phenylhistamine dihydrogenmaleate (Dr. W. Schunack), pcDEF3 (Dr. J. Langer, Ref. 16), and the cDNA encoding the human H_1 receptor (Dr. H. Fukui, Ref. 17) are greatly acknowledged.

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 containing 50 IU/ml penicillin, 50 μ g/ml streptomycin, and 5% (v/v) fetal bovine serum. COS-7 cells were transiently transfected using the DEAE-dextran method as described previously (11).

Site-directed Mutagenesis—The cDNA encoding the human H_1R (17) was subcloned in the pAlter plasmid (Promega), and point mutations were created according to the manufacturer's protocol (Altered Sites® II, Promega). cDNA of all mutant and wild-type receptors were subcloned into the expression plasmid pcDEF3 (16). Mutations in the


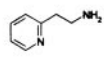
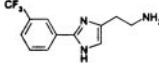
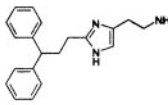
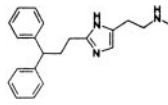
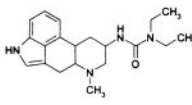
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² The abbreviations used are: RASSL, receptor activated solely by synthetic ligands; H_1R , histamine H_1 receptor; WT, wild-type; HA, histamine; PheHA, 2-phenylhistamine; ClPheHA, 2-(3-chlorophenyl)histamine; Cl₂PheHA, 2-(3,4-dichlorophenyl)histamine; dClPheHA, 2-(3,4-dichlorophenyl)histamine; CF₃PheHA, 2-(3-trifluoromethyl-phenyl)histamine; CxHA, 2-cyclohexyl-histamine; MeCxHA, 2-(methylcyclohexyl)-histamine; EthCxHA, 2-(ethylcyclohexyl)-histamine; COS-7, African green monkey kidney cells; GPCR, G protein-coupled receptors; MD, molecular dynamics.

TABLE ONE

Affinities of H₁R agonists for wild-type and Phe⁴³⁵ Ala mutant H₁Rs

Agonist affinity (pK _i) ^a						
Histamine	Pyridylethylamine	2-(3-trifluoromethyl-phenyl)histamine	Histaprodifen	Suprahistaprodifen	8R-Lisuride	
						
WT	4.1 ± 0.1	3.8 ± 0.1	5.0 ± 0.1	5.6 ± 0.1	5.8 ± 0.1	7.1 ± 0.1
Phe ⁴³⁵ Ala	2.7 ± 0.1 ^c	3.6 ± 0.1	6.8 ± 0.1 ^c	5.3 ± 0.1 ^b	5.5 ± 0.1 ^b	6.7 ± 0.1

^a Affinities are determined by [³H]mepyramine displacement. All data are calculated as the mean ± S.E. of at least three experiments, each performed in triplicate.

^b *p* < 0.01.

^c *p* < 0.001 vs. WT H₁R.

cDNA were verified by DNA sequencing using the dideoxy chain termination method.

NF-κB Reporter Gene Assay—Cells transiently co-transfected with pNF-κB-Luc (125 μg/1 · 10⁷ cells) and pcDEF3 containing mutant or wild-type human H₁R cDNA (25 μg/1 · 10⁷ 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 MgCl₂, 0.78 μM Na₂H₂P₂O₇, 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 Victor² (Wallac).

Histamine H₁R Binding Studies—The transfected COS-7 cells used for radioligand binding studies were harvested after 48 h and homogenized in ice-cold 50 mM Na₂/K phosphate buffer (pH = 7.4) (binding buffer). The COS-7 cell homogenates were incubated for 30 min at 30 °C in H₁R binding buffer in 200 μl with 3 nM [³H]mepyramine. The nonspecific binding was determined in the presence of 1 μM mianserin. The incubations were stopped by rapid dilution with 3 ml of ice-cold H₁R binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filters that had been treated with 0.3% polyethylenimine. Filters were washed twice with 3 ml of H₁R binding buffer, and radioactivity retained on the filters was measured by liquid scintillation counting. Binding data were evaluated by a non-linear, least squares curve-fitting procedure using GraphPad Prism[®] (GraphPad Software, Inc., San Diego, CA).

Analytical Methods—Protein concentrations were determined according to Bradford (18), using bovine serum albumin as a standard. All data shown are expressed as means ± S.E. Statistical analyses 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).

Molecular Modeling—Our H₁R homology model was obtained using the bovine rhodopsin crystal structure (Protein Data Bank entry 1L9H, Ref. 19) as the template. The third intracellular loop that connects transmembranes 5 and 6 was omitted due to its large size. Side chains were added using the homology module of InsightII (20). Water molecules present in the crystal structure were not incorporated. The initial model was refined by a steepest descent energy minimization. The minimized model was placed in a dodecahedral box filled with simple point charge water (21), and a second minimization step using steepest descent was performed. Hereafter, a 20-ps molecular dynamics (MD) run was performed with positions restraints (1000 kJ · mol⁻¹ nm⁻²) on all heavy protein atoms. Finally, the model was refined by a 100-ps MD run. In

this structure, Phe⁴³⁵ was changed to Ala with InsightII, thus creating a model of the F435A H₁R.

Histamine and 2-(3-chlorophenyl)histamine (ClPheHA) were docked in the WT and F435A models using AutoDock 3.0.0 (22) applying default parameters. The monocationic ligands were assigned Löwdin atomic charges obtained after a single point *ab initio* restricted Hartree-Fock calculation using 6-31Gr* with the GAMESS US package (23). The protein was assigned with KOLLUA partial charges using the SYBYL program (version 6, Tripos, St. Louis). Only essential hydrogens were taken into account.

The obtained ligand-receptor complexes were minimized using the steepest descent method. These ligand-receptor complexes were placed in a dodecahedral box, with a minimum distance of 7 Å between protein and the box. The system was solvated with simple point charge water, and the protein partial charges were assigned by GROMACS.

Again, a steepest descent procedure was performed on the system. Subsequently, a set of MD runs with position restraints (1000 kJ · mol⁻¹ nm⁻²) was applied to the system in which a controlled release of the restraints was performed. A run for 50 ps with position restraints on all heavy protein atoms and all ligand atoms was performed, with the protein hydrogens and all water molecules unrestrained. Consecutively, a run was performed for 20 ps with position restraints on all Cα-atoms, all heavy atoms of residues Asp¹⁰⁷ and Asn¹⁹⁸, and all ligand atoms. Finally, a MD run was performed for 1 ns with only position restraints on Cα-atoms. During the last 500 ps of this run, the presence of hydrogen bonds was analyzed using cutoff distance between heavy atoms of 3.5 Å and a cutoff angle between acceptor donor and hydrogen of 60°.

All minimizations and MD simulations were performed using the GROMACS software package and the GROMOS 43a1 force field (24, 25) and LINCS constraints (26) on all bonds. An NPT ensemble was generated using both the Berendsen thermostat and the barostat with default settings for 300 K and 1 bar (25).

RESULTS

Mutation F435A Differentially Modulates Affinities of Several Classes of H₁R Agonists—At the H₁R, several H₁R agonists show a higher affinity than the natural agonist histamine. The histamine analogs 2-(3-trifluoromethyl-phenyl)histamine (CF₃PheHA), histaprodifen, suprahistaprodifen, and the newly characterized H₁R agonist 8R-lisuride bind 9–1000-fold better to the H₁R as compared with histamine (TABLE ONE). Previously, we characterized Phe⁴³⁵ (6.55), as a specific interaction point for histamine (14). Mutation of this residue into alanine resulted in a 25-fold decrease in histamine affinity without drastic alterations in the affinity of the agonists histaprodifen and suprahistaprodifen

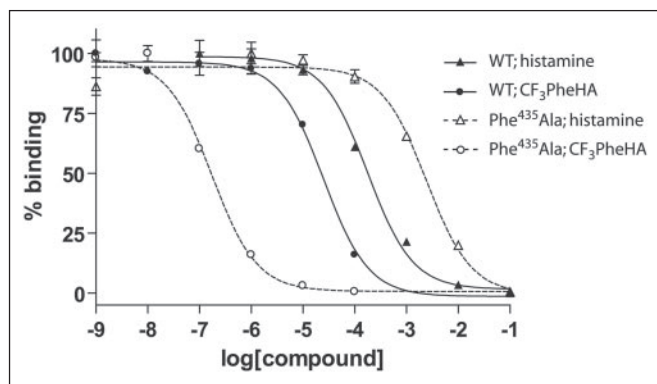


FIGURE 1. Displacement of [³H]mepyramine binding to wild-type H₁R (closed symbols, solid lines) and to mutant receptor H₁R F435A (open symbols, dotted lines) receptors by histamine (triangles), and 2-(3-trifluoromethyl-phenyl)histamine (CF₃PheHA, circles). A representative experiment is shown.

(TABLE ONE) (Ref. 14). In this study, we further studied the role of Phe⁴³⁵ (6.55) in agonist binding to the H₁R. TABLE ONE shows that, similar to histaprodifen and suprahistaprodifen, affinities of pyridylethylamine as well as 8R-lisuride are comparable for WT and F435A mutant H₁Rs. Unexpectedly, for F435A H₁R, the affinity of CF₃PheHA was increased 54-fold as compared with WT H₁R (Fig. 1 and TABLE ONE). Thus, the F435A mutant H₁R has an affinity for CF₃PheHA that exceeds the affinity of histamine more than 10,000-fold.

Basic Characterization of Phe⁴³⁵ Mutant H₁Rs—Phenylalanine at position 6.55 is conserved among all known H₁Rs (27) and is implicated in ligand binding in both human and guinea pig H₁Rs (14, 28). The F435A mutation removes an aromatic ring structure from the binding pocket, and therefore, potential interactions with either ligands or other amino acids in the H₁R-binding pocket may be lost. However, exchanging the bulky Phe⁴³⁵ (6.52) with the smaller alanine may also increase the size of the ligand-binding pocket and thus create space for the trifluoromethyl-phenyl moiety of CF₃PheHA, resulting in an increased affinity for this ligand. To address the latter possibility, we created additional mutant H₁Rs (F435V and F435L) that vary in the size of the side chain at position 6.55.

The generated mutant receptors are expressed at comparable levels ($B_{\max} = 13.6$ – 20.1 pmol/mg of protein) in transiently transfected COS-7 cells and bind the H₁R radioligand [³H]mepyramine with virtually unchanged affinity ($K_d = 1.5$ – 5.7 nM), as determined by radioligand saturation binding experiments (TABLE TWO). Although the expression levels are similar, there is a clear difference in constitutive GPCR activities of the tested H₁R mutants as measured by an NF- κ B-driven reporter gene assay (Fig. 2, white bars). Although the basal activity of H₁R F435L H₁R is comparable with the WT H₁R, the level of constitutive NF- κ B activation of H₁R F435V is twice as high, whereas for mutant H₁R, F435A constitutive activity is hardly detectable. Although the levels of constitutive NF- κ B activation vary between the mutant H₁Rs, their agonist-induced responses are comparable (Fig. 2, black bars), indicating that G protein-coupling of the mutant H₁Rs is not impaired.

Binding Analysis of WT and Phe⁴³⁵ Mutant H₁Rs—Similar to the mutant F435A H₁R ($pK_i = 2.7$), the mutant H₁R F435V ($pK_i = 2.9$) and F435L ($pK_i = 2.9$) exhibit a strong decrease in their affinity for histamine as compared with the WT receptor ($pK_i = 4.1$) (TABLE THREE). Again, these mutant receptors exhibit an increased affinity for CF₃PheHA. The exchange of Phe⁴³⁵ for leucine, valine, or alanine results in mutant H₁Rs with a 47-, 124-, and 54-fold increased affinity for CF₃PheHA respectively, as compared with the WT receptor ($pK_i = 5.0$) (TABLE THREE). For these mutant H₁Rs, the affinity of CF₃PheHA greatly exceeds their

affinity of the endogenous ligand histamine, the mutant F435V H₁R exhibiting the greatest selectivity for the synthetic agonist CF₃PheHA over histamine (17,000-fold).

We tested two other substituted PheHAs, ClPheHA and 2-(3,4-dichlorophenyl)histamine (Cl₂PheHA), on the mutant H₁Rs as well. For the WT H₁R, the affinity of ClPheHA ($pK_i = 5.3$) is slightly higher than that of CF₃PheHA ($pK_i = 5.0$). An (additional) *para*-chloro substituent is not favorable for H₁R affinity ($pK_i = 4.6$), fitting previous findings for both human and rodent H₁Rs (29). Similar to CF₃PheHA, the affinity of ClPheHA is increased upon mutation of Phe⁴³⁵ into leucine ($pK_i = 6.8$), valine ($pK_i = 7.3$), or alanine ($pK_i = 6.7$). Although for Cl₂PheHA, H₁R affinities are lower and increases in affinity are smaller, the same trend upon mutation is observed (pK_i values, 4.6, 5.3, 5.8, and 5.4, respectively). Similar to CF₃PheHA, for ClPheHA and Cl₂PheHA, mutation F435V also results in the largest increase in affinity, as compared with the WT receptor.

Additionally, we tested a series of 2-cyclohexyl-histamines (CxHAs), varying in spacer length between the imidazole ring and the cyclohexyl moieties: CxHA, 2-(methyl-cyclohexyl)-histamine (MeCxHA), and 2-(ethyl-cyclohexyl)-histamine (EthCxHA). The affinities of the CxHAs are approximately equal to the affinity of histamine (TABLE THREE). Again, mutation of Phe⁴³⁵ into alanine, valine, or leucine results in increased affinities as compared with the WT H₁R (TABLE THREE). The increases in affinity, however, are smaller (maximum 10-fold) than those observed for the PheHAs (maximum 120-fold). Although changes in affinity are less prominent, for EthCxHA, we observe a correlation between the space available in the binding pocket (Ala \rightarrow Val \rightarrow Leu \rightarrow Phe) and the affinities for the mutant receptors (F435A, pK_i 4.9; F435V, $pK_i = 4.6$; F435L, $pK_i = 4.3$; and WT, $pK_i = 4.1$, TABLE THREE). For both CxHA and MeCxHA, the highest affinities are observed for the F435V mutant H₁R, following the trend observed for the PheHAs.

Functional Analysis of WT and Phe⁴³⁵ Mutant H₁Rs—Using an NF- κ B-driven reporter gene assay, we evaluated the potencies of several 2-phenylsubstituted histamine analogs for mutant and WT H₁Rs. For the WT H₁R, these analogs are less potent than the endogenous ligand histamine ($pEC_{50} = 6.9$) (TABLE THREE). Fig. 3 clearly illustrates that as compared with the WT H₁R, the potency of HA at the H₁R F435A is decreased 200-fold, whereas the potency of ClPheHA is increased 2,600-fold. Also, for CF₃PheHA and Cl₂PheHA, potencies were increased upon the mutation F435A; again ClPheHA ($pEC_{50} = 9.4$) and CF₃PheHA ($pEC_{50} = 9.2$) are equipotent, and Cl₂PheHA is clearly less potent ($pEC_{50} = 7.9$) (TABLE THREE). For mutant H₁Rs F435L and F435V, similar trends are observed, with PheHA potencies for H₁R F435V being slightly higher than for F435A and F435L H₁Rs. For mutant H₁Rs, F435A, F435V, and F435L ClPheHA and CF₃PheHA are agonists with subnanomolar potencies, which exceed the potency of histamine 70,000–280,000-fold.

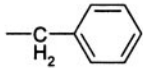
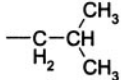


Molecular Modeling of WT and F435A Mutant H₁Rs—Our model of the H₁R, based on the bovine rhodopsin crystal structure, was used to visualize the interactions of either histamine or ClPheHA with the WT and F435A mutant H₁R. Models of both WT and F435A H₁Rs were optimized by molecular dynamics simulations. Hereafter, histamine and ClPheHA were automatically docked in both WT and F435A mutant H₁R models. Finally, a second round of molecular dynamics was used to optimize the receptor-ligand complexes. In the WT H₁R, both histamine and ClPheHA show an ionic interaction between the conserved Asp¹⁰⁷ (3.32) in TM3 (transmembrane domain 3) and their ethylamine group over distances of 2.6 and 3.0 Å, respectively (Fig. 4, A and C). As expected, differences between histamine and ClPheHA can be observed for interaction with Phe⁴³⁵. In binding histamine, Phe⁴³⁵ has

Mutant H₁R F435A, a G_{q/11}-coupled RASSL

TABLE TWO

Characteristics of [³H]mepyramine binding to WT and mutant H₁R

The values are determined by saturation radioligand binding assays. All data are calculated as the mean ± S.E. of at least three experiments, each performed in triplicate.

	Amino acid	K _d [³ H]mepyramine (nM)	H ₁ receptor density (pmol/mg protein)
WT		1.6 ± 0.1	20.1 ± 6.7
Phe ⁴³⁵ Leu		1.5 ± 0.1	15.0 ± 3.7
Phe ⁴³⁵ Val		1.7 ± 0.1	22.0 ± 7.5
Phe ⁴³⁵ Ala		5.7 ± 1.0 ^a	13.6 ± 6.4

^a *p* < 0.01 vs. WT receptor.

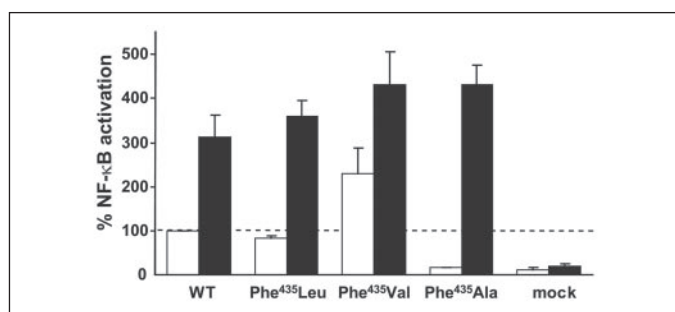


FIGURE 2. Basal H₁R activation (white bars) and maximal histamine-induced NF-κB activation (black bars) in COS-7 cells transfected with cDNA of WT, mutant H₁Rs, or empty vector (mock). Basal activity of WT H₁R is put at 100%. Values are determined by NF-κB-driven reporter gene assays.

an indirect role, keeping Phe⁴³² in an edge-to-face orientation toward the imidazole moiety of histamine (Fig. 4A). In this orientation, a hydrogen bond can be formed between the distal nitrogen of the imidazole ring of histamine and Asn¹⁹⁸ at a distance of 2.7 Å and is observed during 47% of the MD simulation. In binding ClPheHA, Phe⁴³⁵ directly interacts with the ligand by π-π stacking with the phenyl moiety of ClPheHA (Fig. 4B). By this interaction, Phe⁴³⁵ prevents hydrogen bonding of the imidazole ring with Asn¹⁹⁸ since the distance exceeds 5 Å.

The difference in interaction with Phe⁴³⁵ between histamine and ClPheHA is further illustrated by observed changes in receptor-ligand interactions upon the mutation F435A. For histamine, the mutation F435A releases Phe⁴³², thereby allowing histamine to shift toward Asp¹⁰⁷, resulting in an increased distance between the agonist and Asn¹⁹⁸ (7.0 Å) and the loss of the hydrogen bond between Asn¹⁹⁸ and the imidazole ring of histamine (Fig. 4C). Upon the mutation F435A, we observed an opposite effect for ClPheHA; removal of the π-π stacking with Phe⁴³⁵ allows the ligand to move deeper in the binding pocket, enabling hydrogen bonding of the imidazole ring with Asn¹⁹⁸ at a distance of 2.4 Å. This hydrogen bond is observed during 78% of the MD simulation (Fig. 4D).

DISCUSSION

Most H₁R agonists are structurally closely related to histamine and have the imidazole ring either replaced by other heterocycles (pyridylethylamine, thiozylethylamine) or substituted at the 2-position (histaprodifens, PheHAs) (30). Already in 1994, we demonstrated that

H₁R agonists, although structurally similar, appear to have different binding modes (31). Recently, we extended this conclusion to histaprodifens. In contrast to histamine, for this class of compounds, interactions with either Asn¹⁹⁸ or Phe⁴³⁵ are no longer required for high affinity H₁R binding (14). Asn¹⁹⁸, however, still appeared crucial for receptor activation. To establish whether the interaction with Phe⁴³⁵ is specific to histamine alone, we tested several other H₁R agonists for interaction with Phe⁴³⁵. Comparable with histaprodifen, the affinities of suprahistaprodifen, pyridylethylamine, and 8R-lisuride were not altered by the mutation F435A. For CF₃PheHA however, the affinity for H₁R F435A was 54-fold higher than for the WT H₁R. Studying receptor-mediated NF-κB activation, the differences in potency of CF₃PheHA between WT and F435A mutant receptors (1,500-fold) even exceeded the observed differences in affinity. Similar trends are observed for the 3-chloro-substituted PheHA (ClPheHA) and to a lesser extent for 3,4-dichloro-substituted PheHA (dClPheHA). These observations strongly suggested that despite structural (histamine-like moiety) and/or functional similarity (agonism), the precise orientation of H₁R agonists within the H₁R-binding pocket differs.

We used computer modeling to examine the different H₁R binding modes for histamine and the synthetic agonist ClPheHA. For histamine, we observed hydrogen bonding between one of the imidazole nitrogens and Asn¹⁹⁸, whereas for ClPheHA, this interaction is not observed in our molecular dynamics simulations. We previously demonstrated that interaction with Asn¹⁹⁸ appears to be a prerequisite for H₁R activation (14). This explains why although the affinity of histamine for the WT H₁R is lower than that of ClPheHA, histamine is more efficacious as an H₁R agonist.

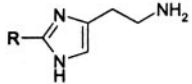






Our computational studies suggested that upon mutation of Phe⁴³⁵ to alanine, the aromatic interaction of Phe⁴³² with the imidazole ring of histamine is lost. Histamine gained in conformational freedom (entropy) and the distance between Asn¹⁹⁸ and the imidazole nitrogen of histamine increased to 7 Å, suggesting the loss of hydrogen bonding. The loss of this interaction and the increase in entropy most likely accounted for the observed decreased in affinity and potency of histamine (as compared with the WT H₁R). In contrast, for ClPheHA, the distance between its imidazole moiety and Asn¹⁹⁸ was decreased in the F435A H₁R, enabling hydrogen bonding and thus facilitating both high affinity binding and potent activation of the F435A H₁R.

To delineate the effect of mutating the bulky, aromatic Phe⁴³⁵ to a

TABLE THREE

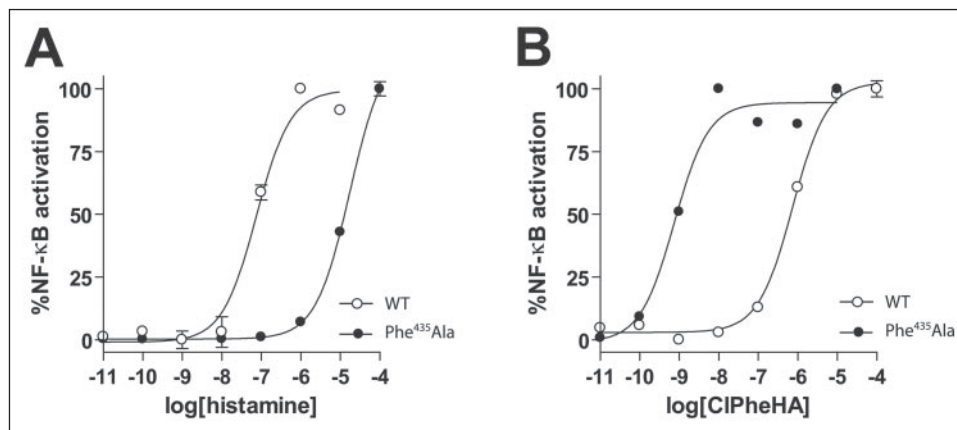
Characterization of H₁R agonists on wild-type and mutant H₁Rs

Potencies of agonists (pEC₅₀) are determined by an NF-κB-driven reporter gene assay. Affinities are determined by [³H]mepyramine displacement. All data are calculated as the mean ± S.E. of at least three experiments, each performed in triplicate. ND indicates that the value could not be determined.

	R	WT		Phe ⁴³⁵ Leu		Phe ⁴³⁵ Val		Phe ⁴³⁵ Ala	
		pK _i	pEC ₅₀	pK _i	pEC ₅₀	pK _i	pEC ₅₀	pK _i	pEC ₅₀
histamine	-	4.1 ± 0.1	6.9 ± 0.1	2.9 ± 0.1 ^c	4.5 ± 0.1 ^c	2.9 ± 0.1 ^c	4.4 ± 0.2 ^c	2.7 ± 0.1 ^c	4.6 ± 0.2 ^c
2-(3-trifluoromethyl-phenyl)histamine		5.0 ± 0.1	6.0 ± 0.1	6.7 ± 0.1 ^c	9.2 ± 0.1 ^c	7.1 ± 0.1 ^c	9.6 ± 0.1 ^c	6.8 ± 0.1 ^c	9.2 ± 0.1 ^c
2-(3-chlorophenyl)histamine		5.3 ± 0.1	6.2 ± 0.1	6.8 ± 0.1 ^c	9.5 ± 0.2 ^c	7.3 ± 0.1 ^c	9.8 ± 0.1 ^c	6.7 ± 0.1 ^c	9.4 ± 0.1 ^c
2-(3,4-dichlorophenyl)histamine		4.6 ± 0.1	5.0 ± 0.1	5.3 ± 0.1 ^c	7.3 ± 0.2 ^c	5.8 ± 0.1 ^c	8.3 ± 0.1 ^c	5.4 ± 0.1 ^c	7.9 ± 0.1 ^c
2-(cyclohexyl)-histamine		3.8 ± 0.1	ND	4.2 ± 0.1 ^c	ND	4.9 ± 0.1 ^c	ND	4.6 ± 0.1 ^c	ND
2-(methylcyclohexyl)-histamine		3.9 ± 0.1	ND	4.3 ± 0.1 ^c	ND	4.9 ± 0.1 ^c	ND	4.7 ± 0.1 ^c	ND
2-(ethylcyclohexyl)-histamine		4.1 ± 0.1	ND	4.3 ± 0.1 ^a	ND	4.6 ± 0.1 ^b	ND	4.9 ± 0.1 ^c	ND

^a *p* < 0.05.^b *p* < 0.01.^c *p* < 0.001 vs. WT receptor.

FIGURE 3. Representative dose response curves of histamine (A) and CIPheHA (B) for wild-type H₁R (○) and mutant receptor H₁R F435A (●) as measured by NF-κB activation.



small, aliphatic alanine, mutant H₁R_s were created in which the side chain at this position have intermediate sizes: Phe⁴³⁵ → Leu → Val → Ala. For none of the PheHAs did we observe gradual size-dependent changes in affinity or potency. Instead, increased affinities and potencies appeared an all-or-nothing phenomenon, correlating with the presence or absence of the aromatic Phe⁴³⁵. The various mutations also affected affinities of MeC_xHA and C_xHA, in a similar but attenuated fashion. Since we did observe a small but clear correlation between affinity and side chain bulk at position 6.55 for EthC_xHA, having an ethyl chain as a spacer between histamine and cyclohexyl moieties, we speculated that only for this ligand, the space in binding pocket is limiting over the full array of receptors. Potentially, increasing the spacer length between histamine moiety and (substituted) phenyl ring would also result in PheHAs that can discriminate between the various mutant H₁R_s.

RASSLs are GPCRs that no longer respond to endogenous ligands but can still be activated by synthetic ligands (5). Previously, such mutant GPCRs have been introduced as new tools to selectively study specific G

protein pathways *in vivo* (1). So far, RASSLs for both G_i and G_s pathways have been described (3–5). For mutant receptors F435A, F435V, and F435L, the affinity and potency of histamine are strongly decreased as compared with the WT H₁R, whereas the affinities and potencies of PheHAs are strongly increased (TABLE THREE). These mutant receptors can therefore be classified as the first G_{α_{q/11}}-coupled RASSLs. It is noteworthy that these mutant H₁R_s differ in their level of constitutive NF-κB activation. The constitutive activity of H₁R F435L was similar to that of the WT H₁R, whereas it is hardly detectable for H₁R F435A and increased for H₁R F435V. Since mutant receptors F435L and F435V displayed basal NF-κB activation, they are not “solely activated by synthetic ligands,” and therefore, only H₁R F435A fits the criteria of RASSL.

It is important to notice that ligand-independent constitutive activity of RASSLs can be apparent *in vivo*. Chronic overexpression of Ro1, a G_{α_i}-coupled RASSL, in transgenic mice caused mortality, independent of stimulation with the synthetic agonist (2). This mortality could be blocked by treatment with either the specific G_i-blocker pertussis toxin

Mutant H₁R F435A, a G_{q/11}-coupled RASSL

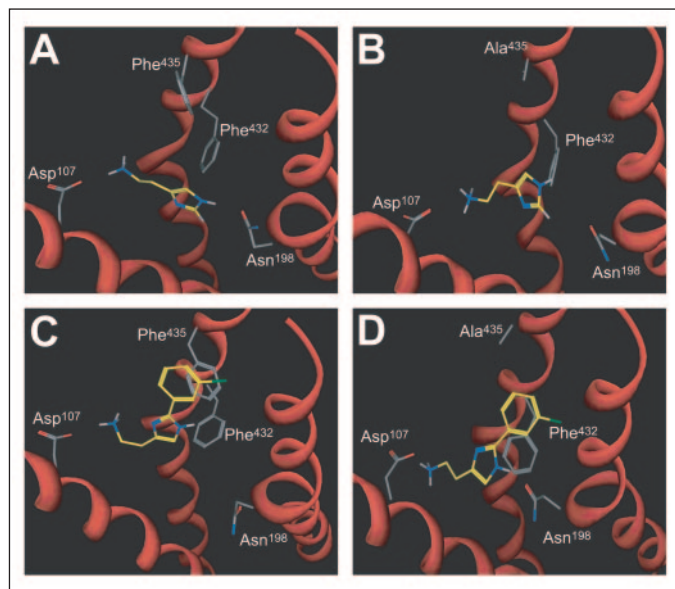


FIGURE 4. Representative snapshots of a 1000-ps molecular dynamics run of the ligand-binding pocket of WT (A and C) or F435A H₁R (B and D) with either histamine (A and B) or 2-(3-chlorophenyl)histamine (C and D).

or the Ro1 antagonist nor-binaltorphimine (2). Apparently, constitutive Ro1-signaling was accountable for this mortality, and this receptor was not a real RASSL. For the F435A-RASSL, we believe that no constitutive activity-related side effects can be expected since, even at receptor expression levels exceeding 10 pmol/mg, no basal NF- κ B signaling is observed using a sensitive reporter gene assay. Besides our G $\alpha_{q/11}$ -coupled RASSL (H₁R F435A), H₁R F435L may also be useful for future *in vivo* use. When replacing the endogenous H₁R, it would create a knock-out system, displaying the H₁R-like constitutive signaling but lacking histamine-induced responses. One would, however, still be able to induce H₁R responses by administration of synthetic agonists.

The *in vivo* use of the RASSL Ro1 has generated considerable knowledge on the effect of G α_q signaling on heart rate (1) and cardiomyopathy (2). Currently, the Ro1 has also been expressed in other tissues of transgenic mice, including several brain areas, to further address the effects of G_i signaling (6). At this moment, much less is known about *in vivo* effects of G $\alpha_{q/11}$ stimulation. Our current G $\alpha_{q/11}$ -coupled RASSL may be employed to fill that gap of *in vivo* knowledge. Although the H₁R RASSL can still be stimulated by the endogenous ligand histamine at high concentrations, plasma levels of histamine in healthy humans, rats, or rabbits are well below that level (2–250 nM) (32–35). When *in vivo* application is limited to tissues in which histamine concentrations are not exceptionally high, potentially excluding, for example, brain (synaptic histamine) and immune system (mast cell contents), the H₁R RASSL is expected to remain quiescent until stimulation with synthetic agonists. Future research should focus on further modifications of our RASSL to make it even less responsive to histamine.

In conclusion, we exploited subtle differences in the binding pockets for different classes of H₁R agonists and showed that the precise orientation of histamine and 2-phenylhistamine in the binding pocket is quite different. As a result, we have thus identified H₁R F435A (6.55) as the first G $\alpha_{q/11}$ -coupled RASSL. This mutant H₁R combines strongly decreased affinities and potencies for the endogenous ligand histamine with improved affinities and potencies for PheHAs, a synthetic class of H₁R agonists. In contrast to mutant H₁Rs F435V and F435L, this recep-

tor lacks constitutive activity, which is a characteristic, we believe, that should be emphasized more strongly in the development of future RASSLs.

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