Delineation of Agonist Binding to the Human Histamine H_4 Receptor Using Mutational Analysis, Homology Modeling, and ab Initio Calculations

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Received December 25, 2007

A three-dimensional homology model of the human histamine H_4 receptor was developed to investigate the binding mode of a series of structurally diverse H_4-agonists, i.e. histamine, clozapine, and the recently described selective, nonimidazole agonist VUF 8430. Mutagenesis studies and docking of these ligands in a rhodopsin-based homology model revealed two essential points of interactions in the binding pocket, i.e. Asp3.32 and Glu5.46 (Ballesteros-Weinstein numbering system). It is postulated that Asp3.32 interacts in its anionic state, whereas Glu5.46 interacts in its neutral form. The hypothesis was tested with the point mutations D3.32N and E5.46Q. For the D3.32N no binding affinity toward any of the ligands could be detected. This is in sharp contrast to the E5.46Q mutant, which discriminates between various ligands. The affinity of histamine-like ligands was decreased approximately a 1000-fold, whereas the affinity of all other ligands remained virtually unchanged. The proposed model for agonist binding as well as ab initio calculations for histamine and VUF 8430 explain the observed differences in binding to the H_4R mutants. These studies provide a molecular understanding for the action of a variety of H_4 receptor−ligands. The resulting H_4 receptor model will be the basis for the development of new H_4 receptor−ligands.

INTRODUCTION

G protein-coupled receptor (GPCR) proteins constitute a large and functionally diverse family of transmembrane proteins that play fundamental roles in the transfer of extracellular stimuli to intracellular signals. They are well-established drug targets, and it is estimated that between 25 and 60% of the currently marketed drugs target GPCRs.[1,2] The biogenic amine receptors form the predominant targeted subfamily of GPCRs. Among these, the histamine receptors have attracted considerable interest as therapeutic targets. Until 2000, only three histamine receptors were known to exist. However, with the unraveling of the human genome, a fourth histamine receptor was identified at the end of 2000 by Oda et al.[3] and later independently confirmed by other groups.[4–6] The histamine H_4 receptor (H_4R) is mainly expressed in bone marrow, eosinophils, and mast cells. Activation of H_4R leads to e.g. chemotaxis of eosinophils and mast cells[7,8] and mediates the production of IL-16 and leukotriene B_4.[9,10] These observations suggest a role for the H_4R in inflammatory diseases, such as asthma and allergy, and thus makes it an attractive target for drug development.[11] The discovery of the first series of H_4 receptor selective antagonists has been described providing evidence for in vivo anti-inflammatory activity.[12–14] To explore the true potential of H_4R ligands, highly potent as well as selective ligands with a variety of scaffolds will be needed. Drug discovery efforts will greatly benefit from a molecular understanding of the interaction of selected ligands with the H_4R. Initial models for the interaction of histamine with the H_4R have been reported by Shin et al.[15] and Kiss et al.[16] Shin et al.[15] manually docked histamine in the H_4R binding pocket with the amine function interacting with Asp94 in TM3 (Asp3.32, according to the Ballesteros-Weinstein numbering system[17]) and the imidazole ring N−H interacting with Glu182 (Glu5.46) in transmembrane domain (TM) 5. Yet, the recent in silico work of Kiss suggests an alternative binding mode and let the authors conclude that more experimental investigations to validate the H_4R-histamine interaction are needed.[16]

GPCRs consist of 7 transmembrane (TM) α-helical domains connected by loops of variable length. So far, GPCR models have been mainly based on the structural information originating from the X-ray structures of bovine rhodopsin[18] in the ground state. The recently elucidated structure of the photoactivated rhodopsin by Salom and co-workers[19] showed high similarity with the X-ray structure of rhodopsin in its ground state,[18] and no major rearrangements of TM helices were observed. Therefore, as the X-ray structure of rhodopsin in the ground state has meanwhile been refined to a significantly higher resolution[20,20] (2.8 Å vs 4.15 Å), this structure has been often used as an appropriate and acceptable starting point for studying agonist−receptor complexes. In the present study we developed a 3D protein homology model for the human histamine H_4R on the basis of this higher resolution X-ray structure of rhodopsin. Docking

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10.1021/ci700474a CCC: $40.75
Published on Web 06/14/2008

studies with various H_4R agonists were supported by mutagenesis studies and the use of a recently developed radioligand [^{3}H]JNJ 7777120, besides [^{3}H]histamine.[12] Moreover, ab initio calculations for histamine and the H_4 receptor selective agonist VUF 8430[21] explain the large differences in binding energy between the wild-type and mutant H_4R and substantiate the proposed binding poses of the various ligands within the H_4R binding pocket.

During the preparation of this manuscript, a crystal structure of the human β_2 adrenergic GPCR was reported.[22] The data show the overall structure to be similar to the bovine rhodopsin crystal structure. However, the β_2 receptor shows a more open structure, especially in the lower ends of helices 3 and 6. The authors suggest that this feature could be the basis for the observed basal activity observed for many GPCRs. This structural feature as well as the interactions between helix 6 and the intracellular loop 2 could become a cornerstone in the true understanding of GPCR activation. As the current paper deals with recognition of endogenous as well as synthetic ligands and does not describe the dynamics of activation, the presented models based upon bovine rhodopsin are in our opinion still valid and provide important insights into protein−ligand recognition.

METHODS

Materials. Dulbecco's modified Eagle medium (DMEM and penicillin and streptomycin were purchased from Life Technologies (Merelbeke, Belgium), fetal bovine serum was purchased from Integro BV (The Netherlands), and cell culture plastics were purchased from Greiner Bio-one (Wemmel, Belgium). Tris base was purchased from AppliChem (Darmstadt, Germany), and linear 25-kDa polyethyleneimine (PEI) was purchased from Polyscience, Inc. (U.S.A.). Histamine dihydrochloride, clozapine, and 750 kDa PEI were purchased from Sigma (U.S.A.), while VUF 8430 (guanidinylethyl isothiourea) and JNJ 7777120 were synthesized at the Department of Medicinal Chemistry, Vrije Universiteit Amsterdam. [^{3}H]Histamine (18.10 Ci/mmol) and 96-well GF/C plates were purchased from Perkin-Elmer Life Science, Inc. (U.S.A.). [^{3}H]JNJ 7777120 was a kind gift from Dr. R. L. Thurmond (Johnson & Johnson Pharmaceutical Research and Development, San Diego, U.S.A.). Oligonucleotides primers for PCR were synthesized by Isogen Bioscience (Maarsen, The Netherlands). Endonuclease restriction enzymes, T_4 DNA ligase, and *Pfu* DNA polymerase were purchased from MBI Fermentas (Germany).

Site-Directed Mutagenesis. The human H_4R cDNA cloned in pcDNA3.1 was purchased from Guthrie cDNA resource center (Sayre, U.S.A.) and subcloned into a mammalian expression vector pcDEF3 (a gift from Dr. J. Langer).[23] The site-directed mutagenesis was performed with the fusion PCR method using oligonucleotide primers containing the indicated mutations. The mutants were verified by sequencing analysis, which was done at ServiceXS (Leiden, The Netherlands).

Cell Culture and Transfection. HEK 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU/mL of penicillin, and 50 μg/mL of streptomycin in 5% CO_2 humidified atmosphere at 37 °C. For transfection, approximately 4 million cells were seeded in a 10-cm dish and cultured overnight. For transfection of each dish of cells, the transfection mixture was prepared in 0.5 mL of serum-free DMEM and contained 5 μg of receptor plasmid and 25 μL of 1 mg/mL 25 kDa linear PEI. The mixture was incubated for 10−15 min at room temperature (RT) before it was added into HEK 293T cell monoloyer culture loaded with 5 mL of fresh cell culture medium. Two days after transfection, the transfected cells were detached from the plastic surface using 5 mL/plate of PBS containing 1 mM EDTA. The cells were then collected as pellets by centrifuging at 200*g* for 3 min at 22 °C and stored at −20 °C until use.

Radioligand Binding Assay. The radioligand binding study was performed using homogenized H_4R-transfected cells in a binding buffer containing 50 mM Tris-HCl, pH 7.4. Saturation binding assay was performed using different concentrations of [^{3}H]JNJ 7777120 (18.10 Ci/mmol), while nonspecific binding was determined by incubation in the presence of 10 μM JNJ 7777120 in a total assay volume of 200 μL. For displacement binding studies, H_4R expressing membranes were typically incubated with 10^{-4} to 10^{-11} M of ligands (stock concentration was 10 mM in DMSO) in the presence of [^{3}H]JNJ 7777120 in a total volume of 200 μL. The reaction mixtures were incubated for 1 h at room temperature (22 °C) and harvested on 96-well GF/C plates that were pretreated with 0.3% 750 kDa PEI. Analysis of [^{3}H]histamine binding was performed under the same experimental conditions as described for [^{3}H]JNJ 7777120. Binding data were analyzed using Prism 4.0 (Graphpad Software Inc., U.S.A.).

Molecular Modeling. A model of the transmembrane domains of the H_4R plus helix 8 that expands parallel to the membrane was constructed by homology modeling using the crystal structure of bovine rhodopsin (PDB code 1L9H) as template.[20] The residues considered to be most conserved in the Class A family of GPCRs were aligned in both sequences.[17] TM3 is slightly bent toward TM5, at position 3.37, as has been suggested for the neurotransmitter family of GPCRs.[24,25] The model also includes the water molecules (Wat_{1a}, Wat_{1b}, and Wat_{1c}) observed in the D2.50/N7.49 environment of rhodopsin.[20] SCWRL-3.0 was employed to add the side chains of the nonconserved residues based on a backbone-dependent rotamer library.[26] The resulting model has been minimized to relieve internal strain using previously published methods.[27] The molecular models for the mutant receptors were built from the derived model of WT H_4R, by changing the atoms implicated in the amino acid substitutions by interactive computer graphics.

Initial docking poses were obtained using the proposed binding mode of histamine in the H_1R as a starting point.[27] Energy minimizations were performed fixing all receptor atoms, except the side chain atoms of the residues in direct contact with the ligand. More specifically, only the side chains of D3.32, Y3.33, C3.36, T5.42, S5.43, E5.46, F5.47, W6.48, Y6.51, S6.52, and Q7.42 were flexible during the energy minimizations. To overcome energetic barriers and explore conformational space, short (10−20 ps) MD simulations were performed in vacuo. All simulations used the Amber99 force field as implemented in MOE 2006.08 (Chemical Computing Group, Montreal, Canada).

The validity of the proposed mode of recognition of histamine and VUF 8430 was subsequently confirmed with

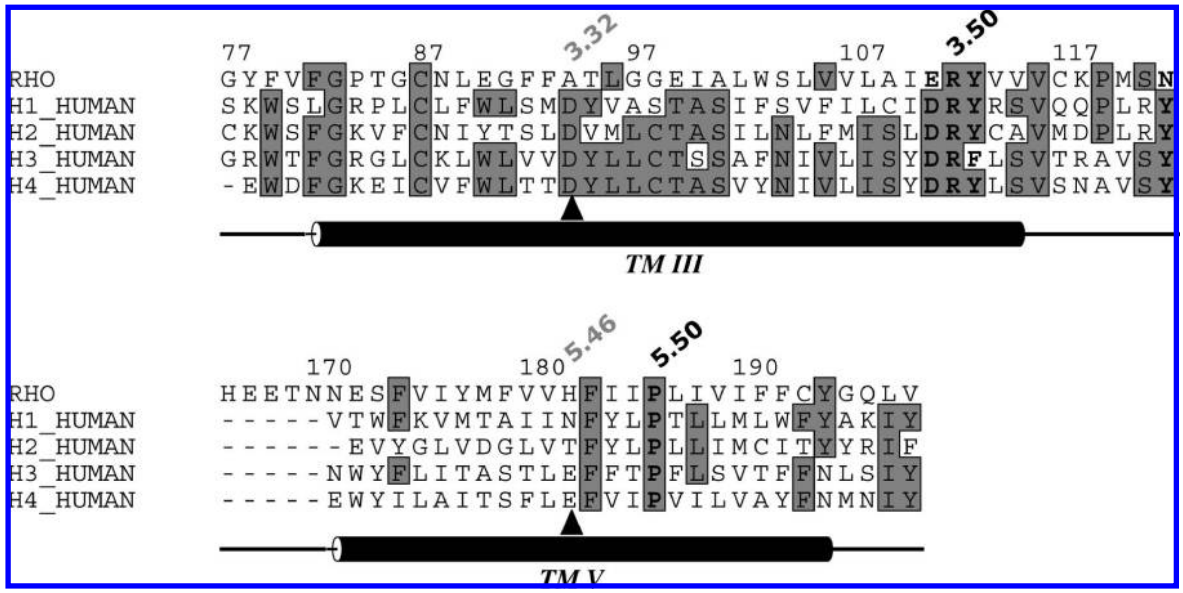

Figure 1. Sequence alignment of TM3 and 5 of the human histamine receptors and bovine rhodopsin. GPCR specific sequence motifs are indicated in **bold** (most conserved residues are denoted using the Ballesteros-Weinstein numbering scheme[17]). Residues mutated in this research are indicated by a triangle. The figure was generated with ALSCRIPT.[50]

ab initio geometry optimizations at the 6−31G(d,p) level of theory. The final model system consisted of D3.32, C3.36, T5.42, E5.46, F5.47, W6.48, Y6.51, S6.52, and Q7.42 (only the C_α atoms of the backbone are included) of the H_4R and the N^τ tautomer of histamine. All free valences were capped with hydrogen atoms. The C_α atoms of the residues were kept fixed at the positions obtained in the H_4R model. The capping hydrogens were aligned to the vectors connecting the C_α atoms to the backbone nitrogen and carbon atoms to preserve the orientation of the backbone. Interaction energies of histamine and VUF 8430 with the model pocket were calculated for the wild-type and the E5.46Q mutant optimized systems by performing single point energy calculations for the complex, the isolated ligand, and the empty pocket.

Molecular modeling was performed using MOE (2006.08) obtained from Chemical Computing Group, Montreal, Canada; quantum mechanical calculations were performed with the GAMESS-US (Nov2004/Sep2006) program.[28] Pictures were generated with PyMol.[29]

Nomenclature of χ1 Rotamer. When the heavy atom at the γ position is at a position opposite to the backbone nitrogen, when viewed from the β-carbon to the α-carbon, the χ1 rotamer is defined as being in the trans (t) position. Similarly, the χ1 rotamer is designated to be in the gauche- (g-) position when the γ atom is opposite the backbone carbon and gauche+ (g+) when opposite the α hydrogen.

RESULTS

Mutational Analysis of H_4R Agonist Binding. The 390-residue amino acid sequence of the H_4R shows all the conserved sequence motifs indicative of a Class-A rhodospin-like GPCR (Figures 1 and 2):[30] N1.50(33) (using the standardized GPCR nomenclature according to Ballesteros-Weinstein;[17] the number between parentheses represents the residue number of the H_4R sequence), D2.50(61), R3.50(112), W4.50(140), P5.50(186), P6.50(318), and P7.50(355). Also, the sequence fingerprint specific for amine-activated GPCRs, D3.32(94), and W7.40(345) is present (Figure 1).[31] D3.32 has been found to be essential for the binding of agonists and basic antagonists by the various aminergic receptors, including the H_4R.[15,32] Residues located in TM5 have also been shown to contribute to the binding of histamine and other compounds to the various histamine receptors.[27,32,33] Whereas in the histamine H_1R a major contribution is made by N5.46,[33,34] in both the H_3R and H_4R, a glutamic acid residue is found in this position, E5.46 (Figures 1 and 2B). To test whether this glutamic acid residue is the source of the increased affinity of histamine observed for both the H_3R and H_4R compared to the H_1R, single point mutations at this position (E5.46A and E5.46Q) were carried out next to a detailed analysis of the D3.32A and D3.32N mutants. Receptor mutants were transiently expressed in HEK 293T cells and analyzed for the binding of [^{3}H]histamine or the H_4R antagonist [^{3}H]JNJ 7777120.[12] As can be seen in Figure 3A, binding of both radiolabels is completely abolished by both the mutations at position 3.32 and the E5.46A mutant, whereas binding of [^{3}H]JNJ 7777120 is hardly affected by the E5.46Q mutation. Saturation analysis indicated that the antagonist radiolabel [^{3}H]JNJ 7777120 bound with high affinity to the H_4R E5.46Q mutant ($K_D = 9.5 \pm 0.5$ nM for H_4R E.46Q, compared to $K_D = 7.5 \pm 1.7$ nM for the WT H_4R, $n = 3$). This particular finding overcomes previous experimental problems encountered with this receptor mutant.[15] It allowed us to specifically determine the effects of this mutation on the affinity of histamine, the recently discovered H_4R agonist VUF 8430,[21] and the trycyclic agonist clozapine.[21,35] Competition binding studies with [^{3}H]JNJ 7777120 revealed a large rightward shift of more than 2.5 log units of the displacement curves for both histamine and the nonimidazole agonist VUF 8430 (Figure 3B,C). In contrast, the effects of the E5.46Q mutation were absent for the tricyclic H_4R agonist clozapine (Figure 3D).

Molecular Modeling of the Histamine H_4R - Binding of Agonists. In Figure 2, the homology model of the H_4R developed in this study is shown. In our H_4R model, TM3 is repositioned relative to the core architecture of rhodospin

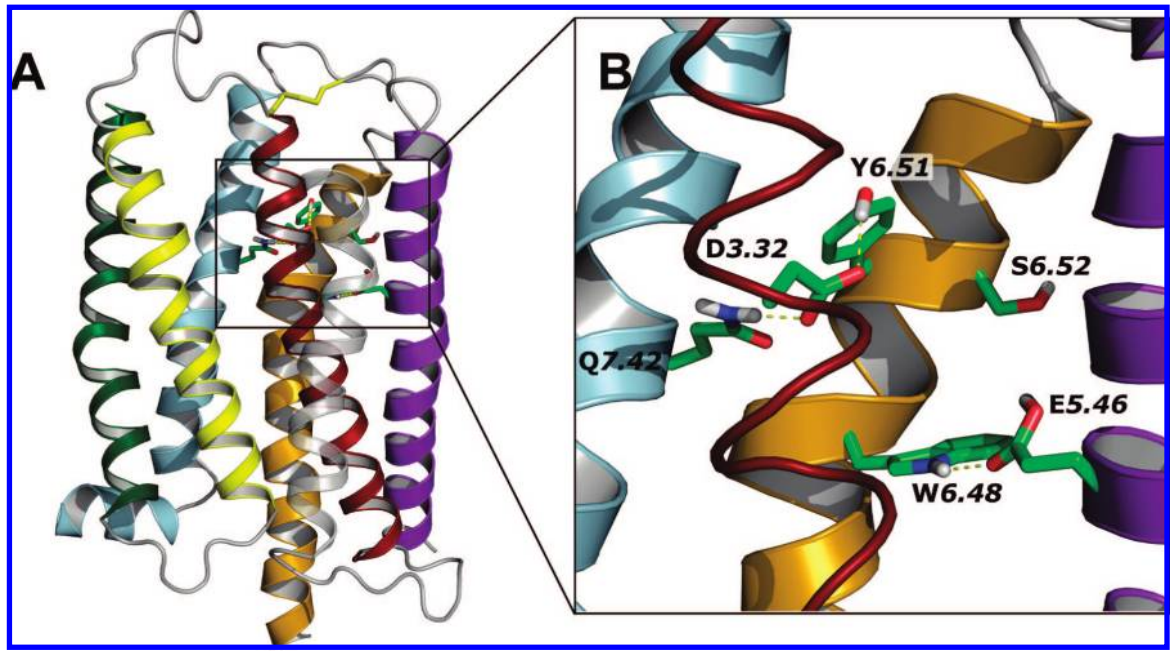

Figure 2. Homology model of the human histamine H_4 receptor (H_4R). (**A**) Schematic representation of the homology model of the human H_4R. (**B**) Closeup of the binding pocket of the H_4R. Selected residues are denoted using the Ballesteros-Weinstein numbering scheme.[17] Figures were generated with PyMol.[29]

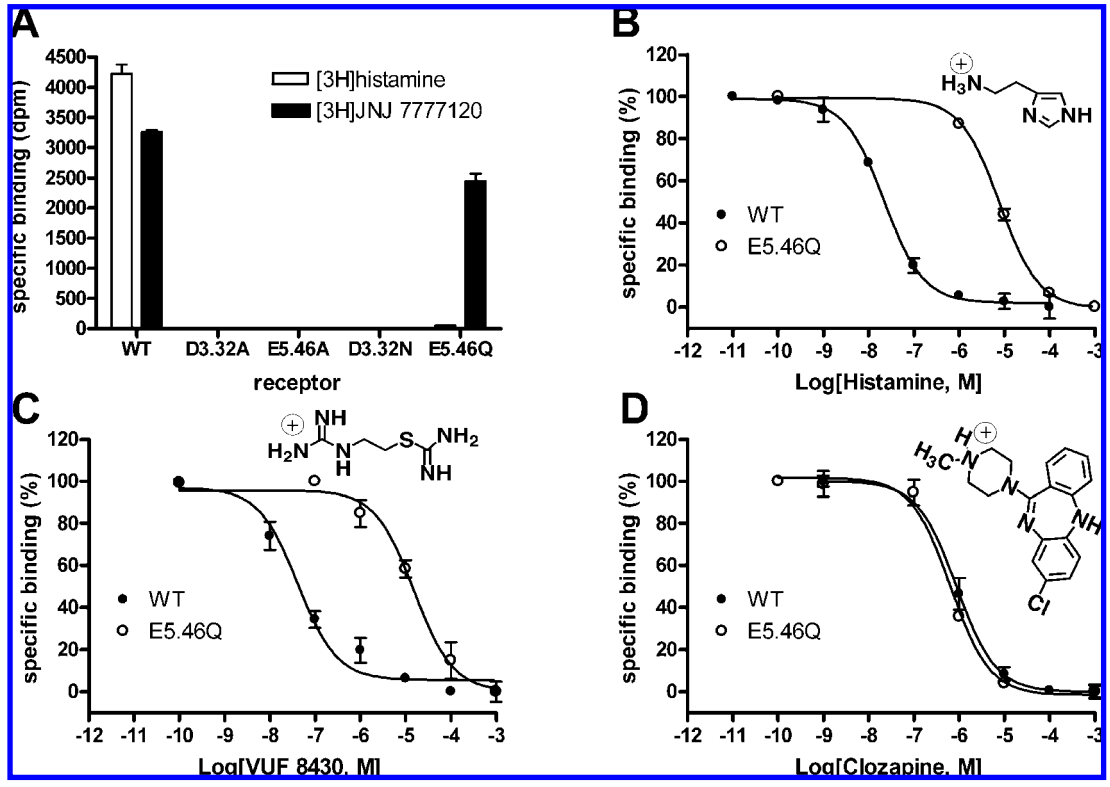

Figure 3. Radioligand binding analysis. (**A**) Binding of [^{3}H]histamine and [^{3}H]JNJ 7777120 to wild-type human H_4R and various human H_4R mutants. (**B, C**, and **D**) Displacement of [^{3}H]JNJ 7777120 binding. Histamine (B), VUF 8430 (C), and clozapine (D) doses dependently displace the binding of [^{3}H]JNJ 7777120 to the wild-type human H_4R (closed squares) and the human H_4R mutant E5.46Q (open circles).

due to the presence of two unique glycine residues in TM2 (2.56 and 2.57, see Figure 1) in the rhodopsin structure as has previously been described for other aminergic GPCRs (including serotonin and dopamine receptors).[24,25] This results in a slight bend of TM3, at position 3.37, toward TM5. D3.32, the major site of interaction for ligands containing a protonated moiety, becomes therefore situated in even closer proximity to E5.46 in TM5 (Figure 2B). W6.48, whose rotamer transition is generally believed to be among the first events occurring upon activation of GPCRs, is in its trans rotameric state, indicative of an 'active' state of the receptor.[27,36,37] The derived homology model was subsequently used in molecular docking studies with the H_4R agonists histamine, clozapine, and VUF 8430. Initially, energy minimization and molecular dynamic runs were performed to test the stability of the proposed binding poses,

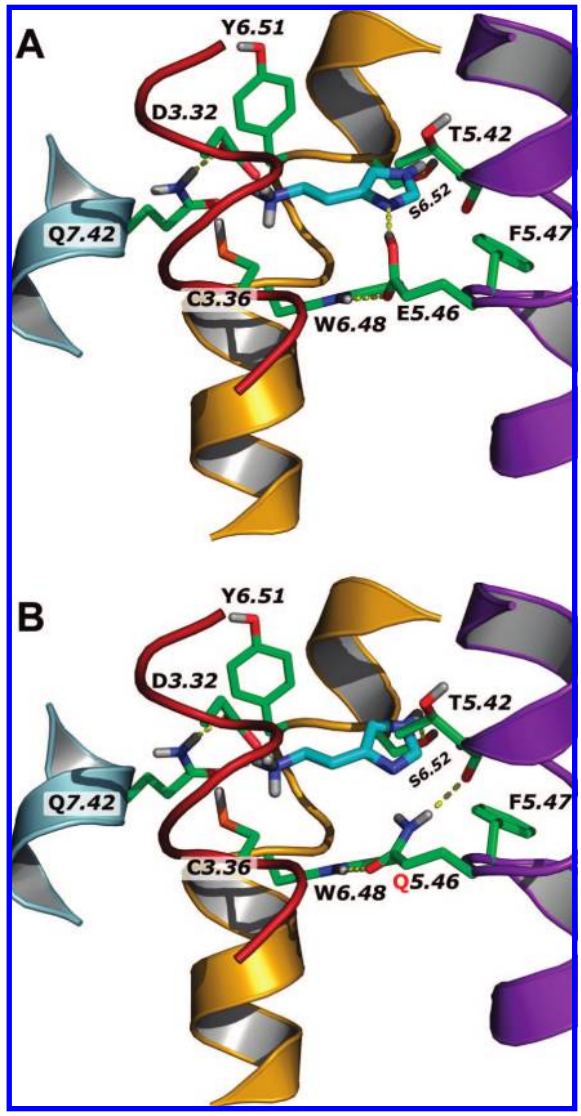

Figure 4. Computational model of the proposed binding of histamine in the binding pocket of the WT hH_4R (**A**) and E5.46Q mutant human H_4R (**B**) after ab initio calculations (6−31G(d,p), GAMESS-US v2006[28]). Only the amino acid residues shown were part of the calculation as was the histamine molecule (carbon atoms in cyan, nitrogen in blue). C_α atoms were fixed to their positions as obtained in the homology model. The helices are shown for clarity but were not part of the calculations. The C_α traces of TMs 3 (dark red), 5 (purple), 6 (orange), and 7 (blue) are colored. The figures were generated with PyMol.[29]

relieve initial restraints, and overcome small energy barriers. The binding poses showed an extensive hydrogen-bonding network in which the imidazole ring of histamine plays an important role. This will result in a conjugation and concomitant shift of electron density between E5.46 and S6.52. The binding affinities were estimated with ab initio calculations as only this method is able to capture the expected electronics effects accurately. The calculations were performed at the 6−31G(d,p) level of accuracy as described in the Methods section.

Histamine. Figure 4 shows a detailed view of the binding pocket of the molecular model of the human histamine H_4 receptor. The main interacting residues in this pocket are the aspartic acid residue (D3.32) in TM3 and the glutamic acid residue (E5.46) in TM5. The tryptophan residue in TM6, W6.48, is in the trans conformation[37] that is generally

Table 1. Binding Affinity (pK_i) of Selected Ligands on the Wild-Type (WT) and E5.46Q Mutant Human H_4R as Determined by Displacement of [^{3}H]JNJ 7777120

compound	WT	E5.46Q
histamine	7.74 ± 0.12	4.95 ± 0.09
clozapine	6.25 ± 0.03	6.15 ± 0.04
VUF 8430	7.57 ± 0.07	5.04 ± 0.04

Table 2. Differences in Experimental Binding Affinities (pK_i) vs Calculated Differences in Interaction Energies (kcal/mol) of Selected Ligands on the Wild-Type (WT) and E5.46Q Mutant Human H_4R

compound	ΔpK_i	$\Delta\Delta E$ (calcd)
histamine	−2.79	−3.4
VUF8430	−2.53	−2.2

assumed to be stabilized by agonist binding and indicative of an activated receptor state.[27,36,37] Histamine was docked with its protonated amine function toward D3.32 and the imidazole moiety directed toward TM5. This orientation is highly stabilized with a perfect match of hydrogen bonds involving D3.32 and Q7.42 and another hydrogen bonding network between the imidazole, E5.46, S6.52, and W6.48 (Figure 4A). E5.46 stabilizes the 'active' rotamer conformation of W6.48 by formation of a hydrogen bond (2.38 Å) between Hϵ1 of W6.48 and Oϵ1 of E5.46. The presence of D3.32 in the vicinity of E5.46 will raise the pK_a of E5.46 and will therefore be in its protonated form. This has been documented for various enzyme and protein systems such as lysozyme,[38] α-amylase,[39] and bacteriorhodopsin.[40,41] In all cases an increase of the pK_a of a glutamic acid of ∼2 pK_a units has been observed, leading to a value close to the pK_a value of the imidazole moiety (∼7). Hence, a perfect hydrogen bonding interaction would be expected between a protonated glutamic acid and the imidazole ring of histamine. The calculated distance between N^π of histamine and the acidic proton of E5.46 is 1.89 Å. Mutation of E5.46 to glutamine weakens this interaction, increasing the distance between N^π and the nearest proton of Q5.46 by 0.4 Å to 2.31 Å (Figure 4B). The hydrogen bond distance to Hϵ1 of W6.48 is slightly decreased from 2.38 to 2.23 Å, while the calculated distance between the N^τ proton of the imidazole ring and the S6.52-OH increases from 2.24 to 2.58 Å (Figure 4B). The mutation of E5.46 to Q causes an overall drop in affinity of ∼2.8 pK_i units (Table 1). This corresponds with the calculated difference in the corresponding stabilization energies of −3.4 kcal/mol at the 6−31G(d,p) level of theory (Table 2).

The model also nicely explains the observed difference in binding affinity for several methylated histamine analogs. The amino function of histamine is involved in a strong H-bonding interaction with D3.32, Q7.42, and C3.36. Upon methylation of the amino function, the additional methyl group in N^α-methylhistamine will result in a loss of an H-bond and a concomitant decrease in binding energy. The observed reduction in pK_i from 7.7 to 6.5 is in nice agreement with the proposed model.[42] The hydrogen at position 4 of the imidazole ring points toward the carbonyl backbone of W6.48. However, based upon the large distance between the oxygen of the backbone and the C4 of the imidazole, 2.9 Å, the introduction of the methyl group at this position in 4-methylhistamine will not impact the binding conformation

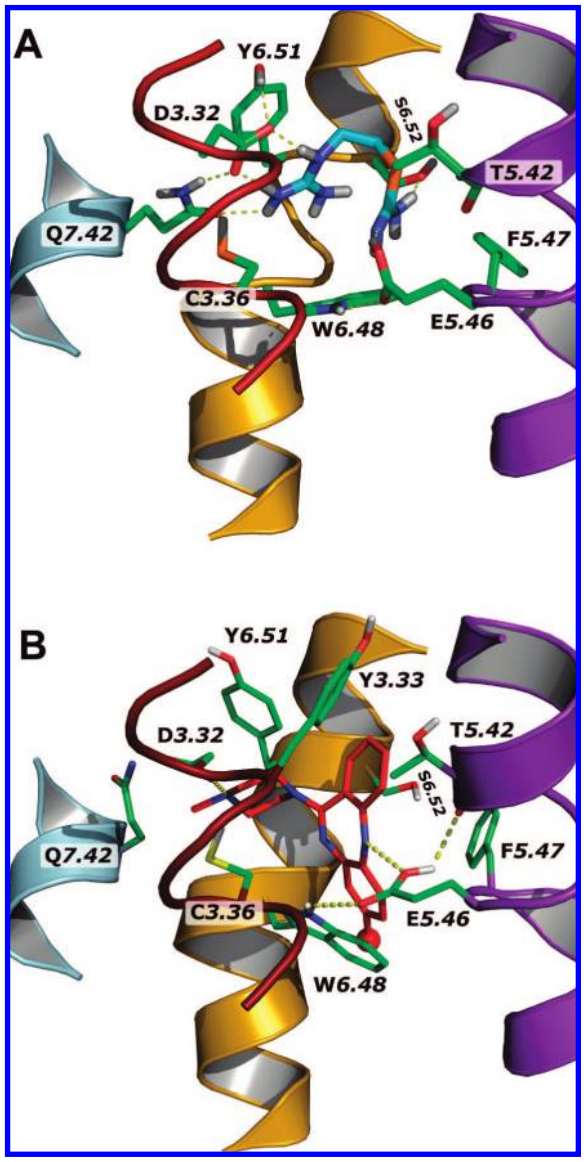

Figure 5. Computational model of the proposed binding of VUF 8430 (**A**) and clozapine (**B**) in the binding pocket of the WT human H_4R. For clozapine (**B**) four conformations based on the CSD file NDNHCL10 were generated and docked. The structure shown in red was chosen based on its various favorable interactions (chlorine in ball and stick representation). For legend on color-coding see Figure 4. The figures were generated with PyMol.[29]

of the corresponding histamine analog. The measured affinity of 4-methylhistamine is comparable to the affinity of histamine, i.e. 7.3 vs 7.7.[42]

VUF 8430. The recently discovered nonimidazole small molecule agonist, *S*-(2-guanidylethyl)isothiourea (VUF 8430),[21] was manually docked in the binding pocket of hH_4R (Figure 5A). A bidentate interaction between the guanidinium moiety and D3.32 is formed (H-bonds of 1.76 Å and 1.88 Å) as well as a hydrogen bond with Oϵ1 of Q7.42 (2.35 Å). The isothiourea group points in the direction of TM5, forming hydrogen bonds with both E5.46 (1.82 Å) and S6.52 (2.21 Å). The resulting H-bonding network is identical to the pattern observed for the imidazole ring of histamine. Upon mutation of E5.46 to Gln, the calculated distance, after minimization, between Q5.46 and the isothiourea group increases to 2.35 Å, signifying a reduction in interaction energy for the H-bond. The distance between S6.52 and the isothiourea increases to 2.31 Å. Also the distance between Oϵ1 *of* Q7.42 with the guanidinium moiety increases slightly to 2.52 Å. The calculated distance between the Hϵ1 of W6.48 and the carbonyl (Oϵ1) of E5.46 slightly decreases upon mutation of Glu to Gln, 2.60 Å in the WT and 2.50 Å in H_4R E5.46Q, respectively. The observed interaction pattern for the isothiourea group strongly resembles the interactions observed for the imidazole ring of histamine. It is therefore expected that the mutation of E5.46Q will display an effect similar to the observed change for histamine. The experimental data are in full agreement with the observed binding mode (Table 2).

Clozapine. The initial structure of clozapine was obtained from the Cambridge Structural Database[43] (entry NDNCL10). Conformations were generated using the stochastic conformation search approach available within MOE. The *N*-methylpiperazine ring of clozapine will predominantly be in the chair conformation, with the methyl group at N-4 of the piperazine ring in equatorial position.[44] The tricyclic ring system mimics the wings of a butterfly and can adopt various conformations around the central 7-membered ring having a large impact on the spatial orientation of the rings. The bulk of the tricyclic headgroup of clozapine enforces strong geometric constraints on the relative position of the *N*-methylpiperazine moiety. Four representative conformations were docked into the model of the human H_4R. Only one conformation was found to have reasonable H-bonding interactions (Figure 5B). In this conformation the *N*-methylpiperazine ring of clozapine interacts as expected with D3.32 in TM3. The N−H in the 7-membered ring was modeled to be in contact with the carbonyl oxygen of E5.46 (Figure 5B). It is important to note that clozapine, in sharp contrast to histamine and VUF 8430, is not involved in a finely balanced H-bonding network with E5.46, S6.52, and W6.48. The lack of this subtle H-bonding network as well as the absence of a weakly basic group in clozapine explains the absence of a strong reduction in affinity upon the introduction of the E5.46Q mutation. In fact, the affinity of the human histamine H_4R for clozapine is rather low (pK_i = 6.7) for a system that has virtually no freely rotatable bond.[42] This can be the consequence of the lack of strong interactions as observed for histamine and VUF 8430. Furthermore, the proposed binding mode indicates that the activation of the H_4R is induced by a steric constrained of the tricyclic ring on W6.48. This differs from the activation mechanism with histamine, which most likely relies on the construction of the energetically favorable H-bonding network described above.

DISCUSSION

The newly discovered histamine H_4R has been identified as a potential new drug target for e.g. allergic asthma and inflammation.[11] As such, there currently exists a keen interest in its function and the binding of potent and selective ligands. Various studies on the characterization of H_4Rs both from human and other animal species have been published.[45–47] Recently, our group has reported 4-methylhistamine as being the first potent and selective H_4R agonist.[42] VUF 8430, or *S*-(2-guanidylethyl)isothuorea, was later reported as the first potent nonimidazole H_4R agonist with a pK_i of 7.5.[21] However, to be able to

design more potent and selective drugs in the future, a good knowledge of the interactions determining the binding of ligands at the atomic level is highly desirable. For this purpose, we combined mutagenesis studies with molecular modeling approaches and quantum chemical calculations.

The primary sequence of the human histamine H_4 receptor contains all of the conserved sequence motifs identified for the Class-A rhodopsin-like GPCRs.[17,30] The overall homology with the histamine H_1, H_2, and H_3 receptors is low (H_3R having the highest degree of sequence identity, 54% within the TM domains[11]), but residues indicated in ligand binding for these receptors are also present in the H_4R. The aspartic acid in TM3 (D3.32), seen to play a critical role in binding of biogenic amines,[32] is also present in the H_4R. The carboxylate group, deprotonated at physiological conditions having a pK_a value of 4.0 in its isolated state,[48] forms an ionic interaction with the protonated nitrogen moieties often necessary for obtaining binding at aminergic receptors. Mutation of this residue completely abolishes binding for all tested ligands (Figure 3). TM5 has also been shown to be of importance in the binding of ligands in various aminergic receptors. For example, in the H_1R, the asparagine (N5.46) has been proposed to interact with the protonated nitrogen of histamine (see ref 33). However, in both the H_3R and the H_4R a glutamic acid residue takes this position. The increased strength of a potential interaction with the protonated nitrogen of the imidazole moiety of histamine by a glutamic acid in comparison with an asparagine might be an important factor in the increase in binding affinity observed for both H_4R and H_3R compared to H_1R. Occurrence of either an aspartic or glutamic acid residue at this position within the family of Class-A rhodopsin-like GPCRs is very low (2% and 0.9%, respectively, within ~1100 sequence in the GPCRDB). Corroborating recent findings of Shin et al. we observed the importance of this position in the binding of histamine.[15] Upon mutation of E5.46 into either Ala or Gln binding of [^{3}H]histamine at feasible concentrations is completely lost. However, the presence of two deprotonated amino acid residues within the same binding pocket is not likely. The pK_a value of E5.46 (pK_a ~ 4.4 in isolation) will certainly be raised in the vicinity of the more acidic aspartic acid D3.32.[39] This has been well documented for other protein systems.[38–41] Recently, we have shown dimaprit, which is present predominantly in its dicationic form[49] (87% at pH 7.4), to bind only with moderate affinity (pK_i ~ 6.5) to H_4R.[21] The presence of two basic centers within the ligand is clearly not a prerequisite for high-affinity binding. Hence, we propose E5.46 to be in its protonated state when binding ligands.

Our mutagenesis data and quantum chemical calculations clearly show the hypothesis to be valid. Upon binding of histamine, anchored by the favorable ionic interaction between the protonated amine function and D3.32, the imidazole moiety points toward TM5 and induces a perfect match of hydrogen bonds between E5.46, S6.52, and W6.48 (Figure 2). The interaction between E5.46 and W6.48 further increases the pK_a of the proton on the carboxylic acid group of E5.46 to a value matching that of the imidazole moiety. Furthermore, this interaction stabilizes the trans-rotamer state of W6.48 indicated to be involved in GPCR activation.[27,36,37] Binding of the small molecule agonist, histamine, thus further stabilizes the active state of the receptor. The proposed binding mode of histamine is further corroborated by the SAR observed for its methylated analogs.[42] The model nicely explains the observed changes in binding affinity.

The nonimidazole containing small molecule agonist VUF 8430 binds in a similar manner within the binding pocket of the H_4R (Figure 5). Again, a perfect orientation of hydrogen bonds is observed, together with the stabilization of the rotamer state of W6.48 indicative of receptor activation. As VUF 8430 fits the site perfectly, shortening as well as elongation of the spacer between the basic moieties (with chain lengths up to 6 CH_2-moieties) will result in less favorable binding modes, corroborating previously published SAR studies.[21]

The docking studies clearly show that only a single conformation of the tricyclic clozapine fits into the active site. Important insights could be obtained from this binding pose. The bulky tricyclic moiety of clozapine fits tightly within the binding pocket. A hydrogen bonding interaction network between the N−H and E5.46 was observed. It is clear from the lack of the conjugated H-bonding network that not only the effect of mutations will differ from histamine but also that the activation principle might be different as well. As such, clozapine might be an interesting template for the generation of new scaffolds, which will be able to modulate H_4 receptor function in a distinct manner.

CONCLUSION

A three-dimensional protein model of the human histamine H_4R has been constructed on the basis of the available structural information of rhodopsin. The derived computational model is able to explain the experimental data obtained for several mutant receptors at the fundamental atomic level. Mutation of E5.46 to Q shows no large differences in affinity for clozapine, containing a bulky, tricyclic moiety. However, a 1000-fold decrease in affinity of small agonistic ligands, i.e. histamine and VUF 8340, is observed. At the atomic level this can be explained by proposing the E5.46 to be in its protonated state. Ab initio calculations at the 6−31G(d,p) level of theory for model systems containing the most important residues of the binding pocket confirm the validity of this hypothesis. In the future, design of effective ligands for the histamine H_4 receptor, a promising drug target for allergy and asthma, should take into account the proposed protonation state of these charged residues within the binding pocket.

ACKNOWLEDGMENT

We thank Dr. R. L. Thurmond (Johnson & Johnson Pharmaceutical Research and Development, San Diego, U.S.A.) for the kind gift of the radioligand [^{3}H]JNJ 7777120.

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CI700474A