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RESEARCH PAPER

Molecular determinants for the high constitutive activity of the human histamine H_4 receptor: functional studies on orthologues and mutants

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Dedicated to Professor Dr Helmut Schönenberger, Regensburg, on the occasion of his 90th birthday.

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BACKGROUND AND PURPOSE

Some histamine H_4 receptor ligands act as inverse agonists at the human H_4 receptor (hH_4R), a receptor with exceptionally high constitutive activity, but as neutral antagonists or partial agonists at the constitutively inactive mouse H_4 receptor (mH_4R) and rat H_4 receptor (rH_4R). To study molecular determinants of constitutive activity, H_4 receptor reciprocal mutants were constructed: single mutants: hH_4R-F169V, mH_4R-V171F, hH_4R-S179A, hH_4R-S179M; double mutants: hH_4R-F169V+S179A, hH_4R-F169V+S179M and mH_4R-V171F+M181S.

EXPERIMENTAL APPROACH

Site-directed mutagenesis with pVL1392 plasmids containing hH_4 or mH_4 receptors were performed. Wild-type or mutant receptors were co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 cells. Membranes were studied in saturation and competition binding assays ([^{3}H]-histamine), and in functional [^{35}S]-GTPγS assays with inverse, partial and full agonists of the hH_4 receptor.

KEY RESULTS

Constitutive activity decreased from the hH_4 receptor via the hH_4R-F169V mutant to the hH_4R-F169V+S179A and hH_4R-F169V+S179M double mutants. F169 alone or in concert with S179 plays a major role in stabilizing a ligand-free active state of the hH_4 receptor. Partial inverse hH_4 receptor agonists like JNJ7777120 behaved as neutral antagonists or partial agonists at species orthologues with lower or no constitutive activity. Some partial and full hH_4 receptor agonists showed decreased maximal effects and potencies at hH_4R-F169V and double mutants. However, the mutation of S179 in the hH_4 receptor to M as in mH_4 receptor or A as in rH_4 receptor did not significantly reduce constitutive activity.

CONCLUSIONS AND IMPLICATIONS

F169 and S179 are key amino acids for the high constitutive activity of hH_4 receptors and may also be of relevance for other constitutively active GPCRs.

LINKED ARTICLES

This article is part of a themed issue on Histamine Pharmacology Update published in volume 170 issue 1. To view the other articles in this issue visit http://onlinelibrary.wiley.com/doi/10.1111/bph.2013.170.issue-1/issuetoc

Abbreviations

ECL, extracellular loop; hH_4R, human H_4 receptor; ICL, intracellular loop; isoloxapine, 8-chloro-11-(4-methylpiperazin-1-yl)dibenzo[b,f][1,4]oxazepine; mH_4R, mouse H_4 receptor; rH_4R, rat H_4 receptor; TM, transmembrane region

Table of Links

TARGETS	LIGANDS
H_4 receptor	Clobenpropit
H_1 receptor	Clozapine
H_3 receptor	Histamine
β_2-adrenoceptor	Immepip
	Thioperamide
	JNJ7777120
	VUF8430
	GTPγS

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

The human histamine H_4 receptor (hH_4R) was independently discovered by several groups (Nakamura *et al.*, 2000; Liu *et al.*, 2001a; Morse *et al.*, 2001; Nguyen *et al.*, 2001; Oda and Matsumoto, 2001; Zhu *et al.*, 2001). The H_4 receptor is coupled to $G\alpha_i$ proteins, leading to inhibition of adenylyl cyclase and, via release of $G\beta\gamma$ complexes, to the activation of phospholipase C (for reviews, see, e.g. Thurmond *et al.*, 2008; Leurs *et al.*, 2009; Seifert *et al.*, 2013). H_4 receptor-mediated $G\alpha_i$ activation in membrane preparation is monitored by agonist-stimulated [^{35}S]-GTPγS binding to $G\alpha_i$ proteins or $G\alpha_i$-mediated [γ-^{32}P]-GTP hydrolysis (Schneider *et al.*, 2009). The H_4 receptor is primarily expressed in cells of the immune system and seems to play a pro-inflammatory role in bronchial asthma, atopic dermatitis and pruritus (de Esch *et al.*, 2005; Dunford *et al.*, 2006; Zampeli and Tiligada, 2009; Dunford and Holgate, 2011; Marson, 2011; Schnell *et al.*, 2011). Human H_4 receptor expression and function has been unequivocally demonstrated by several independent groups in eosinophils (O'Reilly *et al.*, 2002; Buckland *et al.*, 2003; Ling *et al.*, 2004; Reher *et al.*, 2012). However, eosinophils are very difficult to purify in sufficient amounts for pharmacological studies so that experiments with recombinant hH_4 receptor are very important.

A GPCR capable of producing its biological response in the absence of a bound ligand is termed constitutively active (Seifert and Wenzel-Seifert, 2002). Previous studies have shown that the hH_4 receptor possesses an unusually high constitutive activity, resulting in high agonist-independent $G\alpha_i$ protein activation (Morse *et al.*, 2001; Seifert *et al.*, 2013; Strasser *et al.*, 2013). A plausible cause could be the missing ionic lock between an arginine in the DRY motif (TM3) and an acidic amino acid in TM6 (replaced by an alanine in the hH_4 receptor). However, this was not confirmed by reconstitution of this motif in the hH_4 receptor (Schneider *et al.*, 2010). The constitutive activity of canine, murine and rat H_4 receptor species isoforms (cH_4, mH_4 and rH_4 receptor, respectively) is substantially lower (Schneider *et al.*, 2010; Schnell *et al.*, 2011; Strasser *et al.*, 2013). Another striking difference

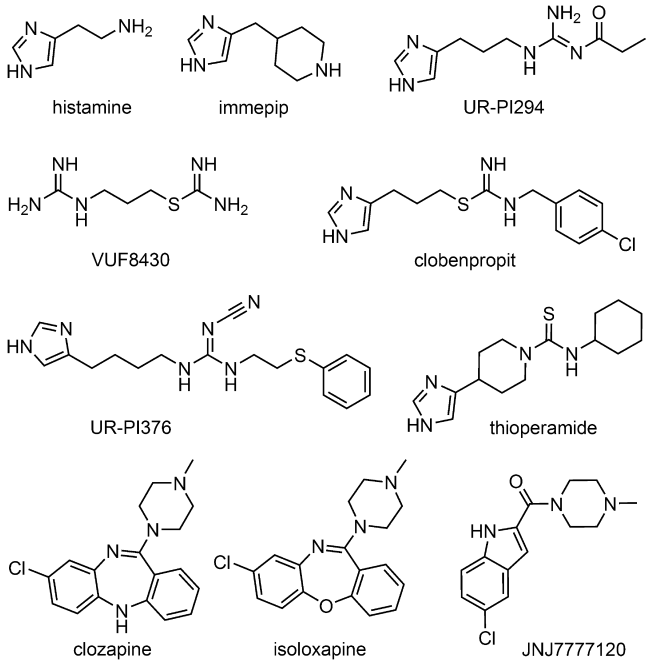

Figure 1
Structures of H_4 receptor ligands investigated.

was observed with the prototypical H_4 receptor antagonist JNJ7777120 (1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine, Figure 1), a partial agonist at the cH_4 receptor, the rH_4 receptor and the mH_4 receptor, but a partial inverse agonist at the hH_4 receptor. Furthermore, H_4 receptor agonists (Igel *et al.*, 2010) from the class of N^G-acylated imidazolylpropylguanidines and cyanoguanidines differed with respect to affinity, potency and efficacy among H_4 receptor species isoforms (Schnell *et al.*, 2011).

Mouse, rat and dog are important laboratory animal species for assessing the pathophysiological role of the H_4 receptor (Liu *et al.*, 2001b; Dunford *et al.*, 2006; Rossbach

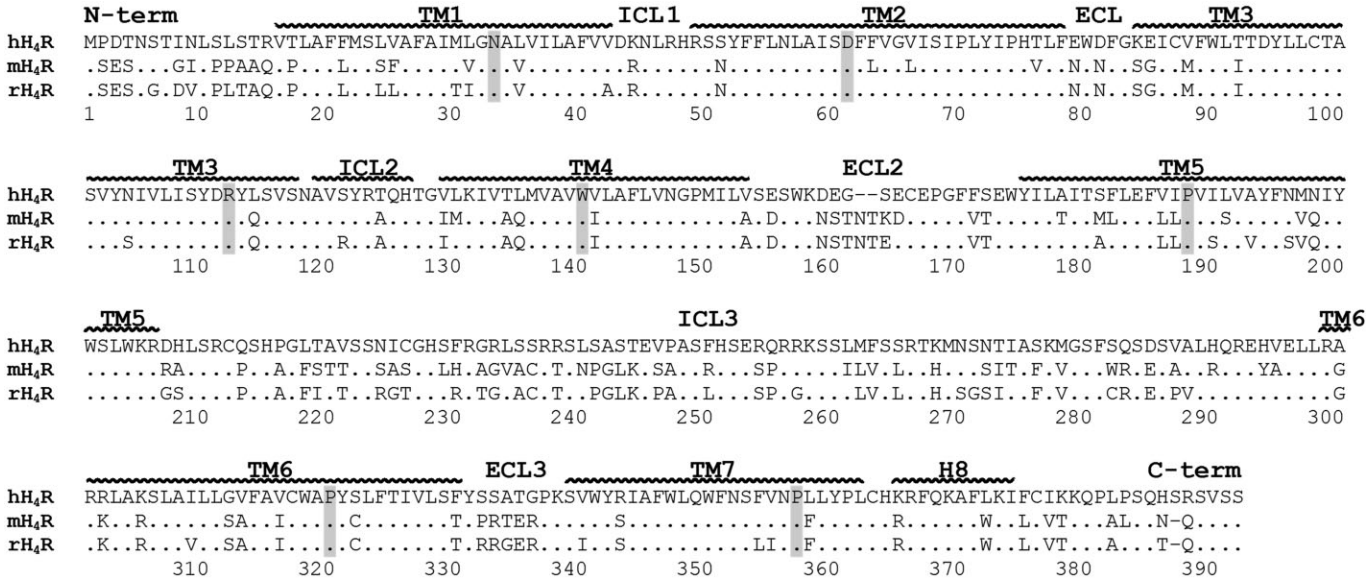

Figure 2

Sequence alignment of hH_4, rH_4 and mH_4 receptors. TMs are indicated by wavy lines. N-term, N-terminus (extracellular); C-term, C-terminus (intracellular); ICL1, ICL2 and ICL3: first, second and third intracellular loops; ECL1, ECL2 and ECL3: first, second and third extracellular loops. Dots in the sequences indicate identity with the hH_4 receptor. The most conserved residues in each TM domain are highlighted by grey shading. TMs were defined according to the hH_4 receptor homology model based on the crystal structure of the hH_1 receptor.

et al., 2009). It is therefore important to characterize the effects of ligands at those H_4 receptor species orthologues in comparison to the hH_4 receptor. Considering the rather low sequence identity of H_4 receptor species isoforms (see alignment of hH_4 receptor, mH_4 receptor and rH_4 receptor, Figure 2), the question arises which molecular determinants account for the species differences in constitutive activity, ligand binding and intrinsic activity.

A systematic investigation with chimeras localized the region between V141$^{4.51}$ and E182$^{5.46}$ [superscripts according to the Ballesteros and Weinstein numbering (Ballesteros and Weinstein, 1995)] involving the second extracellular loop (ECL2) to be responsible for differences in agonist affinity between the hH_4 receptor and the mH_4 receptor (Lim *et al.*, 2008). Moreover, among single hH_4 receptor-mH_4 receptor amino acid exchanges in this region, the hH_4R-F169V mutant resulted in the largest shifts towards the K_d and pK_i values at the mH_4R, suggesting that this residue in ECL2 is 'the key amino acid' for differential interactions of certain agonists with the hH_4 receptor and the mH_4 receptor (Lim *et al.*, 2008). As in the case of the two corresponding consecutive phenylalanine residues in the β_2-adrenoceptor structure (Cherezov *et al.*, 2007), it was assumed that F169 is involved in a network of hydrophobic interactions, stabilizing ECL2 in a conformation, which positions F168 towards the binding pocket (Lim *et al.*, 2008).

To further investigate the role of F169, we generated the single mutants hH_4R-F169V and mH_4R-V171F. Until now, no functional studies on G-protein coupling at these mutants exist to discriminate between agonist, antagonist and inverse agonist effects. We therefore tested H_4 receptor ligands with different qualities of action in functional [^{35}S]-GTPγS assays.

Figure 3 shows the putative histamine binding pocket of the hH_4 receptor and various amino acids in the vicinity of

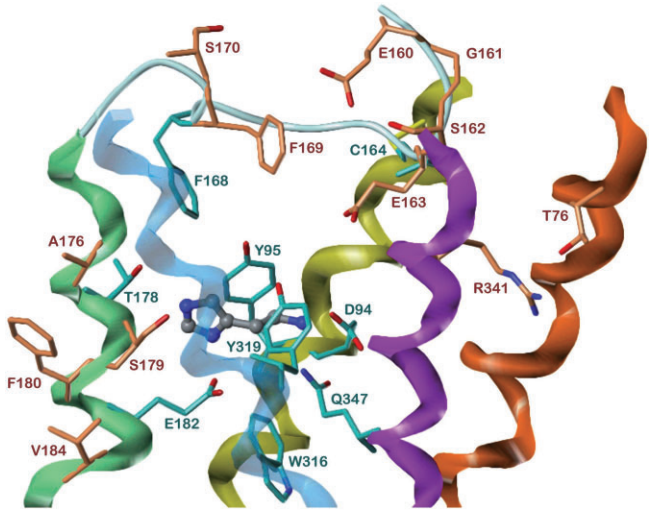

Figure 3

Ligand binding pocket of the hH_4 receptor in complex with histamine. The model is based on the crystal structure of the hH_1 receptor as template (Shimamura *et al.*, 2011). Histamine (ball and stick model) was manually docked considering interactions with the hH_4 receptor suggested from results of *in vitro* mutagenesis. Colours of atoms if not otherwise indicated: C – grey, N – blue, O – red, S – yellow. Carbons and backbone nitrogens of amino acids which are different in the rH_4 receptor and mH_4 receptor are orange-coloured. Other important amino acids of or close to the ligand binding pocket are represented by cyan-coloured C and backbone N atoms. TMs are drawn as ribbons: TM2 – orange, TM3 – yellow, TM5 – green, TM6 – light blue, TM7 – magenta. The C-terminal part of ECL2 is shown as tube.

this pocket which are specific for the hH_4 receptor compared to the mH_4 receptor and the rH_4 receptor. According to results from *in vitro* mutagenesis (Shin *et al.*, 2002), the positively charged amino group of histamine forms a salt bridge with $D94^{3.32}$. The ethylamine side chain is embedded between $Y95^{3.33}$ and $Y319^{6.51}$, whereas the N^{π} nitrogen of the imidazolyl moiety is hydrogen bonded to the side chain of $E182^{5.46}$. F168 (ECL2) points into the binding pocket, albeit direct contacts with histamine are not obvious. Hydrogen bonds of the N^{τ} nitrogen with the hydroxyls of $T178^{5.42}$ and $S179^{5.43}$ are possible, but on the single mutants hH_4R-T178A, hH_4R-S179A (Shin *et al.*, 2002) and hH_4R-S179M (Lim *et al.*, 2008), histamine affinity and activity was only slightly reduced compared to the wild-type hH_4 receptor (factors 2–4). However, $S179^{5.43}$ is mutated in the mH_4 receptor (M) and the rH_4 receptor (A) and therefore a promising candidate for more detailed investigations. In order to study the pharmacological profile including the constitutive activity of the single hH_4R-S179A and hH_4R-S179M mutants, we expressed these constructs in Sf9 cells.

Although our hH_4 receptor model does not indicate direct interactions of $S179^{5.43}$ and F169 (Figure 3), the question arose whether there is an additive effect of both amino acids with respect to the selectivity of ligands for the human H_4 receptor orthologue. We therefore prepared the double mutants of the hH_4 receptor, hH_4R-F169V+S179A and hH_4R-F169V+S179M, corresponding to the rat and mouse H_4 receptor in positions 169 and 179, as well as the reciprocal double mutant of the mH_4 receptor, mH_4R-V171F+M181S.

Methods

Homology model of the hH_4 receptor

To suggest promising mutants and hH_4 receptor-specific intramolecular interactions close to the ligand binding site, a homology model of the hH_4 receptor was generated with the modelling suite Sybyl 7.3 (Tripos Inc., St. Louis, MO, USA) using the crystal structure of the hH_1 receptor (protein databank code 3RZE) as template (Shimamura *et al.*, 2011). For this purpose, the inactive state of the template is not inconsistent with the constitutively active state of the hH_4 receptor since the binding pocket regions and extracellular domains of both states are very similar (Rasmussen *et al.*, 2011). The resulting model contains all extracellular (ECL) and intracellular (ICL) loops except ICL3 (G215-H292). To close the gap between the intracellular parts of TM5 and TM6, eight alanines were inserted in place of ICL3 (and the lysozyme domain of the template structure respectively). Fifteen missing amino acids of the N-terminus were added by a recently established protocol (Strasser and Wittmann, 2013). The E2 loop is not completely resolved in the hH_1 receptor structure. After removing the hH_1 receptor residues W165, N166 and H167, the missing amino acids V153-K158 were included into the hH_4 receptor model using the Loop-Search module within Sybyl. The inserted regions of the N-terminus and ECL2 were separately refined by energy minimization and a short gas phase MD simulation (500 ps). Histamine was manually docked considering interactions with the hH_4 receptor suggested from results of *in vitro* mutagenesis (Shin *et al.*, 2002). Finally, the model was provided with Amber7 FF99 (histamine: Gasteiger-Hueckel) charges and energy minimized with the Amber7 FF99 force field (Cornell *et al.*, 1995) and a dielectric constant of 4 up to a gradient of 0.01 $kcal \cdot mol^{-1} \cdot Å^{-1}$.

Miscellaneous

Protein concentrations of all membrane preparations were determined with the Bio-Rad DC protein assay kit (München, Germany) in one experiment. Because UR-PI376 had to be dissolved in 20% DMSO, the water control and the full agonist histamine ($\alpha = 1.0$), to which all other ligands were referenced, were also dissolved in 20% DMSO in case of this ligand. Data from the [^{3}H]-histamine saturation binding, [^{3}H]-histamine competition binding and the [^{35}S]-GTPγS assays were analysed with the Prism 5.01 software (GraphPad, San Diego, CA, USA). K_b and K_i values were calculated according to the Cheng–Prusoff equation (Cheng and Prusoff, 1973). All values are given as mean ± SEM of at least three (up to nine) independent experiments performed in triplicate. Significances were calculated using one-way ANOVA, followed by Bonferroni's multiple comparison test.

Materials

The pcDNA3.1 vector containing the hH_4 receptor sequence was obtained from the UMR cDNA Resource Center at the University of Missouri-Rolla (Rolla, MO, USA). The cDNAs encoding the mouse and rat H_4 receptors were a kind gift of Dr R. Thurmond (Johnson & Johnson Pharmaceutical R&D, San Diego, CA, USA). The construction of the human, mouse and rat pVL1392-SF-H_4R-His_6 and of the pGEM-3Z-SF-mH_4R-His_6 plasmids, respectively, was described previously (Schneider *et al.*, 2009; Schnell *et al.*, 2011). Baculovirus encoding $G\alpha_{i2}$ was kindly provided by Dr A. G. Gilman (Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX, USA). Recombinant baculovirus encoding the unmodified version of the $G\beta_1\gamma_2$ subunits was a kind gift of Dr P. Gierschik (Department of Pharmacology and Toxicology, University of Ulm, Ulm, Germany). *Pfu* Ultra II DNA polymerase was obtained from Agilent (Böblingen, Germany). The DNA primers for polymerase chain reaction were synthesized by MWG-Biotech (Ebersberg, Germany). Restriction enzymes and T4-DNA ligase were from New England Biolabs (Ipswich, MA, USA). Gradient gels (8–16%, 12 well nUView gels), the 'prestained' peqGOLD protein marker III, used for Western blotting as well as the 'unstained' peqGOLD protein marker I, used for Coomassie brilliant blue R staining, were from Peqlab (Erlangen, Germany). The antibody selective for $G\alpha_{i1/2}$ was from Calbiochem (Darmstadt, Germany). The anti-FLAG M1 antibody, the amino-terminal FLAG-BAP fusion protein and histamine were from Sigma-Aldrich (Taufkirchen, Germany). The binding of secondary antibodies coupled to peroxidase (HRP) was detected with the ECL Western Blotting Substrate (Thermo Scientific, Nidderau, Germany). UR-PI294 and UR-PI376 were synthesized as described previously (Igel *et al.*, 2009a,b). Thioperamide, JNJ7777120 and VUF8430 were synthesized according to Lange *et al.* (1995), Jablonowski *et al.* (2003) and Lim *et al.* (2006). Isoloxapine (Schmutz *et al.*, 1967; Smits *et al.*, 2006) was provided by S. Gobleder

Table 1

Saturation binding data for [^{3}H]-histamine at H_4 receptor wild types and mutants

Receptor	K_d (nM)	B_{max} (pmol·mg^{-1})	*n*
hH_4R-wt	11.16 ± 1.92	1.93 ± 0.32	3
hH_4R-F169V	20.15 ± 4.47	1.92 ± 0.23	3
hH_4R-S179M	17.81 ± 3.26	2.08 ± 0.02	3
hH_4R-F169V+S179M	36.59 ± 4.24	1.52 ± 0.07	3
hH_4R-S179A	14.81 ± 3.84	2.25 ± 0.16	3
hH_4R-F169V+S179A	28.65 ± 3.57	1.46 ± 0.09	3

K_d and B_{max} values are given as mean ± SEM for *n* independent experiments, each performed in triplicate. Non-specific binding, amounting to 6.4–16.0% of total binding at 100 nM [^{3}H]-histamine, was determined in the presence of 10 μM unlabelled histamine. The respective binding curves are available as Supplementary Material (Supporting Information Fig. S3).

(Institute of Pharmacy, University of Regensburg, Regensburg, Germany). All other H_4 receptor ligands were purchased from Tocris (Avonmouth, Bristol, UK). The chemical structures of the ligands are depicted in Figure 1. UR-PI376 (10 mM) was dissolved in 50% (v v^{-1}) DMSO and dilutions were prepared in 20% (v v^{-1}) DMSO in order to attain a final DMSO concentration of 2% (v v^{-1}) in each well. About 10 mM stock solutions of clozapine and isoloxapine were prepared in Millipore water containing 3 and 2 mole equivalents of HCl respectively. All other stock solutions were prepared with Millipore water. [^{35}S]-GTPγS (≥1000 Ci·mmol^{-1}, radiochemical purity >95%) and [^{3}H]-histamine (14.2 Ci·mmol^{-1}) were from Hartmann Analytic (Braunschweig, Germany). All other reagents were from standard suppliers and of the highest purity available.

For the construction of the cDNA for hH_4R-F169V, hH_4R-S179A/M, hH_4R-F169V+S179A/M, mH_4R-V171F and mH_4R-V171F+M181S, SDS-PAGE, Western blotting and cell culture, compare with Supporting Information. Cell culture, the generation of recombinant baculoviruses and membrane preparation (Gether *et al.*, 1995; Schneider *et al.*, 2009; Brunskole *et al.*, 2011) as well as H_4 receptor binding studies with [^{3}H]-histamine and [^{35}S]-GTPγS assays (Geyer and Buschauer, 2011; Geyer *et al.*, 2014) were essentially performed as previously described with minor modifications (cf. Supporting Information).

The drug/molecular target nomenclature conforms to BJP's Concise Guide to Pharmacology (Alexander *et al.*, 2013).

Results

Expression of recombinant proteins

Histamine H_4 receptor wild types (hH_4R, mH_4R and rH_4R) as well as mutants (hH_4R-F169V, mH_4R-V171F, hH_4R-S179A, hH_4R-S179M, hH_4R-F169V+S179A, hH_4R-F169V+S179M and mH_4R-V171F+M181S) were expressed in Sf9 insect cells together with G-protein subunits $G\alpha_{i2}$ and $G\beta_1\gamma_2$ (Schneider *et al.*, 2010). High expression at comparable ratios of both, receptors (wild types and mutants) and G-proteins, was confirmed by SDS-PAGE with Coomassie staining and densitometric analysis referred to the bands with apparent molecular weights of 78, 76, 33 and 30 kDa, respectively, present in all samples including the negative control (cf. Supporting Information Fig. S1A and B), Western blots using anti-FLAG M1 and anti $G\alpha_{i1/2}$ antibodies identified bands at 39 and 71 kDa, probably representing the unglycosylated and the glycosylated or the dimeric form of the receptor, as exemplarily shown for hH_4R-F169V in Supporting Information Fig. S2. The $G\alpha_{i2}$-protein appeared at 41 kDa (Supporting Information Fig. S2). Regardless of the high expression of the mH_4 receptor, the rH_4 receptor and the mH_4R mutants, in these cases almost no specific binding of [^{3}H]-histamine was detectable, which is in agreement with reported data for the mH_4 receptor and rH_4 receptor (Schnell *et al.*, 2011), most probably due to the low affinity of histamine to these receptor proteins. Therefore, competition binding experiments with [^{3}H]-histamine were not feasible at mH_4 receptors, mH_4 receptor mutants and rH_4 receptors.

By contrast, high specific binding of [^{3}H]-histamine to the hH_4 receptors, hH_4R-F169V, hH_4R-S179A, hH_4R-S179M mutant and to the hH_4R-F169V+S179A and hH_4R-F169V+S179M double mutants was detected. B_{max} values ranged from 1.5 to 2.3 pmol [^{3}H]-histamine mg^{-1} of soluble membrane protein and the K_d values of [^{3}H]-histamine from 11.2 to 36.6 nM (Table 1 and Supporting Information Fig. S3).

[^{3}H]-histamine competition binding experiments

The affinity at the hH_4R-F169V mutant was in the same range or lower compared to the data at the wild-type hH_4 receptor (Table 2). The decrease in affinity was pronounced for UR-PI376 (pK_i 6.33 vs. 7.27), clozapine (pK_i 5.51 vs. 6.18), isoloxapine (pK_i 6.05 vs. 6.93) and clobenpropit (pK_i 7.21 vs. 7.73). Effects of a single S179A or S179M mutation on affinity were marginal for most compounds, but higher affinity at hH_4R-S179A compared to the wild type was determined in case of thioperamide, JNJ7777120, clozapine, isoloxapine and UR-PI376. At the double mutants, clozapine, isoloxapine and UR-PI376 showed reduced affinity, whereas the affinity of thioperamide and JNJ7777120 for the hH_4R-F169V+S179A variant was even higher than for the hH_4 receptor. In general, the pK_i values were higher at the hH_4R-S179A than at the

Table 2

[^{35}S]-GTPγS and [^{3}H]-histamine binding on hH_4 receptor wild type and mutants[a]

Ligand		hH_4R	hH_4R-F169V	hH_4R-S179M	hH_4R-F169V+S179M	hH_4R-S179A	hH_4R-F169V+S179A
Histamine	pEC_{50}	8.13 ± 0.06°°	7.72 ± 0.07**,°°	7.48 ± 0.08**,°°	7.24 ± 0.02**,°°	7.50 ± 0.05**,°°	7.36 ± 0.07**,°°
	α	1	1	1	1	1	1
	pK_i	7.89 ± 0.04	7.59 ± 0.05*	7.49 ± 0.03**	7.40 ± 0.06**	7.61 ± 0.07*	7.45 ± 0.07**
UR-PI294	pEC_{50}	8.35 ± 0.04°°	8.00 ± 0.11°°	7.98 ± 0.11°°	7.82 ± 0.02**,°°	8.16 ± 0.04°°	7.84 ± 0.01**,°°
	α	1.02 ± 0.03	1.00 ± 0.07	0.98 ± 0.00	0.94 ± 0.05	0.92 ± 0.03	0.86 ± 0.08
	pK_i	7.84 ± 0.03	7.83 ± 0.04	7.93 ± 0.16	7.81 ± 0.05	7.90 ± 0.09	7.72 ± 0.08
Thioperamide	pEC_{50}	6.58 ± 0.06°°	6.52 ± 0.05°°	6.51 ± 0.04°°	6.60 ± 0.05°°	6.78 ± 0.06	7.28 ± 0.11
	A	–1.39 ± 0.08°°	–0.63 ± 0.06**,°°	–1.19 ± 0.06°°	–0.28 ± 0.04**	–1.12 ± 0.06*,°°	–0.23 ± 0.03**
	pK_b	6.83 ± 0.05	–	–	6.81 ± 0.07	–	7.60 ± 0.10**,°°
	pK_i	6.75 ± 0.07	6.98 ± 0.15	6.67 ± 0.04	6.58 ± 0.06	7.34 ± 0.14*	7.29 ± 0.16
JNJ7777120	pEC_{50}	7.10 ± 0.08°°	6.21 ± 0.12**	7.12 ± 0.03°°	7.28 ± 0.11	7.99 ± 0.08**,°°	n.a.
	A	–0.39 ± 0.03°°	0.43 ± 0.03**	–0.48 ± 0.03°°	0.18 ± 0.04**,°°	–0.66 ± 0.06**,°°	0**,°°
	pK_b	7.60 ± 0.05	–	–	6.85 ± 0.16°°	–	7.47 ± 0.09°°
	pK_i	7.16 ± 0.05	6.83 ± 0.05**	7.23 ± 0.07	6.81 ± 0.02**	7.78 ± 0.02**	7.48 ± 0.04*
Clozapine	pEC_{50}	6.24 ± 0.10°°	5.68 ± 0.12*,°°	6.26 ± 0.12°°	5.25 ± 0.04**	6.59 ± 0.10°°	5.71 ± 0.07*,°°
	α	0.67 ± 0.04°°	0.56 ± 0.03°°	0.49 ± 0.08°°	0.49 ± 0.03°°	0.62 ± 0.09°°	0.36 ± 0.02**,°°
	pK_i	6.18 ± 0.03	5.51 ± 0.16*	6.36 ± 0.12	5.23 ± 0.14**	6.59 ± 0.11	5.48 ± 0.04*
Isoloxapine	pEC_{50}	7.08 ± 0.13°°	6.36 ± 0.10**,°°	7.26 ± 0.08°°	6.24 ± 0.09**,°°	7.36 ± 0.07°°	6.69 ± 0.03°°
	α	0.81 ± 0.03°°	0.85 ± 0.09°°	0.62 ± 0.03°°	0.90 ± 0.03°°	0.77 ± 0.06°°	0.83 ± 0.10°°
	pK_i	6.93 ± 0.02	6.05 ± 0.13**	7.02 ± 0.10	6.24 ± 0.08**	7.47 ± 0.08*	6.68 ± 0.09
Clobenpropit	pEC_{50}	7.65 ± 0.11°°	7.63 ± 0.15°°	6.10 ± 0.15**	n.a.	n.a.	n.a.
	α	0.45 ± 0.04°°	0.27 ± 0.05*	–0.44 ± 0.04**,°°	0**,°	0**,°	0**,°
	pK_b	–	–	–	7.06 ± 0.07	7.42 ± 0.08	7.56 ± 0.16°
	pK_i	7.73 ± 0.07	7.21 ± 0.03**	7.14 ± 0.09**	7.23 ± 0.04**	7.56 ± 0.06	7.22 ± 0.02**
UR-PI376	pEC_{50}	7.79 ± 0.08°°	6.25 ± 0.11**	6.93 ± 0.06**,°°	7.23 ± 0.12	7.28 ± 0.04°°	6.88 ± 0.18**,°°
	α	1.11 ± 0.08°°	0.49 ± 0.02**,°°	0.80 ± 0.04**,°°	0.12 ± 0.01**	1.02 ± 0.06°°	0.25 ± 0.01**,°°
	pK_b	–	–	–	5.82 ± 0.14**	–	6.31 ± 0.22
	pK_i	7.27 ± 0.07	6.33 ± 0.11**	7.10 ± 0.12	6.18 ± 0.06**	7.60 ± 0.04	6.40 ± 0.07**
VUF8430	pEC_{50}	7.42 ± 0.12°°	7.61 ± 0.07°°	7.41 ± 0.08°°	7.06 ± 0.13°°	7.53 ± 0.09°°	7.36 ± 0.09°°
	α	0.84 ± 0.06	0.91 ± 0.06	0.85 ± 0.03	0.86 ± 0.01	0.85 ± 0.05	0.75 ± 0.06
	pK_i	7.84 ± 0.03	7.44 ± 0.02	7.55 ± 0.07	7.42 ± 0.15	7.81 ± 0.14	7.69 ± 0.15
Immepip	pEC_{50}	7.67 ± 0.05°°	7.73 ± 0.19°°	7.45 ± 0.10°°	7.45 ± 0.10°°	7.67 ± 0.09°°	7.68 ± 0.11°°
	α	0.81 ± 0.03	0.85 ± 0.05	0.84 ± 0.09	0.84 ± 0.03	0.85 ± 0.06	0.65 ± 0.08
	pK_i	7.73 ± 0.16	7.47 ± 0.00	7.49 ± 0.09	7.54 ± 0.13	7.44 ± 0.08	7.52 ± 0.08

[a]pEC_{50} values ([^{35}S]-GTPγS agonist mode), pK_b values ([^{35}S]-GTPγS antagonist mode), pK_i values ([^{3}H]-histamine competition binding) and α (intrinsic activity, maximal effect relative to histamine = 1.0) are given as mean ± SEM of at least three (up to nine) independent experiments, performed in triplicate. Results of statistical tests (one-way ANOVA and Bonferroni *post hoc* tests): significant differences with respect to hH_4 receptor – *$P \leq 0.05$, **$P \leq 0.01$; significant differences with respect to mH_4 receptor – °$P \leq 0.05$, °°$P \leq 0.01$. In case of neutral antagonism ($-0.25 \leq \alpha \leq 0.25$), pK_b values were considered for statistical analysis instead of pEC_{50} values. Maximal effect α = 0: neutral antagonism, n.d.: not determined, n.a.: pEC_{50}, pK_b or pK_i not applicable from performed experiments. Functional data for hH_4 receptor compared with Nordemann *et al.* (2013).

hH_4R-S179M single mutants, and higher at the hH_4R-F169V+S179A than at the hH_4R-F169V+S179M double mutants (Table 2).

Functional analysis of wild-type and mutant H_4 receptors in the [^{35}S]-GTPγS assay

We determined potencies (pEC_{50}) and maximal effects (α) as well as antagonist activities (pK_b) at wild-type and mutated receptors in the [^{35}S]-GTPγS assay using agonists and antagonists respectively (Figure 1, Tables 2 and 3). Amounts of [^{35}S]-GTPγS bound were similar except for mH_4R-V171F+M181S, mH_4R-V171F, mH_4 receptor and rH_4 receptor (Figure 4A). To facilitate comparison of the ratio of agonism to inverse agonism at the H_4 receptor orthologues and mutants, the changes in [^{35}S]-GTPγS binding were expressed as relative values in Figure 4B. In this representation, the span between maximal increase in [^{35}S]-GTPγS binding elicited by the full agonist histamine and maximal decrease induced by the inverse agonist thioperamide was set to 100%. [^{35}S]-GTPγS binding in the absence of ligand (water control) was set to zero (Figure 4B). The inverse agonism of thioperamide reflects the extent of constitutive activity of the respective wild-type or mutated H_4 receptor (Figure 4). The response to thioperamide decreased in the order: hH_4 receptor > hH_4R-S179M > hH_4R-S179A > hH_4R-F169V > hH_4R-F169V+S179M > hH_4R-F169V+S179A > mH_4R-V171F+M181S > mH_4R-V171F = rH_4 receptor = mH_4 receptor. Thus, the single mutation F169V significantly decreased the exceptionally high constitutive activity of the hH_4 receptor, and the mutation of hH_4R-F169 and S179 into the corresponding amino acids of the mH_4 and rH_4 receptors caused a further decrease. The single hH_4R-S179A or S179M mutation did not reduce constitutive activity significantly. Accordingly, F169 alone and in concert with S179 contributed to the high constitutive activity of the hH_4 receptor. The mH_4 receptor and the rH_4 receptor did not show constitutive activity under the same conditions; thioperamide behaved as a neutral antagonist in the [^{35}S]-GTPγS assay. This was also the case for the mH_4R-V171F mutant, and there was no significant increase in constitutive activity for the mH_4R-V171F+M181S mutant. The higher the constitutive

Table 3

[^{35}S]-GTPγS binding at mH_4 receptor and rH_4 receptor wild types and mH_4 receptor mutants[a]

Ligand		hH_4R	mH_4R-V171F+M181S	mH_4R-V171F	mH_4R	rH_4R
Histamine	pEC_{50}	8.13 ± 0.06°°	5.87 ± 0.05**,°°	5.95 ± 0.08**,°°	5.17 ± 0.14**	4.28 ± 0.06**,°°
	α	1	1	1	1	1
UR-PI294	pEC_{50}	8.35 ± 0.04°°	6.95 ± 0.11**,°°	7.25 ± 0.02**,°°	6.10 ± 0.11**	5.48 ± 0.08**,°°
	α	1.02 ± 0.03	0.94 ± 0.04	0.99 ± 0.09	0.95 ± 0.03	1.09 ± 0.03
Thioperamide	pEC_{50}	6.58 ± 0.06°°	7.11 ± 0.08	n.a.	n.a.	n.a.
	α	–1.39 ± 0.08°°	–0.20 ± 0.03**	0**	0**	0**
	pK_b	6.83 ± 0.05	7.84 ± 0.04**,°°	7.73 ± 0.09**,°°	7.12 ± 0.09**	6.44 ± 0.09°°
JNJ7777120	pEC_{50}	7.10 ± 0.08°°	n.a.	6.93 ± 0.12°°	6.10 ± 0.07**	6.13 ± 0.14
	α	–0.39 ± 0.03°°	0**,°°	0.42 ± 0.03**	0.44 ± 0.02**	0.24 ± 0.01**,°°
	pK_b	7.60 ± 0.05	5.90 ± 0.03**	–	–	4.93 ± 0.16**,°°
Clozapine	pEC_{50}	6.24 ± 0.10°°	5.71 ± 0.16*,°°	5.35 ± 0.03**	n.a.	n.a.
	α	0.67 ± 0.04°°	0.41 ± 0.08*,°°	0.45 ± 0.04°°	0**	0**
	pK_b	–	–	–	4.92 ± 0.04**	4.90 ± 0.09**
Isoloxapine	pEC_{50}	7.08 ± 0.13°°	6.01 ± 0.05**,°°	5.69 ± 0.16**	n.a.	5.82 ± 0.16
	α	0.81 ± 0.03°°	0.68 ± 0.05°°	0.44 ± 0.01*,°	0**	0.19 ± 0.03**
	pK_b	–	–	–	5.26 ± 0.03**	5.12 ± 0.02**
Clobenpropit	pEC_{50}	7.65 ± 0.11°°	6.72 ± 0.13**	7.00 ± 0.15*	6.07 ± 0.09	n.a.
	α	0.45 ± 0.04°°	0.35 ± 0.03	0.27 ± 0.04*	0.20 ± 0.02**	0**,°
	pK_b	–	–	–	6.79 ± 0.00**	6.28 ± 0.04**
UR-PI376	pEC_{50}	7.79 ± 0.08°°	6.08 ± 0.03**	n.a.	n.a.	n.a.
	α	1.11 ± 0.08°°	0.33 ± 0.04**,°°	0**	0**	0**
	pK_b	–	6.08 ± 0.11	6.30 ± 0.10**	6.06 ± 0.17**	5.48 ± 0.03**
VUF8430	pEC_{50}	7.42 ± 0.12°°	5.83 ± 0.16**,°	5.75 ± 0.18**,°	5.06 ± 0.14**	4.47 ± 0.15**
	α	0.84 ± 0.06	0.73 ± 0.07	0.67 ± 0.05	0.68 ± 0.04	0.43 ± 0.05**
Immepip	pEC_{50}	7.67 ± 0.05°°	5.73 ± 0.06**	6.10 ± 0.12**,°°	5.27 ± 0.06**	4.95 ± 0.07**
	α	0.81 ± 0.03	0.95 ± 0.03	0.66 ± 0.09	0.67 ± 0.08	0.68 ± 0.10

[a]For definition of symbols compare footnote with Table 2; functional data for mH_4 receptor and rH_4 receptor compared with Nordemann *et al.* (2013).

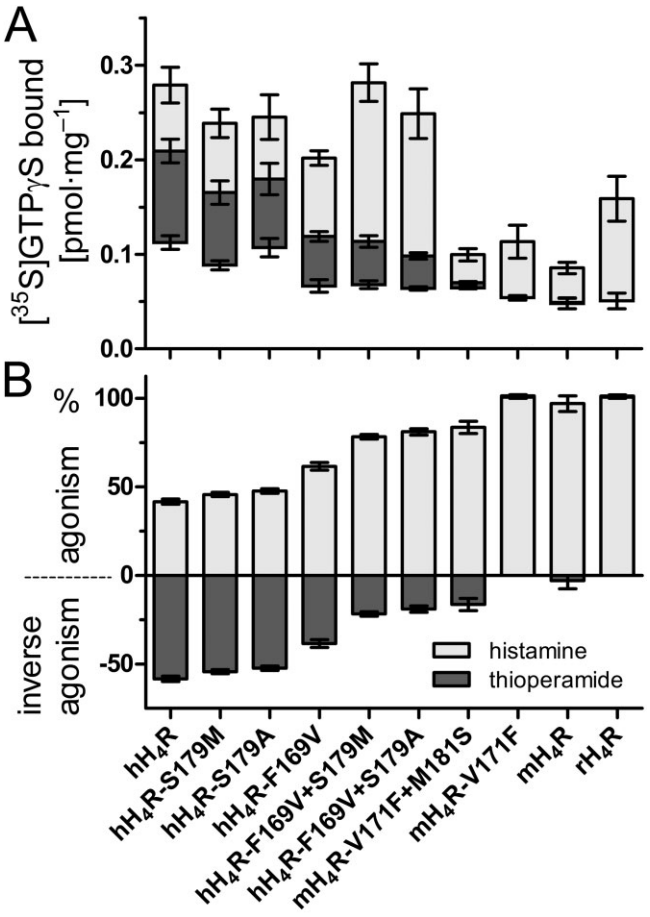

Figure 4

Maximal agonistic effects of histamine and maximal inverse agonistic effects of thioperamide in [^{35}S]-GTPγS assays. (A) Absolute values of bound [^{35}S]-GTPγS (pmol·mg^{-1} protein) in the presence of histamine and thioperamide. Values demarcating light and dark grey bars represent the basal amount (in the absence of ligand) of bound [^{35}S]-GTPγS. (B) For each H_4 receptor species, the sum of the histamine and thioperamide effects was scaled to 100%; the zero line represents the ligand-free control. Significant changes: hH_4R versus hH_4R-F169V ($P < 0.001$), hH_4R versus hH_4R-F169V+S179A ($P < 0.001$), hH_4R versus $hH_4RF169V$+ S179M ($P < 0.001$), hH_4R-F169V versus hH_4R-F169V+S179A ($P < 0.001$), hH_4R-F169V versus $hH_4RF169V$+S179M ($P < 0.001$) and mH_4R versus mH_4R-V171F+M181S ($P < 0.05$).

activity, the lower is the relative 'residual' receptor capacity for activation by agonists (Figure 4B). Thus, the relative maximal response to histamine increased in the order: hH_4 receptor < hH_4R-S179M < hH_4R-S179A < hH_4R-F169V < hH_4R-F169V+S179M < hH_4R-F169V+S179A < mH_4R-V171F+M181S < mH_4R-V171F = rH_4 receptor = mH_4 receptor.

Concentration–response curves of histamine normalized to a percentual scale (maximal effect 100%) are shown in Figure 5A and Supporting Information Fig. S4A. The potency of histamine decreased from the hH_4 receptor via hH_4R-F169V, hH_4R-S179A and hH_4R-S179M mutants to the hH_4 receptor double mutants by up to one order of magnitude (Table 2, Figure 5A and Supporting Information Fig. S4A). The potencies of histamine at the mH_4 receptor and the rH_4 receptor were low (pEC_{50} ~ 4–5, Table 3, Figure 5A and Supporting Information Fig. S4A). Corresponding to the key role of F169 in the hH_4 receptor, the potency was significantly higher at the mH_4R-V171F and mH_4R-V171F+M181S mutant than at the mH_4 receptor wild type.

UR-PI294 (Igel *et al.*, 2009b) was a full agonist with potencies being 5 to 10 times higher than those of histamine at all H_4 receptor species variants (Figure 5B and Supporting Information Fig. S4B; Tables 2 and 3). The rank order at the hH_4 receptor mutants corresponded to that of histamine. The pEC_{50} value at mH_4R-V171F was in between the values at the hH_4 receptor and mH_4 receptor wild types, that is, the presence of F169, making the mH_4 receptor more similar to the hH_4 receptor, substantially increased the potency of UR-PI294, too.

The inverse agonistic response to thioperamide was highest at the hH_4 receptor, slightly smaller at the hH_4R-S179A (for concentration–response curves on this mutant, compare with Supporting Information Fig. S4C) and hH_4R-S179M mutants, significantly reduced at the hH_4R-F169V mutant and, in particular, at the double mutants, hH_4R-F169V+S179A and hH_4R-F169V+S179M (Figure 5C; Table 2). Whereas thioperamide acted as a weak partial inverse agonist at the mH_4R-V171F+M181S mutant, it behaved as a neutral antagonist at the mH_4 receptor, the rH_4 receptor and the mH_4R-V171F mutant with pK_b values of 7.84, 7.12, 6.44 and 7.73 respectively.

JNJ7777120 was a partial inverse agonist at the highly constitutively active hH_4 receptor and hH_4R-S179A/M (Figure 5D and Supporting Information Fig. S4D; Tables 2 and 3) but a partial agonist at the hH_4R-F169V mutant, the mH_4 receptor, the rH_4 receptor and the mH_4R-V171F mutant. At the double mutants as well as at the mH_4R-V171F+M181S mutant, the compound rather behaved as a neutral antagonist.

Clozapine and isoloxapine were weak partial agonists or neutral antagonists at the mH_4 receptor and the rH_4 receptor (Figure 5E; Supporting Information Fig. S4E; Table 3). Introduction of phenylalanine into the mH_4 receptor (mH_4R-V171F mutant) significantly increased partial agonism of both compounds. Furthermore, at the hH_4 receptor and its mutants, clozapine and isoloxapine acted as partial agonists. At the hH_4R-F169V and the double mutants, the potencies were lower than at the wild-type receptor, with the maximal effects only decreasing in case of clozapine. In contrast, at the hH_4R-S179M and S179A mutants, potencies of both clozapine and isoloxapine were similar to those at the hH_4 receptor; maximal effects were reduced only at the S179M mutant. Generally, the potencies and the maximal effects of isoloxapine were higher than those of clozapine.

Both clobenpropit, a partial, and UR-PI376 (Igel *et al.*, 2009a), a full agonist at the hH_4 receptor, showed a considerable decrease in the maximal effects from the hH_4 receptor wild type over the hH_4R-F169V mutant to the double mutants, where clobenpropit revealed neutral antagonism (Figure 5G and H and Supporting Information Fig. S4G and H; Table 2). At the hH_4R-S179M mutant, clobenpropit was a partial inverse agonist. At the hH_4 receptor, the pEC_{50} values of UR-PI376 and clobenpropit were similar, whereas at the

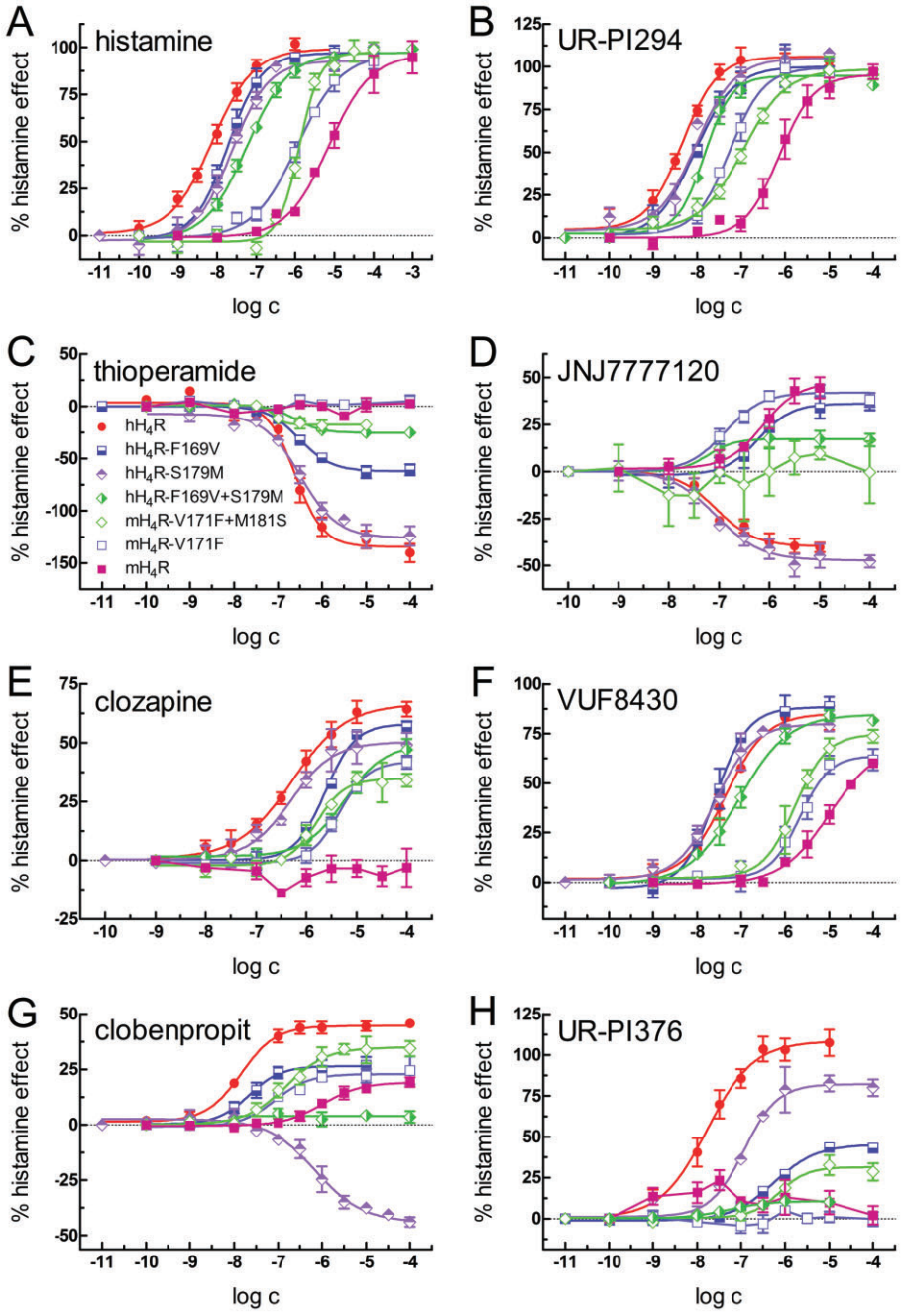

Figure 5

Concentration–response curves of eight ligands investigated in [^{35}S]-GTPγS and [^{3}H]-histamine competition binding assays. All curves are scaled with respect to a maximal histamine effect of 100%. Symbols and colours refer to the species variants and mutants respectively. Filled symbols: wild types; open symbols: mutants. (A) histamine; (B) UR-PI294; (C) thioperamide; (D) JNJ7777120; (E) clozapine; (F) VUF8430; (G) clobenpropit; (H) UR-PI376.

double mutants the pK_b values of UR-PI376 were much lower than those of clobenpropit. At the mH_4 receptor, the rH_4 receptor, the mH_4R-V171F and the mH_4R-V171F+M181S mutant, both compounds behaved as weak partial agonists or neutral antagonists with maximal effects increasing from mH_4 receptor over the mH_4R-V171F to the mH_4R-V171F+M181S mutants (Table 3).

The potent hH_4 receptor agonists VUF8430 (Figures 5F; Supporting Information Fig. S4F; Tables 2 and 3) and immepip showed only little changes in pEC_{50} and α values at the five hH_4 receptor mutants in comparison to the wild type. However, at the mH_4 receptor, the rH_4 receptor and the mH_4R–V171F mutant, potencies and maximal effects were much lower.

Discussion and conclusions

Affinities and potencies of the investigated ligands at H_4 receptor orthologues and mutants

Except for clobenpropit at hH_4R-S179M, binding data were in the same range as the respective EC_{50} values from functional studies in the [^{35}S]-GTPγS-assay (Table 2). Comparing mutant with wild-type receptors, changes in potency (Figure 6B and Supporting Information Fig. S5B) were higher than changes in affinity (Figure 6C and Supporting Information Fig. S5C), for example, in case of histamine and UR-PI294, indicating that the higher potencies of ligands at the hH_4 receptor were a result of the higher constitutive activity. For most agonists, potencies were lower at hH_4R-F169V and/or the double mutants than at the hH_4 receptor, and higher at the mH_4R-V171F and/or mH_4R-V171F+M181S mutant than at the mH_4 receptor (Tables 2 and 3). Remarkable exceptions were VUF8430 and immepip with only minor effects of the F169V and the double mutations. With respect to histamine, clozapine and VUF8430, our results correlate with previous data (Lim *et al.*, 2008), showing markedly reduced affinity for the hH_4R-F169V compared to the wild type in the case of histamine and clozapine, whereas the affinity of VUF8430 was only slightly lowered.

For clozapine and JNJ7777120, binding modes were proposed in which the phenyl and chlorophenyl moieties, respectively, occupy a pocket between TMs 3, 5, 6 and ECL2 (Lim *et al.*, 2010; Kooistra *et al.*, 2013). The phenyl rings of isoloxapine and UR-PI376 may adopt similar positions. For UR-PI294, clobenpropit, VUF8430 and immepip, the potencies at the hH_4R-F169V mutant indicate no influence of F169 on binding. However, at the mH_4R-V171F mutant these compounds are more potent than at the mH_4 receptor wild type. The structures of these ligands suggest a binding mode different to that of JNJ7777120, clozapine and isoloxapine (pK_i values: cf. Table 2). The potencies of histamine, JNJ7777120, clozapine, clobenpropit and UR-PI376 are different on at least one of the double mutants compared to the hH_4R-F169V single mutant (Figure 6B). The additional mutation may either lead to a decrease in potency (histamine) or an increase (JNJ7777120) at both double mutants. The docking poses of histamine (Figure 3), clozapine and JNJ7777120 (Lim *et al.*, 2010; Kooistra *et al.*, 2013) do not indicate direct interactions with F169, but its substitution by valine may alter or destabilize the topology of the ligand binding pocket, in particular the conformation of L175$^{5.39}$, L326$^{6.58}$ and Y340$^{7.35}$ (Figure 7) and, in turn, selectively affect ligand-receptor interactions. Alternatively or additionally, F169 at the entrance of the pocket may be part of the 'optimal' ligand binding path.

In accordance with previous reports (Shin *et al.*, 2002; Lim *et al.*, 2008), the hH_4R-S179A and S179M mutants suggest a minor role of S179$^{5.43}$ on histamine binding. An increase in both potency and affinity (cf. thioperamide, JNJ7777120, clozapine, isoloxapine) due to S179A exchange may be interpreted as a hint that hydrophobic interactions come into play. For most ligands, pEC_{50} and pK_i values are lower at

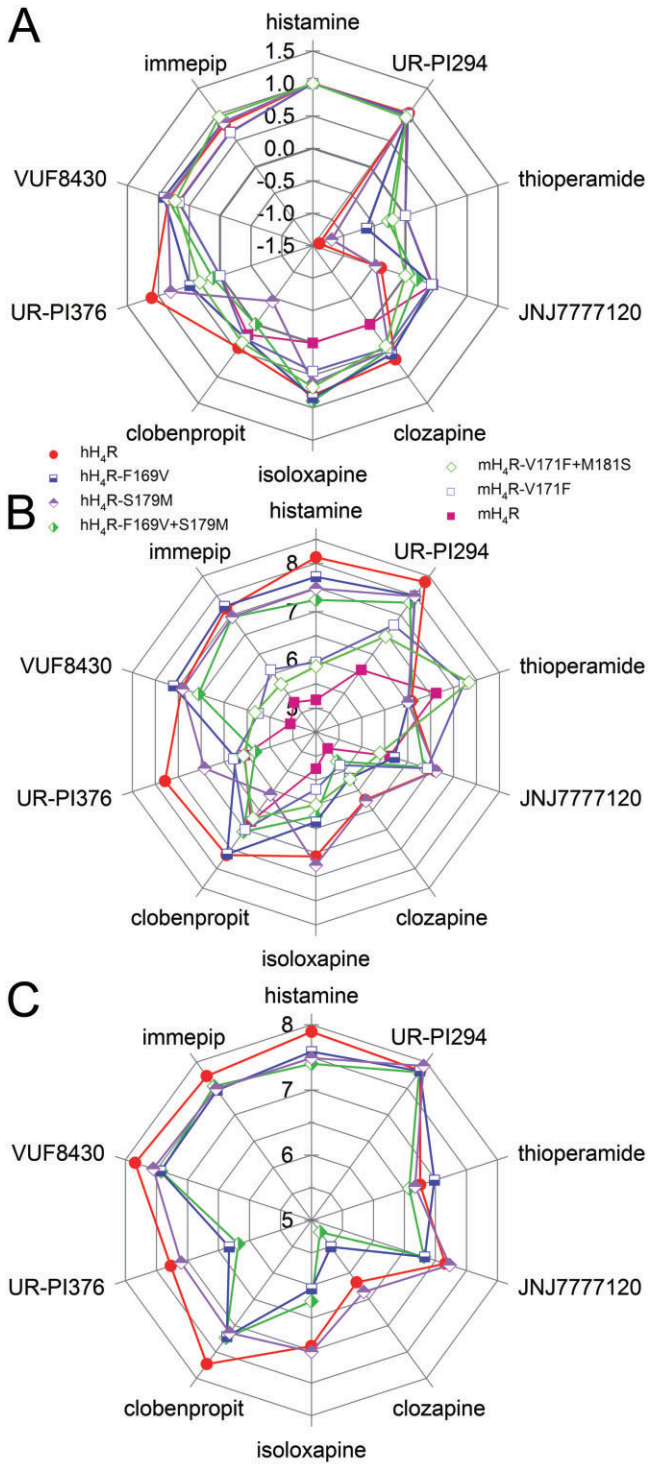

Figure 6

Radar plots of maximal effects, potencies and affinities at wild type and three mutant human → mouse and two mouse → human H_4 receptors. (A) Maximal effects (α values, relative to histamine = 1), (B) pEC_{50} values (or pK_b in case of partial agonists with $-0.25 \leq \alpha \leq 0.25$), (C) pK_i values (n.a. for mH_4R and mH_4R mutants).

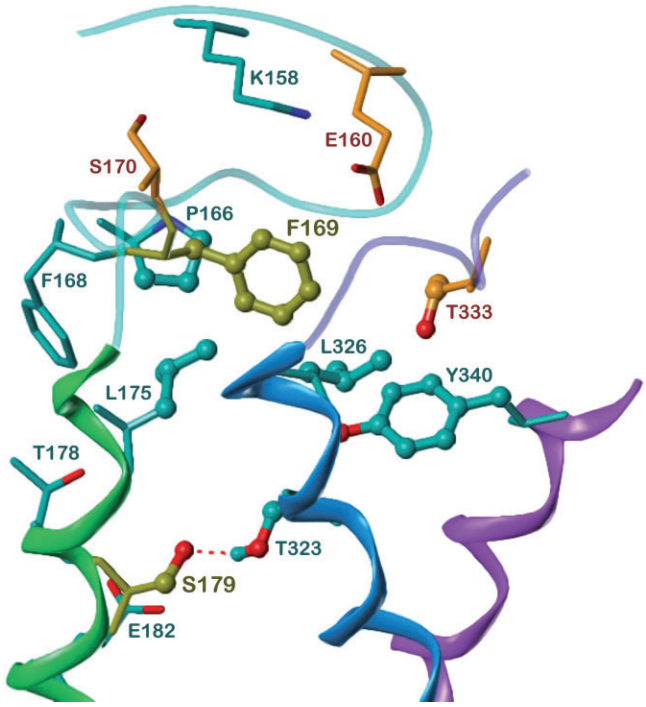

Figure 7

Intramolecular interactions specific for the hH_4 receptor suggested from site-directed mutagenesis – F169V (mH_4R, rH_4R), S179M (mH_4R) and S179A (rH_4R) – and from an hH_4 receptor model based on the crystal structure of the hH_1 receptor. Colours of side chain atoms: N – blue, O – red. Interacting amino acids are represented as ball and stick model. Colours of carbons and backbone nitrogens: F169 and S179 – ochery; other amino acids different in the rH_4 receptor and mH_4 receptor – orange; further residues essential for interactions – cyan. A red dashed line indicates a hydrogen bond between $S179^{5.43}$ and $T323^{6.55}$. TMs are drawn as ribbons: TM5 – green, TM6 – light blue, TM7 – magenta. The C-terminal part of ECL2 and the N-terminal part of ECL3 are shown by tubes (cyan- and violet-coloured respectively).

hH_4R-S179M than at hH_4R-S179A (Table 2), possibly due to steric hindrance of ligand binding by the methionine side chain.

Different quality of action of JNJ7777120

The different degrees of constitutive activity of H_4 receptor species orthologues become obvious from different qualities of action, inverse agonism, neutral antagonism or agonism of one and the same ligand. JNJ7777120 is a partial inverse agonist at the wild-type hH_4 receptor, the hH_4R-S179M and hH_4R-S179A single mutants, and becomes a neutral antagonist at the double mutants hH_4R-F169V+S179M and hH_4R-F169V+S179A as well as at the mH_4R-V171F+M181S mutant and a partial agonist at the mH_4 receptor, the rH_4 receptor and the mH_4R-V171F mutant. Thus, JNJ7777120 fulfils the criteria of a protean agonist: inverse agonism at highly constitutively active receptors and partial agonism at lower or not constitutively active receptors (Kenakin, 2001). A striking exception is the hH_4R-F169V mutant at which JNJ7777120 actually had to be expected to act as a weak partial inverse agonist, but showed partial agonism with similar potency as at the mH_4 receptor and the rH_4 receptor. Possibly, a ligand-specific stabilization of an active state due to the F169V exchange accounts for this apparent discrepancy. The chloro substituent in JNJ7777120 is suggested to interact with the side chain of hH_4R-$L175^{5.39}$ (Lim *et al.*, 2010; Kooistra *et al.*, 2013), which is close to F/V169 (Figure 7). These interactions within the JNJ7777120-occupied binding pocket may result in different qualities of action by stabilizing distinct conformations in wild-type and mutant receptors.

Maximal effects of agonists at H_4 receptor orthologues and mutants

Among the investigated hH_4 receptor agonists, histamine, UR-PI294, isoloxapine, VUF8430 and immepip do not show significantly reduced maximal effects at the hH_4 receptor mutants compared to the wild-type receptor (Figure 6A and Supporting Information Fig. S5A). By contrast, in case of clozapine, clobenpropit and especially UR-PI376, decreasing maximal responses became obvious from the hH_4 receptor over the F169V mutant to the double mutants. Except for UR-PI294 and immepip, which produced responses comparable to that of histamine at all tested H_4 receptor species variants, the maximal agonistic effects (α values) were lowest at the mH_4 receptor and the rH_4 receptor (Figure 6A and Supporting Information Fig. S5A). A significant influence of the mH_4R-V171F mutation was only observed with clozapine and isoloxapine. UR-PI376 was a partial agonist only at the mH_4R-V171F+M181S mutant. Taking the different constitutive activities of the H_4 receptor species variants into consideration, the situation becomes more complex in case of the agonists, too. Equal maximal effects at H_4 receptor orthologues and mutants with high and low constitutive activities, respectively, result from different contributions to the stabilization of the active receptor state by one and the same agonist. Therefore, comparing maximal effects does not allow for drawing conclusions on selective impacts of F169 and/or S179 on receptor activation by different ligands. Furthermore, stabilization of an active state by agonists may be based on interactions different from those in the ligand-free, constitutively active receptor, that is, multiple active states must be taken into consideration. Therefore, beyond the G-protein activation used as readout in the present study, ligand-specific receptor conformations may trigger different signalling pathways according to the concept of functional selectivity [biased signalling; cf. conventional G-protein activation vs. β-arrestin recruitment (Rosethorne and Charlton, 2011; Nijmeijer *et al.*, 2012)].

Constitutive activity

More than 40% of the GPCRs studied *in vitro* have been found to exhibit constitutive activity (Seifert and Wenzel-Seifert, 2002). Active GPCR states may be stabilized by intramolecular interactions in the ligand binding region, also in the absence of agonists. The key result of this study is the fact that the exceptionally high constitutive activity of the hH_4 receptor is significantly reduced by the single F169V and the double F169V+S179M and F169V+S179A mutations, whereas the single S179M and S179A mutations do not significantly reduce constitutive activity. The effect of both amino acids,

F169 (ECL2) and S179$^{5.43}$, on the constitutive activity is cumulative. The mH_4 receptor, the rH_4 receptor and the mH_4R-V171F mutant are not constitutively active. The constitutive activity is slightly increasing at the mH_4R-V171F+M181S mutant. By contrast, high constitutive activity of the hH_4 receptor is reflected by maximal inverse agonism of thioperamide, described as a full (Lim *et al.*, 2005) or partial (Schneider *et al.*, 2009) inverse agonist. In this context, the question arises whether thioperamide is a weaker partial inverse agonist at the hH_4 receptor mutants than at the wild type or whether the maximal inverse agonistic effects only depend on different levels of constitutive activity. The assumption of comparable inverse agonism is supported by the fact that, at the hH_4R-F169V and at the double mutants, but not at the hH_4R-S179A and hH_4R-S179M mutants, the minimum of [^{35}S]-GTPγS binding in the presence of thioperamide approximately corresponds to that at the mH_4 receptor and the rH_4 receptor (Figure 4A). Moreover, in the case of the hH_4 receptor and the double mutants, the pEC_{50}, pK_b and pK_i values are similar (Table 2, Figure 5). All criteria of constitutive activity (Seifert *et al.*, 1998), high basal activity, high intrinsic activity and potency of partial agonists and a high inverse agonistic effect of inverse agonists, are fulfilled.

A possible explanation for the dependence of the high constitutive activity on the presence of F169 and S179 can be derived from a homology model of the hH_4 receptor based on the crystal structure of the hH_1 receptor (Shimamura *et al.*, 2011). Our model indicates that F169 may adopt different conformations. Its phenyl ring may be directed towards the upper part of ECL2 like the corresponding tyrosine in the hH_1 receptor or point to the ligand binding pocket. The first variant is rather unlikely due to an unfavourable polar environment and putative clashes with P166 (ECL2). In the second case shown in Figure 7, F169 is part of a hydrophobic cluster consisting of P166 (ECL2), L175$^{5.39}$, L326$^{6.58}$ and Y340$^{7.35}$. Additionally, F169 contacts T333 (ECL3). A valine side chain as in the mH_4 receptor, rH_4 receptor and the hH_4R-F169V mutants may interact only with P166 and/or L175. Furthermore, S179$^{5.43}$ forms a hydrogen bond with T323$^{6.55}$, which is impossible when S179 is exchanged by alanine or methionine as in the rH_4R and the mH_4R respectively. The cumulative effect on constitutive activity by mutation of both, F169 and S179, indicates that the agonist-free active state of the hH_4 receptor is stabilized by hydrophobic interactions between ECL2 and the extracellular parts of TMs 5, 6 and 7 as well as the hydrogen bond between S179$^{5.43}$ and T323$^{6.55}$. In concert, these contacts favour a specific arrangement in particular of TMs 5 and 6, comparable to the stabilization of an active conformation by an agonist. An inward bulge of TM5 around position 5.46 and smaller inward movements of TMs 6 and 7 are characteristic of the activated β_2-adrenoceptor compared to the inactive state (Rasmussen *et al.*, 2011; Rosenbaum *et al.*, 2011). At the cytoplasmic face of the receptor, an outward move of TM6 and rearrangements of TMs 5 and 7 are necessary for G-protein binding and contribute to the stabilization of active GPCR states. The TMs are suggested to behave as 'oscillating arms'. When they move inwards at the extracellular side, they move outwards at the intracellular side and vice versa. Thus, the inward movement of TM5 and TM6 close to the agonist binding pocket results in an outward movement of these TMs at the 'bottom' of the receptor. In case of the hH_4 receptor, a proximal arrangement of TMs 5 and 6 at the extracellular side becomes possible in the absence of bound agonist due to a network of interactions involving F169 and S179. However, also other amino acids contribute to the agonist-free stabilization of the active state of the hH_4 receptor, since the double mutants still show a moderate degree of constitutive activity.

In case of the β_2-adrenoceptor, an S204A+S207A double mutant showed about 50–60% lower constitutive activity than the β_2-adrenoceptor wild type (Ambrosio *et al.*, 2000). S204$^{5.43}$ forms a hydrogen bond with N293$^{6.55}$ (Rasmussen *et al.*, 2011), corresponding to the suggested interaction of S179$^{5.43}$ with T323$^{6.55}$ in the hH_4 receptor. A contribution of phenylalanine in ECL2 to constitutive activity by a network of hydrophobic interactions with amino acids in TMs 5, 6 and 7 has not been shown for other GPCRs, but may also play a role in other constitutively active receptors such as the hH_3 receptor and the β_2-adrenoceptor , which both contain the same FF motif as the hH_4 receptor.

Conclusions

Until now, most studies on the constitutive activity of GPCRs have focused on the intracellular face, the DRY motif and the N-terminal part of TM6. The present study provides further evidence that intramolecular interactions in the agonist binding region contribute to the stabilization of ligand-free active GPCR states. Key result is the decrease in constitutive activity from the hH_4 receptor over the hH_4R-F169V mutant to the hH_4R-F169V+S179A and hH_4R-F169V+S179M double mutants. Thus, F169 in ECL2 and S179 in TM5 play a major role in stabilizing a ligand-free active state of the hH_4 receptor. Similar results on the β_2-adrenoceptor suggest a common principle that may be of relevance for other GPCRs as well.

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Author contributions

D. W., K. L., G. B., R. S. and A. B. conceived and designed the experiments. D. W., K. L. and U. N. performed the experiments. D. W., S. D. and A. S. performed computational chemistry. D. W., K. L., U. N., S. D., G. B., R. S. and A. B. analysed the data. D. W., G. B., R. S. and A. B. wrote the paper.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Coomassie stained gels.
Figure S2 Western blots.
Figure S3 Saturation binding curves.
Figure S4 Concentration–response curves.
Figure S5 Radar plots.