JPET Fast Forward, Published on March 6, 2003 as DOI:10.1124/jpet.103.049619

Multiple differences in agonist- and antagonist pharmacology between human and guinea pig histamine H_1-receptor

Roland Seifert, Katharina Wenzel-Seifert, Tilmann Bürckstümmer, Heinz H. Pertz, Walter Schunack, Stefan Dove, Armin Buschauer, and Sigurd Elz

Department of Pharmacology and Toxicology, The University of Kansas, Lawrence, KS 66045 (R.S., K.W.-S.)

Department of Molecular Biosciences, The University of Kansas, Lawrence, KS 66045 (T.B.)

Department of Chemistry and Biochemistry, Free University of Berlin, D-14195 Berlin, Germany (T.B.)

Institute of Pharmacy, Free University of Berlin, D-14195 Berlin, Germany (H.H.P., W.S.)

Institute of Pharmacy, University of Regensburg, D-93040 Regensburg, Germany (S.D., A.B., S.E.)

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Differences between human and guinea pig H_1-receptor

Corresponding author:
Dr. Roland Seifert, Department of Pharmacology and Toxicology, The University of Kansas, Malott Hall, Room 5064, 1251 Wescoe Hall Drive, Lawrence, KS 66045-7582; Phone: 785-864-3525; Fax: 785-864-5219. Email: rseifert@ku.edu

Number of text pages: **23**
Number of Tables: **5**
Number of Figures: **5**
Number of words in the Abstract: **231**
Number of words in the Introduction: **702**
Number of words in the Discussion: **1930**
Number of References: **39**

Abbreviations used: GPCR, G-protein-coupled receptor; gpH_1R, guinea pig histamine H_1-receptor; gpH_2R, guinea pig histamine H_2-receptor; gpH_2R-$G_{s\alpha S}$, fusion protein of the guinea pig histamine H_2-receptor and the short splice variant of $G_{s\alpha}$; H_xR, histamine H_1-, H_2-, H_3- or H_4-receptor; hH_1R, human histamine H_1-receptor; hH_2R, human histamine H_2-receptor; hH_2R-$G_{s\alpha S}$, fusion protein of the human histamine H_2-receptor and the short splice variant of $G_{s\alpha}$; hH_1R-F153L, human histamine H_1-receptor bearing a Phe→Leu exchange at position 153; hH_1R-I433V, human histamine H_1-receptor bearing an Ile→Val exchange at position 433; hH_1R-F153L/I433V, human histamine H_1-receptor bearing a Phe→Leu exchange at position 153 and an Ile→Val exchange at position 433; RGS protein, regulator of G-protein signaling; TM transmembrane domain.

Section: Cellular & Molecular

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Species isoforms of histamine H_2-, H_3- and H_4-receptors differ in their pharmacological properties. The study aim was to dissect differences between the human H_1R (hH_1R) and guinea pig H_1R (ghH_1R). We co-expressed hH_1R and gpH_1R with regulators of G-protein signaling in Sf9 insect cells and analyzed the GTPase activity of G_q-proteins. Small H_1R agonists showed similar effects at hH_1R and gpH_1R, whereas bulkier 2-phenylhistamines and histaprodifens were up to ~10-fold more potent at gpH_1R than at hH_1R. Most 2-phenylhistamines and histaprodifens were more efficacious at gpH_1R than at hH_1R. Several first-generation H_1R antagonists were ~2-fold, and arpromidine-type H_1R antagonists up to ~10-fold more potent at gpH_1R than at hH_1R. [^{3}H]Mepyramine competition binding studies confirmed the potency differences of the GTPase studies. Phe-153→Leu-153- or Ile-433→Val-433 exchange in hH_1R (hH_1R→gpH_1R) resulted in poor receptor expression, low [^{3}H]mepyramine-affinity and functional inactivity. The Phe-153→Leu-153/Ile-433→Val-433 double mutant expressed excellently but only partially changed the pharmacological properties of hH_1R. Small H_1R agonists and 2-phenylhistamines interacted differentially with human and guinea pig H_2R in terms of potency and efficacy, respectively. Our data show the following: (***i***) There are differences in agonist- and antagonist-pharmacology of hH_1R and gpH_1R encompassing diverse classes of bulky ligands. These differences may be explained by higher conformational flexibility of gpH_1R relative to hH_1R. (***ii***) Phe-153 and Ile-433 are critical for proper folding and expression of hH_1R. (***iii***) H_2R species isoforms distinguish between H_1R agonists.

Histamine serves as a neurotransmitter and autacoid and acts through specific H_xRs designated as H_1R, H_2R, H_3R and H_4R, respectively (Hill et al., 1997; Hough, 2001). The H_1R couples to G_q-proteins. Numerous H_1R agonists and antagonists are known. H_1R agonists are divided into three classes (Fig. 1); *i.e.* (***i***) small agonists (**2**-**4**) derived from histamine (**1**), (***ii***) histamine derivatives with bulkier aromatic substituents at the 2-position of the imidazole ring (**5**-**18**), and (***iii***) histaprodifens, *e.g.* compounds **19**-**23** (Leschke et al., 1995; Zingel et al., 1995; Elz et al., 2000). H_1R agonists are important experimental tools to analyze H_1R function in cellular and organ systems (Zingel et al., 1995; Hill et al., 1997). H_1R antagonists are commonly divided into sedating (first-generation, **24**-**32**) and non-sedating (second-generation, **41**-**45**) antagonists (Fig. 2). Today, especially the second-generation H_1R antagonists are of great importance for the treatment of allergic diseases (Hill et al., 1997). Guanidines **33**, **34** and **36**-**39** derived from arpromidine (**35**) are dual H_2R agonists/H_1R antagonists (Buschauer, 1989).

The availability of H_xR cDNAs allowed for the comparison of the pharmacological properties of H_xR species isoforms in recombinant systems under identical experimental conditions. Such expression studies uncovered species-differences in the pharmacological properties of hH_2R and gpH_2R (Kelley et al., 2001), rat and human H_3R (Ligneau et al., 2000; Lovenberg et al., 2000) and H_4R from mouse, rat, guinea pig and humans (Liu et al., 2001). Species-differences in the pharmacological properties of H_xRs provided opportunities to analyze the molecular basis of ligand/GPCR interactions (Ligneau et al., 2000; Kelley et al., 2001). From the standpoint of drug design, the pharmacological properties of hH_xRs are important because in the H_xR field, essentially all structures generated so far were derived from animal models, mostly from rat and guinea pig (Zingel et al., 1995; Hill et al., 1997).

The species-differences in pharmacological properties of H_2R, H_3R and H_4R raise the question whether this is a general characteristic of H_xRs. In fact, the K_d values of [^{3}H]mepyramine for H_1Rs from various species differ by ~2-6-fold (Chang et al., 1979). Moreover, histaprodifens exhibit different potencies and efficacies in the guinea pig ileum and rat aorta (Elz et al., 2000). Furthermore, 2-(3-chlorophenyl)histamine (**12**) is a potent H_1R agonist in the guinea pig ileum but failed to exhibit agonistic activity in H_1R-expressing dibutyryl cAMP-differentiated human HL-60 leukemia cells (Seifert et al., 1994). A snake plot of hH_1R depicts the relative positions and topology of amino acid residues in the TM domains, putative agonist- and antagonist binding sites, and differences with respect to the gpH_1R (Fig. 3). Mutagenesis data (Leurs et al., 1994, 1995; Ohta et al., 1994; Nonaka et al., 1998) and modeling approaches (Elz et al., 2000) indicated that histamine and histaprodifens interact with amino acid residues in TMs III, IV, V and VII. Considering the alignment of H_1Rs with bovine rhodopsin (Palczewski et al., 2000) and results of the substituted-cysteine accessibility method with the dopamine D_2-receptor (Ballesteros et al., 2001), there are no amino acid differences in the ligand binding pocket of gpH_1R and hH_1R. The two lipid-directed residues, Phe-153 in TM IV of hH_1R *versus* Leu in gpH_1R and Ile-433 in TM VI of hH_1R *versus* Val in gpH_1R, represent the only differences near the binding site. Although these amino acid exchanges are conservative, the amino acids in hH_1R are bulkier than those in gpH_1R, and such differences could have an impact on the ligand-binding pocket.

The aim of the present study was to compare recombinant hH_1R and gpH_1R expressed in Sf9 insect cells under identical experimental conditions. We also examined the roles of Phe-153 and Ile-433 in hH_1R function. As read-out, we focused on the determination of the GTPase activity of insect cell G_q-proteins in the presence of the RGS proteins RGS4 and GAIP. This co-

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

expression system provides a sensitive model for studying H_1R at the G-protein level (Houston et al., 2002). The GTPase assay is a steady-state method and eliminates the impact of effector availability/compartmentation and pharmacokinetic barriers on the properties of agonists (Buschauer, 1989; Ostrom et al., 2000). Moreover, we conducted [^{3}H]mepyramine binding studies and analyzed the effects of H_1R agonists on recombinant H_2R-$G_{s\alpha}$ fusion proteins, recently verified as sensitive systems for the analysis of H_2Rs (Kelley et al., 2001).

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Materials. Construction of the cDNAs for hH_1R-F153L, hH_1R-I433V and hH_1R-F153L/I433V was performed by overlap-extension PCR following recently described procedures (Houston et al., 2002). Dimethindene enantiomers were a kind gift of Dr. G. Lambrecht (Department of Pharmacology, University of Frankfurt/M., Germany). Ketotifen was a gift from Novartis (Basel, Switzerland), azelastine a gift from Asta Medica (Frankfurt/M., Germany), fexofenadine a gift from Janssen-Cilag (Neuss, Germany), and terfenadine a gift from Aventis (Frankfurt/M., Germany). Guanidines **33**-**38** were synthesized as described (Buschauer, 1989). Guanidine **39** was prepared by analogy to the procedures described for guanidines **33**-**38**. 2-Methylhistamine (**2**) and 2-(2-thiazolyl)ethanamine (**3**) were synthesized using standard procedures. Compounds **5**-**18** were prepared according to published procedures (Zingel et al., 1990; Leschke et al., 1995). Compounds **22**, **23** and **40** were available by synthetic pathways reported for the synthesis of **19**-**21** (Elz et al., 2000). Structures of synthesized compounds were confirmed by elemental analysis (C, H, N), ^{1}H NMR spectroscopy and mass spectrometry. Purity of compounds was >98% as determined by high-performance liquid chromatography or capillary electrophoresis. Tunicamycin, histamine, betahistine, promazine, chlorpromazine, mianserin, cyproheptadine, diphenhydramine, mepyramine, triprolidine, and (+)-chlorpheniramine were from Sigma (St. Louis, MO). Sources of other materials are described elsewhere (Kelley et al., 2001; Houston et al., 2002).

Cell culture and membrane preparation. Recombinant baculoviruses encoding hH_1R-F153L, hH_1R-I433V and hH_1R-F153L/I433V were generated in Sf9 cells using the BaculoGOLD transfection kit (Pharmingen, San Diego, CA) according to the manufacturer's instructions. Infection and culture of Sf9 cells and membrane preparation were performed as

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

described (Kelley et al., 2001; Houston et al., 2002). In some cultures, we added tunicamycin (10 μg/ml) to cultures to inhibit *N*-glycosylation of H_1Rs (Seifert and Wenzel-Seifert, 2001).

[^{3}H]Mepyramine binding assay. Membranes expressing various H_1R constructs plus RGS proteins were thawed and sedimented by a 15 min centrifugation at 4^{o}C and 15,000 x *g*. Membranes were resuspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). Tubes (total volume 500 μl) contained 20-25 μg of membrane protein. Incubations were conducted for 90 min at 25°C and shaking at 250 rpm. For H_1R saturation binding experiments, tubes contained 0.2-20 nM [^{3}H]mepyramine (hH_1R, gpH_1R and hH_1R-F153L/I433V) or 2-100 nM [^{3}H]mepyramine (hH_1R-F153L and hH_1R-I433V). Non-specific binding was routinely determined in the presence of 10 μM mepyramine (**30**). Non-specific binding in the presence of saturating concentrations of compounds **1**, **3**, **12**, **14**, **15**, **19**, **20**, **31**, **35** and **36** was virtually identical to non-specific binding in the presence of compound **30** (data not shown). Competition binding experiments were carried out in the presence of 2 nM [^{3}H]mepyramine and unlabeled ligands at various concentrations. Bound [^{3}H]mepyramine was separated from free [^{3}H]mepyramine by filtration through GF/C filters, followed by three washes with 2 ml of binding buffer (4^{o}C). Filter-bound radioactivity was determined by liquid scintillation counting.

Steady-state GTPase activity assay. Membranes expressing various H_1R constructs plus RGS proteins or H_2R-$G_{s\alpha}$ fusion proteins were thawed, sedimented and resuspended in 10 mM Tris/HCl, pH 7.4. Assay tubes contained Sf9 membranes (10 μg of protein/tube), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 100 nM GTP, 1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 μg creatine kinase and 0.2% (w/v) bovine serum albumin in 50 mM Tris/HCl, pH 7.4, and H_xR ligands at various concentrations. Reaction mixtures (80 μl) were incubated for 3

min at 25°C before the addition of 20 µl [γ-^{32}P]GTP (0.2-0.5 µCi/tube). Reactions were conducted for 20 min at 25°C. Reactions were terminated by the addition of 900 µl slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. Charcoal-quenched reaction mixtures were centrifuged for 15 min at room temperature at 15,000 x *g*. Seven hundred µl of the supernatant fluid of reaction mixtures were removed, and $^{32}P_i$ was determined by liquid scintillation counting.

SDS-PAGE and immunoblot analysis. Membrane proteins were separated on SDS polyacrylamide gels containing 10% (w/v) acrylamide. Proteins were then transferred onto Immobilon-P transfer membranes (Millipore; Bedford, MA). Membranes were reacted with M1 antibody (1 : 1,000). Immunoreactive bands were visualized by sheep anti-mouse IgG (1 : 1,000) coupled to peroxidase, using *o*-dianisidine and H_2O_2 as substrates. Expression of RGS proteins was verified by immunoblot analysis with specific anti-RGS4 IgG and anti-GAIP IgG as described (Houston et al., 2002).

Miscellaneous. Protein concentrations were determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). All analyses of experimental data were performed with the Prism 3.02 software (GraphPad-Prism, San Diego, CA). K_i- and K_B values were calculated according to Cheng and Prusoff (1973). Statistical comparisons were performed with the *t*-test.

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Immunological detection of H_1R constructs. The H_1R constructs analyzed in this study were all *N*-terminally tagged with the FLAG epitope, allowing immunological detection with the M1 monoclonal antibody (Houston et al., 2002). The predicted molecular mass of non-glycosylated hH_1R and gpH_1R is ~56 kDa (Fukui et al., 1994; Traiffort et al., 1994). The FLAG epitope-tagged hH_1R expressed in Sf9 membranes migrated as diffuse ~75 kDa doublet in SDS-PAGE (Figs. 4A and 4B). Treatment of Sf9 cells with the inhibitor of *N*-glycosylation, tunicamycin (Seifert and Wenzel-Seifert, 2001), shifted the majority of the protein towards 70 kDa and rendered the lower band crisper. Migration of FLAG epitope-tagged gpH_1R in SDS-PAGE differed considerably from the migration of hH_1R. In membranes expressing gpH_1R, faint and diffuse bands in the ~36 kDa- and ~50 kDa regions were detected, and tunicamycin treatment had little effect on migration of gpH_1R in SDS-PAGE (Fig. 4A). Additionally, we detected intense and crisp bands of ~16 kDa and ~30 kDa. Both hH_1R-F153L and hH_1R-I433V showed a broad ladder of diffuse bands ranging from ~30-80 kDa, and there was a more intense doublet at 28-29 kDa (Fig. 4B). The hH_1R-F153L/I433V double mutant showed the predicted migration in SDS-PAGE, *i.e.* this mutant migrated as a ~56 kDa band.

Analysis of H_1R constructs in [^{3}H]mepyramine binding assays. The K_d of [^{3}H]mepyramine for hH_1R expressed in Sf9 membranes was 1.8-fold higher than the K_d for gpH_1R (Table 1). The B_{max} values of hH_1R and gpH_1R expression in Sf9 membranes were similar to the expression levels reported for the β_2-adrenoceptor (Seifert et al., 1998). Compared to hH_1R, the [^{3}H]mepyramine-affinities of hH_1R-F153L and hH_1R-I433V were reduced by ~8-12-

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

fold, and the B_{max} values were reduced by ~5-6-fold. The double mutation restored [^{3}H]mepyramine-affinity of hH_1R and efficient expression.

Potencies and efficacies of H_1R and H_2R agonists at H_1R constructs in the GTPase assay. We studied three classes of H_1R agonists in the GTPase assay (Fig. 1). As a control we also studied the H_2R agonists amthamine (**46**) and dimaprit (**47**) (Hill et al., 1997). Table 2 and Fig. 5 summarize the data for hH_1R and gpH_1R co-expressed with RGS4 and GAIP since no significant differences were observed between the two RGS proteins (data not shown). Only histamine and the small histamine derivatives **2** and **3** were full hH_1R agonists, whereas all other modifications resulted in reductions of efficacy. Additionally, compounds **2** and **3** were less potent hH_1R agonists than histamine. We identified only two agonists that were more potent at hH_1R than histamine, *i.e.* the histaprodifens **19** and **20**. However, the moderate increase in potency (1.8-2.7-fold) was accompanied by a significant decrease in efficacy. The introduction of a phenyl group (**6**) or particularly a benzyl group (**5**) at the 2-position of the imidazole ring substantially reduced agonist potency. Introduction of a halogen in the *meta* position of the phenyl ring partially restored agonist potency in the order F < Cl < Br ~ I (compare **6**, **9**, **12**, **14** and **15**). Other hydrogen-donating *meta* substituents (OMe, CF_3) were also favorable (**16** and **17**), whereas a methyl group (**7**) and halogen substitutions in the *ortho*- or *para* position of the phenyl ring (**8** and **13**) further reduced agonist potency. At hH_1R, histaprodifens **21**-**23** were less potent than histamine. The H_2R agonists **46** and **47** were essentially devoid of agonistic activity at the hH_1R (Table 2).

We did not observe significant differences in potency and efficacy of the small H_1R agonists **1**-**4** between hH_1R and gpH_1R (Table 2). This similarity between the H_1R isoforms is

reflected by a linear correlation of the pEC_{50} values of the small agonists at hH_1R and gpH_1R that is close to the theoretical correlation describing identity of H_1R species isoforms (Fig. 5A). However, when the effects of 2-phenylhistamines and histaprodifens were analyzed, significant differences between hH_1R and gpH_1R emerged. All compounds of these two classes were significantly more potent (3.2-9.9-fold) at gpH_1R than at hH_1R. The different interaction of 2-phenylhistamines and histaprodifens with hH_1R and gpH_1R is reflected by a linear correlation of the potencies of each series that is shifted towards the left relative to the theoretical correlation describing pharmacological identity of the GPCR species isoforms (Figs. 5B and 5C). These linear correlations also show that the overall structure/activity relationships of those compounds are similar at both H_1R species isoforms. In addition to the higher potency, most 2-phenylhistamines (**6**, **8**-**12**, **14**-**17**) and 3 out of 5 histaprodifens (**20**, **21**, **23**) were significantly more efficacious at gpH_1R than at hH_1R. Finally, the small H_2R agonist dimaprit (**47**) showed only minimal agonistic effects at gpH_1R, but another small agonist, amthamine (**46**), was a weak partial gpH_1R agonist with significantly higher efficacy at gpH_1R than at hH_1R.

We failed to detect GTPase stimulation by histamine and compounds **3** and **12** in Sf9 membranes expressing hH_1R-F153L and hH_1R-I433V plus RGS proteins (data not shown). In contrast, histamine and compound **3** stimulated GTP hydrolysis in membranes expressing hH_1R-F153L/I433V as potently and efficiently as in membranes expressing hH_1R or gpH_1R. 2-Substituted histamines and histaprodifens tended to be more potent and efficacious at hH_1R-F153L/I433V than at hH_1R, but only the potency and efficacy of compound **12** were significantly increased.

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

Constitutive activity of H_1Rs. hH_1R is constitutively active, and many first- and second-generation H_1R antagonists possess inverse agonistic activity (Bakker et al., 2001; Weiner et al., 2001). However, the extent of constitutive activity of hH_1R is dependent on the specific expression system. All first-generation H_1R antagonists (**24**-**32**), second-generation H_1R antagonists (**41**-**45**) and guanidines (**33**-**39**) examined exhibited only small inverse agonistic activity at hH_1R expressed in Sf9 membranes, *i.e.* the inhibitory effects of compounds amounted to ~5-15% of the stimulatory effect of histamine (data not shown). There were no significant differences in the inverse agonist effects of H_1R antagonists at hH_1R and gpH_1R. These data indicate that the constitutive activity of the two GPCR isoforms is similar.

Potencies of H_1R antagonists at H_1R constructs in the GTPase assay. In agreement with the [^{3}H]mepyramine binding studies (Table 1), mepyramine (**30**) was about two-fold less potent at inhibiting histamine-stimulated GTP hydrolysis in membranes expressing hH_1R than in membranes expressing gpH_1R (Table 3). A similar difference in potency was observed for two other first-generation H_1R antagonists, triprolidine (**31**) and (+)-chlorpheniramine (**32**), whereas the other first-generation antagonists studied (**24**-**28**, dimethindene enantiomers (*R*)-(-)-**29** and (*S*)-(+)-**29**) did not exhibit significantly different potencies at hH_1R and gpH_1R. (*R*)-(-)-Dimethindene was ~30-40-fold more potent than (*S*)-(+)-dimethindene. The stereoselectivity of recombinant H_1Rs for dimethindene enantiomers is in accordance with data for the H_1R expressed in the guinea pig ileum (Pfaff et al., 1995). Among the second-generation H_1R antagonists **41**-**45**, no significant differences in potency between hH_1R and gpH_1R emerged.

Arpromidine (**35**) and arpromidine-derived guanidines (**33**, **34**, **36**-**38**) are not only very potent H_2R agonists but also moderately potent H_1R antagonists (Buschauer, 1989). The

H_1R-antagonistic properties of guanidines are explained by the structural similarity of compounds **33**-**38** and **30**-**32** (Fig. 2). Guanidines **33**-**38** inhibited histamine-stimulated GTP hydrolysis in Sf9 membranes expressing gpH_1R with K_B values of ~50-150 nM (Table 3). Guanidines **33**-**38** were all significantly more potent antagonists at gpH_1R than at hH_1R and showed greater gpH_1R/hH_1R selectivity than compounds **30**-**32**. The difference in potency was most pronounced (~9-fold) for compound **36** that is distinguished from the other guanidines by a *para*-Cl in the phenyl moiety (Fig. 2). In contrast, guanidine **39** that possesses a tri-chlorinated phenyl ring and a thiazole instead of a pyridyl ring (Fig. 2), did not discriminate between hH_1R and gpH_1R. Modifications of the substituents in guanidines **33**-**39** had a considerably larger impact on antagonist potency at gpH_1R (~7-fold) than at hH_1R (~2-fold).

In the 2-phenylhistamine derivative **40**, the free amino group of histamine was integrated into a piperidine ring (Fig. 2). This modification is predicted to interfere with the binding of the basic nitrogen to Asp-107 (hH_1R) (Ohta et al., 1994). In fact, compound **40** exhibited 6.5-8-fold reduced apparent affinity compared to its parent compound (**17**) (Fig. 1) at hH_1R and gpH_1R (Tables 2 and 3). Moreover, introduction of the piperidine ring into **17** conferred antagonistic properties to compound **40** (Table 3). This was also confirmed in the guinea pig ileum assay (K_B of compound **40**, 400 nM). Compound **40** was a several-fold more potent antagonist at gpH_1R than at hH_1R.

In agreement with the binding data (Table 1), mepyramine (**30**) was similarly potent at inhibiting histamine-stimulated GTP hydrolysis in Sf9 membranes expressing hH_1R and hH_1R-F153L/I433V (Table 3). The double mutation exhibited inconsistent effects on the potencies of guanidines **33** and **35**-**38** as well as of the 2-phenylhistamine derivative **40**. Specifically, the

F153L/I433V mutation increased the potency of **36** 1.5-fold, had no effect on the potency of **35** and **37** and decreased the potency of compounds **33**, **38** and **40** by up to 2-fold.

Affinities of H_1R agonists and antagonists at H_1R constructs in the [^{3}H]mepyramine binding assay. Histamine and 2-(3-chlorophenyl)histamine (**12**) inhibited [^{3}H]mepyramine binding in Sf9 membranes expressing hH_1R or gpH_1R plus RGS proteins according to a monophasic function that was not shifted to the right by guanosine 5'-*O*-(3-thiotriphosphate) (10 μM) (data not shown). Thus, we could not detect high-affinity agonist binding. These data were expected since there is a paucity of endogenous G-proteins relative to the expressed mammalian GPCRs in Sf9 membranes (Seifert et al., 1998; Houston et al., 2002). Accordingly, the agonist-affinities determined in the [^{3}H]mepyramine competition binding studies reflect the agonist-affinities of H_1Rs in the G-protein-uncoupled state. In fact, the K_i values of agonists **1**, **3**, **12**, **14**, **15**, **19** and **20** at hH_1R and gpH_1R were all higher than the corresponding EC_{50} values in the GTPase assay (Tables 2 and 4). The K_i value of histamine at hH_1R was 2.3-fold lower than the K_i value of histamine at gpH_1R. Since the amino acids in the histamine-binding H_1R domains are identical in both isoforms (Fig. 3), this difference could point to a better fit of histamine into the G_q-uncoupled hH_1R compared to G_q-uncoupled gpH_1R.

In order to account for the difference in histamine-affinity of H_1R species isoforms, we focused on the comparison of the relative affinities of synthetic agonists at hH_1R and gpH_1R. The relative affinity of the small agonist **3** was similar at hH_1R and gpH_1R, whereas the relative affinities of the 2-phenylhistamines **12**, **14** and **15** and of the histaprodifens **19** and **20** were ~3-7-fold higher at gpH_1R than at hH_1R. These differences fit to the differences in relative agonist potencies observed in the GTPase assay (Table 2). In agreement with the GTPase studies (Table

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

3), too, the H_1R antagonists triprolidine (**31**), arpromidine (**35**) and BU-E 47 (**36**) all exhibited significantly higher binding affinities at gpH_1R than at hH_1R (Table 4).

We also studied the impact of the F153L/I433V mutation in hH_1R on ligand-affinities. The double mutation significantly decreased the affinity of hH_1R for histamine and 2-(2-thiazolyl)ethanamine (**3**) (Table 4). Similar data were obtained for the comparison of hH_1R and gpH_1R. Additionally, in membranes expressing hH_1R-F153L/I433V, the relative affinities of 2-phenylhistamines and histaprodifens were increased relative to hH_1R, but with the exception of methylhistaprodifen (**19**), those changes were not as marked as for the comparison of hH_1R and gpH_1R. The affinities of triprolidine (**31**), arpromidine (**35**) and guanidine **36** at hH_1R and hH_1R-F153L/I433V were similar.

Potencies and efficacies of H_1R agonists at hH_2R and gpH_2R in the GTPase assay. The question arose whether H_1R agonists, originally designed for gpH_1R in comparison to gpH_2R, interact differentially with the corresponding human H_xRs. To address this question, we analyzed the effects of H_1R agonists on GTP hydrolysis in Sf9 membranes expressing H_2R-$G_{s\alpha S}$ fusion proteins. We examined all H_1R agonists shown in Fig. 1 and listed in Table 2 (**1-23**), but included only those compounds into Table 5 that actually exhibited agonistic activity at H_2Rs. In order to account for the fact that the potency of histamine in the GTPase assay in membranes expressing H_1Rs and H_2Rs differs by almost 10-fold (Tables 2 and 5) (Kelley et al., 2001), we focused on the comparison of relative potencies of H_1R agonists.

2-Methylhistamine (**2**) and 2-(2-thiazolyl)ethanamine (**3**) were strong partial agonists at gpH_2R with moderate (2.3-5-fold) gpH_1R/gpH_2R selectivity. The introduction of a (substituted) phenyl group at position 2 of the imidazole ring greatly reduced the efficacy of H_1R

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

agonists at gpH_2R and further increased gpH_1R/gpH_2R selectivity in terms of potency. Several 2-phenylhistamines (**11**, **13**-**15**, **17** and **18**) and histaprodifens **19**-**21** and **23** were devoid of agonistic activity at gpH_2R-$G_{s\alpha S}$.

The analysis of histaprodifens at gpH_2R-$G_{s\alpha S}$ revealed the existence of a strong partial H_1R agonist/moderate partial H_2R agonist, N^{α}-(imidazolylethyl)histaprodifen (**22**) (Tables 2 and 5). The H_2-agonistic activity of this compound can be explained by its structural similarity with guanidines **33**-**38** (Figs. 1 and 2) that are potent H_2R agonists (Buschauer, 1989; Kelley et al., 2001).

Whereas histamine was similarly potent at stimulating GTP hydrolysis in Sf9 membranes expressing hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$, 2-methylhistamine (**2**) and 2-(2-thiazolyl)ethanamine (**3**) were significantly less potent agonists at hH_2R-$G_{s\alpha S}$ than at gpH_2R-$G_{s\alpha S}$ and showed greater hH_1R/hH_2R selectivity (8.4-11.2-fold) than gpH_1R/gpH_2R selectivity (2.3-5-fold). If one considers the absolute EC_{50} values of compound **3** for GTPase activation in membranes expressing hH_1R and hH_2R-$G_{s\alpha S}$, the selectivity for hH_1R becomes even more striking (75-fold *versus* 23-fold for gpH_xRs). In contrast to compound **3**, another small H_1R agonist, betahistine (**4**), exhibited considerably higher gpH_1R/gpH_2R selectivity (10-fold) than hH_1R/hH_2R selectivity (3.5-fold). Similar to the data obtained for gpH_2R, several 2-phenylhistamines (**11**, **13**-**15**, **17** and **18**) and histaprodifens (**19**-**21** and **23**) were devoid of agonistic activity at hH_2R-$G_{s\alpha S}$. As was the case for gpH_xRs, N^{α}-(imidazolylethyl)histaprodifen (**22**) was a strong partial hH_1R agonist/moderate partial hH_2R agonist. There were no significant differences in the interaction of histaprodifens at hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$. Finally, the efficacies of the 2-phenylhistamines **6**-**9** were significantly lower at hH_2R-$G_{s\alpha S}$ than at gpH_2R-$G_{s\alpha S}$ (Table 6) and therefore in the same order as observed for hH_1R and gpH_1R (Table 2).

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Pharmacological differences between hH_1R and gpH_1R. hH_1R is an important drug target for treatment of allergic diseases (second-generation H_1R antagonists) and sedation (first-generation H_1R antagonists) (Hill et al., 1997). Preliminary data indicate that pharmacological differences between H_1R species isoforms exist (Chang et al., 1979; Seifert et al., 1994; Elz et al., 2000), but a systematic analysis of this topic has not yet been conducted. Therefore, we studied recombinant hH_1R and gpH_1R with 23 H_1R agonists (**1**-**23**) (Fig. 1), 22 H_1R antagonists (**24**-**45**) (Fig. 2) and two H_2R agonists (**46**, **47**) under identical experimental conditions, using the GTPase assay (Fig. 5 and Tables 2 and 3) and [^{3}H]mepyramine binding assay (Tables 1 and 4) as read-out.

There were no significant differences between hH_1R and gpH_1R with respect to the potencies and efficacies of small agonists (**1**-**4**) in the GTPase assay (Fig. 5 and Table 2). However, with respect to bulkier ligands, we found significant differences between hH_1R and gpH_1R. Specifically, H_1R agonists of the 2-phenylhistamine class (**6**-**17**) and histaprodifen class (**19**-**23**) were generally more potent and efficacious in the GTPase assay in membranes expressing gpH_1R than in membranes expressing hH_1R (Fig. 5 and Table 2). Additionally, in the binding assay, 2-phenylhistamines and histaprodifens exhibited higher relative affinities for gpH_1R than for hH_1R (Table 4). The differential interaction of 2-phenylhistamine derivatives with gpH_1R and hH_1R is independent of the agonist- or antagonist properties of compounds (compare **17** and **40**, Tables 2 and 3). High constitutive GPCR activity results in high agonist potency and efficacy (Kenakin, 1996; Seifert and Wenzel-Seifert, 2002), but we did not find differences in constitutive activity between hH_1R and gpH_1R studying inverse agonists. Finally, several first-generation H_1R antagonists (**30**-**32**) and particularly arpromidine-type H_1R

antagonists (**33**-**38**) showed higher affinities for gpH_1R than for hH_1R. Our data concerning the affinity of ([^{3}H])mepyramine for hH_1R and gpH_1R (Tables 1 and 3) fit very well to previously published data on H_1R species isoforms expressed in native brain (Chang et al., 1979). Collectively, our data suggest that the ligand-binding site of gpH_1R exhibits a higher conformational flexibility than the ligand-binding site of hH_1R, allowing bulky compounds like 2-phenylhistamines, histaprodifens, mepyramine-type antagonists and guanidines to dock more efficiently into gpH_1R than into hH_1R.

Most of the previous H_1R antagonist development had been conducted with guinea pig models (Hill et al., 1997). Thus, from a therapeutic standpoint, it is fortunate that there are no or only small differences between hH_1R and gpH_1R with respect to commonly used first-generation H_1R antagonists (*e.g.*, **24**-**28**, **30** and **32**) and second-generation antagonists (**41**-**45**). However, with regard to the design of H_1R agonists and guanidine-type H_1R antagonists, which are currently used only as experimental tools (Zingel et al., 1995; Hill et al., 1997), the H_1R species isoform is of much greater relevance.

Differences in electrophoretic mobility between hH_1R and gpH_1R. A previous study showed that H_1R isoforms expressed in brain from various species exhibit different migration in SDS-PAGE (Ruat and Schwartz, 1989). These data prompted us to study the electrophoretic mobility of recombinant FLAG epitope-tagged recombinant hH_1R and gpH_1R (Fig. 4). In agreement with the data concerning native H_1R species isoforms, recombinant H_1R species isoforms showed different migration in SDS-PAGE. hH_1R exhibited a moderately higher molecular mass (~76 kDa) than predicted (~56 kDa) (Fukui et al., 1994). hH_1R migrated as mixture of *N*-glycosylated and non-glycosylated protein as assessed by the effect of the inhibitor

of *N*-glycosylation, tunicamycin (Seifert and Wenzel-Seifert, 2001). Recombinant gpH_1R exhibited very different migration in SDS-PAGE than hH_1R, *i.e.*, we detected faint diffuse ~36 kDa- and ~50 kDa bands and intense crisp ~16 kDa- and ~30 kDa bands in Sf9 membranes expressing gpH_1R. In contrast to the results obtained with hH_1R, tunicamycin had no effect on migration of gpH_1R, pointing to different types of *N*-glycosylation in the two H_1R species isoforms. Currently, we do not know the identity of the multiple bands in Sf9 membranes expressing gpH_1R, but highly atypical migration of GPCRs in SDS-PAGE has been repeatedly observed (Grünewald et al., 1996; Kelley et al., 2001; Seifert and Wenzel-Seifert, 2001). In view of the fact that even complex supramolecular structures such as GPCR dimers are preserved in SDS-PAGE (Fukushima et al., 1997; Hebert and Bouvier, 1998; Kelley et al., 2001), it is possible that the different electrophoretic mobilities of hH_1R and gpH_1R reflect different GPCR conformations. The different GPCR conformations may be associated with the specific pharmacological properties of H_1R species isoforms.

Molecular basis for the pharmacological differences between hH_1R and gpH_1R.

Site-directed mutagenesis was successful at identifying the molecular basis for pharmacological differences between species isoforms of H_2R and H_3R (Ligneau et al., 2000; Kelley et al., 2001). We wished to apply the same strategy to H_1R species isoforms. The pharmacological data discussed above indicate that the ligand-binding pocket of gpH_1R is more flexible than the binding pocket of hH_1R. Thus, gpH_1R may possess smaller amino acid substitutions in the ligand-binding domain than hH_1R so that bulkier structures are accommodated more easily in gpH_1R than in hH_1R. In fact, the amino acid substitutions at positions 153 (TM IV) and 433 in hH_1R (TM VI) are bulkier than the corresponding amino acid substitutions in gpH_1R (Phe→Leu

exchange in TM IV and Ile→Val exchange in TM VI, respectively). However, the Phe→Leu exchange in TM IV and the Ile→Val exchange in TM VI only partially explain the differences in agonist-pharmacology between hH_1R and gpH_1R (Tables 2 and 4). Moreover, with respect to the differences in antagonist-pharmacology, the Phe→Leu- and Ile→Val exchanges between hH_1R and gpH_1R are irrelevant (Tables 3 and 4). Thus, additional mutagenesis studies, targeting the top portions of TM II and TM VII are required to elucidate the molecular basis for the pharmacological differences between hH_1R and gpH_1R (Fig. 3).

Although our mutagenesis studies were disappointing in terms of elucidating the molecular basis for the pharmacological differences between hH_1R and gpH_1R, our studies revealed an unexpected role of Phe-153 and Ile-433 in H_1R expression and folding. Specifically, Phe-153→Leu-153- or Ile-433→Val-433 exchange in hH_1R (hH_1R→gpH_1R) resulted in poor receptor expression, low [^{3}H]mepyramine-affinity and functional inactivity (Table 1). Moreover, the mutations grossly altered the electrophoretic mobility of hH_1R (Fig. 4). The double mutation rescued the single mutants in terms of function (Tables 1-4), and it also changed electrophoretic mobility (Fig. 4). These data suggest that the couples Phe-153/Ile-433 or Leu-153/Val-433 are required for a functionally active H_1R. Thus, even conservative amino acid substitutions in TM regions can have profound effects on antagonist-affinity, expression and folding of a GPCR.

Comparison of the effects of H_1R agonists at recombinant and native gpH_1R.

Historically, the guinea pig ileum has been the standard system for the design of H_1R ligands (Zingel et al., 1995; Hill et al., 1997). Therefore, it is important to compare the intact organ data with the results regarding recombinant H_1R. Whereas many highly potent H_2R- and H_3R agonists, *i.e.* ligands ~50-150-fold more potent than histamine, were developed (Hill et al.,

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

1997), the design of potent H_1R agonists has been a much more difficult task. In fact, the most potent 2-phenylhistamine, 2-(3-trifluoromethylphenyl)histamine (**17**) is only 1.3-fold-, and methylhistaprodifen (**20**) just ~3.5-fold more potent than histamine in the guinea pig ileum (Leschke et al., 1995; Zingel et al., 1995; Elz et al., 2000) (Table 2).

The expression level of H_1R in the guinea pig ileum is much lower than in the Sf9 cell expression system (Table 1) (Hill et al., 1997). If there had been differences in receptor reserves between the two systems, we would have expected higher agonist efficacies in the recombinant system than in the native system (Hoyer and Boddeke, 1993; Kenakin, 1996). However, the opposite was the case (Table 2) (Leschke et al., 1995; Zingel et al., 1995; Elz et al., 2000). Thus, we can rule out differences in receptor reserves accounting for the pharmacological differences between the two systems.

All agonists studied with the exception of **1**, **3**, **22** and **23** were more potent at the recombinant gpH_1R than at the native gpH_1R (Table 2). The increase in potency at the recombinant gpH_1R ranged from ~2-fold to almost 20-fold and was most pronounced for the 2-phenylhistamines **7**, **11** and **13**. Several explanations that are not mutually exclusive could account for the potency differences in the two systems. First, there may be substantial penetration barriers for certain agonists to reach the tunica muscularis of the ileum. Second, compounds may accumulate in certain irrelevant cells, *i.e.* epithelial cells and/or, third, may be subject to degradation. These pharmacokinetic factors are very unlikely to be of relevance when assessing the effects of ligands in membrane fragments of insect cells. Fourth, it is possible that differences in gpH_1R glycosylation in insect cells *versus* native tissue contribute to the pharmacological differences in the two systems. Indeed, changes in glycosylation of H_1R have already been shown to alter the pharmacological properties of the GPCR (Mitsuhashi and Payan,

1989). Fifth, we studied coupling of H_1Rs to insect cell G_q-proteins (Houston et al., 2002), and the specific type of G_q-protein may have an impact on the pharmacological properties of gpH_1R (Wenzel-Seifert and Seifert, 2000). In contrast to the above-discussed data, the high potency of compounds **22** and **23** in the guinea pig ileum does not fit to the results obtained with recombinant gpH_1R. Additional studies with **22**, **23** and closely related new compounds must be performed to clarify this discrepancy.

Collectively, previous studies on the guinea pig ileum resulted in considerably lower potencies of most H_1R agonists than in the recombinant system. While the high potency of H_2R- and H_3R agonists has not yet been achieved for H_1R agonists, our present study shows that gpH_1R agonists with up to ~12-fold higher potency than histamine exist, provided that the GPCR is analyzed in the GTPase assay using membranes. Thus, future studies on the design of H_1R agonists should be complemented with the recombinant system described herein.

Species-differences in pharmacological properties of H_xRs. H_2R, H_3R and H_4R all exhibit species-differences in their pharmacological properties (Ligneau et al., 2000; Lovenberg et al., 2000; Kelley et al., 2001; Liu et al., 2001). Thus, we were not too surprised to uncover differences in the pharmacological properties of H_1R species isoforms. The species-differences in pharmacological properties of H_xRs extend into H_xR subtype-selectivity of compounds. There are numerous efficacious H_1R agonists of the 2-phenylhistamine- and histaprodifen class with high gpH_1R/gpH_2R selectivity (Tables 2 and 5) (Leschke et al., 1995; Zingel et al., 1995; Elz et al., 2000). However, for the analysis of hH_1R one has to consider the fact that 2-phenylhistamines and histaprodifens possess substantially lower efficacies than histamine (Table 2). Unexpectedly, 2-(2-thiazolyl)ethanamine (**3**)**,** a small agonist with full efficacy at hH_1R,

exhibited a larger hH_1R/hH_2R- than gpH_1R/gpH_2R-selectivity (Tables 2 and 5). Thus, for the analysis of hH_1R with a selective hH_1R agonist, compound **3** may be the ligand of choice. These findings emphasize the importance to study hH_xR isoforms for the development of hH_xR ligands. Future studies will have to answer the question whether the species-differences in pharmacological properties of H_xRs reflect species-specific adaptations to as yet unidentified endogenous and/or exogenous H_xR ligands.

Although H_1Rs and H_2Rs are structurally quite distinct from each other (only ~40% homology) (Traiffort et al., 1994; Hill et al., 1997), there is a common aspect in the pharmacological properties of these GPCRs, *i.e.* the preferential interaction of bulky agonists with gpH_xRs relative to hH_xRs. Most notably, arpromidine-derived guanidines represent a class of ligands that exhibit higher affinities for gpH_1R and gpH_2R relative to hH_xRs (Tables 3 and 4) (Kelley et al., 2001). Those differences may indicate that gpH_xRs in general possess a higher conformational flexibility than hH_xRs.

Acknowledgements

We express gratitude to Dr. G. Lambrecht (Department of Pharmacology, University of Frankfurt/M., Germany) for providing dimethindene enantiomers, to Dr. H. Fukui (Department of Pharmacology, University of Tokushima, Japan) for providing cDNAs for hH_1R and gpH_1R and Dr. E. Ross (Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX) for donating baculoviruses encoding RGS proteins. We also thank Dr. F. Schalkhausser (Institute of Pharmacy, University of Regensburg, Germany) for the synthesis of guanidine **39**, Dr. K. Kramer (Institute of Pharmacy, Free University of Berlin, Germany) for the synthesis of compounds **2**, **3**, **22**, **23** and **40**, Dr. W. Toegel (Institute of Pharmacy, Free University of Berlin, Germany) for the preparation of compound **18**, and Mrs. C. Houston (Department of Pharmacology and Toxicology, University of Kansas, Lawrence, KS) for help with the cell culture. Thanks are also due to the Reviewers for their constructive critique.

References

Bakker RA, Schoonus SB, Smit MJ, Timmerman H and Leurs R (2001) Histamine H_1-receptor activation of nuclear factor-κ-b: roles for Gβγ- and $G\alpha_{q/11}$-subunits in constitutive and agonist-mediated signaling. *Mol Pharmacol* 60:1133-1142.

Ballesteros JA, Shi L and Javitch JA (2001) Structural mimicry in G protein-coupled receptors: implications of the high-resolution structure of rhodopsin for structure-function analysis of rhodopsin-like receptors. *Mol Pharmacol* 60:1-19.

Buschauer A (1989) Synthesis and *in vitro* pharmacology of arpromidine and related phenyl(pyridylalkyl)guanidines, a potential new class of positive inotropic drugs. *J Med Chem* 32:1963-1970.

Chang RS, Tran VT and Snyder SH (1979) Heterogeneity of histamine H_1-receptors: species variations in [^{3}H]mepyramine binding of brain membranes. *J Neurochem* 32:1653-1663.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* 22:3099-3108.

Elz S, Kramer K, Pertz HH, Detert H, ter Laak AM, Kühne R and Schunack W (2000) Histaprodifens: synthesis, pharmacological in vitro evaluation, and molecular modeling of a new class of highly active and selective histamine H_1-receptor agonists. *J Med Chem* 43:1071-1084.

Fukui H, Fujimoto K, Mizuguchi H, Sakamoto K, Horio Y, Takai S, Yamada K and Ito S (1994) Molecular cloning of the human histamine H_1 receptor gene. *Biochem Biophys Res Commun* 201:894-901.

Fukushima Y, Asano T, Saitoh T, Anai M, Funaki M, Ogihara T, Katagiri H, Matsuhashi N, Yazaki Y and Sugano K (1997) Oligomer formation of histamine H_2 receptors expressed in Sf9 and COS7 cells. *FEBS Lett* 409:283-286.

Grünewald S, Haase W, Reiländer H and Michel H (1996) Glycosylation, palmitoylation, and localization of the human D_{2S} receptor in baculovirus-infected insect cells. *Biochemistry* 35:15149-15161.

Hebert TE and Bouvier M (1998) Structural and functional aspects of G protein-coupled receptor oligomerization. *Biochem Cell Biol* 76:1-11.

Hill SJ, Ganellin CR, Timmerman H, Schwartz JC, Shankley NP, Young JM, Schunack W, Levi R and Haas HL (1997) International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol Rev* 49:253-278.

Hough LB (2001) Genomics meets histamine receptors: new subtypes, new receptors. *Mol Pharmacol* 59:415-419.

Houston C, Wenzel-Seifert K, Bürckstümmer T and Seifert R (2002) The human histamine H_2-receptor couples more efficiently to Sf9 insect cell G_s-proteins than to insect cell G_q-proteins: limitations of Sf9 cells for the analysis of receptor/G_q-protein coupling. *J Neurochem* 80:678-696.

Hoyer D and Boddeke HW (1993) Partial agonists, full agonists, antagonists: dilemmas of definition. *Trends Pharmacol Sci* 14:270-275.

Kelley MT, Bürckstümmer T, Wenzel-Seifert K, Dove S, Buschauer A and Seifert R (2001) Distinct interaction of human and guinea pig histamine H_2-receptor with guanidine-type agonists. *Mol Pharmacol* 60:1210-1225.

Kenakin T (1996) The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol Rev* 48:413-463.

Leschke C, Elz S, Garbarg M and Schunack W (1995) Synthesis and histamine H_1 receptor agonist activity of a series of 2- phenylhistamines, 2-heteroarylhistamines, and analogues. *J Med Chem* 38:1287-1294.

Leurs R, Smit MJ, Tensen CP, Ter Laak AM and Timmerman H (1994) Site-directed mutagenesis of the histamine H_1-receptor reveals a selective interaction of asparagine^{207} with subclasses of H_1-receptor agonists. *Biochem Biophys Res Commun* 201:295-301.

Leurs R, Smit MJ, Meeder R, Ter Laak AM and Timmerman H (1995) Lysine200 located in the fifth transmembrane domain of the histamine H_1 receptor interacts with histamine but not with all H_1 agonists. *Biochem Biophys Res Commun* 214:110-117.

Ligneau X, Morisset S, Tardivel-Lacombe J, Gbahou F, Ganellin CR, Stark H, Schunack W, Schwartz JC and Arrang JM (2000) Distinct pharmacology of rat and human histamine H_3 receptors: role of two amino acids in the third transmembrane domain. *Br J Pharmacol* 131:1247-1250.

Liu C, Wilson SJ, Kuei C and Lovenberg TW (2001) Comparison of human, mouse, rat, and guinea pig histamine H_4 receptors reveals substantial pharmacological species variation. *J Pharmacol Exp Ther* 299:121-130.

Lovenberg TW, Pyati J, Chang H, Wilson SJ and Erlander MG (2000) Cloning of rat histamine H_3 receptor reveals distinct species pharmacological profiles. *J Pharmacol Exp Ther* 293:771-778.

Mitsuhashi M and Payan DG (1989) Receptor glycosylation regulates the affinity of histamine H_1 receptors during smooth muscle cell differentiation. *Mol Pharmacol* 35:311-318.

Nonaka H, Otaki S, Ohshima E, Kono M, Kase H, Ohta K, Fukui H and Ichimura M (1998) Unique binding pocket for KW-4679 in the histamine H_1 receptor. *Eur J Pharmacol* 345:111-117.

Ohta K, Hayashi H, Mizuguchi H, Kagamiyama H, Fujimoto K and Fukui H (1994) Site-directed mutagenesis of the histamine H_1 receptor: roles of aspartic acid[107], asparagine[198] and threonine[194]. *Biochem Biophys Res Commun* 203:1096-1101.

Ostrom RS, Post SR and Insel PA (2000) Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving G_s. *J Pharmacol Exp Ther* 294:407-412.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289:739-745.

Pfaff O, Hildebrandt C, Waelbroeck M, Hou X, Moser U, Mutschler E and Lambrecht G (1995) The (*S*)-(+)-enantiomer of dimethindene: a novel M_2-selective muscarinic receptor antagonist. *Eur J Pharmacol* 286:229-240.

Ruat M and Schwartz JC (1989) Photoaffinity labeling and electrophoretic identification of the H_1-receptor: comparison of several brain regions and animal species. *J Neurochem* 53:335-339.

Seifert R and Wenzel-Seifert K (2001) Defective G_i-protein coupling in two formyl peptide receptor mutants associated with localized juvenile periodontitis. *J Biol Chem* 276:42043-42049.

Seifert R and Wenzel-Seifert K (2002) Constitutive activity of G-protein-coupled receptors: Cause of disease and common property of wild-type receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* 366:381-416.

Seifert R, Lee TW, Lam VT and Kobilka BK (1998) Reconstitution of β_2-adrenoceptor-GTP-binding-protein interaction in Sf9 cells: High coupling efficiency in a β_2-adrenoceptor-$G_{s\alpha}$ fusion protein. *Eur J Biochem* 255:369-382.

Seifert R, Hagelüken A, Höer A, Höer D, Grünbaum L, Offermanns S, Schwaner I, Zingel V, Schunack W and Schultz G (1994) The H_1 receptor agonist 2-(3-chlorophenyl)histamine activates G_i proteins in HL-60 cells through a mechanism that is independent of known histamine receptor subtypes. *Mol Pharmacol* 45:578-586.

Traiffort E, Leurs R, Arrang JM, Tardivel-Lacombe J, Diaz J, Schwartz JC and Ruat M (1994) Guinea pig histamine H_1 receptor. I. Gene cloning, characterization, and tissue expression revealed by in situ hybridization. *J Neurochem* 62:507-518.

Weiner DM, Burstein ES, Nash N, Croston GE, Currier EA, Vanover KE, Harvey SC, Donohue E, Hansen HC, Andersson CM, Spalding TA, Gibson DF, Krebs-Thomson K, Powell SB, Geyer MA, Hacksell U and Brann MR (2001) 5-Hydroxytryptamine$_{2A}$ receptor inverse agonists as antipsychotics. *J Pharmacol Exp Ther* 299:268-276.

Wenzel-Seifert K and Seifert R (2000) Molecular analysis of β_2-adrenoceptor coupling to G_s-, G_i-, and G_q-proteins. *Mol Pharmacol* 58:954-966.

Wieland K, Laak AM, Smit MJ, Kühne R, Timmerman H and Leurs R (1999) Mutational analysis of the antagonist-binding site of the histamine H_1 receptor. *J Biol Chem* 274:29994-30000.

Zingel V, Elz S and Schunack W (1990) Histamine analogues. 33rd communication: 2-phenylhistamines with high histamine H_1-agonistic activity. *Eur J Med Chem* 25:673-680.

Zingel V, Leschke C and Schunack W (1995) Developments in histamine H_1-receptor agonists. *Prog Drug Res* 44:49-85.

Footnotes

This work was supported by the National Institutes of Health COBRE award 1 P20 RR15563 and matching support from the State of Kansas and the University of Kansas (R.S.), a grant from the Army Research Office (DAAD 19-00-1-006) (R.S.), grants of the Deutsche Forschungsgemeinschaft (W.S. and A.B.) Fonds der Chemischen Industrie (T.B., W.S., A.B. And S.E.), the Bundesministerium für Bildung und Forschung (T.B.) and the Deutscher Akademischer Austauschdienst within the International Quality Network “Medicinal Chemistry” (S.E. and A.B.).

Figure legends

Fig. 1. Structures of H_1R agonists. 1, Histamine; **2-4**, small histamine-derived agonists; **5-17**, 2-phenylhistamine derivatives; **18**, thioether compound related to **5-17**; **18-23**, histaprodifens. For agonist names, see Table 2.

Fig. 2. Structures of H_1R antagonists. 24-32, First-generation H_1R antagonists; **33-39**, arpromidine (**35**)-derived dual H_2R agonists/H_1R antagonists; **40**, H_1R antagonist derived from 2-phenylhistamines; **41-45**, second-generation H_1R antagonists. For antagonist names, see Table 3.

Fig. 3. Snake plot of hH_1R showing the relative positions and topology of amino acid residues in the TM domains, putative agonist- and antagonist binding sites and differences with respect to the gpH_1R. Fig. 3 considers results of the substituted-cysteine accessibility method by which the dopamine D_2-receptor binding site was mapped (Ballesteros et al., 2001). *Red circles*, amino acids predicted to face the interior of the TM helix bundle based on corresponding positions in bovine rhodopsin (Palczewski et al., 2000). *Blue circles*, amino acids predicted to face lipid. *Red letters*, the corresponding amino acids in the dopamine D_2-receptor are water-accessible and protected by ligand binding in substituted-cysteine accessibility experiments (Ballesteros et al., 2001). *Magenta letters*, amino acids that are water-accessible but not protected by ligand binding in substituted-cysteine accessibility experiments. *Red shading*, histamine binding site residues according to site-directed mutagenesis data (Leurs et al., 1994, 1995; Ohta et al., 1994; Nonaka et al., 1998). *Green shading*, amino acids of the H_1R antagonist binding site based on mutagenesis studies (Ohta et al., 1994; Nonaka et al., 1998; Wieland et al., 1999). *Yellow shading*, amino acids that are essential for both histamine and H_1R antagonist

binding (Wieland et al., 1999). *Orange shading*, additional amino acid residues of the histaprodifen binding site based on molecular modeling approaches (Elz et al., 2000). Note that Lys-191 does not interact with histaprodifens. *Blue shading*, amino acids targeted by site-directed mutagenesis in the present paper. *Grey shading*, other amino acid residues different between hH_1R and gpH_1R. *Brown line*, conserved disulfide bridge between Cys-100 and Cys-180. Numbers designate the first and last amino acids, respectively, of each TM domain.

Fig. 4. Analysis of the expression of H_1R constructs in Sf9 cell membranes. A, Sf9 cells were cultured in the absence (-) of tunicamycin (Tuna.) or in the presence (+) of tunicamycin (10 μg/ml). Sf9 cell membranes expressing hH_1R or gpH_1R were separated by SDS-PAGE on a gel that contained 10% (w/v) acrylamide. Membranes were probed with the anti-FLAG Ig (M1 antibody). Each membrane preparation was analyzed in two different amounts (25 μg and 50 μg of protein per lane, respectively). **B**, Sf9 cell membranes expressing hH_1R-F153L, hH_1R-I433V, hH_1R-F153L/I433V and hH_1R were separated by SDS-PAGE on a gel that contained 10% (w/v) acrylamide. Membranes (75 μg of protein/lane each) were probed with the anti-FLAG Ig. *Numbers* on the *left* of the immunoblots in **A** and **B** indicate molecular masses of marker proteins. The horseradish peroxidase-reacted Immobilon P membranes of representative gels are shown. Similar results were obtained with 3-6 other membrane preparations of hH_1R constructs. Expression levels of H_1R constructs in terms of [^{3}H]mepyramine saturation binding are given in Table 1.

Fig. 5. Relations between the potencies of H_1R agonists at hH_1R and gpH_1R expressed in Sf9 membranes in the GTPase assay. pEC_{50} values of agonists at hH_1R and gpH_1R were

derived from EC_{50} values shown in Table 1 and analyzed by linear regression. Solid lines represent the actual correlations obtained. Dashed lines represent the 95% confidence intervals of the correlations. The straight dotted lines represent the theoretical correlations describing pharmacological identity between the H_1R species isoforms. The theoretical curves have a slope of 1.00. **A**, Correlation of the potencies of small agonists (**1**-**4**) at hH_1R *versus* gpH_1R. Slope, 0.74 ± 0.03; r^2, 0.99; $p < 0.001$ (significant). **B**, Correlation of potencies of 2-phenylhistamines (**6**-**18**) at hH_1R *versus* gpH_1R. Slope, 0.96 ± 0.07; r^2, 0.95; $p < 0.001$ (significant). **C**, Correlation of potencies of histaprodifens (**19**-**23**) at hH_1R *versus* gpH_1R. Slope, 0.54 ± 0.08; r^2, 0.96; $p < 0.001$ (significant).

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. [^{3}H]Mepyramine saturation binding in Sf9 membranes expressing hH$_1$R, gpH$_1$R, hH$_1$R-F153L, hH$_1$R-I433V and hH$_1$R-F153L/I433V

H$_1$R construct	**K_d (nM)**	**B_{max} (pmol/mg)**
hH$_1$R	4.49 ± 0.35	5.85 ± 1.67
gpH$_1$R	2.53 ± 0.23*	3.94 ± 0.83
hH$_1$R-F153L	37.8 ± 15.8*	0.88 ± 0.18*
hH$_1$R-I433V	53.7 ± 26.2*	1.13 ± 0.03*
hH$_1$R-F153L/I433V	4.33 ± 0.40	15.7 ± 6.4*

Sf9 membranes expressing various H$_1$R constructs (+ RGS4 or GAIP) were incubated with 0.2-100 nM [^{3}H]mepyramine as appropriate according to the protocol described in *Methods*. Nonspecific binding was determined in the presence of 10 μM mepyramine and was subtracted from total [^{3}H]mepyramine binding. Binding data were analyzed by non-linear regression and were best fit to monophasic saturation curves. Data shown are the means ± SD of 3-4 membrane preparations analyzed in triplicates each. *, $p < 0.05$ for comparison of hH$_1$R *versus* other H$_1$R constructs.

Table 2. Potencies and efficacies of H_1R- and H_2R agonists in the GTPase assay in Sf9 membranes expressing hH_1R, gpH_1R and hH_1R-F153L/I433V: Comparison with the guinea pig ileum

		hH_1R			gpH_1R			hH_1R-F153L/I433V			gp-ileum		
Cpd.	**agonist**	**EC_{50} (μM)**	**rel. pot.**	**E_{max}**	**EC_{50} (μM)**	**rel. pot.**	**E_{max}**	**pot. rat. gp/h**	**EC_{50} (μM)**	**rel. pot.**	**E_{max}**	**pot. rat. m/h**	**rel. pot.**	**pot. rat. rec/il**
1	Histamine	0.184 ± 0.094	100	1.00	0.220 ± 0.047	100	1.00	0.84	0.163 ± 0.029	100	1.00	1.13	100	1.00
2	2-Methylhistamine	0.837 ± 0.110	22.0	0.98 ± 0.03	0.708 ± 0.181	31.1	0.97 ± 0.04	0.87	n. d.	n. d.	n. d.	n. d.	14.0	2.22
3	2-(2-Thiazolyl)-ethanamine	0.440 ± 0.116	41.8	0.97 ± 0.13	0.433 ± 0.133	50.8	1.00 ± 0.08	1.02	0.373 ± 0.076	43.7	1.08 ± 0.05*	1.17	45.0	1.13
4	Betahistine	1.438 ± 0.368	12.8	0.86 ± 0.10	0.963 ± 0.277	22.8	0.86 ± 0.09	1.49	n. d.	n. d.	n. d.	n. d.	8.0	2.85
5	2-Benzylhistamine	6.518 ± 0.482	2.80	0.75 ± 0.10	5.234 ± 1.432	4.20	0.88 ± 0.11	1.24	3.130 ± 0.060*	5.21	0.74 ± 0.01	2.08	2.5	1.68
6	2-Phenylhistamine	0.877 ± 0.201	21.0	0.79 ± 0.07	0.160 ± 0.010*	137.5	0.98 ± 0.09*	5.48	0.638 ± 0.100	25.5	0.91 ± 0.06	1.37	31.0	4.44
7	2-(3-Methylphenyl)-histamine	1.088 ± 0.240	17.0	0.77 ± 0.11	0.134 ± 0.065*	164.2	0.89 ± 0.05	8.06	n. d.	n. d.	n. d.	n. d.	14.7	11.2
8	2-(2-Fluorophenyl)-histamine	2.783 ± 1.092	6.6	0.71 ± 0.04	0.766 ± 0.231*	28.9	0.88 ± 0.14*	3.66	n. d.	n. d.	n. d.	n. d.	5.8	4.98
9	2-(3-Fluorophenyl)-histamine	0.683 ± 0.303	26.9	0.75 ± 0.04	0.073 ± 0.022*	301.4	0.89 ± 0.08*	9.39	0.263 ± 0.079	62.0	0.85 ± 0.09	2.60	85.0	3.55
10	2-(4-Fluorophenyl)-histamine	3.220 ± 0.569	5.5	0.60 ± 0.12	0.587 ± 0.127*	37.5	0.80 ± 0.07*	5.65	n. d.	n. d.	n. d.	n. d.	12.5	3.00
11	2-(3,5-Difluoro-phenyl)histamine	0.765 ± 0.133	24.0	0.59 ± 0.10	0.132 ± 0.029*	166.7	0.83 ± 0.11*	5.80	n. d.	n. d.	n. d.	n. d.	15.0	11.1
12	2-(3-Chlorophenyl)-	0.434 ± 0.128	42.4	0.71 ± 0.05	0.081 ± 0.026*	271.6	0.89 ± 0.10*	5.35	0.160 ± 0.056*	101.9	0.81 ± 0.08*	2.71	96.0	2.83

	histamine													
13	2-(4-Chlorophenyl)-histamine	16.440 ± 3.880	1.1	0.54 ± 0.11	2.313 ± 0.788*	9.5	0.56 ± 0.11	7.11	n. d.	n. d.	n. d.	n. d.	0.5	19.0
14	2-(3-Bromophenyl)-histamine	0.210 ± 0.065	87.6	0.73 ± 0.01	0.053 ± 0.002*	415.1	0.95 ± 0.11*	3.96	0.143 ± 0.032	114.0	0.75 ± 0.05	1.47	112	3.71
15	2-(3-Iodophenyl)-histamine	0.220 ± 0.118	83.6	0.70 ± 0.08	0.040 ± 0.021*	550.0	0.86 ± 0.05*	5.50	0.133 ± 0.051	122.6	0.70 ± 0.09	1.65	96.0	5.73
16	2-(3-Methoxyphenyl)-histamine	0.262 ± 0.100	70.2	0.74 ± 0.05	0.060 ± 0.017*	366.7	1.00 ± 0.09*	4.37	n. d.	n. d.	n. d.	n. d.	42.1	8.71
17	2-(3-Trifluoromethyl-phenyl)histamine	0.243 ± 0.091	41.8	0.74 ± 0.04	0.057 ± 0.023*	386.0	0.91 ± 0.12*	4.26	n. d.	n. d.	n. d.	n. d.	129	2.99
18	2-(4-Methylphenylthio-methyl)histamine	2.54 ± 0.36	7.2	0.50 ± 0.05	0.77 ± 0.18*	29.9	0.80 ± 0.11*	3.18	n. d.	n. d.	n. d.	n. d.	12.7	2.35
19	Methylhistaprodifen	0.068 ± 0.018	270.5	0.77 ± 0.10	0.019 ± 0.006*	1157.8	0.87 ± 0.06	3.57	0.040 ± 0.011	407.5	0.88 ± 0.06	1.70	343	3.38
20	Dimethylhistaprodifen	0.100 ± 0.026	184.0	0.64 ± 0.09	0.027 ± 0.012*	814.8	0.84 ± 0.06*	3.70					242	3.37
21	Pyrrolidinohistaprodifen	0.293 ± 0.120	62.8	0.19 ± 0.02	0.044 ± 0.013*	500.0	0.40 ± 0.03*	6.66	0.258 ± 0.217	63.2	0.34 ± 0.05	1.14	67.0	7.46
22	N^{α}-(Imidazolylethyl)-histaprodifen	0.238 ± 0.138	77.3	0.84 ± 0.08	0.050 ± 0.025*	440.0	0.89 ± 0.09	4.76	n. d.	n. d.	n. d.	n. d.	3630	0.12
23	Dimeric histaprodifen	0.653 ± 0.210	28.2	0.65 ± 0.11	0.066 ± 0.033*	333.3	0.92 ± 0.08*	9.85	n. d.	n. d.	n. d.	n. d.	1680	0.20
46	Amthamine	-	-	0.01 ± 0.02	150 ± 56	-	0.14 ± 0.05*	-	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
47	Dimaprit	-	-	0.06 ± 0.04	-	-	0.06 ± 0.03	-	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.

Steady-state GTPase activity in Sf9 membranes expressing H_1R constructs (+ RGS4 or GAIP) was determined as described in *Methods*. Reaction mixtures contained H_xR ligands at concentrations from 1 nM - 1 mM as appropriate to generate saturated concentration/response curves. Data were analyzed by non-linear regression and were best fit to sigmoid concentration/response curves. Typical basal GTPase activities ranged between 1.5-2.5 pmol/mg/min, and the maximum stimulatory effect of histamine (10 μM) amounted to 125-175% above basal (Houston et al., 2002). The efficacy (E_{max}) of histamine was determined by non-linear regression and was set 1.00 The E_{max} values of other agonists were referred to this value. Data shown are the means ± SD of 5-8 experiments performed in duplicates each. * $p < 0.05$ for comparison of hH_1R *versus* other H_1R constructs. The relative potency (rel. pot.) of histamine was set 100, and the potencies of other agonists were referred to this value. We also calculated the ratio of the EC_{50} values of H_1R agonists for hH_1R and gpH_1R (pot. rat. gp/h) and the ratio of the EC_{50} values of H_1R agonists for hH_1R and hH_1R-F153L/I433V (pot. rat. m/h). Table 2 also shows the relative potencies of H_1R agonists in the standard system for the analysis of the H_1R, the guinea pig ileum. The EC_{50} of histamine in this system is ~0.15-0.20 μM (Zingel et al., 1990; Elz et al., 2000). The relative potency (rel. pot.) of histamine was set 100, and the potencies of other agonists were referred to this value. Finally, we calculated the ratio of the relative potency of H_1R agonists at the native gpH_1R expressed in the ileum and recombinant gpH_1R expressed in Sf9 membranes (pot. rat. rec/il). The relative potencies of compounds **2**-**23** in the guinea pig ileum were calculated from isotonically recorded cumulative concentration/response curves on whole segments of ileum (preload 0.5 g) in the continuous presence of atropine (100 nM) as described (Elz et al., 2000). gp-ileum, guinea pig ileum; n. d., not determined; -, not applicable because stimulatory effects of agonists were too small; Cpd., compound.

Table 3. Potencies of H_1R antagonists in the GTPase assay in Sf9 membranes expressing hH_1R, gpH_1R and hH_1R-F153L/I433V

Cpd.	antagonist	K_B hH_1R (nM)	K_B gpH_1R (nM)	pot. rat. gpH_1R/hH_1R	hH_1R-F153L/I433V	pot. rat. mut./hH_1R
24	Promazine	1.08 ± 0.11	1.30 ± 0.19	0.83	n. d.	n. d.
25	Chlorpromazine	2.55 ± 0.54	2.85 ± 1.34	0.89	n. d.	n. d.
26	Mianserin	2.13 ± 1.17	3.16 ± 1.88	0.67	n. d.	n. d.
27	Cyproheptadine	1.92 ± 0.13	2.64 ± 0.94	0.73	n. d.	n. d.
28	Diphenhydramine	15.5 ± 1.77	16.4 ± 1.99	0.95	n. d.	n. d.
(*R*)-29	(*R*)-(-)-Dimethindene	2.68 ± 1.52	2.97 ± 1.49	0.90	n. d.	n. d.
(*S*)-29	(*S*)-(+)-Dimethindene	113.2 ± 30.9	94.5 ± 38.8	1.20	n. d.	n. d.
30	Mepyramine	5.67 ± 0.88	2.30 ± 0.52*	2.47	5.76 ± 1.10	0.99
31	Triprolidine	4.37 ± 1.61	1.73 ± 0.32*	2.52	n. d.	n. d.
32	(+)-Chlorpheniramine	9.85 ± 2.32	4.97 ± 1.37*	1.98	n. d.	n. d.
33	BU-E 42	554 ± 36.8	98.6 ± 19.7*	5.62	698 ± 31.8*	0.80
34	BU-E 43	332 ± 74.0	142 ± 37.7*	2.34	n. d.	n. d.
35	Arpromidine	332 ± 89.6	48.9 ± 12.9*	6.79	465 ± 88.3	0.72
36	BU-E 47	724 ± 180	80.0 ± 16.8*	9.05	477 ± 61.0*	1.51

37	BU-E 48	543 ± 198	116 ± 31.0*	4.68	528 ± 29.7	1.03
38	BU-E 75	499 ± 122	131 ± 10.4*	3.81	1180 ± 64*	0.42
39	D281	562 ± 123	352 ± 79	1.60	n. d.	n. d.
40	*N*-{2-[2-(3-Trifluoromethylphenyl)-1*H*-imidazol-4-yl]ethyl}piperidine	1570 ± 148	454 ± 155*	3.46	2310 ± 210*	0.68
41	Terfenadine	28.1 ± 4.97	38.5 ± 6.08	0.73	n. d.	n. d.
42	Fexofenadine	226 ± 50.2	258 ± 61.0	0.88	n. d.	n. d.
43	Astemizole	10.4 ± 1.42	12.7 ± 3.25	0.82	n. d.	n. d.
44	Azelastine	1.71 ± 0.16	1.89 ± 0.17	0.90	n. d.	n. d.
45	Ketotifen	0.96 ± 0.03	1.09 ± 0.30	0.88	n. d.	n. d.

Steady-state GTPase activity in Sf9 membranes expressing H_1R constructs (+ RGS4 or GAIP) was determined as described in *Methods*. Reaction mixtures contained 1 μM histamine and H_1R antagonists at concentrations from 1 nM - 100 μM as appropriate to generate saturated concentration/response curves. Data were analyzed by non-linear regression and were best fit to sigmoid concentration/response curves. Typical GTPase activities in the presence of 1 μM histamine between 2.5-3.0 pmol/mg/min. Data shown are the means ± SD of 3-5 experiments performed in duplicates each. * $p < 0.05$ for comparison of hH_1R *versus* other H_1R constructs. We also calculated the ratio of the K_B values for hH_1R and gpH_1R (pot. rat. gpH_1R/hH_1R) and the ratio of the K_B values for hH_1R and hH_1R-F153L/I433V (pot. rat. mut./hH_1R). n. d., not determined. Cpd., compound.

Table 4. Agonist- and antagonist-affinities of hH_1R, gpH_1R and hH_1R-F153L/I433V expressed in Sf9 membranes in the [^{3}H]mepyramine competition binding assay

		hH_1R		gpH_1R			hH_1R-F153L/I433V		
Cpd.	**ligand**	**K_i**	**rel. aff.**	**K_i**	**rel. aff.**	**aff. rat. gp/h**	**K_i**	**rel. aff.**	**aff. rat. m/h**
1	Histamine	2.06 ± 0.18 µM	100	4.65 ± 0.26 µM*	100	0.44	3.30 ± 0.60 µM*	100	0.62
3	2-(2-Thiazolyl)ethanamine	4.60 ± 1.93 µM	44.8	8.49 ± 3.53 µM	54.8	0.54	12.7 ± 1.70 µM*	26	0.36
12	2-(3-Chlorophenyl)histamine	1.78 ± 0.30 µM	115.7	0.60 ± 0.17 µM*	775.0	2.97	1.53 ± 0.18 µM	215.7	1.16
14	2-(3-Bromophenyl)histamine	2.22 ± 0.30 µM	107.8	0.70 ± 0.10 µM*	668.1	3.19	1.43 ± 0.24 µM	230.8	1.55
15	2-(3-Iodophenyl)histamine	1.76 ± 0.24 µM	117.1	0.61 ± 0.14 µM*	759.8	2.88	1.38 ± 0.02 µM	239.1	1.28
19	Methylhistaprodifen	0.37 ± 0.07 µM	552.3	0.29 ± 0.06 µM	1603.4	1.29	0.24 ± 0.04 µM*	1375	1.54
20	Dimethylhistaprodifen	0.40 ± 0.06 µM	509.9	0.31 ± 0.08 µM	1480.9	1.29	0.36 ± 0.02 µM	916.7	1.11
31	Triprolidine	3.01 ±0.54 nM	-	1.15 ± 0.02 nM*	-	2.62	2.88 ± 0.15 nM	-	1.05
35	Arpromidine	353 ± 71 nM	-	33.3 ± 10.1 nM*	-	10.6	282 ± 57 nM	-	1.25
36	BU-E 47	255 ± 68 nM	-	53.9 ± 14.8 nM*	-	4.73	321 ± 56 nM	-	0.79

[^{3}H]Mepyramine competition binding in Sf9 membranes expressing H_1R constructs (+ RGS4 or GAIP) was determined as described in *Methods*. Reaction mixtures contained 2 nM [^{3}H]mepyramine and unlabeled H_1R ligands at concentrations of 0.1 nM - 10 mM as appropriate to generate saturated competition curves. Data were analyzed by non-linear regression and were best fit to one-site (monophasic) competition curves. Data shown are the means ± SD of 3-5 experiments performed in duplicates each. * $p < 0.05$ for comparison of hH_1R *versus* other H_1R constructs. The relative affinity of histamine (rel. aff.) was set 100, and the affinities of other agonists were referred to this value. We also calculated the ratio of the K_i values for hH_1R and gpH_1R (aff. rat. gp/h) and the ratio of the K_i values for hH_1R and hH_1R-F153L/I433V (aff. rat. m/h). -, not applicable. Cpd., compound.

Table 5. Potencies and efficacies of H_1R agonists in the GTPase assay in Sf9 membranes expressing hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$

		hH_2R				gpH_2R				
Cpd.	**Agonist**	**EC_{50} (μM)**	**rel. pot.**	**E_{max}**	**rel. pot. rat. hH_1R/hH_2R**	**EC_{50} (μM)**	**rel. pot.**	**E_{max}**	**rel. pot. rat. gpH_1R/gpH_2R**	**pot. rat. gpH_2R/hH_2R**
1	Histamine	1.26 ± 0.25	100	1.00	1.00	1.20 ± 0.24	100	1.00	1.00	1.05
2	2-Methylhistamine	47.9 ± 18.5*	2.63	0.93 ± 0.05	8.36	8.82 ± 1.48	13.6	0.87 ± 0.06	2.29	5.43
3	2-(2-Thiazolyl)ethanamine	33.9 ± 9.21*	3.72	0.91 ± 0.11	11.2	11.8 ± 1.12	10.2	0.90 ± 0.16	4.98	2.87
4	Betahistine	33.6 ± 7.23	3.75	0.73 ± 0.07	3.41	51.4 ± 8.3	2.33	0.73 ± 0.06	9.79	0.65
5	2-Benzylhistamine	27.9 ± 3.33	4.52	0.37 ± 0.04*	0.62	28.5 ± 15.6	4.21	0.54 ± 0.04	1.00	0.98
6	2-Phenylhistamine	38.7 ± 6.12	3.26	0.20 ± 0.03*	6.44	59.6 ± 20.5	2.01	0.38 ± 0.05	68.4	0.65
7	2-(3-Methylphenyl)histamine	-	-	0.06 ± 0.03*	-	12.1 ± 4.5	9.92	0.16 ± 0.04	16.6	-
8	2-(2-Fluorophenyl)histamine	63.8 ± 22.6	1.98	0.23 ± 0.03*	3.33	89.9 ± 6.1	1.33	0.43 ± 0.02	21.7	0.71
9	2-(3-Fluorophenyl)histamine	25.6 ± 10.6	4.92	0.17 ± 0.04*	5.47	42.3 ± 9.6	2.84	0.28 ± 0.02	227	0.61
10	2-(4-Fluorophenyl)histamine	11.5 ± 3.7	10.6	0.10 ± 0.02	0.52	10.8 ± 4.3	11.1	0.14 ± 0.05	13.2	1.06
12	2-(3-Chlorophenyl)histamine	-	-	0.08 ± 0.04	-	41.4 ± 14.8	2.90	0.15 ± 0.03	93.7	-
16	2-(3-Methoxyphenyl)-histamine	-	-	0.08 ± 0.05	-	90.4 ± 23.8	1.32	0.13 ± 0.04	278	-

22	N^{α}-(Imidazolylethyl)-histaprodifen	0.570 ± 0.133	221	0.39 ± 0.06	0.35	0.470 ± 0.085	255	0.46 ± 0.05	1.73	1.21

Steady-state GTPase activity in Sf9 membranes expressing H_2R-$G_{s\alpha}$ fusion proteins was determined as described in *Methods*. Reaction mixtures contained H_1R ligands at concentrations from 10 nM - 1 mM as appropriate to generate saturated concentration/response curves. Data were analyzed by non-linear regression and were best fit to sigmoid concentration/response curves. Typical basal GTPase activities ranged between 1.0-2.0 pmol/mg/min, and the maximum stimulatory effect of histamine (100 μM) amounted to 200-300% above basal (Kelley et al., 2001). The efficacy (E_{max}) of histamine was determined by non-linear regression and was set 1.00 The E_{max} values of other H_1R agonists were referred to this value. Data shown are the means ± SD of 3-4 experiments performed in duplicates each. * $p < 0.05$ for comparison of hH_2R *versus* gpH_2R. The relative potency (rel. pot.) of histamine was set 100, and the potencies of other agonists were referred to this value. We also calculated the ratio of the relative potencies of H_1R agonists at hH_1R (taken from Table 2) and hH_2R-$G_{s\alpha S}$ (rel. pot. rat. hH_1R/hH_2R) and the ratio of the relative potencies of H_1R agonists at gpH_1R (taken from Table 2) and gpH_2R-$G_{s\alpha S}$ (rel. pot. rat. gpH_1R/gpH_2R). Additionally, we calculated the ratio of the EC_{50} values of H_1R agonists for hH_2R-$G_{s\alpha S}$ and gpH2R-$G_{s\alpha S}$ (pot. rat. gpH_2R/hH_2R). Data for compounds **1** and **4** were taken from Kelley et al. (2001). Cpd., compound; -, not applicable because stimulatory effects of agonists were too small. Compounds 11, **13**-**15**, 17–21 and 23 at concentrations from 10 μM – 1 mM were devoid of any stimulatory effect on GTPase activity in membranes expressing hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ and, therefore, not shown in the Table.

1 (R = H, histamine)
2 (R = CH_3)

3

4

5

no.	R
6	H
7	3-CH_3
8	2-F
9	3-F
10	4-F
11	3,5-F_2
12	3-Cl
13	4-Cl
14	3-Br
15	3-I
16	3-OCH_3
17	3-CF_3

18

21

22

23

no.	R
19	H
20	CH_3

24 (R = H)
25 (R = Cl)

26

27

28

(*R*)-(–)-**29**
(*S*)-(+)-**29**

30

31

32

no.	n	R
33	2	H
34	3	H
35	2	4-F
36	2	4-Cl
37	2	4-Br
38	2	3,4-Cl_2

40

41 (R = CH_3)
42 (R = COOH)

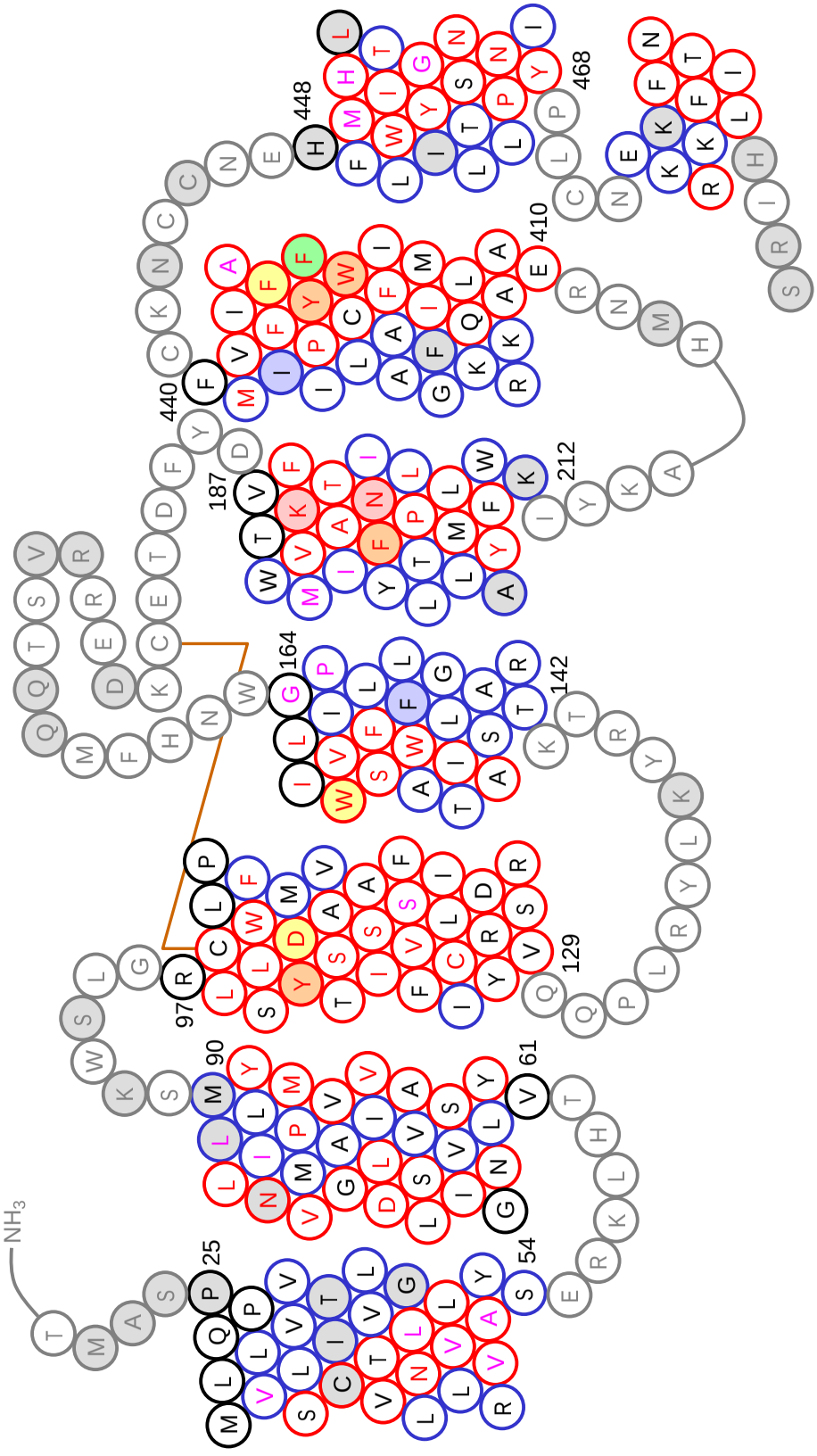
43

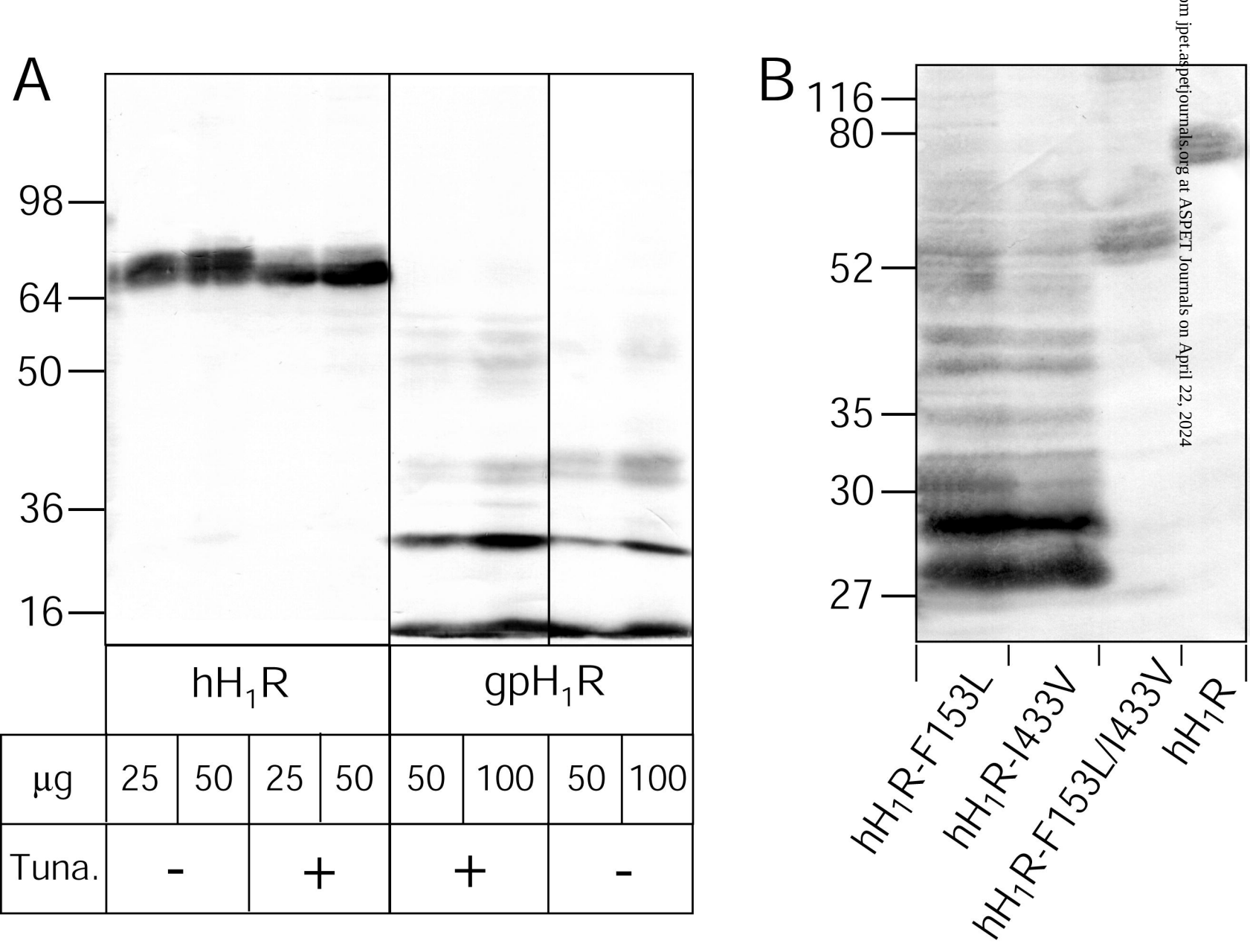
39

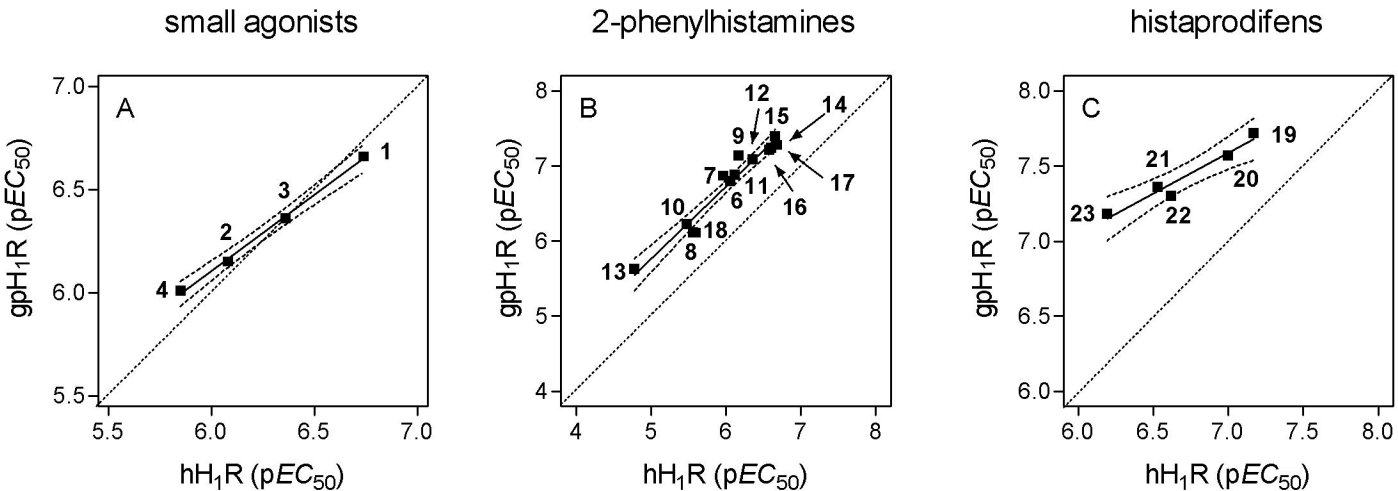
44

45

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.103.049619
This article has not been copyedited and formatted. The final version may differ from this version.






small agonists
2-phenylhistamines
histaprodifens
A
B
C
gpH1R (pEC50)
hH1R (pEC50)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23