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Mutations of Cys-17 and Ala-271 in the Human Histamine H_2 Receptor Determine the Species Selectivity of Guanidine-Type Agonists and Increase Constitutive Activity

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ABSTRACT

In a steady-state GTPase activity assay, *N*-[3-(1*H*-imidazol-4-yl)propyl)]guanidines and N^G-acylated derivatives are more potent and efficacious at fusion proteins of guinea pig (gpH_2R-$G_{s\alpha S}$) than human (hH_2R-$G_{s\alpha S}$) histamine H_2 receptor, coupled to the short splice variant of $G_{s\alpha}$, $G_{s\alpha S}$. Whereas Ala-271 (hH_2R) and Asp-271 (gpH_2R) in transmembrane domain 7 were identified to determine the potency differences of guanidine-type agonists, the molecular basis for the efficacy differences remains to be elucidated. A homology model of the gpH_2R suggested that an H-bond between Tyr-17 and Asp-271 stabilizes an active receptor conformation of the gpH_2R. In the present study, we generated a mutant hH_2R-$G_{s\alpha S}$ with Cys-17→Tyr-17/Ala-271→Asp-271 exchanges (hH_2R→gpH_2R) that exhibited an enhanced level of constitutive GTPase activity and adenylyl cyclase activity compared with wild-type hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$. Potencies and efficacies of guanidines and N^G-acylguanidines were increased at this mutant receptor compared with hH_2R-$G_{s\alpha S}$, but they were still lower than at gpH_2R-$G_{s\alpha S}$, suggesting that aside from Tyr-17 and Asp-271 additional amino acids contribute to the distinct pharmacological profiles of both species isoforms. Another hH_2R-$G_{s\alpha S}$ mutant with a Cys-17→Tyr-17 exchange showed inefficient coupling to $G_{s\alpha S}$ as revealed by reduced agonist-stimulated GTPase and basal adenylyl cyclase activities. Collectively, our present pharmacological study confirms the existence of an H-bond between Tyr-17 and Asp-271 favoring the stabilization of an active receptor conformation. Distinct potencies and efficacies of agonists and inverse agonists further support the concept of ligand-specific conformations in wild-type and mutant H_2R-$G_{s\alpha S}$ fusion proteins.

The histamine H_2 receptor (H_2R) is a biogenic amine receptor that belongs to the class A of the family of GPCRs. After stimulation by histamine (HA; Fig. 1, **1**), the H_2R couples to G_s proteins to activate adenylyl cyclase (AC). H_2Rs mediate regulation of gastric acid secretion in parietal cells, cardiac contractility, and myeloid cell differentiation (Del Valle and Gantz, 1997).

N-[3-(1*H*-Imidazol-4-yl)propyl]guanidines are the most potent agonists at the H_2R known so far (up to 400 times more active than HA at the guinea pig right atrium), and they are possibly useful as positive inotropic drugs for the treatment of severe congestive heart failure, as agents inducing cell differentiation in acute myelogenous leukemia, and as anti-inflammatory drugs (Dove et al., 2004). Guanidines are less potent and efficient agonists at the

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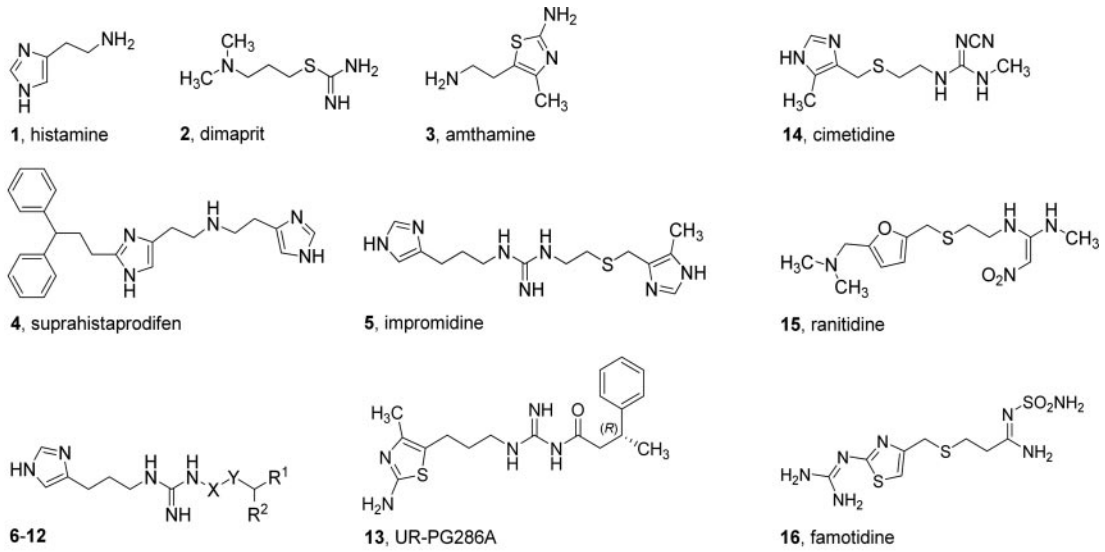
ABBREVIATIONS: H_2R, histamine H_2 receptor; GPCR, G protein-coupled receptor; HA, histamine; AC, adenylyl cyclase; $G_{s\alpha}$, α-subunit of the G_s protein that mediates adenylyl cyclase activation; $G_{s\alpha S}$, short splice variant of the G_s protein $G_{s\alpha}$; gpH_2R, guinea pig histamine 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_1R, 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_2R-C17Y-$G_{s\alpha S}$, fusion protein of the human histamine H_2 receptor bearing a Cys→Tyr mutation at position 17 and the short splice variant of $G_{s\alpha}$; hH_2R-C17Y-A271D-$G_{s\alpha S}$, fusion protein of the human histamine H_2 receptor bearing a Cys→Tyr mutation at position 17 and an Ala→Asp mutation at position 271 and the short splice variant of $G_{s\alpha}$; DIM, dimaprit; AMT, amthamine; TM, transmembrane domain of a G protein-coupled receptor; IMP, impromidine; ARP, arpromidine; CIM, cimetidine; RAN, ranitidine; FAM, famotidine; APT, aminopotentidine; IAPT, iodoaminopotentidine; PCR, polymerase chain reaction; S, signal peptide from influenza hemagglutinin; F, FLAG epitope; PAGE, polyacrylamide gel electrophoresis; AR, adrenoceptor; β_2AR-$G_{s\alpha}$, fusion protein of the β_2-adrenoceptor and $G_{s\alpha}$.



Cpd.		X	Y	R^1	R^2
6,	arpromidine	CH_2	CH_2	4-F-C_6H_4	2-pyridyl
7,	BU-E-43	CH_2	$(CH_2)_2$	phenyl	2-pyridyl
8,	UR-PG214	CO	CH_2	CH_3	2-thiazolyl
9,	UR-PG215	CO	CH_2	phenyl	2-thiazolyl
10,	UR-PG222A*	CO	CH_2	CH_3	phenyl
11,	UR-PG136	CO	CH_2	4-F-C_6H_4	2-pyridyl
12,	UR-AK57	CO	CH_2	CH_3	c-hexyl

* *(R)* configuration

Fig. 1. Structures of H_2R agonists and inverse agonists. **1** to **3**, small H_2R agonists; **4**, H_1R agonist with partial agonism at the H_2R; **5** to **7**, guanidine-type H_2R agonists; **8** to **12**, N^G-acylated *N*-[3-(1*H*-imidazol-4-yl)propyl]guanidines with agonistic H_2R activity; **13**, (*R*)-*N*-[3-(2-amino-4-methylthiazol-5-yl)propyl]-*N′*-(3-phenylbutanoyl)guanidine, an H_2R agonist; and **14** to **18**, H_2R inverse agonists.

H_2R of human neutrophils than at the H_2R of the guinea pig right atrium (Burde et al., 1989, 1990). In a membrane steady-state GTPase activity assay with fusion proteins of H_2R and the short splice variant of $G_{s\alpha}$, $G_{s\alpha S}$, these compounds are considerably more potent and efficacious at gpH_2R-$G_{s\alpha S}$ than at hH_2R-$G_{s\alpha S}$ (Kelley et al., 2001). Recently, a novel class of N^G-acylated imidazolylpropylguanidines was developed (Ghorai, 2005). The introduction of an electron-withdrawing carbonyl group adjacent to the guanidine moiety reduces the basicity of the compounds (pK_a of ~8). This structural modification does not change the species selectivity between hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ (Xie et al., 2006a). By contrast, HA and the small H_2R agonists dimaprit (DIM; Fig. 1, **2**) and amthamine (AMT; Fig. 1, **3**) do not exhibit species selectivity.

A three-dimensional homology model of the gpH_2R suggested that the nonconserved Asp-271 in transmembrane domain (TM) 7 confers high potency to the guanidines, which was subsequently confirmed by an Ala-271→Asp-271 mutation in hH_2R-$G_{s\alpha S}$ (hH_2R-A271D-$G_{s\alpha S}$) (Kelley et al., 2001). However, the efficacies of guanidines at this mutant and at hH_2R/gpH_2R chimeras were lower than at gpH_2R, demonstrating that guanidine efficacy depends on additional or other interactions. As a rationale, an interhelical H-bond between Tyr-17 in TM1 and Asp-271 was predicted from the model, stabilizing an active guanidine-bound conformation only in gpH_2R but not in hH_2R (containing Cys-17 and Ala-271) (Kelley et al., 2001).

To test this hypothesis, we generated an hH_2R-$G_{s\alpha S}$ mutant with a Cys-17→Tyr-17 exchange and a double mutant with Cys-17→Tyr-17 and Ala-271→Asp-271 exchanges in the sequence of hH_2R. Sf9 cell membranes expressing mutant and wild-type H_2R-$G_{s\alpha S}$ were used to measure steady-state GTPase activity, because this system was previously shown to be reliable and very sensitive to analyze ligand potencies and efficacies (Seifert et al., 1999; Milligan, 2000). due to the defined 1:1 stoichiometry of receptor and $G_{s\alpha}$ in fusion proteins, ligand potencies and efficacies in the steady-state GTPase assay are independent of the expression levels, allowing for the comparison of various membrane preparations with different expression levels. We also assessed AC activity in Sf9 membranes as a sensitive readout to compare distinct levels of constitutive activity of mutant and wild-type H_2R-$G_{s\alpha S}$ fusion proteins. Figure 1 shows the structures of H_2R agonists examined in the present study. Impromidine (IMP; **5**), arpromidine (ARP; **6**), and BU-E-43 (**7**) are representatives of *N*-[3-(1*H*-imidazol-4-yl)propyl]guanidines. Their N^G-acylated derivatives contain diverse diarylpropanoyl (**9** and **11**), 3-(hetero)arylbutanoyl (**8** and **10**), and 3-(cyclohexylbutanoyl) (**12**) groups. Compound **13** contains a 2-amino-4-methylthiazol-5-yl group and exhibits enhanced selectivity relative to the H_3R (Ghorai, 2005). Compounds **10** and **13** are the pure (*R*)-enantiomers. In addition, the inverse agonists cimetidine (CIM; **14**), ranitidine (RAN; **15**), famotidine (FAM; **16**), aminopotentidine (APT; **17**), and iodoaminopotentidine (IAPT; **18**) were studied (Hill et al., 1997; Dove et al., 2004).

Materials and Methods

Materials. The generation of pGEM-3Z-SF-hH_2R-$G_{s\alpha S}$, pGEM-3Z-SF-hH_2R-A271D-$G_{s\alpha S}$, and pVL1392-SF-hH_2R-$G_{s\alpha S}$ was described previously (Kelley et al., 2001). The generation of the baculoviruses encoding hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ was described previously (Kelley et al., 2001; Houston et al., 2002). Compounds **8** and **11** and **13** (Ghorai, 2005; Xie et al., 2006a) and compound **12** (Xie et al., 2006b) were prepared as described. IMP was synthesized as described previously (Durant et al., 1978). ARP and BU-E-43 were synthesized as described previously (Buschauer, 1989). APT and IAPT were prepared as described previously (Hirschfeld et al., 1992). Suprahistaprodifen was synthesized as described previously (Elz et al., 2000). The structures of compounds were confirmed by elemental analysis (C, H, N), ^{1}H NMR, and mass spectrometry. Purity of compounds was >98% as determined by high-performance liquid chromatography or capillary electrophoresis. The anti-FLAG Ig (M1 monoclonal antibody) was from Sigma-Aldrich (St. Louis, MO), and the anti-His_6 Ig was from Clontech (Mountain View, CA). [γ-^{32}P]GTP was synthesized through phosphorylation of GDP by enzymatic conversion of L-α-glycerol phosphate to 3-phosphoglycerate following a procedure described previously (Walseth and Johnson, 1979). [^{32}P]P_i (8500–9100 Ci/mmol orthophosphoric acid), [α-^{32}P]ATP (800 Ci/mmol), and [^{3}H]dihydroalprenolol (85–90 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Boston, MA). All unlabeled nucleotides, glycerol-3-phosphate dehydrogenase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase were from Roche Diagnostics (Indianapolis, IN). 3-Phosphoglycerate kinase, L-α-glycerol phosphate, HA, CIM, RAN, and FAM were from Sigma-Aldrich. AMT was from Tocris Cookson Inc. (Ballwin, MO). DIM was from Sigma/RBI (Natick, MA). All restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA). Cloned *Pfu* DNA polymerase was from Stratagene (La Jolla, CA).

Construction of the cDNA for hH_2R-C17Y-$G_{s\alpha S}$. The Cys-17→Tyr-17 exchange in hH_2R was generated by sequential overlap-extension PCRs. With pGEM-3Z-SF-hH_2R-$G_{s\alpha S}$ as template, PCR 1A was used to amplify a DNA fragment consisting of the cleavable signal peptide from influenza hemagglutinin (S), the FLAG epitope (F) recognized by the M1 monoclonal antibody, and the N-terminal portion of the hH_2R. The sense primer annealed with 18 base pairs of pGEM-3Z before the 5′ end of SF. The antisense primer encoded the sequence 5′-GATCTTATATGCGGTAGAGTCTAGACAAAAGGAAGAGGCTG-3′ to generate the Cys-17→Tyr-17 exchange and a new XbaI site (TCTAGA). In PCR 1B, the DNA sequence of the hH_2R, a hexahistidine tag, and the entire sequence of $G_{s\alpha S}$ was amplified using pGEM-3Z-SF-hH_2R-$G_{s\alpha S}$ as template. The sense primer encoded the sequence ′-CTTTTGTCTAGACTCTACCGCATATAAGATCACCATCACCG-3′ to generate the Cys-17→Tyr-17 exchange and the new XbaI site. The antisense primer annealed with the cDNA encoding the five C-terminal amino acids of $G_{s\alpha S}$, the stop codon, and an XbaI site. In PCR 2, the products of PCR 1A and 1B annealed in the region encoding the newly created Cys-17→Tyr-17 exchange and the new XbaI site. Here, the sense primer of PCR 1A and the antisense primer of PCR 1B were used. In that way, the complete cDNA for the hH_2R-C17Y-$G_{s\alpha S}$ fusion protein was amplified. The product of PCR 2 was digested with SacI and KpnI and cloned into pGEM-3Z-SF-hH_2R-$G_{s\alpha S}$ digested with SacI and KpnI. pGEM-3Z-SF-hH_2R-C17Y-$G_{s\alpha S}$ was digested with SacI and EcoN I and cloned into the baculovirus transfer vector pVL1392-SF-hH_2R-$G_{s\alpha S}$ digested with SacI and EcoNI. PCR-generated DNA sequences were confirmed by extensive restriction enzyme analysis and enzymatic sequencing.

Construction of the cDNA for hH_2R-C17Y-A271D-$G_{s\alpha S}$. To generate the DNA for fusion proteins with two amino acid exchanges Cys-17→Tyr-17 and Ala-271→Asp-271, pGEM-3Z-SF-hH_2R-A271D-$G_{s\alpha S}$ was digested with KpnI and BglII and cloned into pGEM-3Z-SF-hH_2R-C17Y-$G_{s\alpha S}$ digested with KpnI and BglII. pGEM-3Z-SF-hH_2R-C17Y-A271D-$G_{s\alpha S}$ was digested with NcoI and BglII and cloned into the baculovirus transfer vector pVL1392-SF-hH_2R-$G_{s\alpha S}$ digested with NcoI and BglII.

Generation of Recombinant Baculoviruses, Cell Culture, and Membrane Preparation. Recombinant baculoviruses encoding hH_2R-C17Y-$G_{s\alpha S}$ and hH_2R-C17Y-A271D-$G_{s\alpha S}$ were generated in Sf9 cells using the BaculoGOLD transfection kit (BD Biosciences PharMingen, San Diego, CA) according to the manufacturer's instructions. After initial transfection, high-titer virus stocks were generated by two sequential virus amplifications. Sf9 cells were cultured in 250-ml disposable Erlenmeyer flasks at 28°C under rotation at 125 rpm in SF 900 II medium (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) fetal calf serum (Cambrex Bio Science Walkersville Inc., Walkersville, MD) and 0.1 mg/ml gentamicin (Cambrex Bio Science Walkersville Inc.). Cells were maintained at a density of 0.5 to 6.0 $\times$ 10^6 cells/ml. For infection, cells were sedimented by centrifugation and suspended in fresh medium. Cells were seeded at 3.0 $\times$ 10^6 cells/ml and infected with a 1:100 dilution of high-titer baculovirus stocks encoding H_2R-$G_{s\alpha S}$ fusion proteins. Cells were cultured for 48 h before membrane preparation. Sf9 membranes were prepared as described previously (Seifert et al., 1998a), using 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine, and 10 μg/ml leupeptin as protease inhibitors. Membranes were suspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA, and 75 mM Tris-HCl, pH 7.4) and stored at −80°C until use.

SDS-PAGE and Immunoblot Analysis. Membrane proteins were separated on SDS polyacrylamide gels containing 12% (w/v) acrylamide. Proteins were transferred onto Immobilon-P membranes (Millipore Corporation, Bedford, MA) and reacted with M1 antibody, or anti-His_6 Ig (1:1000 each). Protein bands were visualized by enhanced chemoluminescence (Pierce Chemical, Rockford, IL) using sheep anti-mouse IgG, coupled to peroxidase.

Steady-State GTPase Activity Assay. Membranes were thawed, sedimented, and resuspended in 10 mM Tris-HCl, pH 7.4. Assay tubes contained Sf9 membranes expressing H_2R-$G_{s\alpha S}$ fusion proteins (10 μg of protein/tube), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 100 nM GTP, 0.1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, and 0.2% (w/v) bovine serum albumin in 50 mM Tris-HCl, pH 7.4, and H_2R ligands at various concentrations. Reaction mixtures (80 μl) were incubated for 2 min at 25°C before the addition of 20 μl of [γ-^{32}P]GTP (0.1 μCi/tube). All stock and work dilutions of [γ-^{32}P]GTP were prepared in 20 mM Tris-HCl, pH 7.4. Reactions were conducted for 20 min at 25°C. Preliminary studies under basal conditions and with HA, IMP, and ARP showed that under these conditions, GTP hydrolysis was linear. Reactions were terminated by the addition of 900 μl of slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. Charcoal absorbs nucleotides but not P_i. Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at 15,000*g*. Six hundred microliters of the supernatant fluid of reaction mixtures was removed, and $^{32}P_i$ was determined by liquid scintillation counting. Enzyme activities were corrected for spontaneous degradation of [γ-^{32}P]GTP. Spontaneous [γ-^{32}P]GTP degradation was determined in tubes containing all of the above-described components plus a very high concentration of unlabeled GTP (1 mM) that, by competition with [γ-^{32}P]GTP, prevents [γ-^{32}P]GTP hydrolysis by enzymatic activities present in Sf9 membranes. Spontaneous [γ-^{32}P]GTP degradation was <1% of the total amount of radioactivity added using 20 mM Tris-HCl, pH 7.4, as solvent for [γ-^{32}P]GTP. The experimental conditions chosen ensured that not more than 10% of the total amount of [γ-^{32}P]GTP added was converted to $^{32}P_i$.

AC Activity Assay. AC activity in Sf9 membranes was determined as described previously (Houston et al., 2002). In brief, membranes were thawed and sedimented by a 15-min centrifugation at 4°C and 15,000*g* to remove residual endogenous guanine nucleotides as far as possible, and they were subsequently resuspended in binding buffer. Tubes contained Sf9 membranes expressing H_2R-$G_{s\alpha S}$

fusion proteins (20 μg of protein/tube), additionally 5 mM $MgCl_2$, 0.4 mM EDTA, and 30 mM Tris-HCl, pH 7.4. Assay tubes containing membranes and various additions in a total volume of 30 μl were incubated for 3 min at 37°C before starting reactions by the addition of 20 μl of reaction mixture containing (final) [α-^{32}P]ATP (0.3 μCi/tube) plus 40 μM unlabeled ATP, 2.7 mM mono(cyclohexyl)ammonium phosphoenolpyruvate, 0.125 IU of pyruvate kinase, 1 IU of myokinase, and 0.1 mM cAMP. Reactions were conducted for 20 min at 37°C. Reactions were terminated by the addition of 20 μl of 2.2 N HCl. Denatured protein was sedimented by a 3-min centrifugation at 25°C and 15,000*g*. Sixty-five microliters of the supernatant fluid was applied onto disposable columns filled with 1.3 g of neutral alumina (A-1522, super I, WN-6; Sigma-Aldrich). [^{32}P]cAMP was separated from [α-^{32}P]ATP by elution of [^{32}P]cAMP with 4 ml of 0.1 M ammonium acetate, pH 7.0. Recovery of [^{32}P]cAMP was ~80%. Blank values were routinely ~0.01% of the total amount of [α-^{32}P]ATP added. [^{32}P]cAMP was determined by liquid scintillation counting. The experimental conditions chosen ensured that not more than 1 to 3% of the total amount of [α-^{32}P]ATP added was converted to [^{32}P]cAMP.

Miscellaneous. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA). [^{3}H]Dihydroalprenolol saturation binding was performed as described previously (Seifert et al., 1998a). All analyses of experimental data were performed with the Prism 4 program (GraphPad Software Inc., San Diego, CA). K_B values were calculated using the Cheng and Prusoff (1973) equation. Expression levels of recombinant proteins were determined using the GS-710 calibrated imaging densitometer and the software tool Quantity One version 4.0.3 (Bio-Rad).

Results

Immunological Detection of Recombinant Proteins in Sf9 Cell Membranes. In Sf9 cells hH_2R-C17Y-$G_{s\alpha S}$ and hH_2R-C17Y-A271D-$G_{s\alpha S}$ were well expressed (Fig. 2, A and B). Monomeric nonfused H_2R expressed in Sf9 cells migrates as an ~33-kDa band in SDS-PAGE (Fukushima et al., 1997; Houston et al., 2002), and the apparent molecular mass of $G_{s\alpha S}$ is ~45 kDa (Graziano et al., 1989). SDS-PAGE analysis of membranes expressing hH_2R-C17Y-A271D-$G_{s\alpha S}$ yielded intense bands at ~80 kDa, recognized by both the anti-FLAG and the anti-His_6 antibodies, that coincide with the expected apparent molecular masses of H_2R-$G_{s\alpha S}$ monomers (Kelley et al., 2001; Houston et al., 2002). Both bands seemed somewhat diffuse, representing different glycosylation forms of the proteins. With the anti-His_6 antibody, an additional doublet band was detected at ~45 kDa not recognized by the anti-FLAG antibody, which is presumably due to a lack of epitope exposure. By contrast, SDS-PAGE of membranes expressing hH_2R-C17Y-$G_{s\alpha S}$ yielded strong and diffuse bands at ~40 kDa and lacked the expected bands at ~80 kDa. These bands could either represent atypically migrating glycosylated forms of H_2R-$G_{s\alpha S}$ monomers or degraded proteins. Because the anti-FLAG Ig recognizes the N terminus and the anti-His_6 Ig the C terminus of the H_2R, it can be concluded that for either case the complete amino acid sequence of hH_2R-C17Y was expressed. Additional diffuse bands at ~110 kDa may correspond to GPCR dimers or higher oligomers and were also observed in wild-type hH_2R-$G_{s\alpha S}$ fusion proteins (Kelley et al., 2001). Comparison with the peak intensities of calibrated Sf9 membranes expressing the β_2-adrenergic receptor (AR) at 7.5 pmol mg^{-1} (as determined by [^{3}H]dihydroalprenolol saturation binding) revealed approximately similar expression levels of ~2 pmol mg^{-1} for hH_2R-C17Y-$G_{s\alpha S}$ and hH_2R-C17Y-A271D-$G_{s\alpha S}$.

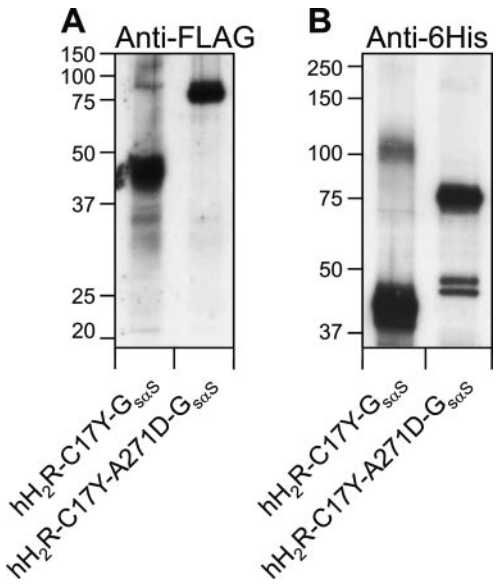


Fig. 2. Immunological detection of recombinant proteins in Sf9 cells. Sf9 membranes expressing various proteins were prepared, separated by SDS-PAGE on gels containing 12% (w/v) acrylamide, transferred onto Immobilon P membranes, and probed with the respective Ig indicated on top of each panel. In each lane, 10 μg of membrane protein was loaded onto the gel. Numbers on the left of membranes designate masses of marker proteins in kilodaltons.

Agonist and Inverse Agonist Effects on GTPase Activities in Sf9 Membranes Expressing hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, hH_2R-C17Y-$G_{s\alpha S}$, and hH_2R-C17Y-A271D-$G_{s\alpha S}$. The basal GTPase activity of hH_2R-$G_{s\alpha S}$ amounted to 0.66 ± 0.09 pmol mg^{-1} min^{-1} (n = 10). Compared with it, the data were similar in membranes expressing gpH_2R-$G_{s\alpha S}$ (0.69 ± 0.19 pmol mg^{-1} min^{-1}; n = 8; $p > 0.05$) and hH_2R-C17Y-$G_{s\alpha S}$ (0.78 ± 0.10 pmol mg^{-1} min^{-1}; n = 9; $p > 0.05$), respectively, but significantly increased at hH_2R-C17Y-A271D-$G_{s\alpha S}$ (1.67 ± 0.38 pmol mg^{-1} min^{-1}; n = 9; $p < 0.01$). At the fusion proteins of both wild-type receptors and at hH_2R-C17Y-A271D-$G_{s\alpha S}$, stimulation with 100 μM HA yielded GTPase activities 400 to 600% of the basal levels. By contrast, at hH_2R-C17Y-$G_{s\alpha S}$, maximal HA GTPase activities amounted to just 140% of the basal signal, thereby providing an insufficiently low signal-to-noise ratio for detailed analysis of agonists (Fig. 3). Thus, for a comparative analysis of efficacies and potencies of compounds **1** to **13**, only membranes expressing both wild-type receptors and the double mutant hH_2R-C17Y-A271D-$G_{s\alpha S}$ were considered (Table 1). The efficacies of the small agonists DIM (**2**) and AMT (**3**) were slightly increased at gpH_2R-$G_{s\alpha S}$ and hH_2R-C17Y-A271D-$G_{s\alpha S}$, relative to hH_2R-$G_{s\alpha S}$. The potencies of HA (**1**) (Fig. 3), DIM (**2**), and AMT (**3**) were increased at hH_2R-C17Y-A271D-$G_{s\alpha S}$ compared with the wild-type receptors. The H_1R-selective agonist suprahistaprodifen (**4**) (Seifert et al., 2003) acted as a partial agonist with similar efficacies and potencies at hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, and hH_2R-C17Y-A271D-$G_{s\alpha S}$. In agreement with previous studies (Kelley et al., 2001; Xie et al., 2006a,b), *N*-[3-(1*H*-imidazol-4-yl)propyl]guanidines **5** to **7** and most of their N^G-acylated derivatives **8** to **13** were more efficacious and more potent at gpH_2R-$G_{s\alpha S}$ than at hH_2R-$G_{s\alpha S}$. Except for IMP (**5**) being more efficacious at hH_2R-C17Y-A271D-$G_{s\alpha S}$, the efficacies of **5** to **7** were not significantly changed at the mutant receptor compared with

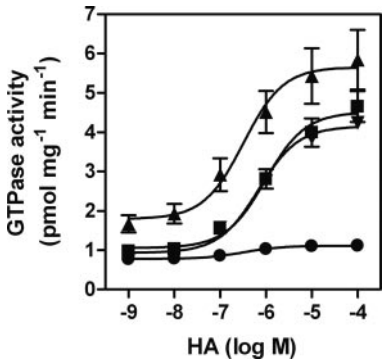


Fig. 3. Concentration-dependent increase of GTPase activity by HA in membranes expressing hH_2R-$G_{s\alpha S}$ (■), gpH_2R-$G_{s\alpha S}$ (▼), hH_2R-C17Y-$G_{s\alpha S}$ (●), and hH_2R-C17Y-A271D-$G_{s\alpha S}$ (▲). GTPase activity in Sf9 membranes was determined as described under *Materials and Methods*. Reaction mixtures contained membranes (10 μg of protein/tube) expressing fusion proteins and HA at concentrations indicated on the abscissa. Data shown are the means ± S.E.M. of three independent experiments performed in duplicates. Data were analyzed by nonlinear regression and were best fitted to sigmoidal concentration-response curves.

wild-type hH_2R-$G_{s\alpha S}$. Compound **11** but not ARP (**6**) was more efficacious and both were more potent at hH_2R-C17Y-A271D-$G_{s\alpha S}$ than at wild-type hH_2R-$G_{s\alpha S}$. Compounds **8** and **9** share a 2-thiazolyl moiety and were more potent at the double mutant compared with hH_2R-$G_{s\alpha S}$, although for compound **9** the difference was not significant. When the 2-thiazolyl group was replaced by a cyclohexyl group (**12**), the selectivity for the mutant was lost. Taken together, the small H_2R agonists **1** to **3** were considerably more potent at hH_2R-C17Y-A271D-$G_{s\alpha S}$ than at the wild-type human and guinea pig H_2R-$G_{s\alpha S}$. Some guanidines and N^G-acylated guanidines displayed enhanced potencies at the mutant receptor compared with hH_2R-$G_{s\alpha S}$. However, these compounds were all less potent at hH_2R-C17Y-A271D-$G_{s\alpha S}$ than at gpH_2R-$G_{s\alpha S}$, and the efficacies varied between the corresponding values at both wild-type receptors.

At wild-type H_2R-$G_{s\alpha S}$ and hH_2R-C17Y-A271D-$G_{s\alpha S}$ CIM (**14**), RAN (**15**), FAM (**16**), APT (**17**), and IAPT (**18**) decreased GTPase activities below basal and thus acted as inverse agonists (Table 2). At hH_2R-C17Y-A271D-$G_{s\alpha S}$ inverse agonist efficacies of **14** to **18** were significantly increased relative to wild-type receptors. Because inverse agonists stabilize an inactive receptor conformation (Milligan et al., 1995), the differences in inverse agonist efficacies reflect an increased level of constitutive activity of hH_2R-C17Y-A271D-$G_{s\alpha S}$ relative to wild-type receptors. The magnitudes of constitutive activity measured critically depend on the relative stoichiometry of GPCR and G protein in the system (Kenakin, 2001). Physical tethering of H_2R with $G_{s\alpha S}$ in the fusion proteins used provides a fixed 1:1 stoichiometry of both partners, allowing for a direct comparison of the efficacies in an expression-independent manner (Milligan, 2000). Compounds **16** and **18** were slightly more potent at hH_2R-C17Y-A271D-$G_{s\alpha S}$ than at the wild-type receptors, whereas no significant differences in the K_B values were observed for **14**, **15**, and **17**.

Regulation of AC Activities in Membranes Expressing hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, hH_2R-C17Y-$G_{s\alpha S}$, and hH_2R-C17Y-A271D-$G_{s\alpha S}$. AC activities were measured in Sf9 membranes expressing hH_2R-C17Y-$G_{s\alpha S}$ and hH_2R-C17Y-A271D-$G_{s\alpha S}$, and they were compared with results at wild-type human and guinea pig H_2R-$G_{s\alpha S}$ (Table 3). hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ were similarly expressed in Sf9 cells (at ~3 and 1 pmol mg^{-1}, respectively) and produced similar basal AC activities. By contrast, basal AC activities were increased ~3-fold at hH_2R-C17Y-A271D-$G_{s\alpha S}$. At both mutant and both wild-type receptors, 10 μM GTP by itself increased AC activities above the basal level (Fig. 4), indicating constitutive activity of these receptors (Seifert et al., 1998a,b; Gille and Seifert, 2003). Accordingly, at all four H_2Rs, the inverse agonist IAPT (**18**) reduced this GTP-dependent AC activity. At hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$, AC activity increases by 10 μM GTP achieved 73 and 77%, respectively, of the signal increases by 10 μM GTP plus 100 μM HA. Strikingly, at hH_2R-C17Y-A271D-$G_{s\alpha S}$, HA did not further enhance the GTP effect. Both higher basal AC activity and a strong stimulation by GTP caused exhaustion of the limiting

TABLE 1

Agonist efficacies and potencies at hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, and hH_2R-C17Y-A271D-$G_{s\alpha S}$ in the GTPase assay

Steady-state GTPase activity in Sf9 membranes expressing hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, and hH_2R-C17Y-A271D-$G_{s\alpha S}$ was determined as described under *Materials and Methods*. Reaction mixtures contained Sf9 membranes expressing fusion proteins and agonists at concentrations from 1 nM to 1 mM as appropriate to generate saturated concentration-response curves. Curves were analyzed by nonlinear regression and were best fitted to sigmoidal concentration-response curves. The maximal stimulatory effect of 100 μM HA amounted to 400 to 600% above basal. To calculate agonist efficacies, the maximum stimulatory effect of HA was set at 1.00, and the stimulatory effects of other agonists were referred to this value. Data shown are the means ± S.D. of three to six experiments performed in duplicate or triplicate. Efficacies and potencies, respectively, of ligands at hH_2R-$G_{s\alpha S}$ were compared with the corresponding parameters at gpH_2R-$G_{s\alpha S}$, and hH_2R-C17Y-A271D-$G_{s\alpha S}$, respectively, using the *t* test. The control data for hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ are identical with the control data for these constructs in Table 1 of Preuss et al. (2007).

	Compound	hH_2R-$G_{s\alpha S}$		gpH_2R-$G_{s\alpha S}$		hH_2R-C17Y-A271D-$G_{s\alpha S}$	
		Efficacy	EC_{50}	Efficacy[a]	EC_{50}[b]	Efficacy[a]	EC_{50}[b]
			nM		*nM*		*nM*
1	HA	1.00	990 ± 92	1.00	850 ± 340	1.00	$320 \pm 9^{+++}$
2	DIM	0.85 ± 0.02	910 ± 430	0.94 ± 0.06*	740 ± 360	0.98 ± 0.01***	$370 \pm 36^{+}$
3	AMT	0.91 ± 0.02	190 ± 50	1.04 ± 0.01**	190 ± 42	0.97 ± 0.01**	$65 \pm 6^{+}$
4	Suprahistaprodifen	0.54 ± 0.08	240 ± 41	0.43 ± 0.02	310 ± 62	0.61 ± 0.02	$320 \pm 11^{+}$
5	IMP	0.82 ± 0.02	160 ± 40	0.96 ± 0.06*	$18 \pm 9^{++}$	0.95 ± 0.02**	$37 \pm 5^{+}$
6	ARP	0.84 ± 0.03	72 ± 9	0.94 ± 0.05*	$7 \pm 1^{+++}$	0.87 ± 0.02	$39 \pm 4^{++}$
7	BU-E-43	0.71 ± 0.11	130 ± 13	0.87 ± 0.05	$43 \pm 10^{+++}$	0.73 ± 0.01	$150 \pm 6^{+}$
8	UR-PG214	0.91 ± 0.08	130 ± 45	0.94 ± 0.05	$25 \pm 10^{++}$	0.92 ± 0.09	$44 \pm 13^{+}$
9	UR-PG215	0.80 ± 0.04	120 ± 45	0.94 ± 0.05*	$14 \pm 4^{++}$	0.83 ± 0.04	46 ± 21
10	UR-PG222A	0.90 ± 0.04	18 ± 6	1.18 ± 0.08**	$5 \pm 1^{+}$	1.01 ± 0.04**	13 ± 5
11	UR-PG136	0.82 ± 0.05	100 ± 9	1.02 ± 0.11*	$29 \pm 10^{+++}$	0.92 ± 0.04*	$66 \pm 5^{++}$
12	UR-AK57	0.86 ± 0.05	15 ± 4	0.97 ± 0.18	14 ± 6	0.78 ± 0.03	21 ± 10
13	UR-PG286A	0.55 ± 0.06	49 ± 9	0.82 ± 0.02***	$12 \pm 8^{++}$	0.68 ± 0.06	29 ± 16

[a] Comparison with the efficacy at hH2R-$G_{s\alpha S}$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
[b] Comparison with the EC_{50} value at hH_2R-$G_{s\alpha S}$; $^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$; $^{+++}$, $p < 0.001$.

TABLE 2

Potencies and inverse agonist efficacies of antagonists at hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, and hH_2R-C17Y-A271D-$G_{s\alpha S}$ in the GTPase assay

Steady-state GTPase activity in Sf9 membranes expressing hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, and hH_2R-C17Y-A271D-$G_{s\alpha S}$ was determined as described under *Materials and Methods*. Reaction mixtures contained Sf9 membranes expressing fusion proteins, 1 μM HA as agonist and antagonists at concentrations from 1 nM to 1 mM as appropriate to generate saturated competition curves. Competition curves were analyzed by nonlinear regression. To determine the inverse agonist efficacies (Inv. Ago. Eff.), the effects of antagonists at fixed concentrations (10 μM RAN, FAM, APT, and IAPT; 100 μM CIM) on basal GTPase activity were assessed and referred to the stimulatory effect of 100 μM HA (=1.00). Data shown are the means ± S.D. of three experiments performed in duplicates. K_B values and inverse agonist efficacies, respectively, of antagonists at hH_2R-$G_{s\alpha S}$ were compared with the corresponding parameters at gpH_2R-$G_{s\alpha S}$ and hH_2R-C17Y-A271D-$G_{s\alpha S}$, respectively, using the *t* test. The control data for hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ are identical with the control data for these constructs in Table 2 of Preuss et al. (2007).

Compound		hH_2R-$G_{s\alpha S}$		gpH_2R-$G_{s\alpha S}$		hH_2R-C17Y-A271D-$G_{s\alpha S}$	
		K_B	Inv. Ago. Eff.	K_B[a]	Inv. Ago. Eff.	K_B[a]	Inv. Ago. Eff.
		nM		*nM*		*nM*	
14	CIM	1700 ± 430	−0.08 ± 0.01	1300 ± 270	−0.09 ± 0.02	1400 ± 19	$-0.25 \pm 0.04^{++}$
15	RAN	840 ± 94	−0.09 ± 0.01	1000 ± 170	−0.08 ± 0.01	1000 ± 64	$-0.21 \pm 0.05^{++}$
16	FAM	48 ± 10	−0.10 ± 0.02	38 ± 3	−0.10 ± 0.01	29 ± 2*	$-0.27 \pm 0.06^{++}$
17	APT	180 ± 12	−0.09 ± 0.01	260 ± 43*	−0.09 ± 0.01	200 ± 31	$-0.26 \pm 0.05^{++}$
18	IAPT	35 ± 7	−0.10 ± 0.01	26 ± 4	−0.10 ± 0.01	12 ± 2**	$-0.27 \pm 0.05^{+++}$

[a] Comparison with the K_B value at hH_2R-$G_{s\alpha S}$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
[b] Comparison with Inv. Ago. Eff. at hH_2R-$G_{s\alpha S}$; $^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$; $^{+++}$, $p < 0.001$.

TABLE 3

AC activities in Sf9 membranes expressing hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, hH_2R-C17Y-$G_{s\alpha S}$, and hH_2R-C17Y-A271D-$G_{s\alpha S}$

Basal AC activities and the effects of GTP and HA on AC activities in membranes expressing hH_2R-C17Y-$G_{s\alpha S}$ and hH_2R-C17Y-A271D-$G_{s\alpha S}$ were assessed and compared with the corresponding values at hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$. AC activity in Sf9 membranes was determined as described under *Materials and Methods*. Reaction mixtures contained Sf9 membranes (20 μg protein/tube) expressing fusion proteins and distilled water (basal), 10 μM GTP, or 10 μM GTP plus 100 μM HA. Data shown are the means ± S.D. of three experiments performed in triplicates. To calculate the stimulatory effect of GTP (Rel. GTP Effect), the effect of 10 μM GTP was referred to the effect of 10 μM GTP plus 100 μM HA. The control data for hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ are identical with the control data for these constructs in Table 3 of Preuss et al. (2007).

Construct	Basal, AC Activity	10 μM GTP, AC Activity	10 μM GTP + 100 μM HA, AC Activity	Rel. GTP Effect
	pmol mg^{-1} min^{-1}			%
hH_2R-$G_{s\alpha S}$	12.1 ± 2.1	24.3 ± 4.8	28.7 ± 5.3	73
gpH_2R-$G_{s\alpha S}$	13.4 ± 3.1	25.0 ± 0.6	28.5 ± 2.2	77
hH_2R-C17Y-$G_{s\alpha S}$	0.8 ± 0.2	2.4 ± 0.4	7.0 ± 0.8	26
hH_2R-C17Y-A271D-$G_{s\alpha S}$	42.5 ± 2.2	66.9 ± 4.2	66.4 ± 3.3	100

pool of AC molecules in Sf9 cells and reflect an increased level of constitutive activity of hH_2R-C17Y-A271D-$G_{s\alpha S}$, compared with the wild-type fusion proteins. Similar reduced agonist-responsiveness due to high constitutive activity was shown for other aminergic GPCRs, e.g., β_2AR-$G_{s\alpha}$ fusion proteins (Seifert et al., 1998a) and mutants of the 5-HT_4 receptor (Claeysen et al., 1999). At hH_2R-C17Y-$G_{s\alpha S}$ much lower basal AC activities and a much smaller stimulatory effect of GTP were determined. In this case, GTP on its own caused only 26% of the effect with HA addition.

At hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, and hH_2R-C17Y-A271D-$G_{s\alpha S}$, 100 μM HA reduced the basal AC activities in the absence of added GTP (Fig. 4, A–C). Similar effects were observed at β_2AR-$G_{s\alpha S}$ fusion proteins (Seifert et al., 1998b), and they are due to dissociation of GDP from $G_{s\alpha S}$ following agonist binding to the receptor without subsequent binding of GTP. $G_{s\alpha\text{-}GDP}$ is more effective in activating AC than nucleotide-free $G_{s\alpha}$; therefore, AC activities were decreased.

Discussion

Impaired Coupling in Membranes Expressing hH_2R-C17Y-$G_{s\alpha S}$. In Sf9 cells expressing hH_2R-C17Y-$G_{s\alpha S}$, the anti-FLAG and the anti-His_6 antibodies recognized similarly migrating proteins in SDS-PAGE that did not coincide with the expected bands for monomeric H_2R-$G_{s\alpha S}$ fusion proteins. Measurement of GTP hydrolysis at hH_2R-C17Y-$G_{s\alpha S}$ yielded HA responses, but the increases in GTPase activity upon agonist stimulation were much lower than in the wild-type H_2R-$G_{s\alpha S}$ species isoforms. Moreover, with this receptor mutant, substantial stimulatory effects of HA and inhibitory effects of IAPT on the GTP-dependent AC activity increases were observed, but the basal AC activities and the stimulatory effects of GTP were largely reduced relative to wild-type H_2R-$G_{s\alpha S}$ species isoforms, and they were similar to the values typical for Sf9 membranes expressing nonfused H_2R species (Houston et al., 2002). These controversial results of hH_2R-C17Y-$G_{s\alpha S}$ relative to hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ imply the following conclusions: The proteins expressed in Sf9 cells made up the amino acid sequence for hH_2R-C17Y, and they were functional in the test systems used. However, coupling of hH_2R-C17Y to $G_{s\alpha S}$ was much less efficient than is characteristic for GPCR-$G_{s\alpha}$ fusion proteins (Seifert et al., 1999; Gille and Seifert, 2003). As a rationale, $G_{s\alpha S}$ could be incorrectly expressed or degraded in Sf9 cells. Instead, hH_2R-C17Y possibly coupled to only a fraction of recombinant $G_{s\alpha S}$ or to endogenous $G_{s\alpha}$-like G proteins with much lower efficiency.

Increased Constitutive Activity in Membranes Expressing hH_2R-C17Y-A271D-$G_{s\alpha S}$. In membranes expressing hH_2R-C17Y-A271D-$G_{s\alpha S}$, high-efficiency coupling was observed as GTPase activities were increased upon agonist stimulation similar to hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$. Moreover, with this receptor mutant enhanced basal GTPase activities, increased potencies of the agonists as well as increased inverse agonist efficacies of antagonists were detected, representing the hallmarks of enhanced constitutive activity compared with the wild-type proteins (Lefkowitz et al., 1993). The determination of AC activity in Sf9 cell membranes has previously shown to be an alternative and sensitive system to quantify differences in the constitutive activities of GPCRs (Seifert et al., 1998a). In membranes expressing hH_2R-C17Y-A271D-$G_{s\alpha S}$ the high basal AC activ-

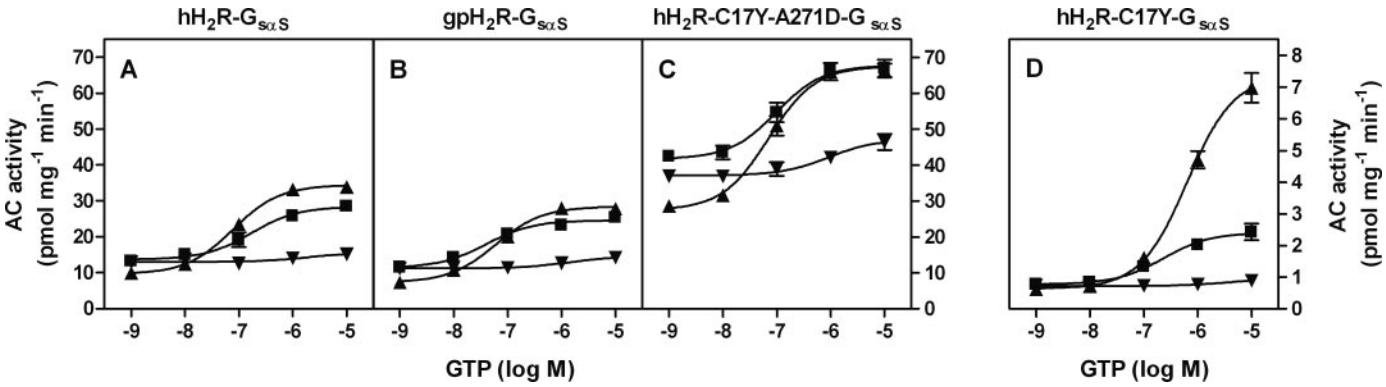


Fig. 4. Regulation of AC activities in Sf9 membranes expressing hH_2R-$G_{s\alpha S}$ (A), gpH_2R-$G_{s\alpha S}$ (B), hH_2R-C17Y-A271D-$G_{s\alpha S}$ (C), and hH_2R-C17Y-$G_{s\alpha S}$ (D). AC activity in Sf9 membranes was determined as described under *Materials and Methods*. Reaction mixtures contained Sf9 membranes expressing the proteins indicated on top of each panel and GTP at concentrations indicated at the abscissa. Reaction mixtures additionally contained H_2O (■), 100 μM HA (▲), or 10 μM IAPT (▼). Data shown are the means ± S.E.M. of one representative experiment performed in triplicates. The statistical analysis of AC activities is provided in Table 3. Data were analyzed by nonlinear regression and were best fitted to sigmoidal concentration-response curves. Please note the different scale of the ordinate in D. The control data for hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ are identical with the control data for these constructs in Fig. 6 of Preuss et al. (2007).

ities and the strong AC activity increases upon stimulation with GTP additionally reflect high constitutive activity compared with hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, and hH_2R-C17Y-$G_{s\alpha S}$.

The discovery of increased constitutive activity at hH_2R-C17Y-A271D-$G_{s\alpha S}$ further supports the concept of an H-bond between Tyr-17 in TM1 and Asp-271 in TM7 (Kelley et al., 2001) as basis for the distinct pharmacological properties of human and guinea pig H_2Rs. Our data suggest that this interhelical interaction stabilizes an active receptor conformation not only when agonists are bound but also when ligands are absent. However, gpH_2R-$G_{s\alpha S}$ containing Tyr-17 and Asp-271 also was similarly constitutively active as hH_2R-$G_{s\alpha S}$ which is presumably due to additional intramolecular interactions constraining the gpH_2R in an inactive conformation and thereby compensating for the activating function of both residues.

Of interest, the tertiary structure of the α_{1b}-AR contains Lys-331 in TM7 corresponding to Ala-271 in hH_2R. Strikingly, α_{1b}-AR mutants with Lys-331 exchanged by alanine or glutamate were more constitutively active than wild-type α_{1b}-AR (Porter et al., 1996), suggesting a general role of an amino acid at this position for the activation mechanism of related GPCRs.

Species Selectivity of Guanidines and N^G-Acylguanidines at Wild-Type and Mutant H_2R-$G_{s\alpha S}$. The main intention of this study was to elucidate the impact of Cys-17/Tyr-17 and Ala-271/Asp-271 on the species selectivity of *N*-[3-(1*H*-imidazol-4-yl)propyl]guanidines and N^G-acylated imidazolypropylguanidines between hH_2R and gpH_2R. In our GTPase activity experiments, some of these agonists were more potent and more efficacious at hH_2R-C17Y-A271D-$G_{s\alpha S}$ than at hH_2R-$G_{s\alpha S}$, and some compounds were not selective. Overall, the potencies and efficacies of the agonists were still higher at gpH_2R-$G_{s\alpha S}$ than at hH_2R-C17Y-A271D-$G_{s\alpha S}$. The following conclusions can be drawn from these results.

First, both Tyr-17 and Asp-271 contribute to the enhanced potencies and efficacies of guanidines and N^G-acylguanidines at the gpH_2R. This investigation adds to a previous study at an hH_2R-A271D-$G_{s\alpha S}$ mutant conferring high potency to guanidines without affecting the efficacies (Kelley et al., 2001). However, the pharmacological differences between hH_2R-C17Y-A271D-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ indicate that more than these two amino acids determine the species selectivity of agonists and will have to be identified in future mutagenesis studies.

Second, the concept of ligand-specific conformations in H_2R species (Kelley et al., 2001; Kenakin, 2003; Xie et al., 2006a) is further supported. The variable side chains of the compounds distinctly interact with wild-type and mutant H_2R-$G_{s\alpha S}$, which is represented by compounds **8** and **9** containing a 2-thiazolyl group and being more potent at hH_2R-C17Y-A271D-$G_{s\alpha S}$ than at hH_2R-$G_{s\alpha S}$ in contrast to compound **10** with a cyclohexyl group being similarly potent at both proteins. The 5-methyl-1*H*-imidazol-4-yl group in IMP (**5**) presumably directly interacts with Asp-271 (Kelley et al., 2001), yielding the high-potency increase of ~4-fold at hH_2R-C17Y-A271D-$G_{s\alpha S}$ versus hH_2R-$G_{s\alpha S}$.

GPCRs with enhanced constitutive activity exhibit an increased affinity for agonists with the affinity increase being correlated with the efficacy of the ligand (Samama et al., 1993). Accordingly, the parameter of constitutive activity not only affects elevated potencies of small H_2R agonists at hH_2R-C17Y-A271D-$G_{s\alpha S}$ but also potency increases of the guanidines and N^G-acylguanidines. Different magnitudes of constitutive activity therefore add to the complexity of the system for the analysis of species-selective ligand/GPCR interactions. Moreover, inverse agonists are less potent at constitutively active than at quiescent GPCRs (Kenakin, 2001). Accordingly, **14** to **18** were expected to be less potent at the more constitutively active hH_2R-C17Y-A271D-$G_{s\alpha S}$ than at hH_2R-$G_{s\alpha S}$. However, the potencies of inverse agonists were not decreased, and **16** and **18** were even more potent at the mutant receptor, assuming that not only guanidine-type agonists but also inverse agonists could stabilize ligand-specific conformations in H_2R species isoforms.

Conclusions

In the present study, we demonstrate that an hH_2R-$G_{s\alpha S}$ fusion protein with mutations of Cys-17→Tyr-17 in TM1 and Ala-271→Asp-271 in TM7 displayed enhanced constitutive activity compared with hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$. We additionally showed that an interaction between Tyr-17 and Asp-271 in gpH_2R contributes to the species-selective action

of *N*-[3-(1*H*-imidazol-4-yl)propyl]guanidines and their N^G-acylated derivatives. Distinct potencies and efficacies of agonists and inverse agonists further support the concept of ligand-specific conformations in wild-type and mutant H_2R-$G_{s\alpha S}$ fusion proteins. A single point mutation of Cys-17→Tyr-17 was devoid of efficient GPCR-G protein coupling. By analogy, point mutations of Phe-153→Leu-153 or Ile-433→Val-433 in the hH_1R (hH_1R→gpH_1R) resulted in functional inactivity, whereas a Phe-153→Leu-153/Ile-433→Val-433 double mutant was functionally active (Seifert et al., 2003). The reasons for the annihilating effects of the single point mutations hH_1R and hH_2R are not known, but they illustrate the limitations of site-directed mutagenesis experiments. The characterization of closely related wild-type GPCR species isoforms is, therefore, an important alternative approach to relate distinct pharmacological properties to relatively few molecular determinants.

Taken together, our mutational studies provide unique insight into the molecular mechanisms of H_2R functions and will help us to find potent and selective agonists for the hH_2R that may be useful as positive inotropic drugs for the treatment of severe congestive heart failure, as agents inducing cell differentiation in acute myelogenous leukemia, and as anti-inflammatory drugs.

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References

Burde R, Buschauer A, and Seifert R (1990) Characterization of histamine H_2-receptors in human neutrophils with a series of guanidine analogues of impromidine. Are cell type-specific H_2-receptors involved in the regulation of NADPH oxidase? *Naunyn-Schmiedeberg's Arch Pharmacol* **341:**455–461.

Burde R, Seifert R, Buschauer A, and Schultz G (1989) Histamine inhibits activation of human neutrophils and HL-60 leukemic cells via H_2-receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* **340:**671–678.

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.

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.

Claeysen S, Sebben M, Becamel C, Bockaert J, and Dumuis A (1999) Novel brain-specific 5-HT_4 receptor splice variants show marked constitutive activity: role of the C-terminal intracellular domain. *Mol Pharmacol* **55:**910–920.

Del Valle J and Gantz I (1997) Novel insights into histamine H_2 receptor biology. *Am J Physiol* **273:**G987–G996.

Dove S, Elz S, Seifert R, and Buschauer A (2004) Structure-activity relationships of histamine H_2 receptor ligands. *Mini Rev Med Chem* **4:**941–954.

Durant GJ, Duncan WA, Ganellin CR, Parsons ME, Blakemore RC, and Rasmussen AC (1978) Impromidine (SK&F 92676) is a very potent and specific agonist for histamine H_2 receptors. *Nature (Lond)* **276:**403–405.

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.

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.

Ghorai P (2005) *Arpromidine-Related Acylguanidines: Synthesis and Structure-Activity Relationships of a New Class of Guanidine-Type Histamine H_2 Receptor Agonists with Reduced Basicity*. Ph.D. thesis, University of Regensburg, Regensburg, Germany.

Gille A and Seifert R (2003) Co-expression of the β_2-adrenoceptor and dopamine D_1-receptor with $G_{s\alpha}$ proteins in Sf9 insect cells: limitations in comparison with fusion proteins. *Biochim Biophys Acta* **1613:**101–114.

Graziano MP, Freissmuth M, and Gilman AG (1989) Expression of $G_{s\alpha}$ in *Escherichia coli*. Purification and properties of two forms of the protein. *J Biol Chem* **264:**409–418.

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.

Hirschfeld J, Buschauer A, Elz S, Schunack W, Ruat M, Traiffort E, and Schwartz JC (1992) Iodoaminopotentidine and related compounds: a new class of ligands with high affinity and selectivity for the histamine H_2 receptor. *J Med Chem* **35:**2231–2238.

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.

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 (2001) Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J* **15:**598–611.

Kenakin T (2003) Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol Sci* **24:**346–354.

Lefkowitz RJ, Cotecchia S, Samama P, and Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* **14:**303–307.

Milligan G (2000) Insights into ligand pharmacology using receptor-G-protein fusion proteins. *Trends Pharmacol Sci* **21:**24–28.

Milligan G, Bond RA, and Lee M (1995) Inverse agonism: pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol Sci* **16:**10–13.

Porter JE, Hwa J, and Perez DM (1996) Activation of the α_{1b}-adrenergic receptor is initiated by disruption of an interhelical salt bridge constraint. *J Biol Chem* **271:**28318–28323.

Preuss H, Ghorai P, Kraus A, Dove S, Buschauer A, and Seifert R (2007) Constitutive activity and ligand selectivity of human guinea pig, rat, and canine histamine H_2 receptors. *J Pharmacol Exp Ther* **321:**983–995.

Samama P, Cotecchia S, Costa T, and Lefkowitz RJ (1993) A mutation-induced activated state of the β_2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* **268:**4625–4636.

Seifert R, Lee TW, Lam VT, and Kobilka BK (1998a) 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, Wenzel-Seifert K, Bürckstümmer T, Pertz HH, Schunack W, Dove S, Buschauer A, and Elz S (2003) Multiple differences in agonist and antagonist pharmacology between human and guinea pig histamine H_1-receptor. *J Pharmacol Exp Ther* **305:**1104–1115.

Seifert R, Wenzel-Seifert K, and Kobilka BK (1999) GPCR-G_α fusion proteins: molecular analysis of receptor-G-protein coupling. *Trends Pharmacol Sci* **20:**383–389.

Seifert R, Wenzel-Seifert K, Lee TW, Gether U, Sanders-Bush E, and Kobilka BK (1998b) Different effects of $G_s\alpha$ splice variants on β_2-adrenoreceptor-mediated signaling. The β_2-adrenoreceptor coupled to the long splice variant of $G_s\alpha$ has properties of a constitutively active receptor. *J Biol Chem* **273:**5109–5116.

Walseth TF and Johnson RA (1979) The enzymatic preparation of [α-^{32}P] nucleoside triphosphates, cyclic [^{32}P] AMP, and cyclic [^{32}P] GMP. *Biochim Biophys Acta* **562:**11–31.

Xie SX, Ghorai P, Ye QZ, Buschauer A, and Seifert R (2006a) Probing ligand-specific histamine H_1- and H_2-receptor conformations with N^G-acylated Imidazolylpropylguanidines. *J Pharmacol Exp Ther* **317:**139–146.

Xie SX, Kraus A, Ghorai P, Ye QZ, Elz S, Buschauer A, and Seifert R (2006b) N^1-(3-cyclohexylbutanoyl)-N^2-[3-(1*H*-imidazol-4-yl)propyl]guanidine (UR-AK57), a potent partial agonist for the human histamine H_1- and H_2-receptors. *J Pharmacol Exp Ther* **317:**1262–1268.

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