Mutations of Cys-17 and Ala-271 in the Human Histamine H₂ 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-(1H-imidazol-4-yl)propyl]guanidines and Nα-acylated derivatives are more potent and efficacious at fusion proteins of guinea pig (gpH₂R-GsS) than human (hH₂R-GsS) histamine H₂ receptor, coupled to the short splice variant of GsS, GαS. Whereas Ala-271 (hH₂R) and Asp-271 (gpH₂R) 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 gpH₂R suggested that an H-bond between Tyr-17 and Asp-271 stabilizes an active receptor conformation of the gpH₂R. In the present study, we generated a mutant hH₂R-GsS with Cys-17 → Tyr-17/Ala-271 → Asp-271 exchanges (hH₂R→gpH₂R) that exhibited an enhanced level of constitutive GTPase activity and adenylyl cyclase activity compared with wild-type hH₂R-GsS and gpH₂R-GsS. Potencies and efficacies of guanidines and Nα-acylguanidines were increased at this mutant receptor compared with hH₂R-GsS but they were still lower than at gpH₂R-GsS, suggesting that aside from Tyr-17 and Asp-271 additional amino acids contribute to the distinct pharmacological profiles of both species isoforms. Another hH₂R-GsS mutant with a Cys-17 → Tyr-17 exchange showed inefficient coupling to GαS 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 H₂R-GsS fusion proteins.

The histamine H₂ receptor (H₂R) is a biogenic amine receptor that belongs to the class A of the family of GPCRs. After stimulation by histamine (HA; Fig. 1, I), the H₂R couples to Gα proteins to activate adenylyl cyclase (AC). H₂Rs mediate regulation of gastric acid secretion in parietal cells, cardiac contractility, and myeloid cell differentiation (Del Valle and Gantz, 1997). N-[3-(1H-Imidazol-4-yl)propyl]guanidines are the most potent agonists at the H₂R 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...
H₂R of human neutrophils than at the H₂R of the guinea pig right atrium (Burde et al., 1989, 1990). In a membrane steady-state GTPase activity assay with fusion proteins of H₂R and the short splice variant of Gₛ/H₉₂₅₁, Gₛ/H₉₂₅₁S, these compounds are considerably more potent and efficacious at gpH₂R-Gₛ/H₉₂₅₁S than at hH₂R-Gₛ/H₉₂₅₁S (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 (pKₐ of ~8). This structural modification does not change the species selectivity between hH₂R-Gₛ/H₉₂₅₁S and gpH₂R-Gₛ/H₉₂₅₁S (Xie et al., 2006a). By contrast, HA and the small H₂R agonists dimaprit (DIM; Fig. 1, 2) and amthamine (AMT; Fig. 1, 3) do not exhibit species selectivity.

A three-dimensional homology model of the gpH₂R 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 hH₂R-Gₛ/H₉₂₅₁S (hH₂R-A₂₇₁D-Gₛ/H₉₂₅₁S) (Kelley et al., 2001). However, the efficacies of guanidines at this mutant and at hH₂R/gpH₂R chimeras were lower than at gpH₂R, 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 gpH₂R but not in hH₂R (containing Cys-17 and Ala-271) (Kelley et al., 2001).

To test this hypothesis, we generated an hH₂R-Gₛ/H₉₂₅₁S 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 hH₂R. SF9 cell membranes expressing mutant and wild-type H₂R-Gₛ/H₉₂₅₁S 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 Gₛ 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 H₂R-Gₛ/H₉₂₅₁S fusion proteins. Figure 1 shows the structures of H₂R agonists examined in the present study. Imprnidine (IMP; 5), arpromidine (ARP; 6), and BU-E-43 (7) are representatives of N-[3-(1H-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-methylthiazole-5-yl group and exhibits enhanced selectivity relative to the H₂R (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-hH2R-Gs

Construction of the cDNA for hH2R-C17Y-Gs

Materials. The generation of pGEM-3Z-SF-hH2R-Gs

Construction of the cDNA for hH2R-C17Y-A271D-Gs

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fusion proteins (20 μg of protein/tube), additionally 5 mM MgCl₂, 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 of the addition of 20 μl of reaction mixture containing (final) [α-32P]ATP (0.3 μCi/tube) plus 40 μM unlabeled ATP, 2.7 mM monocylohexylammonium 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, Sigma-Aldrich). [32P]cAMP was separated from [α-32P]ATP by elution of [32P]cAMP with 4 ml of 0.1 M ammonium acetate, pH 7.0. Recovery of [32P]cAMP was ~90%. Blank values were routinely ~0.01% of the total amount of [α-32P]ATP added. [32P]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-32P]ATP added was converted to [32P]cAMP.

**Miscellaneous.** Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA). [3H]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). Ki 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 hH₂R-C17Y-Gₛₛₛ and hH₂R-C17Y-A271D-Gₛₛₛ were well expressed (Fig. 2, A and B). Monomeric nonfused H₂R 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 Gₛₛₛ is ~45 kDa (Graziano et al., 1989). SDS-PAGE analysis of membranes expressing hH₂R-C17Y-A271D-Gₛₛₛ yielded intense bands at ~80 kDa, recognized by both the anti-FLAG and the anti-Hisₙ antibodies, that coincide with the expected apparent molecular masses of H₂R-Gₛₛₛ monomers (Kelley et al., 2001; Houston et al., 2002). Both bands showed somewhat diffuse, representing different glycosylation forms of the proteins. With the anti-Hisₙ 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 hH₂R-C17Y-Gₛₛₛ 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 H₂R-Gₛₛₛ monomers or degraded proteins. Because the anti-FLAG Ig recognizes the N terminus and the anti-Hisₙ Ig the C terminus of the H₂R, it can be concluded that for either case the complete amino acid sequence of hH₂R-C17Y was expressed. Additional diffuse bands at ~110 kDa may correspond to GPCR dimers or higher oligomers and were also observed in wild-type hH₂R-Gₛₛₛ fusion proteins (Kelley et al., 2001). Comparison with the peak intensities of calibrated Sf9 membranes expressing the β₂-adrenergic receptor (AR) at 7.5 pmol mg⁻¹ (as determined by [3H]dihydroalprenolol saturation binding) revealed approximately similar expression levels of ~2 pmol mg⁻¹ for hH₂R-C17Y-Gₛₛₛ and hH₂R-C17Y-A271D-Gₛₛₛ.

**Agonist and Inverse Agonist Effects on GTPase Activities in Sf9 Membranes Expressing hH₂R-Gₛₛₛ, gpH₂R-Gₛₛₛ, hH₂R-C17Y-Gₛₛₛ, and hH₂R-C17Y-A271D-Gₛₛₛ.**

The basal GTPase activity of hH₂R-Gₛₛₛ amounted to 0.66 ± 0.09 pmol mg⁻¹ min⁻¹ (n = 10). Compared with it, the data were similar in membranes expressing gpH₂R-Gₛₛₛ (0.69 ± 0.19 pmol mg⁻¹ min⁻¹; n = 8; p < 0.05) and hH₂R-C17Y-Gₛₛₛ (0.78 ± 0.10 pmol mg⁻¹ min⁻¹; n = 9; p > 0.05), respectively, but significantly increased at hH₂R-C17Y-A271D-Gₛₛₛ (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 hH₂R-C17Y-A271D-Gₛₛₛ, stimulation with 100 μM HA yielded GTPase activities 400 to 600% of the basal levels. By contrast, at hH₂R-C17Y-Gₛₛₛ, 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 hH₂R-C17Y-A271D-Gₛₛₛ were considered (Table 1). The efficacies of the small agonists DIM (2) and AMT (3) were slightly increased at gpH₂R-Gₛₛₛ and hH₂R-C17Y-A271D-Gₛₛₛ, relative to hH₂R-Gₛₛₛ. The potencies of HA (1) (Fig. 3), DIM (2), and AMT (3) were increased at hH₂R-C17Y-A271D-Gₛₛₛ compared with the wild-type receptors. The H₂R-selective agonist supraphastaprodifen (4) (Seifert et al., 2003) acted as a partial agonist with similar efficacies and potencies at hH₂R-Gₛₛₛ, gpH₂R-Gₛₛₛ, and hH₂R-C17Y-A271D-Gₛₛₛ. In agreement with previous studies (Kelley et al., 2001; Xie et al., 2006a,b), N-[3-(1H-imidazol-4-yl)propyllguanidines 5 to 7 and most of their N₄-acylated derivatives 8 to 13 were more efficacious and more potent at gpH₂R-Gₛₛₛ than at hH₂R-Gₛₛₛ. Except for IMP (5) being more efficacious at hH₂R-C17Y-A271D-Gₛₛₛ, the efficacies of 5 to 7 were not significantly changed at the mutant receptor compared with...
was determined as described under Methods. Reaction mixtures contained membranes (10 μg of protein/tube) expressing fusion proteins and HA at concentrations indicated on the abscissa. Data shown were 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.

At wild-type hH2R-GsS and hH2R-C17Y-A271D-GsS, HA did not further enhance AC activities above the basal level (Fig. 4), indicating constitutive activity of these constructs in Table 3 of Preuss et al. (2007). Accordingly, at all four H2Rs, the inverse agonist IAPT (18) reduced this GTP-dependent AC activity. At hH2R-GsS and gpH2R-GsS, 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 hH2R-C17Y-A271D-GsS, HA did not further enhance the GTP effect. Both higher basal AC activity and a strong stimulation by GTP caused exhaustion of the limiting GTPase activities below basal and thus acted as inverse agonists (Table 2). At hH2R-C17Y-A271D-GsS, 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 hH2R-C17Y-A271D-GsS 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 H2R with GsS 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 hH2R-C17Y-A271D-GsS than at the wild-type receptors, whereas no significant differences in the Kp values were observed for 14, 15, and 17.

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>hH2R-GsS</th>
<th>gpH2R-GsS</th>
<th>hH2R-C17Y-A271D-GsS</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Efficacy</td>
<td>EC50 nM</td>
<td>Efficacy</td>
</tr>
<tr>
<td>1</td>
<td>HA</td>
<td>1.00</td>
<td>990 ± 92</td>
</tr>
<tr>
<td>2</td>
<td>DIM</td>
<td>0.85 ± 0.02</td>
<td>910 ± 430</td>
</tr>
<tr>
<td>3</td>
<td>AMT</td>
<td>0.91 ± 0.02</td>
<td>190 ± 50</td>
</tr>
<tr>
<td>4</td>
<td>Supra</td>
<td>0.84 ± 0.02</td>
<td>200 ± 44</td>
</tr>
<tr>
<td>5</td>
<td>IMP</td>
<td>0.82 ± 0.02</td>
<td>160 ± 40</td>
</tr>
<tr>
<td>6</td>
<td>ARF</td>
<td>0.84 ± 0.02</td>
<td>72 ± 53</td>
</tr>
<tr>
<td>7</td>
<td>BU-E-43</td>
<td>0.71 ± 0.11</td>
<td>130 ± 13</td>
</tr>
<tr>
<td>8</td>
<td>UR-PG214</td>
<td>0.91 ± 0.08</td>
<td>130 ± 45</td>
</tr>
<tr>
<td>9</td>
<td>UR-PG215</td>
<td>0.80 ± 0.04</td>
<td>120 ± 45</td>
</tr>
<tr>
<td>10</td>
<td>UR-PG222A</td>
<td>0.90 ± 0.04</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>11</td>
<td>UR-PG136</td>
<td>0.82 ± 0.05</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>12</td>
<td>UR-AK7</td>
<td>0.86 ± 0.05</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>13</td>
<td>UR-PG286A</td>
<td>0.53 ± 0.06</td>
<td>49 ± 9</td>
</tr>
</tbody>
</table>

### Notes

- a Comparison with the efficacy at hH2R-GsS: *p < 0.05, **p < 0.01, ***p < 0.001.
- b Comparison with the EC50 value at hH2R-GsS: *p < 0.05, **p < 0.01, ***p < 0.001.
TABLE 2

Potencies and inverse agonist efficacies of antagonists at hH₂R-Gₛₛ, gpH₂R-Gₛₛ, and hH₂R-C17Y-A271D-Gₛₛ in the GTPase assay

Steady-state GTPase activity in Sf9 activity expressing hH₂R-Gₛₛ, gpH₂R-Gₛₛ, and hH₂R-C17Y-A271D-Gₛₛ 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 μM 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. Bₐ values typical for Sf9 membranes expressing nonfused H₂R species isoforms, and they were similar to the values typical for Sf9 membranes expressing nonfused H₂R species (Houston et al., 2002). These controversial results of hH₂R-C17Y-Gₛₛ relative to hH₂R-Gₛₛ and gpH₂R-Gₛₛ imply the following conclusions: The proteins expressed in Sf9 cells made up the amino acid sequence for hH₂R-C17Y, and they were functional in the test systems used. However, coupling of hH₂R-C17Y to Gₛₛ was much less efficient than is characteristic forGPCR-Gₛₛ fusion proteins (Seifert et al., 1999; Gille and Seifert, 2003). As a rationale, Gₛₛ could be incorrectly expressed or degraded in Sf9 cells. Instead, hH₂R-C17Y possibly coupled to only a fraction of recombinant Gₛₛ or to endogenous Gₛₛ-like G proteins with much lower efficiency.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Basal, AC Activity</th>
<th>10 μM GTP, AC Activity</th>
<th>10 μM GTP + 100 μM HA, AC Activity</th>
<th>Rel. GTP Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>hH₂R-Gₛₛ</td>
<td>12.1 ± 2.1</td>
<td>24.5 ± 4.8</td>
<td>28.7 ± 5.3</td>
<td>73</td>
</tr>
<tr>
<td>gpH₂R-Gₛₛ</td>
<td>13.4 ± 3.1</td>
<td>25.0 ± 0.6</td>
<td>28.5 ± 2.2</td>
<td>77</td>
</tr>
<tr>
<td>hH₂R-C17Y-Gₛₛ</td>
<td>0.8 ± 0.2</td>
<td>2.4 ± 0.4</td>
<td>7.0 ± 0.8</td>
<td>26</td>
</tr>
<tr>
<td>hH₂R-C17Y-A271D-Gₛₛ</td>
<td>42.5 ± 2.2</td>
<td>66.9 ± 4.2</td>
<td>66.4 ± 3.3</td>
<td>100</td>
</tr>
</tbody>
</table>

TABLE 3

AC activities in Sf9 membranes expressing hH₂R-Gₛₛ, gpH₂R-Gₛₛ, hH₂R-C17Y-Gₛₛ, and hH₂R-C17Y-A271D-Gₛₛ

Basal AC activities and the effects of GTP and HA on AC activities in membranes expressing hH₂R-C17Y-Gₛₛ and hH₂R-C17Y-A271D-Gₛₛ were assessed and compared with the corresponding values at hH₂R-Gₛₛ and gpH₂R-Gₛₛ. 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 hH₂R-Gₛₛ and gpH₂R-Gₛₛ are identical with the control data for these constructs in Table 3 of Preuss et al. (2007).

**Discussion**

**Impaired Coupling in Membranes Expressing hH₂R-C17Y-Gₛₛ**

In Sf9 cells expressing hH₂R-C17Y-Gₛₛ, the anti-FLAG and the anti-His₉ antibodies recognized similarly migrating proteins in SDS-PAGE that did not coincide with the expected bands for monomeric H₂R-Gₛₛ fusion proteins. Measurement of GTP hydrolysis at hH₂R-C17Y-Gₛₛ yielded HA responses, but the increases in GTPase activity upon agonist stimulation were much lower than in the wild-type H₂R-Gₛₛ 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 H₂R-Gₛₛ species isoforms, and they were similar to the values typical for Sf9 membranes expressing nonfused H₂R species (Houston et al., 2002). These controversial results of hH₂R-C17Y-Gₛₛ relative to hH₂R-Gₛₛ and gpH₂R-Gₛₛ imply the following conclusions: The proteins expressed in Sf9 cells made up the amino acid sequence for hH₂R-C17Y, and they were functional in the test systems used. However, coupling of hH₂R-C17Y to Gₛₛ was much less efficient than is characteristic for GPCR-Gₛₛ fusion proteins (Seifert et al., 1999; Gille and Seifert, 2003). As a rationale, Gₛₛ could be incorrectly expressed or degraded in Sf9 cells. Instead, hH₂R-C17Y possibly coupled to only a fraction of recombinant Gₛₛ or to endogenous Gₛₛ-like G proteins with much lower efficiency.

**Increased Constitutive Activity in Membranes Expressing hH₂R-C17Y-A271D-Gₛₛ**

In membranes expressing hH₂R-C17Y-A271D-Gₛₛ, high-efficiency coupling was observed as GTPase activities were increased upon agonist stimulation similar to hH₂R-Gₛₛ and gpH₂R-Gₛₛ. 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 hH₂R-C17Y-A271D-Gₛₛ the high basal AC activ-
of both residues. conformation and thereby compensating for the activating more potent and more efficacious at hH2R-C17Y-A271D-Gs GTPase activity experiments, some of these agonists were at the gpH2R. This investigation adds to a previous study at amino acid at this position for the activation mechanism of glutamate were more constitutively active than wild-type G-acylguanidines. Different magnitudes of the variable side chains of the compounds distinctly interact with wild-type and mutant H2R-Gs, which is represented by compounds 8 and 9 containing a 2-thiazolyl group and being more potent at hH2R-C17Y-A271D-Gs than at hH2R-Gs in contrast to compound 10 with a cyclohexyl group being similarly potent at both proteins. The 5-methyl-1H-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 hH2R-C17(Y-A271D-Gs versus hH2R-Gs.

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 H2R agonists at hH2R-C17Y-A271D-Gs but also potency increases of the guanidines and N4-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 hH2R-C17Y-A271D-Gs than at hH2R-Gs. 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 H2R species isoforms.

Conclusions

In the present study, we demonstrate that an hH2R-Gs fusion protein with mutations of Cys-17→Tyr-17 in TM1 and Ala-271→Asp-271 in TM7 displayed enhanced constitutive activity compared with hH2R-Gs and gpH2R-Gs. We additionally showed that an interaction between Tyr-17 and Asp-271 in gpH2R contributes to the species-selective action.
of \(N-[3-(1H-imidazol-4-yl)propyl]guanidines\) and their \(N^2\)-acylated derivatives. Distinct potencies and efficacies of agonists and inverse agonists further support the concept of ligand-specific conformations in wild-type and mutant \(H_2\)-G\(_{\alpha}\) 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 h\(H_2\)R (h\(H_2\)R→gpH\(H_2\)R) 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 h\(H_2\)R and h\(H_2\)R 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 \(H_2\)R functions and will help us to find potent and selective agonists for the h\(H_2\)R 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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