No Evidence for Functional Selectivity of Proxyfan at the Human Histamine H3 Receptor Coupled to Defined G\textsubscript{i}/G\textsubscript{o} Protein Heterotrimers

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ABSTRACT

Numerous structurally diverse ligands were developed to target the human histamine H3 receptor (hH3R), a presynaptic G\textsubscript{i}/G\textsubscript{o}-coupled auto- and heteroreceptor. Proxyfan was identified to be functionally selective, with different efficacies toward G\textsubscript{i}/G\textsubscript{o}-dependent hH3R signaling pathways. However, the underlying molecular mechanism of functional selectivity of proxyfan is still unclear. In the current study, we investigated the role of different \( \alpha_2 \)G\textsubscript{o}/G\textsubscript{i} proteins in hH3R signaling, using a baculovirus/Sf9 cell expression system. We tested the hypothesis that ligand-specific coupling differences to defined G\textsubscript{i}/G\textsubscript{o}-heterotrimers are responsible for functional selectivity of proxyfan at hH3R. In Sf9 membranes, full-length hH3R (445 amino acids) was expressed in combination with an excess of different mammalian G proteins (\( \alpha_2 \), \( \alpha_3 \), \( \alpha_5 \), \( \alpha_6 \), and \( \beta_1 \gamma_2 \) dimers, respectively). In addition, we constructed the fusion proteins hH3R-G\textsubscript{\alpha_2} and hH3R-G\textsubscript{\alpha_6} to ensure clearly defined receptor/G protein stoichiometries. Steady-state GTPase experiments were performed to directly measure the impact of each G protein on hH3R signal transduction. The hH3R coupled similarly to all G proteins. We also observed similar ligand-independent or constitutive activity. Proxyfan and various other imidazole-containing ligands, including full agonists, partial agonists, and inverse agonists, showed very similar pharmacological profiles not influenced by the type of G protein coexpressed. Selected ligands, examined in membranes expressing the fusion proteins hH3R-G\textsubscript{\alpha_2} and hH3R-G\textsubscript{\alpha_6} (plus \( \beta_1 \gamma_2 \) dimers), yielded very similar results. Collectively, our data indicate that hH3R couples similarly to different \( \alpha_2 \)-subunits and that ligand-specific active receptor conformations, resulting in G protein-coupling preferences, do not exist for proxyfan or other imidazole compounds investigated.

The histamine H3 receptor (H3R) is currently one of the most targeted biogenic amine receptors because it participates in important physiological processes such as the sleep-wake cycle, eating behavior, and cognition. The H3R is a promising drug target for many diseases, including obesity; sleep disorders such as narcolepsy; and cognitive problems associated with Alzheimer's disease, attention deficit-hyperactivity disorder, and schizophrenia. This work was supported by the Research Training Program (Graduiertenkolleg) [Grant GRK780] “Medicinal Chemistry: Molecular Recognition—Ligand-Receptor Interactions” of the German Research Foundation (to D.S.); the “Research Internships in Science and Engineering program of the German Academic Exchange service (Deutsche Akademische Austauschdienst)” (to K.B. and J.T.); and COST Action [Grant BM0806] (to R.S. and D.S.).

ABBREVIATIONS: H3R, histamine H3 receptor; GPCR, G protein-coupled receptor; h, human; \( \beta_2 \)AR, \( \beta_2 \)-adrenoceptor; H3R, histamine H3 receptor; aa, amino acids; PCR, polymerase chain reaction; JNN-U773707, (4-fluorophenyl)(1-methyl-2-an-1H-imidazol-5-yl)methanone; GTP-S, guanosine 5’-O-(3-thio)triphosphate; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; r, rat.
Many GPCRs are able to signal through various intracellular pathways. Depending on the specific G proteins to which the GPCR is coupled functional ligand selectivity has been frequently observed (Kenakin, 2001, 2007). In this case, the ligand preferentially activates specific signaling pathways mediated by a single GPCR in a manner that challenges the above-introduced two-state model. Based on such findings, a multiple-state model, implying the existence of ligand-specific conformational states, has been developed (Kenakin, 2001, 2007; Koblika and Deupi, 2007). It is interesting to note that even stereoisomers of one and the same compound can show different functional selectivities, providing an additional opportunity to control receptor-mediated effects (Seifert and Dove, 2009).

Protein agonism is considered a special case of functional selectivity. A protein agonist presumably stabilizes a receptor conformation with a lower efficacy toward the G protein than the agonist-free constitutively active or agonist-stabilized GPCR state. The ligand then acts as an inverse agonist. In a quiscent system with low constitutive activity, the protein ligand can act as an agonist (Gbahou et al., 2003; Kenakin, 2007).

Numerous structurally diverse H3R ligands have been synthesized as potential drug candidates or as pharmacological tools. Almost all H3R agonists are imidazole-containing small molecules, derived from the endogenous agonist histamine (Leurs et al., 2005). H3R antagonists/inverse agonists can be differentiated into imidazole-containing antagonists and nonimidazole antagonists (Cowart et al., 2004; Leurs et al., 2005). Proxyfan is a prototypical, imidazole-containing H3R ligand that was initially characterized as an antagonist (Hüls et al., 1996) (Fig. 1). Subsequent studies revealed a more complex pharmacological profile and proxyfan was re-classified as a protein agonist (Gbahou et al., 2003). However, the systems examined were all very different, rendering data interpretation difficult. In brief, proxyfan was examined in different species and measuring various parameters. In addition, the parameters were often quite distal and the G protein constructs used to transfect recombinant cell lines do not represent the physiological coupling partners or were chimeric to redirect the signaling cascade (Krueger et al., 2005). Collectively, due to the large differences between the systems examined it is very difficult to precisely define the molecular mechanism for the pleiotropic effects of proxyfan.

By studying nine imidazole-containing H3R ligands (Fig. 1), we wanted to obtain more direct evidence for the existence of different ligand-specific H3R conformations. Most importantly, we aimed at probing the hypothesis that the type of Gi2/Go protein α-subunit to which H3R couples is responsible for the differential effects of proxyfan. Therefore, we established a baculovirus/Sf9 cell expression system for the full-length hH3R (445 aa), in which the receptor can be expressed either alone or coexpressed with different Gα protein α-subunits (Gα1, Gα12, Gα13, or Gα17, and β1/γ2 dimers, respectively). Sf9 cells have already been successfully used for reconstitution of several Gi/Go-coupled GPCRs (Wenzel-Seifert et al., 1998; Kleemann et al., 2008). The hH3R-expressing membranes were then studied under identical experimental conditions, focusing on steady-state GTPase activity, a proximal parameter of GPCR/G protein coupling. Moreover, we examined the fusion proteins hH3R-Gα1 and hH3R-Gα12-GPCR-Gα1 fusion proteins ensure proximity and defined 1:1 stoichiometry of the signaling partners (Seifert et al., 1999b), ruling out the possibility that differences in receptor-to-G protein ratio account for potential differences in pharmacological properties of ligands at hH3R.

**Materials and Methods**

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**Materials.** The cDNA for the hH3R was kindly provided by Dr. T. Lovenberg (Johnson and Johnson Pharmaceutical R&D, San Diego, CA). All restriction enzymes and T4 DNA ligase were from New England Biolabs (Frankfurt, Germany). Moloney murine leukemia virus reverse transcriptase was from Invitrogen (Carlsbad, CA). Cloned Pfu polymerase was obtained from Stratagene (La Jolla, CA). The DNA primers for PCR were synthesized by MWG Biotech (Ebersberg, Germany). Baculoviruses for Gα17, Gα12, and Gα13 were donated by Dr. A. G. Gilman (Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX). Baculovirus for rat Gα11 was donated by Dr. J. C. Garrison (University of Virginia, Charlottesville, VA). Recombinant baculovirus encoding the unmodified length hH3R (445 aa), in which the receptor can be expressed either alone or coexpressed with different Gα protein α-subunits (Gα1, Gα12, Gα13, or Gα17, respectively). Sf9 cells have already been successfully used for reconstitution of several Gi/Go-coupled GPCRs (Wenzel-Seifert et al., 1998; Kleemann et al., 2008). The hH3R-expressing membranes were then studied under identical experimental conditions, focusing on steady-state GTPase activity, a proximal parameter of GPCR/G protein coupling. Moreover, we examined the fusion proteins hH3R-Gα1 and hH3R-Gα12-GPCR-Gα1 fusion proteins ensure proximity and defined 1:1 stoichiometry of the signaling partners (Seifert et al., 1999b), ruling out the possibility that differences in receptor-to-G protein ratio account for potential differences in pharmacological properties of ligands at hH3R.

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![Fig. 1. Structures of imidazole-containing H3R ligands: full agonists 1 to 3, partial agonists 4 to 6, and antagonists/inverse agonists, 7 to 9.](image-url)
the cDNA for G was generated. In PCR 3B, a fragment encoding the hexahistidine tag, the baculovirus transfer vector pHI1392-SF-hH4R-His6 (Schneider construct was fully sequenced. Finally, the construct was cloned into fusion proteins between the hH3R and G proteins (Seifert et al., 1998). The sense primer HUMAN HRH3-F (5'-GAC GAT GAC GCC ATG GAG CCG CCG CC-3') consisted of 15 bp of the 3'-end of SF and the first 17 bp of the 5'-end of the hH3R. The antisense primer HUMAN HRH3-RV (5'-GA TCC TCT AGA TTA GTG ATG ATG ATG ATG GTG ATG TCT CCA GCA GTG TGT-3') consisted of 15 bp of the C terminus of the hH3R, the hexahistidine tag, the stop codon, and an XbaI site. As template, a plasmid (pCIneo) containing the sequence of hH3R was used. In PCR 2, the products of PCR 1A and PCR 1B were used. In that way, a fragment encoding SF, the hH3R sequence, the hexahistidine tag, the stop codon, and an XbaI site were obtained. The fragment was digested with SacI and XbaI and cloned into pGEM-3Z-SF-hH3R-His6, digested with the same restriction enzymes, to yield pGEM-3Z-SF-hH3R-His6. After transformation of chemically competent bacteria (JM 109), amplification of the plasmids and analytical restriction digestion, the subcloned hH3R construct was fully sequenced. Finally, the construct was cloned into the baculovirus transfer vector pVL1392-SF-hH4R-His6 via SacI and XbaI restriction sites. Again, competent bacteria (Top 10) were transformed, the plasmids were amplified, and the accuracy of the resulting MaxiPrep-DNA was checked by extensive restriction digestion analysis and sequencing.

**Generation of Recombinant Baculoviruses, Cell Culture, and Membrane Preparation.** Baculoviruses encoding recombinant proteins were generated in SF9 cells using the BaculoGOLD transfection kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions. SF9 cells cultured in 250- or 500-ml disposable Erlenmeyer flasks at 28°C under rotation at 150 rpm in SF9 900 II medium (Invitrogen) supplemented with 5% (v/v) fetal calf serum (Biochrom, Berlin, Germany) and 0.1 mg/ml gentamycin (Lonza Walkersville, Inc., Walkersville, MD). Cells were maintained at a density of 0.5 to 6.0 × 10⁶ cells/ml. After initial transfection, high-titer virus stocks were generated by two sequential virus amplifications. In the first amplification, cells were seeded at 2.0 × 10⁶ cells/ml and infected with a 1:100 dilution of the supernatant from the initial transfection. Cells were cultured for 7 days, resulting in the death of virtually the entire cell population. The supernatant fluid of this infection was harvested and stored under light protection at 4°C. In a second amplification, cells were seeded at 3.0 × 10⁶ cells/ml and infected with a 1:20 dilution of the supernatant fluid from the first amplification. Cells were cultured for 48 h, and the supernatant fluid was harvested. After the 48-h culture period, the majority of cells showed signs of infections (e.g., altered morphology, viral inclusion bodies), but most of the cells were still intact. The supernatant fluid from the second amplification was stored under light protection at 4°C and used as routine virus stock for membrane preparations. To ensure the purity and identity of the viruses, the total RNA of infected SF9 cells was isolated (RNeasy kit; QIAGEN GmbH, Hilden, Germany), the cDNA was derived via reverse transcription, and fragments representative for the constructs were PCR-amplified and analyzed by restriction digestion. For infection, cells were sedimented by centrifugation and suspended in fresh medium. Cells were seeded at 3.0 × 10⁶ cells/ml and infected with a 1:100 dilution of high-titer baculovirus stocks encoding hH3R constructs, Gαi2 proteins and Gβi2γ2 dimers. Cells were cultured for 48 h before membrane preparation. SF9 membranes were prepared as described previously, 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 MgCl₂, 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 diluted in Laemmli buffer and separated on SDS polyacrylamide gels containing 12% (w/v) acrylamide. The purified G protein standards were handled in dilution buffer (25 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 100 mM NaCl, 0.1% Lubrol PX (v/v), 25 mM MgCl₂, and 1 mM EDTA). Proteins were transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) and then reacted with anti-hH3R (1:1000), M1 antibody (1:1000), anti-Gα (1:1000), anti-Gαi2 (1:1000), anti-Gβi1γ2 (1:1000), anti-Gαi3 (1:1000), anti-Gβ1γ2 (1:1000), and anti-Gβi3 (1:1200) IgGs. Immunoreactive bands were visualized by enhanced chemoluminescence (Pierce Chemical, Rockford, IL), using anti-mouse and anti-rabbit IgGs coupled to peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Electrophoremluminoscintially stained blots were exposed to X-ray films (GE Healthcare). The expression level of proteins were roughly estimated by using appropriate dilutions of a reference membrane expressing defined levels of hβ3AR protein or purified G proteins. hβ3AR expression levels were determined by radioligand binding with [3H]hydroalprenolol. Immunoblots were scanned with a GS-710-calibrated imaging densitometer (Bio-Rad Laboratories). The intensity
of the bands was analyzed with the Quantity One 4.0.3 software (Bio-Rad Laboratories).

[3H]HJN3-7753707 Binding Assay. Before experiments, membranes were sedimented by a 10-min centrifugation at 4°C and 15,000 g and resuspended in binding buffer (12.5 mM MgCl₂, 1 mM EDTA, and 75 mM Tris-HCl, pH 7.4), to remove residual endogenous guanine nucleotides as much as possible. Each tube (total volume, 250 or 500 μl) contained 10 to 50 μg of protein. Nonspecific binding was determined in the presence of [3H]HJN3-7753707 at various concentrations plus 10 μM theophylline and amounted to ~20 to 30% of total binding at saturating concentrations (10 nM). Incubations were conducted for 60 min at room temperature and shaking at 250 rpm. Saturation binding experiments were carried out using 0.3 to 10 nM [3H]HJN3-7753707. Bound [3H]HJN3-7753707 was separated from free [3H]HJN3-7753707 by filtration through 0.3% (m/v) polyethylenimine-pretreated GF/C filters (Whatman, Maidstone, UK), followed by three washes with 2 ml of binding buffer (4°C). Filter-bound radioactivity was determined by liquid scintillation counting. The experimental conditions chosen ensured that not more than 10% of the total amount of radioactivity added to binding tubes was bound to filters.

[35S]GTPγS Binding Assay. Membranes were thawed, sedimented by a 10-min centrifugation at 4°C and 15,000 g to remove residual endogenous guanine nucleotides as far as possible. Membranes were resuspended in binding buffer, supplemented with 0.05% (m/v) bovine serum albumin. Each tube (total volume of 250 or 500 μl) contained 10 to 20 μg of protein. In saturation binding experiments, tubes contained 0.2 to 2 nM [35S]GTPγS plus unlabeled GTPγS to give the desired final ligand concentrations (0.2–50 nM). Neither GDP nor H₃R ligands were included in assays. Nonspecific binding was determined in the presence of 100 μM unlabeled GTPγS and amounted to less than 1% of total binding. Incubations were conducted for 90 min at 25°C and shaking at 250 rpm. Bound [35S]GTPγS was separated from free [35S]GTPγS by filtration through GF/C filters, followed by three washes with 2 ml of binding buffer (4°C). Filter-bound radioactivity was determined by liquid scintillation counting. The experimental conditions chosen ensured that not more than 10% of the total amount of radioactivity added to binding tubes was bound to filters.

Steady-State GTPase Activity Assay. Membranes were thawed, sedimented, and resuspended in 10 mM Tris-HCl, pH 7.4. Assay tubes contained 90 μg of protein/tube), 5.0 mM MgCl₂, 0.1 mM EDTA, 0.1 mM ATP, 100 μM GTP, 0.1 mM adenylyl imidodiphosphate, 1.2 mM creatine phosphate, 1 μg of creatine kinase, and 0.2% (w/v) bovine serum albumin in 50 mM Tris-HCl, pH 7.4, and H₃R ligands at various concentrations. Reaction mixtures (80 μl) were incubated for 2 min at 25°C before the addition of 20 μl of [γ²-³²P]GTP (0.1 μCi/tube). All stock and work dilutions of [γ²-³²P]GTP were prepared in 20 mM Tris-HCl, pH 7.4. Reactions were conducted for 20 min at 25°C. Reactions were terminated by the addition of 900 μl of slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH₂PO₄, pH 2.0. Charcoal absorbs nucleotides but not Pᵢ. Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at 15,000g. Six hundred microliters of the supernatant fluid of reaction mixtures were removed, and ³²P was determined by liquid scintillation counting. Enzyme activities were corrected for spontaneous degradation of [γ²-³²P]GTP. Spontaneous [γ²-³²P]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 [γ²-³²P]GTP, prevents [γ²-³²P]GTP hydrolysis by enzymatic activities present in SF9 membranes. Spontaneous [γ²-³²P]GTP degradation was <1% of the total amount of radioactivity added using 20 mM Tris-HCl, pH 7.4, as solvent for [γ²-³²P]GTP. The experimental conditions chosen ensured that not more than 10% of the total amount of [γ²-³²P]GTP added was converted to ³²Pᵢ.

Miscellaneous. Molecular biology was planned with GenScript 2.5 (Textco BioSoftware, West Lebanon, NH). Ligand structures were illustrated using ChemDraw Ultra 8.0 (CambridgeSoft Corporation, Cambridge, MA). Protein was determined using the DC protein assay kit (Bio-Rad Laboratories). [3H]Dihydroalprenolol saturation binding was performed as described previously (Seifert et al., 1998). All analyses of experimental data were performed with the Prism 5 (GraphPad Software Inc., San Diego, CA). When expression levels of recombinant proteins were determined by Western blot, the GS-710 calibrated imaging densitometer and Quantity One version 4.0.3 (Bio-Rad Laboratories) were used.

Results

Immunological Detection of Recombinant Proteins Expressed in SF9 Cell Membranes. Membranes from the same batch of SF9 cells infected with recombinant N- and C-terminally tagged hH₃R-baculoviruses alone or in combination with baculoviruses encoding different mammalian G proteins (G_q₁₅, G_q₁₂, G_o₃ or G_o₁₁ and/or β₁y₂ dimers, respectively) were prepared and subject to immunological analysis. The predicted molecular mass of the hH₃R is ~49 kDa. We used anti-hH₃R Ig, recognizing an 18-aa peptide within the extracellular N terminus of the hH₃R to confirm expression (Fig. 2A). Indeed, hH₃R migrated as the expected band for a monomeric GPCR. The results were confirmed by the use of anti-FLAG Ig (Fig. 2B), recognizing the N-terminal FLAG-epitope and anti-His Ig (Fig. 2C), recognizing the C-terminal hexahistidine tag. The bands were doublets, probably representing different glycosylated forms of hH₃R. hH₃R possesses one putative N-glycosylation site (Asn11), located in the N terminus. The receptor expression levels were similar in all membrane batches and estimated to be ~1 to 2 pmol/mg, using anti-FLAG Ig and h₃AR as standards (Fig. 2B). The expression level of the h₃AR was 7.5 pmol/mg, as determined by [H]dihydroalprenolol saturation binding. Thus, hH₃R was properly expressed in SF9 cells and not proteolytically degraded after membrane preparation.

To visualize the coexpressed Gs-subunits, we used an antibody recognizing all Gₛ isoforms (Fig. 2D). Gₛₐ₁-subunits appeared at the expected molecular mass (~40–41 kDa) as very intense bands, although the expression level of Gₛ₁ was lower compared with the other G protein subunits. This is an intrinsic property of mammalian Gₛ₁, heterologously expressed in SF9 cells, as already shown in a previous study (Kleemann et al., 2008). Unfortunately, this problem could not be overcome by an optimization of the expression process. However, it was also shown that a low expression level of Gₛ₁ could not be overcome by an optimization of the expression process. Unfortunately, this problem could not be overcome by an optimization of the expression process. However, it was also shown that a low expression level of Gₛ₁ could not be overcome by an optimization of the expression process.
Due to the fact that the determination of expression levels by immunoblotting does not discriminate between functional and nonfunctional proteins, we also quantified the hH3R and Gα proteins by a combination of antagonist [3H]JNJ-7753707- and [35S]GTPγS saturation binding and calculated the functional GPCR/Gα protein ratios (Table 1). The membranes were from the same batch as those studied by immunoblot to ensure maximal comparability and data accuracy.

In [35S]GTPγS saturation binding experiments neither GDP nor H3R ligands were present. The maximal number of Gαi/o-related GTPγS binding sites in membranes expressing hH3R plus Gα-subunits plus βγ2 was corrected by the binding determined in parallel in membranes expressing hH3R plus Gαi1 + βγ2 was analyzed to quantify the Gα-subunits, using 2, 4, 7.5, 15, and 30 pmol of purified Gαi2 as standard. G, 0.5, 1.0, 1.5, and 2.0 μg of a corresponding membrane of the same batch expressing hH3R + Gαi1 + βγ2 was analyzed to quantify the Gα-subunits, using 2, 4, 7.5, 15, and 30 pmol of purified Gαi2 an almost identical splice variant of Gαi1 as standard.

[3H]JNJ-7753707 and [35S]GTPγS Binding: Quantitative Analysis of Receptor-to-G Protein Stoichiometry. Due to the fact that the determination of expression levels by immunoblotting does not discriminate between functional and nonfunctional proteins, we also quantified the hH3R and Gα proteins by a combination of antagonist [3H]JNJ-7753707- and [35S]GTPγS saturation binding and calculated the functional GPCR/Gα protein ratios (Table 1). The membranes were from the same batch as those studied by immunoblot to ensure maximal comparability and data accuracy.

In [35S]GTPγS saturation binding experiments neither GDP nor H3R ligands were present. The maximal number of Gαi/o-related GTPγS binding sites in membranes expressing hH3R plus Gα-subunits plus βγ2 was corrected by the binding determined in parallel in membranes expressing hH3R plus βγ2 alone. To ensure the same viral load in the reference membrane, Sf9 cells were infected with baculoviruses encoding hH3R, βγ2 and virus encoding no recombinant protein at all. In this manner, only the number of functionally intact and heterologously expressed mammalian Gαi/o-subunits was quantified.
The experiments revealed that the number of $[^3H]$JNJ-7753707 binding sites was very similar to the hH$_3$R protein expression levels determined via immunoblot. Thus, most hH$_3$R molecules were correctly folded in Sf9 cell membranes. However, the number of specific $[^35S]$GTP$_S$ binding sites for mammalian Go proteins was much smaller compared with the Go protein expression levels determined via immunoblot. Nevertheless, there were still more functionally intact mammalian Go proteins than receptors in the membranes and the functional receptor-to-G protein ratios ranged between 1:2 and 1:11. Similar ratios were also found by other investigators, using the same methodology (Gazi et al., 2003). These data also fit to the linear and noncatalytic signal transfer observed for several GPCR/G-protein pairs in Sf9 cell membranes (Wenzel-Seifert et al., 1998, 1999; Wenzel-Seifert and Seifert, 2000).

**Steady-State GTPase Assay: hH$_3$R Coupling to Different Go-Subunits.** To investigate the G protein-coupling profile of the hH$_3$R, we measured the receptor-dependent $[^35S]$GTP hydrolysis of different Go-subunits (Fig. 3). The experiments were performed under steady-state conditions. Thus, multiple G protein activation/deactivation cycles were assessed, eliminating the inherent bias of kinetic $[^35S]$GTP$_S$ binding studies. GTP hydrolysis was determined in parallel under basal conditions, maximal stimulation with the physiological (and full) agonist histamine (10 $\mu$M) and a saturating concentration of the inverse agonist thioperamide (10 $\mu$M) in Sf9 cell membranes expressing the hH$_3$R alone or coexpressing the hH$_3$R and different G proteins.

In membranes expressing the hH$_3$R alone, basal GTPase activity was low, and the stimulatory and inhibitory effects of histamine and thioperamide, respectively, were small (Table 2). This reflects only very weak coupling of the hH$_3$R to insect cell G proteins. The structurally related hH$_3$R also couples only weakly to insect cell G proteins (Schneider et al., 2009). hH$_3$R coupled efficiently to all coexpressed mammalian Go$_{i/o}$-subunits (Go$_{i1}$, Go$_{i2}$, Go$_{o1}$, Go$_{o3}$, and Go$_{o4}$, respectively) as was evident by the higher basal GTPase activity and the larger absolute stimulatory and inhibitory effects of histamine and thioperamide, respectively. The relative stimulatory effects of histamine and the relative inhibitory effects of thioperamide based on total ligand-regulated GTPase activity were similar for each of the five systems studied, indicating that the constitutive activity of hH$_3$R was comparable and not substantially influenced by the type of G protein (Seifert and Wenzel-Seifert, 2002). In contrast to hH$_3$R, the short and long splice variants of Go$_{o4}$ had a large impact on the constitutive activity of the hB$_2$AR (Seifert et al., 1998; Seifert, 2001). The constitutive activity of hH$_3$R coupled to cognate Gi/Go proteins was rather high and comparable with the constitutive activity of hH$_3$R (Schneider et al., 2009) and the human formyl peptide receptor (Wenzel-Seifert et al., 1998, 1999). However, some Gi/Go-coupled GPCRs expressed in Sf9 cells exhibit only low or no constitutive activity, indicating that the expression system per se does not give rise to high constitutive activity (Seifert and Wenzel-Seifert, 2002; Kleemann et al., 2008).

**Ligand Potencies and Efficacies in the Steady-State GTPase Assay at hH$_3$R Coexpressed with Different Go-Subunits.** Next, we examined a variety of imidazole-based ligands, including the functionally selective proxyfan, in Sf9 cell membranes expressing the hH$_3$R and different Go$_{i/o}$ proteins in the steady-state GTPase assay (Fig. 1; Table 3).

The endogenous agonist histamine (1) and the standard H$_3$R ligand N$^\alpha$-methylhistamine (2) and (R)-$\alpha$-methylhistamine (3) were full agonists and equally potent in all membranes. The highly potent standard H$_3$R agonist imetit (4)
TABLE 3

GTPase activities in Sf9 membranes expressing hH3R and different G<sub>i/o</sub> proteins

Steady-state GTPase experiments were performed as described under Materials and Methods. Reaction mixtures contained 0.1 µCi [γ-<sup>32</sup>P]GTP and 100 nM unlabeled GTP in the presence of solvent (basal), 10 µM histamine (+ ago.), or 10 µM thioperamide (+ inv. ago.). Data shown are the means ± S.E.M. of three to four independent experiments for each membrane preparation performed in duplicates. The absolute agonist-stimulation (Δ ago.) and inverse agonist-inhibition (Δ inv. ago.) of GTP hydrolysis, as well as the relative agonist stimulation and inverse agonist inhibition of GTP hydrolysis (percentage of basal) were calculated.

<table>
<thead>
<tr>
<th>Condition</th>
<th>GTPase Activity (× 10&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.94 (pmol GTP/min/mg)</td>
</tr>
<tr>
<td>+ ago</td>
<td>1.06 (pmol GTP/min/mg)</td>
</tr>
<tr>
<td>Δ ago</td>
<td>0.09 (pmol GTP/min/mg)</td>
</tr>
<tr>
<td>+ inv. ago</td>
<td>0.93 (pmol GTP/min/mg)</td>
</tr>
</tbody>
</table>

Table 2

Ligand potencies and efficacies in the GTPase assay

Steady-state GTPase activity in Sf9 membranes expressing hH3R, different G<sub>i/o</sub> subunits and β<sub>1</sub>γ<sub>2</sub> was determined as described under Materials and Methods. Reaction mixtures contained ligands at concentrations from 0.1 µM to 10 µM as appropriate to generate saturated concentration/response curves. Data were analyzed by nonlinear regression and were best fit to sigmoid concentration/response curves. Typical basal GTPase activities ranged between 2.0 and 5.0 pmol × mg<sup>-1</sup> × min<sup>-1</sup> and the maximal stimulatory effect of histamine (10 µM) amounted to ~30 to ~50% above basal. The efficacy (E<sub>max</sub>) of histamine was determined by nonlinear regression and was set to 1.00. The E<sub>max</sub> values of other agonists and inverse agonists were referred to this value. The abbreviations are shown in Fig. 1.

<table>
<thead>
<tr>
<th>Condition</th>
<th>GTPase Activity (× 10&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>0.94 (µM)</td>
</tr>
<tr>
<td>NAMH</td>
<td>0.94 (µM)</td>
</tr>
<tr>
<td>RAMH</td>
<td>0.94 (µM)</td>
</tr>
<tr>
<td>IME</td>
<td>0.94 (µM)</td>
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<tr>
<td>PRO</td>
<td>0.94 (µM)</td>
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<tr>
<td>IMP</td>
<td>0.94 (µM)</td>
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<tr>
<td>CIP</td>
<td>0.94 (µM)</td>
</tr>
<tr>
<td>CLOB</td>
<td>0.94 (µM)</td>
</tr>
<tr>
<td>THIO</td>
<td>0.94 (µM)</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01; and *** P < 0.001.
* No asterisk(s) after a value indicates not significant.

was almost a full partial agonist in this assay. It was most interesting that the protean agonist proxyfan (5) was a strong partial agonist in all systems, independent of the G protein subtype coexpressed. Impentamine (6) was a moderate partial agonist in all experimental settings. The inverse agonists ciproxynan (7), clenobropin (8), and thioperamide (9) each were also equally potent in the various systems, although they significantly differed in efficacy with the various G<sub>i/o</sub> proteins. However, the rank orders of potency and efficacy of compounds 7 to 9 did not change under the various conditions. Thus, the pharmacological profile of the hH<sub>3</sub>R is very similar under the various experimental conditions. In addition, there is a strong linear correlation between potencies and efficacies of H<sub>3</sub>R ligands at membranes expressing different G<sub>i/o</sub> subunits (Fig. 4). An increase in constitutive activity of hH<sub>3</sub>R coupled to one G protein relative to another G protein would have been reflected in increased agonist potency and efficacy (Seifert and Wenzel-Seifert, 2002), but such an observation was not made. Moreover, differences in constitutive activity of hH<sub>3</sub>R under the various conditions would have resulted in systematic changes in inverse agonist potency, i.e., an increase in constitutive activity would have resulted in decreased inverse agonist potency (Seifert and Wenzel-Seifert, 2002). Again, no such data were obtained. Collectively, these results corroborate the findings regarding the relative stimulatory and inhibitory effects of histamine and thioperamide, respectively (Table 2) based on total ligand-regulated GTPase activity and are indicative for similar constitutive activity of hH<sub>3</sub>R under all experimental conditions (Seifert and Wenzel-Seifert, 2002).

Studies with hH<sub>3</sub>R-G<sub>i</sub> and hH<sub>3</sub>R-G<sub>α</sub>, Fusion Proteins. The use of coexpression systems is often hampered by the fact that it is difficult, if not impossible, to control the expression levels of different signaling partners exactly (Fig. 2, Table 1) (Gille and Seifert, 2003; Kleemann et al., 2008). Table 1 shows that also for hH<sub>3</sub>R, identical GPCR/G<sub>α</sub> ratios could not be achieved. The efficiency of interactions between GPCRs and heterotrimeric G proteins can be influenced by the absolute and relative densities of these proteins in the plasma membrane (Kenakin, 1997; Gille and Seifert, 2003). Fortunately, the analysis of these interactions is greatly facilitated by the use of GPCR-G<sub>α</sub> fusion proteins (Seifert et al., 1999b). Fusion proteins ensure a defined 1:1 stoichiometry of GPCR to G<sub>α</sub> and ensure physical proximity of the signaling partners. Nonetheless, fusion proteins are not physiologically occurring and therefore, the academically best procedure is to compare coexpression systems directly with fusion proteins (Wenzel-Seifert et al., 1998, 1999; Wenzel-Seifert and Seifert, 2000).

Pertussis toxin-sensitive G proteins consist of different G<sub>α</sub> family members and βγ-complexes (Birnbaumer, 2007). The three G<sub>α</sub> subunits are all very similar in structure, whereas the two G<sub>γ</sub>-splice variants are less conserved. The largest structural differences in this G protein family exist...
No Functional Selectivity of Proxyfan at hH3R

The imidazole-containing H3R ligand proxyfan exhibits pleiotropic effects, ranging from inverse agonism to agonism, depending on the system studied (Gabahou et al., 2003; Krueger et al., 2005). An explanation for these findings could be that the proxyfan-bound H3R exhibits different affinities and efficacies for coupling to various G proteins (Kenakin, 2001, 2007; Kobilka and Deupi, 2007). Thus, the observed proxyfan effects could be due to functional selectivity. We tested this hypothesis by studying coupling of the hH3R to four different Gαo4 proteins under clearly defined experimental conditions, measuring one and the same parameter of GPCR/G-protein coupling, i.e., steady-state GTPase activity. However, we did not obtain evidence for functional selectivity of proxyfan. In our hands, proxyfan was a strong partial agonist at the full-length hH3R (445 aa) in all experimental settings. For eight other hH3R ligands, we did not obtain evidence for functional selectivity either. Moreover, we could not find differences in constitutive activity of hH3R coupled to Gαo4 proteins that would have been important for detecting protean agonism (Gabahou et al., 2003). Those "negative"

detail. We hypothesized that, if there were any hH3R/G protein-coupling differences, then potencies and efficacies of ligands should diverge most prominently at this fusion protein pair. The assessment of GTPase activity at different GPCR-Gαo4 fusion proteins is the most accurate measure of the pharmacological profile of a given receptor because GTPase activities are determined under steady-state conditions, rendering potency and efficacy values of agonists and inverse agonists expression level-independent (Seifert et al., 1999a,b; Wenzel-Seifert et al., 1999, 2000). The pharmacological profiles of histamine, imetit, proxyfan, clobenpropit, and thiopemide in the GTPase assay were very similar at the hH3R-Gαo4 and hH3R-Gαo1 fusion proteins both in terms of potency and efficacy (Table 4). Moreover, there was no evidence for differences in constitutive activity of the two fusion proteins. These results fit very well to the data obtained with the corresponding coexpression systems (Table 3) and render it unlikely that the nonidentical GPCR/Gα stoichiometries in the coexpression studies documented in Table 1 had a major impact on the pharmacological profile of hH3R. Actually, the stoichiometry issue would have been of greater concern if the pharmacological profiles of the hH3R had been different with the various coexpressed Gαo4 proteins. This was, however, not the case (Table 3).

Discussion

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### Table 4

| Potencies and efficacies of selected ligands in the GTPase assay at fusion proteins |
|---------------------------------|-----------------|-----------------|
|                                | pEC_{50} = S.E.M. | E_{max} = S.E.M. |
| **hH3R-Gαo4 + β1γ2**           | **hH3R-Gαo1 + β1γ2** |
| HA                             | 7.40 ± 0.10     | 1.00            |
| IME                            | 8.55 ± 0.19     | 0.96 ± 0.08     |
| PRO                            | 7.65 ± 0.23     | 0.81 ± 0.08     |
| CLOB                           | 8.72 ± 0.25     | -0.60 ± 0.07    |
| THIO                           | 7.00 ± 0.18     | -0.76 ± 0.07    |
data were obtained in a coexpression system and a fusion protein system. Thus, the crucial question is how the discrepancies between our present study and the studies of Gbahou et al. (2003) and Krueger et al. (2005) could be explained.

Defined ligands stabilize distinct conformations in the human dopamine D2 receptor that result in the activation of only one specific Gαi/o-subunit when expressed in Sf9 cells (Gazi et al., 2003). In addition, endogenous catecholamines and synthetic ligands stabilize distinct ligand-specific active states in human β-adrenergic receptors (Seifert et al., 1999a; Wenzel-Seifert and Seifert, 2000; Weitl and Seifert, 2008).

Moreover, ligand-specific conformations were readily unmasked for histamine H2 and H4 receptors expressed in Sf9 cells (Preuss et al., 2007; Wittmann et al., 2009). These data show that the baculovirus/Sf9 cell system is sufficiently sensitive at detecting ligand-specific GPCR conformations so that we should have been able to detect functional selectivity of proxfan. However, it should also be emphasized that for some GPCRs expressed in Sf9 cells, e.g., the human formyl peptide receptor, no evidence for ligand-specific receptor conformations could be obtained despite intense efforts (Wenzel-Seifert et al., 1999). These data indicate that not all GPCRs exhibit ligand-specific conformations. It is noteworthy that like hH3R, the formyl peptide receptor couples to Gi/Go proteins (Wenzel-Seifert et al., 1999).

In the study by Gbahou et al. (2003), proxfan was a partial agonist in [35S]GTPγS binding, cAMP accumulation and mitogen-activated protein kinase assays, but an inverse agonist in phospholipase A2 assays, some parameters representing distal consequences of Gαi/o protein activation. It is possible that different combinations of Gαi/o proteins are involved in the responses, that the G protein/effector stoichiometry is different in the pathways (Ostrom and Insel, 2004) and that there is cross-talk between the mitogen-activated protein kinase and phospholipase A2. In contrast, we studied clearly defined G protein heterotrimmers (although at somewhat different GPCR/G protein ratios) and a proximal parameter of GPCR/G-protein coupling, avoiding complications of G protein/effector stoichiometry and cross-talk of signaling pathways.

In the study of Krueger et al. (2005), proxfan exhibited little activity in neurotransmitter release assays, full agonism in cAMP accumulation assays, and partial agonism in [35S]GTPγS binding assays. In addition, in transfected human embryonic kidney cells proxfan displayed differential activity in cAMP accumulation and calcium mobilization assays dependent on the type of G protein coexpressed (Gαi6 or Gxi6i6). The authors concluded that the type of G protein determines the pharmacological properties of proxfan. Although it is known that Gαi6 has an impact on the pharmacological properties of GPCRs (Wenzel-Seifert and Seifert, 2000), Gαi6 is certainly not a cognate G protein of hH3R, but rather a G protein expressed in hematopoietic cells and not in neuronal cells (Birnbaumer, 2007). Moreover, Gxi6i6 is not a physiological G protein, but a chimeric G protein used to direct Gi-coupled GPCRs toward Gαi6 and phospholipase C coupling (Coward et al., 1999). We studied only cognate G proteins of hH3R, i.e., Gαi6i6 proteins, and did not obtain evidence for ligand-specific hH3R conformations.

Gbahou et al. (2003) expressed H3R in Chinese hamster ovary cells. These cells express some of the cognate Gαi6i6-proteins of H3R, but the specific expression pattern of Gαi6i6-subunits was not defined in the previous study. Moreover, it is unknown whether the proxfan-bound H3R interacted differentially with various Gαi6i6 proteins in Chinese hamster ovary cells. Such an interaction can be studied by photoaffinity labeling with [α-32P]GTP azidoanilide (Woo et al., 2009). However, such data are not available. Moreover, various βγi-complexes affect on GPCR/G protein coupling (Birnbaumer, 2007). However, in the previous studies on proxfan βγi-complexes were not defined. We studied a single βγ-complex that is broadly expressed, i.e., β2γi (Birnbaumer, 2007), but we did not examine other βγi-complexes. It is possible that distinct βγi-complexes account for the protean agonism of proxfan observed in the previous studies, but those βγi-complexes remain to be identified. It is also possible that differential compartmentation of G protein heterotrimmers into specific membrane domains, resulting in different GPCR/G protein stoichiometries (Ostrom and Insel, 2004) contributed to protean agonism of proxfan observed in previous studies. In our coexpression system, we cannot exclude different compartmentation of signaling partners either, but the GPCR-Gα fusion protein approach circumvented this problem (Seifert et al., 1999b). With hH3R-Gα fusion proteins, like with the corresponding coexpression systems, there was no evidence for ligand-specific GPCR conformations.

Another issue is the fact that some of the previous assays were performed with intact cells and some assays with membranes. In experiments with intact cells, the precise ionic and nucleotide environments of G proteins are unknown, but both ionic and nucleotide composition can largely affect GPCR/G protein coupling, constitutive GPCR activity and pharmacological GPCR profile (Seifert et al., 1999a; Seifert, 2001; Gille et al., 2002). Moreover, in intact cells, specifically native tissues, relevant for neurotransmitter release studies, it cannot be excluded that endogenous histamine is present, thereby changing the apparent agonistic/inverse agonistic activities of ligands. We performed experiments with extensively washed membranes, excluding the presence of contaminating histamine and conducted experiments under clearly defined ionic conditions and nucleotide composition. We are aware of the fact that our experimental conditions do not represent physiological conditions, but our conditions are defined and allow direct comparison with data from our group for other GPCRs over a period of a decade (Seifert et al., 1999a; Seifert, 2001; Gille et al., 2002; Preuss et al., 2007; Schneider et al., 2009). It is noteworthy that under our experimental conditions, different degrees of constitutive activity of a GPCR can be readily detected (Wenzel-Seifert and Seifert, 2000; Seifert, 2001; Preuss et al., 2007), supporting the principal suitability of our system for the hypothesis tested.

It should be also noted that the study of Gbahou et al. (2003) was performed with rH3R. Species-specific pharmacology of the H3R has been mainly attributed to two aa differences in transmembrane domain 3, which are part of the ligand binding site, and this leads to changes in antagonist affinities (Yao et al., 2003). However, it is possible that the rH3R also shows a different G protein coupling profile compared with the hH3R. Here, we studied only the full-length hH3R (445 aa). Future studies will have to examine rH3R as well.

Another point is the fact that Gbahou et al. (2003) used
a truncated splice variant of the full-length hH3R (413 aa) in their experiments. This short splice variant lacks 32 amino acids in the 3rd intracellular loop of the receptor, which is an important interaction site for G proteins (Seifert et al., 1999b; Leurs et al., 2005). It is possible that the truncated hH3R possesses an altered G protein coupling profile compared with the full-length hH3R. The detailed coupling profiles of various H3R splice variants are not yet known, but should be addressed in future investigations. The pattern of H3R splice variant expression differs between species and brain regions (Bongers et al., 2007) and splice variants differentially regulate signal transduction pathways (Drutel et al., 2001). These data are indicative for differences in G protein coupling of H3R splice variants. We studied only the full-length hH3R (445 aa) without considering splice variants.

In conclusion, we have shown that the full-length hH3R (445 aa) couples similarly to four defined Gs/Gi proteins heterotrimers expressed in S9F cells. We did not obtain evidence in favor of the hypothesis that proxyfan or eight other H3R ligands are functionally selective in a coexpression and a fusion protein system. Moreover, we did not find differences in constitutive activity of hH3R under various experimental conditions. These “negative” results cannot be attributed to unsuitability of our expression system for exclusion of ligand functional selectivity. However, our system is not suitable to definitely exclude agonist at hH3R because that would require a systematic and precise variation of receptor-to-G protein stoichiometries (Kenakin 2001, 2007). In addition, we discussed several possibilities that could account, fully or partially, for the differences between the results of our study and the previous studies of Gbahou et al. (2003) and Krueger et al. (2005). Extensive systematic studies under clearly defined experimental conditions are required to reconcile the discrepancies. Thus, presently, a specific and generally applicable mechanistic explanation for the previously observed pleiotropic effects of proxyfan cannot yet be provided.

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References


