No Evidence for Functional Selectivity of Proxyfan at the Human Histamine H₃ Receptor Coupled to Defined Gᵢ/Gₒ Protein Heterotrimers

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ABSTRACT

Numerous structurally diverse ligands were developed to target the human histamine H₃ receptor (hH₃R), a presynaptic Gᵢ/Gₒ-coupled auto- and heteroreceptor. Proxyfan was identified to be functionally selective, with different efficacies toward Gᵢ/Gₒ-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 Gᵢ₁,Gₒ₁ proteins in hH₃R signaling, using a baculovirus/Sf9 cell expression system. We tested the hypothesis that ligand-specific coupling differences to defined Gᵢ₁,Gₒ₁-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 (Gᵢ₁, Gₒ₁, Gᵢ₀, or Gₒ₀ and β₁γ₂ dimers, respectively). In addition, we constructed the fusion proteins hH₃R-Gᵢ₁ and hH₃R-Gₒ₀ 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-Gᵢ₁ and hH₃R-Gₒ₀ (plus β₁γ₂ dimers), yielded very similar results. Collectively, our data indicate that hH₃R couples similarly to different Gᵢ₀ 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 (Leurs et al., 2005). Discovered pharmacologically in the early 1980s and cloned almost 20 years later (Lovenberg et al., 2000), the H₃R was shown to be a presynaptic auto- and heteroreceptor, regulating the release of neurotransmitters including histamine, dopamine, norepinephrine, serotonin, and acetylcholine via negative feedback mechanisms (Haas et al., 2008). Thus, 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 (Bonaventure et al., 2007; Esbenshade et al., 2008).

According to the two-state model of receptor activation, G protein-coupled receptors (GPCRs) isomerize from an inactive (R) state to an active (R*) state (Kenakin, 2001; Seifert and Wenzel-Seifert, 2002). In the R* state, GPCRs activate G proteins. Agonist-independent R to R* isomerization is referred to as constitutive activity and results in an increase in basal G protein activity. Agonists stabilize the R* state and further increase, whereas inverse agonists stabilize the R state and decrease, basal G protein activity. The H₃R is constitutively active (Leurs et al., 2005) and couples to Gᵢ₀ proteins in native tissues (Clark and Hill, 1996).

ABBREVIATIONS: H₃R, histamine H₃ receptor; GPCR, G protein-coupled receptor; h, human; β₁AR, β₂-adrenoceptor; H₂R, histamine H₂ receptor; aa, amino acids; PCR, polymerase chain reaction; JNN-7753707, (4-fluorophenyl)(1-methyl-2-an-1H-imidazol-5-yl)methanone; GTP-γ-S, guanosine 5’-[(γ-thio)triphosphate; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; r, rat.

[References and further details]
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 quiescent 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 protean 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 Gₓ/Gₙ 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 Gₓ/Gₙ protein α-subunits (Gₓ₁, Gₓ₂, Gₓ₃, or Gₓ₄, and β₁γ₂ dimers, respectively). Sf9 cells have already been successfully used for reconstitution of several Gₓ/Gₙ-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-Gₓ₁, hH₃R-Gₓ₂, hH₃R-Gₓ₃, GPCR-Ga 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-G protein ratio account for potential differences in pharmacological properties of ligands at hH₃R.

Materials and Methods

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 Gₓ₁, Gₓ₂, and Gₓ₄ were donated by Dr. A. G. Gilman (Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX). Baculovirus for rat Gₓ₅₁ was donated by Dr. J. C. Garrison (University of Virginia, Charlottesville, VA). Recombinant baculovirus encoding the unmodified version of Gₓ₁,γ₂-subunits was a kind gift from Dr. P. Gierschik (Department of Pharmacology and Toxicology, University of Ulm, Ulm, Germany). Anti-hH₃R Ig was from Bio-Trend (Cologne, Germany). The anti-FLAG Ig (M1 monoclonal antibody) and anti-His6 Ig were from Sigma-Aldrich (St. Louis, MO). The antibodies recognizing Gₓ₁,γ₂-subunits (Gₓ₁,γ₂; AS 266) and Gβ-subunits (Gβ; AS 398/9), as well as purified Gx₁,γ₂ and Gx₄,γ₂ protein, were kindly provided by Dr. B. Nürnberg (Institute of Pharmacology, University of Tübingen, Tübingen, Germany). The Gx₂,γ₂ and Gx₄,γ₂-selective antibodies were from Calbiochem (San Diego, CA). Histamine, (R)-α-methylhistamine, N,N'-methylhistamine, imetit, clobenpropit, and thioperamide were from Teccis Bioscience (Avonmouth, Bristol, UK). Impentamine, imiproxyfan, and ciproxyfan were kind gifts from Dr. S. Elz (Department of Pharmaceutical/Medicinal Chemistry I, University of Regensburg, Regensburg, Germany). Proxyfan was synthesized by Dr. P. Igel (Department of Pharmaceutical/Medicinal Chemistry II, University of Regensburg, Regensburg, Germany). Ligand structures are depicted in Fig. 1. Stock solutions (10 mM) of all H₃R ligands described in this article were prepared in distilled water and stored at −20 °C. [³H]JNJ-7753707 ([³H]RWJ-422475) (30 Ci/mmol) was kindly donated by Dr. P. Bonaventure (Johnson and Johnson Pharmaceutical R&D). [³H]Dihydroalprenolol (85–90 Ci/mmol) and [³⁵S]GTPγS (1100 Ci/ mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). [³²P]GTP was prepared using GDP and [³²P]P³ (8500–9120 Ci/mmol orthophosphoric acid) (PerkinElmer Life and Analytical Sciences) according to a previously described enzymatic labeling procedure (Walseth and Johnson, 1979). Unlabeled nucleotides were from Roche Diagnostics (Indianapolis, IN).
IN), and all other reagents were of the highest purity available and from standard suppliers.

**Construction of FLAG Epitope- and Hexahistidine-Tagged cDNA for hH₃R.** The cDNA for the tagged receptor protein was generated by sequential overlap-extension PCR. With pGEM-3Z-SF-hH₃R-His₆ as template (Schneider et al., 1998), the sense primer human HHR3-F (5'-GAC CAT GAT GAC GCC AGC CCT CCA GTA GTG ATG ATG ATG AGT CCT CCA GTA GTG CTC-3') annealed with 19 bp of pGEM-3Z before the 5'-end of SF. The antisense primer hH₃R-FLAG (5'-CAT GGC GTC ATC ATC GTC-3') annealed with 15 bp of the 3'-end of SF and with ATG. In PCR 1B, the cDNA encoding the hH₃R, followed by the start codon, and five N-terminal codons of G₁₄, or G₁₄-His₆, digested with the SacI and XbaI sites. As template, a plasmid (pCIneo containing the sequence of hH₃R was used. In PCR 2, the products of PCR 1A and PCR 1B 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-G₁₄-His₆ and pGEM-3Z-SF-hH₃R-G₁₄-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-hH₃R-His₆ (Schneider et al., 2009) via SacI and XbaI restriction sites. After transformation of chemically competent bacteria (Top 10) were transformed, the plasmid was amplified, and the accuracy of the resulting MaxiPrep-DNA was checked by extensive restriction digestion analysis and sequencing.

**Construction of the cDNAs for hH₃R-G₁₄ and hH₃R-G₁₄.** The cDNAs for the tagged fusion proteins were also generated by sequential overlap-extension PCR. With pGEM-3Z-SF-hH₃R-His₆ as template, a prolonged sense primer from PCR 1A F1 (5'-GAC CAT GAG ATC TTT AGG TGA CAG TAT AGA ATA GTC TCT CCA GTA GTG ATG ATG AGT CCT CCA GTA GTG CTC-3') and an antisense primer a6His_H3R (5'-GAT TAC GCC AAG CTA TTT AGG TGA CAC TAT AGA ATA CTC TCA-3') annealed with 19 bp of the 5'-end of SF. The antisequence primer C3 (5'-CAT GGC GTC ATC ATC GTC-3') containing the sequence of hH₃R was used. In PCR 2, the products of PCR 1A and the antisense primer of PCR 1B were used. In that way, a fragment encoding SF, 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 and 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-hH₃R-His₆ (Schneider et al., 2009) via SacI and XbaI restriction sites. Again, competent bacteria (Top 10) were transformed, the plasmid was amplified, and the accuracy of the resulting MaxiPrep-DNA was checked by extensive restriction digestion analysis and sequencing.
of the bands was analyzed with the Quantity One 4.0.3 software (Bio-Rad Laboratories).

\[^{3}H\]HJNJ-7753707 Binding Assay. Before experiments, membranes were sedimeted by a 10-min centrifugation at 4°C and 15,000g and resuspended in binding buffer (12.5 mM MgCl\(_2\), 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 \(\mu\)l) contained 10 to 50 \(\mu\)g of protein. Nonspecific binding was determined in the presence of \[^{3}H\]HJNJ-7753707 at various concentrations plus 10 \(\mu\)M thioperamide and amounted to \(\approx 20\) 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 0.1 to 10 nM \[^{3}H\]HJNJ-7753707. Bound \[^{3}H\]HJNJ-7753707 was separated from free \[^{3}H\]HJNJ-7753707 by filtration through 0.2% (w/v) polyethyleneimine-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.

\[^{35}S\]GTP\(_{\gamma}\)S Binding Assay. Membranes were thawed, sedimented by a 10-min centrifugation at 4°C and 15,000g to remove residual endogenous guanine nucleotides as far as possible. Membranes were resuspended in binding buffer, supplemented with 0.05\% (v/v) bovine serum albumin. Each tube (total volume of 250 or 500 \(\mu\)l) contained 10 to 20 \(\mu\)g of protein per tube. In saturation binding experiments, tubes contained 0.2 to 2 nM \[^{35}S\]GTP\(_{\gamma}\)S plus unlabeled GTP\(_{\gamma}\)S to give the desired final ligand concentrations (0.2–50 nM). Neither GDP nor H\(_2\)R ligands were included in assays. Nonspecific binding was determined in the presence of 100 \(\mu\)M unlabeled GTP\(_{\gamma}\)S and amounted to less than 1\% of total binding. Incubations were conducted for 90 min at 25°C and shaking at 250 rpm. Bound \[^{35}S\]GTP\(_{\gamma}\)S was separated from free \[^{35}S\]GTP\(_{\gamma}\)S 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 50 \(\mu\)l of protein/tube, 5.0 mM MgCl\(_2\), 0.1 mM EDTA, 0.1 mM ATP, 100 nM GTP, 0.1 mM adenyllyimidodiphosphate, 1.2 mM creatine phosphate, 1 \(\mu\)g of creatine kinase, and 0.2\% (w/v) bovine serum albumin in 50 mM Tris-HCl, pH 7.4, and H\(_2\)R ligands at various concentrations. Reaction mixtures (80 \(\mu\)l) were incubated for 2 min at 25°C before the addition of 20 \(\mu\)l of \([\gamma-\text{32P}]\)GTP (0.1 \(\mu\)Ci/tube). All stock and work dilutions of \([\gamma-\text{32P}]\)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 \(\mu\)l of slurry consisting of 5\% (w/v) activated charcoal and 50 mM NaH\(_2\)PO\(_4\), pH 2.0. Charcoal absorbs nucleotides but not P\(_i\). Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at 15,000g.

Six hundred microliters of the supernatant fluid of reaction mixtures was removed, and \(\text{32P}\) was determined by liquid scintillation counting. Enzyme activities were corrected for spontaneous degradation of \([\gamma-\text{32P}]\)GTP. Spontaneous \([\gamma-\text{32P}]\)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 \([\gamma-\text{32P}]\)GTP, prevents \([\gamma-\text{32P}]\)GTP hydrolysis by enzymatic activities present in Sf9 membranes. Spontaneous \([\gamma-\text{32P}]\)GTP degradation was <1\% of the total amount of radioactivity added using 20 mM Tris-HCl, pH 7.4, as solvent for \([\gamma-\text{32P}]\)GTP. The experimental conditions chosen ensured that not more than 10\% of the total amount of \([\gamma-\text{32P}]\)GTP added was converted to \(\text{32P}\).

Miscellaneous. Molecular biology was planned with GCCK 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). \[^{3}H\]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 h\(_{H4R}\)-baculoviruses alone or in combination with baculoviruses encoding different mammalian G proteins (G\(_{\alpha 11}\), G\(_{\alpha 12}\), G\(_{\alpha 13}\) or G\(_{\alpha 14}\) and/or \(\beta y_{1,2}\) dimers, respectively) were prepared and subjected to immunological analysis. The predicted molecular mass of the h\(_{H4R}\) is \(\sim 49\) kDa. We used anti-h\(_{H4R}\) Ig, recognizing an 18-aa peptide within the extracellular N terminus of the h\(_{H4R}\) to confirm expression (Fig. 2A). Indeed, h\(_{H4R}\) 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\(_4\) Ig (Fig. 2C), recognizing the C-terminal hexahistidine tag.

The bands were doublets, probably representing differently glycosylated forms of h\(_{H4R}\). h\(_{H4R}\) 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 \(\approx 1\) to 2 pmol/mg, using anti-FLAG Ig and h\(_{H2AR}\) as standards (Fig. 2B).

The expression level of the h\(_{H2AR}\) was 7.5 pmol/mg, as determined by \[^{3}H\]dihydroalprenolol saturation binding. Thus, h\(_{H4R}\) was properly expressed in Sf9 cells and not proteolytically degraded after membrane preparation.

To visualize the coexpressed G\(_{\alpha}\)-subunits, we used an antibody recognizing all G\(_{\alpha}\)-proteins (Fig. 2D). G\(_{\alpha}\)-subunits appeared at the expected molecular mass (\(\sim 40–41\) kDa) as very intense bands, although the expression level of G\(_{\alpha 11}\) was lower compared with the other G protein subunits. This is an intrinsic property of mammalian G\(_{\alpha 11}\), 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 G\(_{\alpha 11}\) does not influence its ability to effectively interact with GPCRs (Kleemann et al., 2008). It is likely that G\(_{\alpha 11}\) accumulates in GPCR-expressing membrane domains. G\(_{\beta\gamma}\)-subunits were expressed at similar levels in all membranes studied (Fig. 2E).

The GPCR/G protein ratio can alter the pharmacological properties of ligands (Kenakin, 1997). Therefore, we quantified G\(_{\alpha 12}\) and G\(_{\alpha 14}\) using purified protein as reference. These semiquantitatively determined expression levels for the G\(_{\alpha}\)-subunits were in the range of 50 to 100 pmol/mg, resulting in receptor-to-G protein ratios of \(\sim 1:50\) to 1:100 (Fig. 2, F and G). This is in good agreement with ratios determined for other G\(_{\alpha}\)-coupled receptors in Sf9 cell membranes, for example the h\(_{H2R}\) (Schneider et al., 2009), human cannabinoid receptors CB\(_1\) and CB\(_2\) (Nickel et al., 2008), and the human fornyl peptide receptor (Wenzel-Seifert et al., 1998).
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 Go subunits by a combination of antagonist [³H]JNJ-7753707- and [³⁵S]GTPᵽ/S saturation binding and calculated the functional GPCR/Go protein ratios (Table 1). The membranes were from the same batch as those studied by immunoblot to ensure maximal comparability and data accuracy.


Fig. 2. Immunological detection of recombinant proteins expressed in Sf9 cells. Each lane of the gels was loaded with 10 μg of membrane protein, unless otherwise indicated below the film. Numbers on the left designate masses of marker proteins in kilodaltons. A, a membrane expressing the hH₃R alone was loaded onto the gels. B1 and B2, 2, 4, 6, 8, and 10 μg of protein of Sf9 membranes expressing hβ₂AR at 7.5 pmol/mg (as determined by [³H]dihydroalprenolol saturation binding) were analyzed to quantify the Go-subunits, using 2, 4, 7.5, 15, and 30 pmol of purified Go₃₂ as standard. G, 0.5, 1.0, 1.5, and 2.0 μg of a corresponding membrane of the same batch expressing hH₃R plus Gα₁Gβ₂γ₂ was analyzed to quantify the Go-subunits, using 2, 4, 7.5, 15, and 30 pmol of purified Gα₁Gβ₂γ₂ an almost identical splice variant of Gα₁ as standard.

In [³⁵S]GTPᵽ/S saturation binding experiments neither GDP nor H₂R ligands were present. The maximal number of Gα₀/Gᵽ₁-related GTPᵽ/S binding sites in membranes expressing hH₃R plus Go-subunits plus β₁γ₂ was corrected by the binding determined in parallel membranes expressing hH₃R plus β₁γ₂ alone. To ensure the same viral load in the reference membrane, Sf9 cells were infected with baculoviruses encoding hH₃R, β₁γ₂, and virus encoding no recombinant protein at all. In this manner, only the number of functionally intact and heterologously expressed mammalian Gα₀/Gᵽ₁-subunits was quantified.
The experiments revealed that the number of \(^{[3]H}\)JNJ-7753707 binding sites was very similar to the hH3R protein expression levels determined via immunoblot. Thus, most hH3R molecules were correctly folded in Sf9 cell membranes. However, the number of specific \(^{[35]S}\)GTP\(_\gamma\)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-Go 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 profile of the hH3R, we measured the receptor-dependent constitutive activity of the hH3R (Schneider et al., 2009) and the human formyl peptide receptor (Wenzel-Seifert et al., 1998; Seifert, 2001). The constitutive activity of endogenous agonist histamine (10 \(\mu M\)) and a saturating concentration of the inverse agonist thioperamide (10 \(\mu M\)) in Sf9 cell membranes expressing the hH3R alone or coexpressing the hH3R and different G proteins.

In membranes expressing the hH3R 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 hH3R to insect cell G proteins. The structurally related hH4R also couples only weakly to insect cell G proteins (Schneider et al., 2009). hH3R coupled efficiently to all coexpressed mammalian Go\(_{i/o}\)-subunits (Go\(_{1,3}\), Go\(_{2,4}\), Go\(_{13}\), and Go\(_{11}\), 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 hH3R was comparable and not substantially influenced by the type of G protein (Seifert and Wenzel-Seifert, 2002). In contrast to hH3R, the short and long splice variants of Go\(_2\) had a large impact on the constitutive activity of the h\(\beta_3\)AR (Seifert et al., 1998; Seifert, 2001). The constitutive activity of hH3R coupled to cGMP/Gi proteins was rather high and comparable with the constitutive activity of hH3R (Schneider et al., 2009) and the human formyl peptide receptor (Wenzel-Seifert et al., 1998, 1999; Wenzel-Seifert and Seifert, 2000).

**Steady-State GTPase Assay: hH3R Coupling to Different Go-Subunits.** To investigate the G protein-coupling profile of the hH3R, we measured the receptor-dependent GTP\(_\gamma\) 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 \(^{[35]S}\)GTP\(_\gamma\)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 hH3R alone or coexpressing the hH3R and different G proteins.

**TABLE 1**

<table>
<thead>
<tr>
<th>Membrane</th>
<th>(\text{R}_{\text{max}} \pm \text{S.E.M.} \text{ pmol} \cdot \text{mg}^{-1})</th>
</tr>
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<tbody>
<tr>
<td>(^{[3]H})JNJ-7753707</td>
<td>0.6 ± 0.04</td>
</tr>
<tr>
<td>(^{[35]S})GTP(_\gamma)S</td>
<td>1.40 ± 0.57</td>
</tr>
<tr>
<td>R/G ratio</td>
<td>~1.2</td>
</tr>
</tbody>
</table>

**Fig. 3.** Comparison of the effects of histamine, proxyfan, and thioperamide in membranes expressing the hH3R, different Go\(_{i/o}\)-subunits [Go\(_{1,3}\) (A), Go\(_{2,4}\) (B), Go\(_{13}\) (C), and Go\(_{11}\) (D)], and \(\beta_3\)Y2 dimers. Steady-state GTPase activity in Sf9 membranes was determined as described under Materials and Methods. Reaction mixtures contained histamine (HA), proxyfan (PRO), or thioperamide (THIO) at the concentrations indicated on the abscissa to achieve saturation. Data were analyzed by nonlinear regression and were best fit to sigmoidal concentration-response curves. Data points shown are the means ± S.E.M. of three to four independent experiments performed in duplicate. A summary of all results is shown in Table 3.
TABLE 3

GTPase activities in Sf9 membranes expressing hH3R and different Gαi/o proteins

Steady-state GTPase experiments were performed as described under Materials and Methods. Reaction mixtures contained 0.1 μCi [γ-32P]GTP and 100 nM unlabelled GTP in the presence of solvent (basal), 10 μM histamine (+ agon.), or 10 μM thioperamide (+ inv. ago.). Data shown are the means ± S.E.M. of four to five independent experiments for each membrane preparation performed in duplicates. Minor differences in regulation parameters might be caused by the use of GPCR-Gαi/o fusion proteins (Seifert et al., 1998, 1999; Seifert and Wenzel-Seifert, 1998). Again, no such data were obtained. Statistical analysis was performed using one-way ANOVA followed by Dunnet’s multiple comparison test using the values determined at hH3R-Gαi1 and hH3R-Gαi3 as a reference. Significant differences to the membrane expressing Gαi1 are shown following comparison with other Gαi/o subunits. Structures of ligands and definitions of abbreviations are shown in Fig. 1.

<table>
<thead>
<tr>
<th>hH3R + Gαi1</th>
<th>hH3R + Gαi2</th>
<th>hH3R + Gαq1</th>
<th>hH3R + Gαq1 + β1γ2</th>
<th>hH3R + Gαi2 + β1γ2</th>
<th>hH3R + Gαq1 + β1γ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEC50 ± S.E.M.</td>
<td>Emax ± S.E.M.</td>
<td>pEC50 ± S.E.M.</td>
<td>Emax ± S.E.M.</td>
<td>pEC50 ± S.E.M.</td>
<td>Emax ± S.E.M.</td>
</tr>
<tr>
<td>HA</td>
<td>8.02 ± 0.07*</td>
<td>1.00</td>
<td>7.90 ± 0.05</td>
<td>1.00</td>
<td>7.88 ± 0.09</td>
</tr>
<tr>
<td>NAMH</td>
<td>8.95 ± 0.12</td>
<td>1.04 ± 0.07</td>
<td>8.86 ± 0.07</td>
<td>1.00 ± 0.04</td>
<td>8.83 ± 0.11</td>
</tr>
<tr>
<td>RAMH</td>
<td>8.78 ± 0.21</td>
<td>1.08 ± 0.12</td>
<td>8.81 ± 0.16</td>
<td>1.16 ± 0.10</td>
<td>8.82 ± 0.12</td>
</tr>
<tr>
<td>ME</td>
<td>9.61 ± 0.19</td>
<td>0.94 ± 0.09</td>
<td>9.68 ± 0.13</td>
<td>0.92 ± 0.06</td>
<td>9.63 ± 0.16</td>
</tr>
<tr>
<td>PRO</td>
<td>8.34 ± 0.15</td>
<td>0.88 ± 0.06</td>
<td>8.19 ± 0.11</td>
<td>0.88 ± 0.05</td>
<td>8.26 ± 0.15</td>
</tr>
<tr>
<td>IMP</td>
<td>8.68 ± 0.21</td>
<td>0.50 ± 0.05</td>
<td>8.25 ± 0.20</td>
<td>0.33 ± 0.03</td>
<td>8.97 ± 0.49</td>
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<tr>
<td>CIP</td>
<td>7.21 ± 0.22</td>
<td>-0.71 ± 0.08*</td>
<td>7.03 ± 0.18</td>
<td>-1.08 ± 0.11</td>
<td>7.04 ± 0.19</td>
</tr>
<tr>
<td>CLOB</td>
<td>9.08 ± 0.22</td>
<td>-1.00 ± 0.11*</td>
<td>9.14 ± 0.12</td>
<td>-1.37 ± 0.08</td>
<td>9.18 ± 0.23</td>
</tr>
<tr>
<td>THIO</td>
<td>6.97 ± 0.13</td>
<td>-0.93 ± 0.06</td>
<td>6.93 ± 0.14</td>
<td>-1.02 ± 0.07</td>
<td>6.77 ± 0.18</td>
</tr>
</tbody>
</table>

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

TABLE 4

Ligand potencies and efficacies in the GTPase assay

Steady-state GTPase activity in Sf9 membranes expressing hH3R, different Gαi/o subunits, and β1γ2 was determined as described under Materials and Methods. Reaction mixtures contained ligands at concentrations from 0.1 nM to 10 μM as appropriate to generate saturated concentration/response curves. Data were analyzed by nonlinear regression and were best fit to sigmoidal concentration/response curves. Typical basal GTPase activities ranged between 2.0 and 5.0 pmol x mg⁻¹ x min⁻¹ and the maximal stimulatory effect of histamine (10 μM) amounted to ~30 to ~50% above basal. The efficacy (Emax) of histamine was determined by nonlinear regression and was set to 1.00. The Emax values of other agonists and inverse agonists were referred to this value. Data shown are the means ± S.E.M. of three to four experiments performed in duplicates each. Statistical analysis was performed using one-way ANOVA followed by Dunnet’s multiple comparison test using the values determined at hH3R-Gαi1 and hH3R-Gαi3 as a reference. Significant differences to the membrane expressing Gαi1 are shown following comparison with other Gαi/o subunits. Structures of ligands and definitions of abbreviations are shown in Fig. 1.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EC50 (nM)</th>
<th>Max effect (pmol/mg/min)</th>
<th>Emax ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>0.74 ± 0.12</td>
<td>29.01 ± 3.98</td>
<td>1.00 ± 0.02</td>
</tr>
<tr>
<td>Ciproxyfan</td>
<td>7.56 ± 0.19</td>
<td>2.59 ± 0.05</td>
<td>0.94 ± 0.04</td>
</tr>
<tr>
<td>Impentamine</td>
<td>3.10 ± 0.16</td>
<td>3.98 ± 0.22</td>
<td>0.89 ± 0.09</td>
</tr>
</tbody>
</table>
| Pertussis toxin-sensitive G proteins consist of different Gαi/o family members and βγ-complexes (Birnbgaumer, 2007). The three Gαi/o subunits are all very similar in structure, whereas the two Gαq splice variants are less conserved. The largest structural differences in this G protein family exist

was almost a full partial agonist in this assay. It was most interesting that the protein 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 agonist ciproxyfan (7), clobenpropit (8), and thioperamide (9) each were also equally potent in the various systems, although they significantly differed in efficacy with the various Gαi/o 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 hH3R is very similar under the various experimental conditions. In addition, there is a strong linear correlation between potencies and efficacies of H3R ligands at membranes expressing different Gαi/o subunits (Fig. 4). An increase in constitutive activity of hH3R 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 hH3R under the various conditions would have resulted in systematic changes of 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 H3R under all experimental conditions (Seifert and Wenzel-Seifert, 2002).

Studies with hH3R-Gα12 and hH3R-Gαq1, 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 hH3R, identical GPCR/Gα 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-Gα fusion proteins (Seifert et al., 1999b). Fusion proteins ensure a defined 1:1 stoichiometry of GPCR to Gα 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 Gαi/o family members and βγ-complexes (Birnbgaumer, 2007). The three Gαi/o subunits are all very similar in structure, whereas the two Gαq splice variants are less conserved. The largest structural differences in this G protein family exist...
between \(\alpha_i\) and \(\alpha_4\) (Birnbaumer, 2007). Thus, we constructed \(\alpha_i\) and \(\alpha_i\) fusion proteins as representative pair to study \(\alpha_i\) protein coupling in more detail. We hypothesized that, if there were any \(\alpha_i\) 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-

\(\alpha_i\) 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; Wenzel-Seifert et al., 1999, 2000). The pharmacological profiles of histamine, imetit, proxyfan, clofenpropit, and thio-

eramide in the GTPase assay were very similar at the \(\alpha_i\) and \(\alpha_i\) 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/\(\alpha_i\) stoichiometries in the coexpression studies documented in Table 1 had a major impact on the pharmacological profile of \(\alpha_i\). Actually, the stoichiometry issue would have been of greater concern if the pharmacological profiles of the \(\alpha_i\) had been different with the various coexpressed \(\alpha_4\) proteins. This was, however, not the case (Table 3).

Discussion

The imidazole-containing \(\alpha_i\) ligand proxyfan exhibits pleiotropic effects, ranging from inverse agonism to agonism, depending on the system studied (Gabhou et al., 2003; Krueger et al., 2005). An explanation for these findings could be that the proxyfan-bound \(\alpha_i\) exhibits different affinities and efficacies for coupling to various \(\alpha_i\) 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 \(\alpha_i\) to four different \(\alpha_4\) proteins under clearly defined experimental conditions, measuring one and the same parameter of GPCR/\(\alpha_i\)-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 \(\alpha_i\) (445 aa) in all experimental settings. For eight other \(\alpha_i\) ligands, we did not obtain evidence for functional selectivity either. Moreover, we could not find differences in constitutive activity of \(\alpha_i\) coupled to \(\alpha_i\) proteins that would have been important for detecting protean agonism (Gabhou et al., 2003). Those “negative”

| Potencies and efficacies of selected ligands in the GTPase assay at fusion proteins |
|------------------|------------------|------------------|------------------|
|                  | \(pEC_{50} \pm S.E.M.\) | \(E_{max} \pm S.E.M.\) | \(pEC_{50} \pm S.E.M.\) | \(E_{max} \pm S.E.M.\) |
| **hH3R-\(\alpha_i\) + \(\beta_1\)** |                  |                  |                  |                  |
| HA               | 7.40 ± 0.10       | 1.00             | 7.46 ± 0.11       | 1.00             |
| IME              | 9.55 ± 0.19       | 0.96 ± 0.08      | 9.62 ± 0.09       | 0.95 ± 0.04      |
| PRO              | 7.65 ± 0.23       | 0.81 ± 0.08      | 7.76 ± 0.13       | 0.74 ± 0.05      |
| CLOB             | 8.72 ± 0.25       | −0.60 ± 0.07     | 8.45 ± 0.25       | −0.86 ± 0.09     |
| THIO             | 7.00 ± 0.18       | −0.76 ± 0.07     | 7.05 ± 0.15       | −0.74 ± 0.05     |
| **hH3R-\(\alpha_i\) + \(\beta_3\)** |                  |                  |                  |                  |
| HA               | 7.40 ± 0.10       | 1.00             | 7.46 ± 0.11       | 1.00             |
| IME              | 9.55 ± 0.19       | 0.96 ± 0.08      | 9.62 ± 0.09       | 0.95 ± 0.04      |
| PRO              | 7.65 ± 0.23       | 0.81 ± 0.08      | 7.76 ± 0.13       | 0.74 ± 0.05      |
| CLOB             | 8.72 ± 0.25       | −0.60 ± 0.07     | 8.45 ± 0.25       | −0.86 ± 0.09     |
| THIO             | 7.00 ± 0.18       | −0.76 ± 0.07     | 7.05 ± 0.15       | −0.74 ± 0.05     |

Materials and Methods

Steady-state GTPase activity in S8 membranes expressing \(\alpha_i\) or \(\alpha_i\) proteins, plus \(\beta_1\), was determined as described under Materials and Methods. Reaction mixtures contained ligands at concentrations from 0.1 nM to 10 μM as appropriate to generate saturated concentration/response curves. Data were analyzed by nonlinear regression and were best fit sigmoid GTPase activities ranging between 1.0 and 1.5 pmol × mg⁻¹ × min⁻¹, and the maximal stimulatory effect of HA (10 μM) amounted to ~50 to ~80% above basal. The efficacy (\(B_{max}\)) of HA was determined by nonlinear regression and was set to 1.00. The \(B_{max}\) values of other agonists and inverse agonists were referred to this value. Data shown are the means ± S.E.M. of three to four experiments performed in duplicates each. Statistical analysis was performed using the t-test (\(P < 0.05\)). Significant differences were not found for the data analyzed. Structures of ligands and definitions of abbreviations are shown in Fig. 1.

Fig. 4. Correlation of potency and efficacy of ligands at the \(\alpha_i\) in the presence of different coexpressed \(\alpha_4\) proteins. Data shown in Table 3 were analyzed by linear regression. A, B, C, and E, potencies of ligands at membranes coexpressing the \(\alpha_i\), \(\alpha_4\), or \(\alpha_4\), and \(\beta_1\) dimers, respectively, were correlated with values determined at the reference membrane expressing \(\alpha_4\). A, \(r^2 = 0.97\); slope = 0.93 ± 0.06. B, \(r^2 = 0.93\); slope = 1.02 ± 0.10. E, \(r^2 = 0.92\); slope = 0.96 ± 0.11. B, D, and F, the efficacies of ligands at membranes coexpressing the \(\alpha_i\), \(\alpha_4\), \(\alpha_4\), or \(\alpha_4\), and \(\beta_1\) dimers, respectively, were correlated with values determined at the reference membrane expressing \(\alpha_4\). B, \(r^2 = 0.99\); slope = 0.87 ± 0.03. D, \(r^2 = 0.99\); slope = 0.78 ± 0.03. F, \(r^2 = 0.99\); slope = 0.85 ± 0.03. The dotted lines indicate the 95% confidence intervals of the regression lines. The diagonal dashed line has a slope of 1 and represents a theoretical curve for identical values.
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 D_{2} receptor that result in the activation of only one specific G_{\alpha_{i/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 \beta-adrenergic receptors (Seifert et al., 1999a; Wenzel-Seifert and Seifert, 2000; Weitl and Seifert, 2008). Moreover, ligand-specific conformations were readily unmasked for histamine H_{3} and H_{4} 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 hH_{3}R, the formyl peptide receptor couples to G_{i/o} proteins (Wenzel-Seifert et al., 1999).

In the study by Gbahou et al. (2003), proxfan was a partial agonist in [\textsuperscript{35}S]GTP\gammaS binding, cAMP accumulation and mitogen-activated protein kinase assays, but an inverse agonist in phospholipase A\_2 assays, some parameters representing distal consequences of G_{\alpha_{i/o}} protein activation. It is possible that different combinations of G_{\alpha_{i/o}} proteins are involved in the responses, that the G protein/effecter stoichiometry is different in the pathways (Ostrom and Insel, 2004) and that there is cross-talk between the mitogen-activated protein kinase and phospholipase A\_2. In contrast, we studied clearly defined G protein heterotrimers (although at somewhat different GPCR/G protein ratios) and a proximal parameter of GPCR/G-protein coupling, avoiding complications of G protein/effecter 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 [\textsuperscript{35}S]GTP\gammaS 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 (G_{\alpha_{16}} or G_{\alpha_{q16}}). The authors concluded that the type of G protein determines the pharmacological properties of proxfan. Although it is known that G_{\alpha_{16}} has an impact on the pharmacological properties of GPCRs (Wenzel-Seifert and Seifert, 2000), G_{\alpha_{16}} is certainly not a cognate G protein of hH_{3}R, but rather a G protein expressed in hematopoietic cells and not in neuronal cells (Birnbaumer, 2007). Moreover, G_{\alpha_{q16}} is not a physiological G protein, but a chimeric G protein used to direct G\_coupled GPCRs toward G_{\alpha_{i}} and phospholipase C coupling (Coward et al., 1999). We studied only cognate G proteins of hH_{3}R, i.e., G_{\alpha_{i/o}} proteins, and did not obtain evidence for ligand-specific hH_{3}R conformations.

Gbahou et al. (2003) expressed hH_{3}R in Chinese hamster ovary cells. These cells express some of the cognate G_{\alpha_{i/o}} proteins of H_{3}R, but the specific expression pattern of G_{\alpha_{q16}}-subunits was not defined in the previous study. Moreover, it is unknown whether the proxfan-bound H_{3}R interacted differentially with various G_{\alpha_{i/o}} proteins in Chinese hamster ovary cells. Such an interaction can be studied by photoaffinity labeling with [\textsuperscript{32}P]GTP azidoanilide (Woo et al., 2009). However, such data are not available. Moreover, various \beta_{\gamma_{i/o}}-complexes affect on GPCR/G protein coupling (Birnbaumer, 2007). However, in the previous studies on proxfan \beta_{\gamma_{i/o}}-complexes were not defined. We studied a single \beta_{\gamma}-complex that is broadly expressed, i.e., \beta_{\gamma_{2}} (Birnbaumer, 2007), but we did not examine other \beta_{\gamma_{i/o}}-complexes. It is possible that distinct \beta_{\gamma_{i/o}}-complexes account for the protean agonism of proxfan observed in the previous studies, but those \beta_{\gamma_{i/o}}-complexes remain to be identified. It is also possible that differential compartmentation of G protein heterotrimers 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-Ga fusion protein approach circumvented this problem (Seifert et al., 1999b). With hH_{3}R-G_{\alpha_{i/o}} 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 rH_{3}R. Species-specific pharmacology of the H_{3}R 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 rH_{3}R also shows a different G protein coupling profile compared with the hH_{3}R. Here, we studied only the full-length hH_{3}R (445 aa). Future studies will have to examine rH_{3}R as well.

Another point is the fact that Gbahou et al. (2003) used...
a truncated splice variant of the full-length hH3R (445 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 protein heterotrimeric expressed in Sf9 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 agonism 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


D1-receptor with Gi protein.