
Pharmacological Characterization of Human Histamine Receptors and Histamine Receptor Mutants in the Sf9 Cell Expression System

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Abstract

A large problem of histamine receptor research is data heterogeneity. Various experimental approaches, the complex signaling pathways of mammalian cells, and the use of different species orthologues render it difficult to compare and interpret the published results. Thus, the four human histamine receptor subtypes were analyzed side-by-side in the Sf9 insect cell expression system, using radioligand binding assays as well as functional readouts proximal to the receptor activation event (steady-state GTPase assays and [³⁵S]GTPγS assays). The human H₁R was co-expressed with the regulators of G protein signaling RGS4 or GAIP, which unmasked a productive interaction between hH₁R and insect cell Gα_q. By contrast, functional expression of the hH₂R required the generation of

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an hH₂R-Gs α fusion protein to ensure close proximity of G protein and receptor. Fusion of hH₂R to the long (Gs α_L) or short (Gs α_S) splice variant of Gs α resulted in comparable constitutive hH₂R activity, although both G protein variants show different GDP affinities. Medicinal chemistry studies revealed profound species differences between hH₁R/hH₂R and their guinea pig orthologues gpH₁R/gpH₂R. The causes for these differences were analyzed by molecular modeling in combination with mutational studies. Co-expression of the hH₃R with G α_{i1} , G α_{i2} , G α_{i3} , and G $\alpha_{i/o}$ in Sf9 cells revealed high constitutive activity and comparable interaction efficiency with all G protein isoforms. A comparison of various cations (Li⁺, Na⁺, K⁺) and anions (Cl⁻, Br⁻, I⁻) revealed that anions with large radii most efficiently stabilize the inactive hH₃R state. Potential sodium binding sites in the hH₃R protein were analyzed by expressing specific hH₃R mutants in Sf9 cells. In contrast to the hH₃R, the hH₄R preferentially couples to co-expressed G α_{i2} in Sf9 cells. Its high constitutive activity is resistant to NaCl or GTP γ S. The hH₄R shows structural instability and adopts a G protein-independent high-affinity state. A detailed characterization of affinity and activity of a series of hH₄R antagonists/inverse agonists allowed first conclusions about structure/activity relationships for inverse agonists at hH₄R. In summary, the Sf9 cell system permitted a successful side-by-side comparison of all four human histamine receptor subtypes. This chapter summarizes the results of pharmacological as well as medicinal chemistry/molecular modeling approaches and demonstrates that these data are not only important for a deeper understanding of H_xR pharmacology, but also have significant implications for the molecular pharmacology of GPCRs in general.

Keywords

[³⁵S]GTP γ S binding • GPCRs • Histamine receptors • Radioligand binding • Sf9 insect cells • Steady-state GTPase assay

Abbreviations

[³ H]histamine	Tritiated histamine
[³ H]NAMH	Tritiated <i>N</i> ^{α} -methylhistamine
[³⁵ S]GTP γ S	GTP γ S, labeled with ³⁵ S
α_2 AR	α -Adrenoceptor, subtype 2
β_1 AR, β_2 AR	β -Adrenoceptor subtypes
β_2 AR _{CAM}	β_2 -Adrenoceptor, constitutively active mutant
[γ - ³² P]GTP	GTP, γ -labeled with ³² P
A _{2a} R	Adenosine receptor subtype 2A
AC	Adenylyl cyclase
ACKR1	Atypical chemokine receptor 1

AIPGs	N^G -acylated imidazolylpropylguanidines
B ₂ R	Bradykinin B ₂ receptor
Balb/C, C57Bl/6	Mouse strains
cAMP	3',5'-Cyclic adenosine monophosphate
CCR5	C–C chemokine receptor type 5
CNS	Central nervous system
D ₁ R, D ₂ R	Dopamine receptor subtypes
DRY	Aspartate–arginine–tyrosine motif at the bottom of the third transmembrane helix of a GPCR
e	Extracellular loop (e.g. e2)
EAE	Experimental autoimmune encephalitis
ECL	Extracellular loop
FLAG	Peptide tag (DYKDDDDK)
FPR1	Formyl peptide receptor 1
FPR26	FPR1 isoform
GABA _B R	Receptor for γ -amino butyric acid, subtype B
GAIP	G α -interacting protein (= regulator of G protein signaling RGS19)
GDP	Guanosine-5'-diphosphate
gp	Guinea pig (prefix)
GPCR	G protein-coupled receptor
Gs α_L	Stimulatory G protein, long splice variant
Gs α_S	Stimulatory G protein, short splice variant
GTP	Guanosine-5'-triphosphate
GTP γ S	Guanosine 5'-O-[γ -thio]triphosphate (non-hydrolysable GTP derivative)
G α_{i1} , G α_{i2} , G α_{i3} , G $\alpha_{i/o}$	Inhibitory G protein isoforms
G α_q	G protein isoform activating phospholipase C
G α_s	Stimulatory G protein
G $\beta_1\gamma_2$	G protein complex, consisting of G β_1 and G γ_2
h	Human (prefix)
h(gpE2)H ₁ R	Chimeric receptor (human H ₁ R with second extracellular loop from guinea pig H ₁ R)
h(gpNgpE2)H ₁ R	Chimeric receptor (human H ₁ R with N-terminus and second extracellular loop from guinea pig H ₁ R)
H ₁ R, H ₂ R, H ₃ R, H ₄ R	Histamine receptor subtypes
HDC	Histidine decarboxylase
HeLa	Cervix carcinoma cell line
His ₆	Hexahistidine tag
HL-60	Human promyelocytic leukemia cell line
HPLC-MS/MS	High performance liquid chromatography-coupled tandem mass spectrometry
K _D	Ligand dissociation constant
K _M	Michaelis–Menten constant, substrate concentration resulting in 50% of maximum enzymatic reaction speed
LH/CG receptor	Receptor for luteinizing hormone/choriogonadotropin

m	Murine (prefix)
M ₃ R	Muscarinic receptor subtype 3
NAMH	N ^α -methylhistamine
NgpChH ₂ R-Gsα ₅	Fusion protein of Gsα ₅ with a chimeric receptor (N-terminus to transmembrane domain 3 from guinea pig H ₂ R plus transmembrane domain 4 to C-terminus from human H ₂ R)
NhCgpH ₂ R-Gsα ₅	Fusion protein of Gsα ₅ with a chimeric receptor (N-terminus to transmembrane domain 3 from human H ₂ R plus transmembrane domain 4 to C-terminus from guinea pig H ₂ R)
pEC ₅₀	Negative decadic logarithm of the agonist concentration that causes 50% of the maximum effect
pIC ₅₀	Negative decadic logarithm of the antagonist concentration that causes 50% inhibition
pK _b	Negative decadic logarithm of a dissociation constant determined in a functional assay
PKC	Protein kinase C
pK _i	Negative decadic logarithm of a dissociation constant determined in a competition binding assay
PLC	Phospholipase C
PTX	Pertussis toxin
r	Rat (prefix)
RAMH	(R)-α-methylhistamine
RGS4	Regulator of G protein signaling 4
SAR	Structure-activity relationship
S49	Murine lymphoma cell line
Sf9, Sf21	Insect cell lines originating from ovarian cells of <i>Spodoptera frugiperda</i>
Th1, Th2	Differentially polarized T helper cell subgroups
TM	Transmembrane helix of a G protein-coupled receptor
TMN	Tuberomamillary nucleus
U373 MG	Human astrocytoma cell line
V _{max}	Maximum enzymatic reaction speed in the presence of saturating substrate concentrations

1 Principles of GPCR Analysis in the Sf9 Cell Expression System

1.1 The Sf9 Cell Expression System

Pharmacological characterization of GPCRs is commonly performed in transfected mammalian cells or in cells that endogenously express the receptor of interest (Kenakin 1996). There are, however, several problems of mammalian cell systems.

First, mammalian cells normally express various additional GPCRs, which may result in GPCR heteromerization or signaling crosstalk (Breitwieser 2004; Prezeau et al. 2010; Gomes et al. 2016). For example, signaling crosstalk between GPCRs has been described for the $G\alpha_i$ -coupled $GABA_B$ R and the $G\alpha_q$ -coupled $mGlu_{1A}$ R (Rives et al. 2009). Another example is ACKR1 (atypical chemokine receptor 1), which has been shown to functionally antagonize CCR5 by forming ACKR1/CCR5 heterodimers (Chakera et al. 2008). Second, the presence of other constitutively active receptors may interfere with the analysis of agonist-independent activity of the receptor of interest. For example, the inverse FPR1 agonist cyclosporin H failed to inhibit basal $G\alpha_i$ protein activity in HL-60 cells, indicating that these cells additionally express other constitutively active receptors different from FPR1 (Wenzel-Seifert and Seifert 1993; Seifert and Wenzel-Seifert 2003). Third, promiscuous G protein coupling of GPCRs in the presence of several G protein subtypes may preclude the analysis of GPCR-G protein selectivity (Woehler and Ponimaskin 2009). Finally, some GPCRs are only expressed at low levels in mammalian cells, rendering it difficult to obtain a sufficiently high signal-to-noise ratio in functional and ligand binding assays.

As discussed in a comprehensive review article (Schneider and Seifert 2010c), the problems listed above are effectively addressed by using the Sf9 cell expression system. Sf9 cells are derived from the Sf21 cell line, which had been originally isolated from the pupal ovarian tissue of the American fall army worm (*Spodoptera frugiperda*). The protein of interest is expressed by infecting Sf9 cells with baculoviruses encoding the corresponding gene. Although Sf9 cells express $G\alpha_i$ -, $G\alpha_q$ -, and $G\alpha_s$ -like proteins, insect cell $G\alpha_i$ is not activated by mammalian GPCRs. This renders Sf9 cells a functionally “ $G\alpha_i$ -free” system and permits the analysis of $G\alpha_i$ -coupled receptors without the necessity of pertussis toxin (PTX)-mediated GPCR/ $G\alpha_i$ uncoupling. Also, PTX would not be active in Sf9 cells, because it does not enter the cells (Wenzel-Seifert et al. 1998). By contrast, uncoupling of $G\alpha_i$ -coupled GPCRs by PTX in mammalian cells is problematic. Despite entering mammalian cells, PTX is not capable of completely inactivating all $G\alpha_i$ proteins (Wenzel-Seifert and Seifert 1990).

Moreover, Sf9 cells do not express constitutively active GPCRs and therefore provide a low-background environment for the analysis of agonist-independent receptor activity. Furthermore, the highly efficient baculovirus promoters lead to very high expression levels of GPCRs in Sf9 cells. This results in high signal-to-noise ratios in binding assays and allows the purification of receptor protein, e.g. for crystallization purposes. Finally, as explained below, Sf9 cell membranes expressing large amounts of GPCRs and G proteins can be used to study G protein activation in steady-state GTPase assays and experiments with [35 S]GTP γ S ([35 S]-labeled guanosine 5'-O-[γ -thio]triphosphate).

For the preparation of baculoviruses encoding the gene of interest, several straightforward methods are established. The Sf9 cell studies discussed in this chapter were performed by using the BaculoGold™ kit from Invitrogen. As explained in Fig. 1, the gene of interest (in this example hH_4 R) is cloned into a pVL1392 baculovirus transfer vector, which is transfected into Sf9 cells together with the missing part of the baculovirus genome (BaculoGold™ DNA).

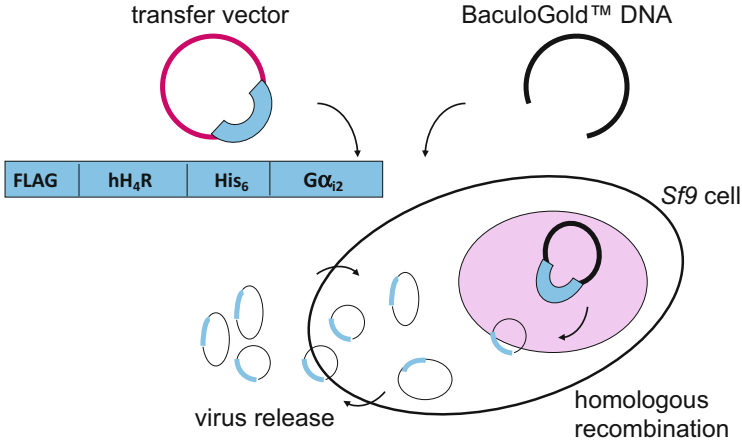


Fig. 1 Preparation of baculoviruses for the expression of a GPCR (example for H₄R-Gα₁₂ fusion protein). The gene of interest (in this case a FLAG-tagged hH₄R fused to Gα₁₂ via a His₆ linker) is cloned into a pV11392 transfer vector. The plasmid and the missing part of the baculovirus DNA (BaculoGold™ DNA) are co-transfected into Sf9 cells. The full baculovirus genome is completed in Sf9 cells by homologous recombination. The cells start to release virus particles into the surrounding medium

After that, the full baculovirus genome with the integrated receptor gene is reconstituted in the host cells by homologous recombination. The cell then releases virus particles into the surrounding medium which is harvested and used for further infections. A detailed protocol for the production and maintenance of genetically modified baculoviruses was published in *Methods in Enzymology* (Schneider and Seifert 2010a). Numerous examples of the characterization of Gα_q-, Gα_s-, and Gα_i-coupled receptors reconstituted in Sf9 insect cells were documented by Schneider and Seifert (2010c). In this chapter, an in-depth discussion of the pharmacological characterization of histamine receptors in Sf9 cell membranes is provided.

1.2 Methods for the Characterization of Histamine Receptors in Sf9 Cell Membranes

1.2.1 The G Protein Cycle

The G protein activation cycle (Gilman 1987; Oldham and Hamm 2008), which is explained in the following, is the basis for the methods used to generate the functional histamine receptor data discussed in this chapter. When histamine binds to the hH₄R, the receptor protein undergoes a conformational change and interacts with an inactive GDP-bound heterotrimeric G protein (Fig. 2 step 1). This induces GDP release and the formation of the so-called ternary complex, which contains agonist, receptor and guanine-nucleotide-free G protein (Fig. 2, step 2). It is generally accepted that a GPCR exhibits its highest agonist-binding affinity, when it is part of the ternary complex. The interaction between agonist-bound

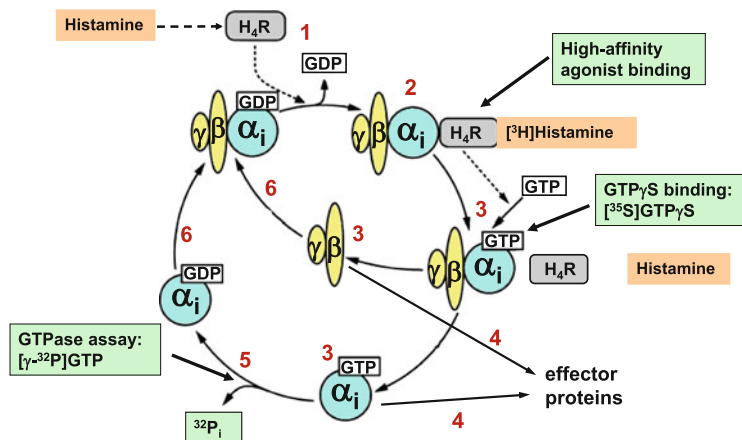


Fig. 2 Stimulation of $G\alpha_i$ -proteins by the histamine H_4R and resulting G protein cycle. The numbers designate the different stages of the cycle and are explained in detail in Sect. 1.2.1

GPCR and G protein promotes GTP binding to the $G\alpha$ -subunit. This weakens the intermolecular interactions in the G protein and in the ternary complex, breaking the complex up into agonist and GPCR as well as $G\alpha$ - and $G\beta\gamma$ subunit (Fig. 2, step 3).

After their dissociation from the receptor, the active GTP-loaded $G\alpha$ subunit and the $G\beta\gamma$ part interact with various effector proteins (Fig. 2, step 4) and induce numerous biochemical processes. Such effects include activation ($G\alpha_s$) or inhibition ($G\alpha_i$) of membranous adenylyl cyclase (AC), modulation of ion channel activity ($G\beta\gamma$, $G\alpha_i$) or stimulation of phospholipase C (PLC) activity followed by intracellular Ca^{2+} mobilization ($G\beta\gamma$, $G\alpha_q$). As long as GTP is bound to $G\alpha$, the $G\alpha$ and $G\beta\gamma$ subunits are active. To terminate signaling, the $G\alpha$ subunit inactivates itself by its intrinsic GTPase activity, resulting in conversion of the bound GTP to GDP and release of inorganic phosphate (Fig. 2, step 5). The inactive GDP-bound $G\alpha$ subunit re-associates with $G\beta\gamma$ and becomes available for another cycle (Fig. 2, step 6).

1.2.2 High Affinity Radioligand Binding

High affinity radioligand binding with histamine receptors is performed with radiolabeled agonists, e.g. tritiated histamine ($[^3H]$ histamine). Normally, agonists show their highest affinity to the ternary complex (Fig. 2, step 2) and stabilize the active receptor conformation. Thus, agonistic radioligands preferentially label the G protein-coupled high-affinity receptor population. When two populations of GPCRs with different G protein coupling states occur simultaneously, the saturation or competition curves with agonistic radioligands may become biphasic, which allows the determination of high-affinity and a low-affinity binding constants. This was, e.g., demonstrated for histamine H_2R (Houston et al. 2002) as well as for the β_2 -adrenergic receptor (β_2AR) or the dopamine D_1R (Gille and Seifert 2003). By contrast, inverse agonists interact preferentially with the inactive receptor state and

therefore show increased affinity to uncoupled GPCRs. Neutral antagonists do not discriminate between active and inactive receptor states and label both receptor conformations with comparable affinity.

For some experiments it may be required to convert GPCRs to their inactive conformation by disrupting receptor-G protein interactions. This is achieved by addition of GTP γ S (guanosine 5'-O-[γ -thio]triphosphate), which binds to the G α -subunit like GTP (Fig. 2, step 3), but cannot be hydrolyzed by the G α subunit (Gilman 1987). Thus, no GDP-loaded G protein is available anymore for the formation of new ternary complexes resulting in uncoupling of the entire GPCR population. This is normally reflected by a dramatic reduction in the binding affinity of agonistic radioligands. A detailed protocol for high-affinity agonist binding assays as well as example data for various receptor/G protein systems is provided in book chapters about GPCR/G protein co-expression and fusion protein systems in Sf9 cell membranes (Schneider and Seifert 2010a, b).

1.2.3 Steady-State GTPase Assays

In steady-state GTPase assays, the intrinsic GTPase activity (Fig. 2, step 5) of the active GTP-bound G α -subunit is determined (Gilman 1987; Schneider and Seifert 2010a). This is achieved by quantitating radioactive inorganic phosphate released after G α -mediated hydrolysis of [γ -³²P]GTP. The steady-state GTPase assay represents a very proximal readout of GPCR activation, which directly reflects GPCR-mediated G protein stimulation. By contrast, functional assays analyzing more distal parameters (e.g., Ca²⁺-, cAMP- or reporter gene assays) are often influenced by signal amplification processes, making valid conclusions about the original extent of receptor activation difficult. Technical details of the steady-state GTPase assay were explained in two book chapters about GPCR/G protein co-expression and fusion protein systems in Sf9 cells (Schneider and Seifert 2010a, b).

Steady-state GTPase assays can be used for the functional characterization of ligands in medicinal chemistry projects. In addition, these assays provide information about the efficacy of receptor-G protein interactions. In Michaelis–Menten kinetics experiments with increasing concentrations of the substrate [γ -³²P]GTP, the K_M and V_{max} value of the G α -GTPase can be determined (Schneider and Seifert 2009, 2010a). Subtraction of the GTPase activity in the presence of a full inverse agonist from the activity elicited by a full agonist yields the total receptor-regulated GTPase activity (ΔV_{max}). Dividing the ΔV_{max} value by B_{max} (maximum number of radiolabeled receptor proteins) provides the so-called turnover number, which signifies the number of GTP molecules hydrolyzed per minute, resulting from the activation of a single GPCR protein (Schneider and Seifert 2010a).

1.2.4 [³⁵S]GTP γ S Binding Assays

The [³⁵S]GTP γ S binding assay is another method to determine the functional effect of a ligand at a very proximal level of GPCR signal transduction. As depicted in Fig. 2, GTP γ S binds to the activated G α -subunit instead of GTP, resulting in the dissociation of the ternary complex (Fig. 2, step 3). Unlike GTP, however, GTP γ S

cannot be hydrolyzed by the intrinsic GTPase activity of $G\alpha$ (Gilman 1987), resulting in an accumulation of GTP γ S-bound $G\alpha$ -subunits. When radiolabeled [35 S]GTP γ S is used, the amount of activated $G\alpha$ subunits can be quantitated by scintillation counting, allowing the characterization of $G\alpha$ activation kinetics (time course of [35 S]GTP γ S- $G\alpha$ accumulation) and the determination of agonist- and inverse-agonist modulated $G\alpha$ activation. When the total ligand-regulated $G\alpha$ activation (maximum effect of full agonist minus activation level in the presence of a full inverse agonist) is divided by the B_{\max} value from radioligand binding, the so-called coupling factor is obtained. Similar to the aforementioned turnover number, the coupling factor provides information about the number of $G\alpha$ subunits stimulated by a single GPCR protein.

Furthermore, saturation binding experiments with increasing concentrations of [35 S]GTP γ S yield information about alterations of $G\alpha$ affinity to [35 S]GTP γ S under various conditions (e.g., constitutive receptor activity, agonist- or inverse agonist-induced effects). Finally, [35 S]GTP γ S binding assays are useful to pharmacologically characterize new ligands synthesized during the course of medicinal chemistry projects. A detailed experimental protocol of [35 S]GTP γ S binding assays as well as an explanation of how to analyze and interpret the data is provided in comprehensive book chapters about the characterization of GPCR/ $G\alpha$ co-expression and fusion protein systems in Sf9 cell membranes (Schneider and Seifert 2010a, b).

1.2.5 Fusion Protein Systems

Mammalian GPCRs and G protein $G\alpha$ and $G\beta\gamma$ subunits can be readily co-expressed in the baculovirus/Sf9 cell system yielding useful systems for the pharmacological characterization of GPCR ligands and receptor-G protein interactions. However, sometimes co-expression systems produce only insufficient GPCR-mediated $G\alpha$ activation (Seifert et al. 1998a; Gille and Seifert 2003). Specifically, $G\alpha_s$ proteins rapidly dissociate from the plasma membrane (Yu and Rasenick 2002) and therefore cannot be efficiently activated by a co-expressed GPCR. This problem is solved by constructing GPCR- $G\alpha$ fusion proteins (Fig. 3) that guarantee close proximity of receptor and G protein.

This approach was successfully used for the pharmacological characterization of $G\alpha_s$ -coupled receptors like the β_2 AR (Bertin et al. 1994; Seifert et al. 1998a) or the histamine H_2 R (Wenzel-Seifert et al. 2001). GPCR- $G\alpha$ fusion proteins of β_2 AR, FPR1 or dopamine D_1 R allowed a detailed examination of $G\alpha$ -isoform specificity of these receptors (Wenzel-Seifert et al. 1999; Wenzel-Seifert and Seifert 2000; Gille and Seifert 2003). GPCR- $G\alpha$ fusion proteins are also useful controls to exclude activation of Sf9 cell G proteins by a specific mammalian GPCR. Normally, the turnover number from steady-state GTPase assays or the coupling factor from [35 S]GTP γ S binding experiments should be around unity in fusion protein systems, corresponding to linear signaling. A coupling factor >1 in a GPCR- $G\alpha$ fusion protein system, however, indicates additional activation of insect cell proteins.

The fusion protein approach can also be applied to generate GPCR-RGS fusion proteins. RGS proteins (regulators of G protein signaling) activate the intrinsic GTPase activity of $G\alpha$ proteins. GPCR-RGS fusion proteins bring the RGS protein in close proximity to receptor and G protein. This may enhance signal intensity in

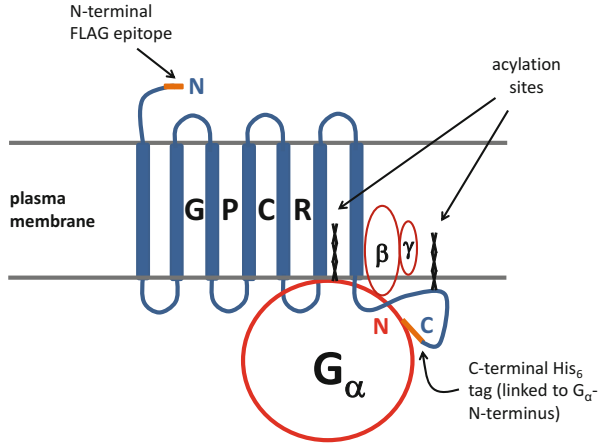


Fig. 3 Structure of a GPCR-G α fusion protein. The GPCR is N-terminally tagged with a FLAG epitope, which allows detection by an anti-FLAG antibody, and connected to the N-terminus of a G α -subunit via a His₆ linker. G α proteins are anchored in the plasma membrane via their acylation sites. The interaction between G α_s proteins and the plasma membrane is only weak in co-expression systems, but can be significantly improved in GPCR-G α_s fusion proteins

steady-state GTPase assays. The first GPCR-RGS fusion proteins were constructed in 2003 (Bahia et al. 2003). A detailed discussion of various aspects of co-expression and fusion protein systems was provided in *Methods in Enzymology* (Schneider and Seifert 2010a, b).

2 Pharmacological Characterization of Human Histamine Receptors in Sf9 Insect Cells

The biogenic amine histamine is formed by histidine decarboxylase (HDC)-mediated decarboxylation of the precursor amino acid histidine. Histamine is stored in granula of mast cells and basophils and occurs in enterochromaffin-like cells of the stomach (Panula et al. 2015). Moreover, by means of a highly sensitive HPLC-MS/MS-based detection method, histamine was identified in lymph nodes and thymus of C57Bl/6 and Balb/c mice (Zimmermann et al. 2011). In the central nervous system (CNS), histamine occurs as a neurotransmitter. It is synthesized in histaminergic neurons that emerge from the tuberomammillary nucleus (TMN) in the posterior hypothalamus and spread to numerous regions throughout the brain (Schneider et al. 2014a, b; Panula et al. 2015). The distribution of histamine in the body indicates its most important functions, namely the regulation of inflammatory/allergic reactions, stimulation of gastric acid secretion and neurotransmission. Most of the histamine effects are mediated by four G protein-coupled receptors, H₁R, H₂R, H₃R, and H₄R (Seifert et al. 2013; Panula et al. 2015). Additionally histamine acts on some non-histaminergic targets, e.g. at NMDA

receptors (Vorobjev et al. 1993; Panula et al. 2015), which, however, is not in the focus of mainstream histamine research.

This section addresses the results obtained from the pharmacological characterization of the four human histamine receptor isoforms in Sf9 cells. Other species variants will only be mentioned, when this is required by the context (e.g., comparisons of human and guinea pig H₁R or H₂R). Moreover, data from the characterization of ligands in medicinal chemistry projects will only be discussed, when they lead to new insights about structure and conformation of the corresponding receptor. Finally, publications that contain only “in silico” results without experimental verification will be omitted, since the purpose of this chapter is specifically the expression and characterization of human histamine receptors in the Sf9 cell system. For detailed information on the analysis of histamine receptor species variations in Sf9 cells or for the characterization of histamine receptor subtypes in cellular systems other than insect cells, the reader is referred to comprehensive review articles (Seifert et al. 2013; Strasser et al. 2013; Panula et al. 2015).

2.1 The Histamine H₁ Receptor

2.1.1 General Information About the Histamine H₁R

The H₁R is ubiquitously expressed, specifically in lung, CNS, and blood vessels. It preferentially couples to G $\alpha_{q/11}$ proteins, causing PLC and protein kinase C (PKC) activation as well as inositol-1,4-5-trisphosphate (IP₃) formation and intracellular Ca²⁺ release (Seifert et al. 2013; Panula et al. 2015). The typical signs of a type I allergic reaction like pruritus, increased vascular permeability, and edema are caused by H₁R activation. Therefore, administration of H₁R antagonists (so-called antihistamines) belongs to the most important anti-allergic therapeutic interventions (Simons and Simons 2011), e.g. for the treatment of allergic rhinitis. The H₁R is expressed on various types of immune cells, specifically on T cell subsets and dendritic cells and influences T cell polarization (Neumann et al. 2014). Moreover, as indicated by results from H₁R-deficient mice, the H₁R plays a role in various models of inflammatory diseases, e.g. nasal allergy, Th2-driven allergic asthma, atopic dermatitis or experimental autoimmune encephalitis (EAE) (Neumann et al. 2014). In the CNS, H₁R is involved in the regulation of locomotor activity, emotions, cognitive functions, arousal, sleep and circadian rhythm or pain perception (Schneider et al. 2014a). Moreover, the H₁R participates in the modulation of energy consumption, food intake, and respiration. H₁R blockade with antagonists increases susceptibility to seizures (Schneider et al. 2014a). Sedation, the most important side effect of brain-penetrating first-generation antihistamines, is caused by antagonism at H₁R in the CNS (Simons and Simons 2011; Neumann et al. 2014). The human H₁R (hH₁R) is endogenously expressed by various human cell lines. HeLa cervix carcinoma cells as well as U373 MG astrocytoma cells are used since more than two decades to study hH₁R pharmacology and signal transduction (Seifert et al. 2013). In the following, the

results from the characterization of the human H₁R in the Sf9 insect cell expression system will be discussed.

2.1.2 Characterization of the hH₁R in Sf9 Cell Membranes

The hH₁R was extensively characterized in Sf9 cells with regard to ligand pharmacology, and activation of G proteins. Moreover, the pharmacological differences between the hH₁R and its guinea pig orthologue (gpH₁R) were addressed by mutational and molecular modeling studies. An overview of the most important results is provided in Table 1.

Although Sf9 cells contain an endogenous PLC-stimulating G α_q -like protein (Hepler et al. 1993), histamine does not induce a significant rise in steady-state GTPase activity in Sf9 cell membranes expressing the hH₁R alone (Houston et al. 2002). Only co-expression of the hH₁R with the regulators of G protein signaling RGS4 and GAIP (G-alpha-interacting protein, RGS19) unmasks an interaction of hH₁R with insect cell G α_q , resulting in histamine-induced stimulatory effects of 142% (RGS4) and 126% (GAIP) (Houston et al. 2002). These results indicate that the intrinsic GTPase activity of Sf9 cell G α_q is rate-limiting for hH₁R-mediated G protein activation in Sf9 cell membranes. This is probably due to a low number of G proteins relative to hH₁R molecules. RGS proteins commonly accelerate the intrinsic GTPase activity of G α proteins, which results in a higher turnover and in increased availability of inactive GDP-bound G α subunits (Fig. 2).

Due to its favorable properties, the Sf9 cell hH₁R/RGS protein co-expression system is routinely used to characterize affinity (radioligand binding), activity (steady-state GTPase assays), and binding mode of hH₁R ligands in medicinal chemistry projects. This revealed major pharmacological differences between H₁R species isoforms. Specifically, some agonistic bulky 2-phenylhistamines and histaprodifens exhibited increased efficacy and up to tenfold higher potency at gpH₁R as compared to hH₁R (Seifert et al. 2003). Such differences were also observed for antagonists. Most notably, the potency of several arpromidine-type H₁R antagonists was up to tenfold higher at gpH₁R than at hH₁R (Seifert et al. 2003). Mutagenesis experiments were performed to elucidate the molecular basis of these pharmacological species differences. Basing on the hypothesis that smaller amino acid substitutions render the gpH₁R binding pocket more flexible than the corresponding site at the hH₁R, the amino acids 153 or 433 of the hH₁R were mutated into "gpH₁R direction" (Phe-153 \rightarrow Leu 153 or Ile-433 \rightarrow Val 433) (Seifert et al. 2003). Although this attempt was unsuccessful in terms of generating gpH₁R-like pharmacology, the mutations dramatically decreased hH₁R receptor expression, function, electrophoretic mobility as well as [³H]mepyramine (triated 2-((2-(Dimethylamino)ethyl)(p-methoxybenzyl)amino)-pyridine) affinity, suggesting that these amino acid positions are essential for correct folding and expression of the H₁R (Seifert et al. 2003). In addition, the hH₁R-F153L/I433V double mutant was studied. Although this protein was excellently expressed in Sf9 cell membranes, there were only partial changes in pharmacology. Thus, Phe-153 and Ile-433 cannot fully explain the species difference between hH₁R and gpH₁R (Seifert et al. 2003).

Table 1 Overview on the pharmacological characterization of the human histamine H₁R in the Sf9 cell expression system

Expressed proteins	Most important new findings	Reference
Only hH ₁ R	No histamine-induced signal in steady-state GTPase assays → no interaction with Sf9 cell G proteins	
hH ₁ R + RGS4 hH ₁ R + GAIP	<ul style="list-style-type: none"> • Interaction of hH₁R with insect cell Gα_q unmasked • Intrinsic GTPase activity of Sf9 cell Gα_q is rate-limiting for hH₁R-mediated G protein activation in Sf9 cell membranes. • Histamine-induced stimulation in steady-state GTPase assay: 142% with RGS4 and 126% with GAIP 	Houston et al. (2002)
hH ₁ R, gpH ₁ R + RGS4 or GAIP	<ul style="list-style-type: none"> • Higher efficacy and up to tenfold higher potency of bulky 2-phenylhistamines and histaprodidens at gpH₁R than at hH₁R • Potency of several arpromidine-type H₁R antagonists up to tenfold higher at gpH₁R than at hH₁R 	
hH ₁ R-F153L ^a hH ₁ R-I433V ^a + RGS4 or GAIP	<p><i>Compared to wild-type hH₁R:</i></p> <ul style="list-style-type: none"> • Dramatic reduction of expression, function and [³H]mepyramine affinity, altered electrophoretic mobility • Mutated amino acid positions required for correct folding and expression of the H₁R 	Seifert et al. (2003)
hH ₁ R-F153L/I433V ^a double mutant + RGS4 or GAIP	<ul style="list-style-type: none"> • Excellent expression, but only partial change of pharmacological properties (compared to wild-type hH₁R) • Mutated amino acid positions not solely responsible for the pharmacological difference between hH₁R and gpH₁R 	
hH ₁ R, gpH ₁ R, rH ₁ R, bH ₁ R + RGS4	<ul style="list-style-type: none"> • Differential interaction of chiral histaprodidens with hH₁R, gpH₁R, rH₁R, and bH₁R • Two compounds showed agonism at gpH₁R, but antagonism at hH₁R, bH₁R, and rH₁R. • Potency rank order of histaprodidens: hH₁R < bH₁R < rH₁R < gpH₁R; structure and pharmacology of hH₁R similar to bH₁R; gpH₁R resembles rH₁R • Docking studies (active-state model of gpH₁R): multiple interaction sites between dimeric histaprodiden and gpH₁R (Asp-116, Ser-120, Lys-187, Glu-190, and Tyr-432) 	Strasser et al. (2008a)
hH ₁ R, gpH ₁ R h(gpNgpE2)H ₁ R ^b h(gpE2)H ₁ R ^c + RGS4	<ul style="list-style-type: none"> • Higher maximum G_q-activation and lower potency of histamine at h(gpNgpE2)H₁R as compared to hH₁R or h(gpE2)H₁R • Differences between hH₁R and gpH₁R in N-terminus and ECL2 not responsible for pharmacological species differences • Unexpected reduction of pK_i and pEC₅₀ in the series hH₁R > h(gpE2)H₁R > h(gpNgpE2)H₁R for three phenoprodidens (change of ligand orientation?) 	Strasser et al. (2008b)

(continued)

Table 1 (continued)

Expressed proteins	Most important new findings	Reference
hH ₁ R, gpH ₁ R h(gpNgpE2)H ₁ R h(gpE2)H ₁ R + RGS4	<ul style="list-style-type: none"> • Association rate constants for h(gpNgpE2)H₁R significantly different from the constants for hH₁R and gpH₁R. • Extracellular surface of the H₁R influences ligand binding and recognition and guiding of the ligand into the binding pocket. 	Wittmann et al. (2011)
hH ₁ R, gpH ₁ R, bH ₁ R, rH ₁ R + RGS4	<ul style="list-style-type: none"> • Identification of bulky phenylhistamines with higher potency and affinity at hH₁R than at gpH₁R • Molecular modeling: higher hH₁R potency possibly due to a more effective van der Waals interaction with Asn^{2.61} of hH₁R as compared to Ser^{2.61} of gpH₁R • Two distinct binding modes of phenoprodifens cause Trp^{6.48} (part of the rotamer toggle switch activation mechanism) to assume either an active or an inactive conformation 	Strasser et al. (2009)
hH ₁ R, gpH ₁ R + RGS4 or GAIP	<i>N</i> ^G -acylated imidazolylpropylguanidines are partial H ₁ R agonists with higher efficacies at hH ₁ R than at gpH ₁ R	Xie et al. (2006a, b)

^aMutations were performed to make the hH₁R “more similar” to gpH₁R and to investigate the resulting alterations of receptor pharmacology

^bHuman H₁R with N-terminus and ECL2 of guinea pig H₁R

^cHuman H₁R with ECL2 of the guinea pig H₁R

A series of chiral histaprodifens was pharmacologically characterized at hH₁R and gpH₁R as well as rat (r) and bovine (b) H₁R, revealing differential interaction with H₁R species isoforms. Two of the compounds showed agonism at gpH₁R, but were antagonists at hH₁R, bH₁R, and rH₁R. The histaprodifens followed the rank order of potency hH₁R < bH₁R < rH₁R < gpH₁R. The hH₁R was pharmacologically and structurally similar to bH₁R, while gpH₁R resembled rH₁R (Strasser et al. 2008a). Docking studies with an active-state model of the gpH₁R and dimeric histaprodifen revealed multiple interaction sites, involving hydrogen bonds and electrostatic interactions with Asp-116, Ser-120, Lys-187, Glu-190 and Tyr-432 (Strasser et al. 2008a).

Since the amino acid sequence of the N-terminus and the second extracellular loop (ECL2) exhibit major differences between hH₁R and gpH₁R, it was hypothesized that these structures may be responsible for the preferred binding of bulky agonists to gpH₁R as compared to hH₁R. To address this hypothesis, wild-type hH₁R and gpH₁R as well as the chimeric receptors h(gpE2)H₁R (hH₁R with ECL2 from gpH₁R) and h(gpNgpE2)H₁R (hH₁R with N-terminus and ECL2 from gpH₁R) were co-expressed with RGS4 in Sf9 cells and compared in radioligand binding and steady-state GTPase assays (Strasser et al. 2008b). A small inverse agonistic effect of mepyramine suggests that all four receptors show only low constitutive activity. Histamine potency in steady-state GTPase assays decreased in the series hH₁R > h(gpE2)H₁R > h(gpNgpE2)H₁R. Maximum G_q-protein activation by histamine and the $\Delta V_{\max}/B_{\max}$ ratio (turnover number) was significantly enhanced at

h(gpNgpE2)H₁R as compared to hH₁R, gpH₁R, and h(gpE2)H₁R, despite a very low expression level of h(gpNgpE2)H₁R. This indicates that histamine induces a h(gpNgpE2)H₁R conformation which is specifically efficient at activating G proteins (Strasser et al. 2008b). Molecular dynamics simulations suggest that the replacement of N-terminus and ECL2 affect the network of hydrogen bonds between N-terminus, ECL1 and ECL2 and alter the conformation and flexibility of ECL2. Thus, either the replacement of the N-terminus or the combined exchange of N-terminus and ECL2 induces conformational alterations that increase the stimulatory effect of histamine and reduce its potency (Strasser et al. 2008b).

The hypothesis that major differences of N-terminus and ECL2 cause the distinct pharmacology of hH₁R and gpH₁R, however, had to be rejected, since neither binding assays nor steady-state GTPase assays revealed more pronounced “gpH₁R-like” properties of h(gpNgpE2)H₁R and h(gpE2)H₁R (Strasser et al. 2008b). Instead, three members of a new class of histaprodidifens (phenoprodidifens) even exhibited a *reduction* of pK_i and pEC₅₀ values in the series hH₁R > h(gpE2)H₁R > h(gpNgpE2)H₁R (Strasser et al. 2008b). Previous molecular dynamics simulations with these compounds had suggested that they can adopt two distinct orientations in the gpH₂R binding pocket (Strasser et al. 2008a). Thus, the data may be explained by a change in ligand orientation in the series hH₁R – h(gpE2)H₁R – h(gpNgpE2)H₁R. Such changes, however, are probably determined early in ligand binding, which can only be addressed by kinetic binding studies (Strasser et al. 2008b).

Such experiments were performed with the antagonist [³H]mepyramine and the partial agonist phenoprodidifen using Sf9 cell membranes expressing RGS4 together with hH₁R, gpH₁R as well as the chimeric receptors h(gpNgpE2)H₁R and h(gpE2)H₁R (Wittmann et al. 2011). With regard to the association rate constant, h(gpNgpE2)H₁R significantly differed from both hH₁R and gpH₁R. Molecular dynamics simulations helped to explain, how the extracellular surface of the H₁R influences ligand binding kinetics, recognition of the ligand and guiding of the ligand into the binding pocket (Wittmann et al. 2011).

There are also exceptions, where bulky agonists do not interact more efficiently with gpH₁R than with hH₁R. Specifically, N^G-acylated imidazolylpropylguanidines (AIPGs) are partial H₁R agonists that exhibit higher efficacies at hH₁R as compared to gpH₁R (Xie et al. 2006a, b). Moreover, another study addressing the pharmacology of phenylhistamines and phenoprodidifens at human, guinea pig, bovine, and rat H₁R identified bulky phenylhistamines with higher potency and affinity at hH₁R as compared to gpH₁R (Strasser et al. 2009). A comparison of the hypothesized binding modes of these compounds with the binding mode of the previously characterized N^G-acylated imidazolylpropylguanidine UR-AK57 (N¹-(3-Cyclohexylbutanoyl)-N²-[3-(1*H*-imidazol-4-yl)propyl]guanidine) (Xie et al. 2006b) suggests that the higher potency at the hH₁R is caused by a more pronounced van der Waals interaction with Asn^{2.61} of hH₁R as compared to Ser^{2.61} of gpH₁R (Strasser et al. 2009). Moreover, phenoprodidifens seem to adopt two distinctly oriented binding modes that cause the highly conserved Trp^{6.48}, which is part of the toggle switch mechanism of GPCR activation (Shi et al. 2002), to assume either an active or an inactive conformation (Strasser et al. 2009).

2.2 The Histamine H₂ Receptor

2.2.1 General Information About the Histamine H₂R

The H₂R is ubiquitously expressed, most importantly in stomach, heart, and CNS (Seifert et al. 2013; Schneider et al. 2014a; Panula et al. 2015). Agonist binding to this receptor results in activation of G α_s -proteins that stimulate the adenylyl-cyclase-mediated production of the second messenger cAMP (Panula et al. 2015). The central role of the H₂R in the regulation of gastric acid production is the basis for the therapeutic use of H₂R antagonists to treat gastroesophageal reflux disease (Schubert and Peura 2008). The function of the H₂R in the brain is less well documented as for H₁R, but includes, e.g. modulation of cognitive processes and of circadian rhythm (Schneider et al. 2014a). Moreover, H₂R influences glucose metabolism and food intake (Schneider et al. 2014a).

Experiments with knockout mice have revealed that the histamine H₂R is involved in the regulation of immune responses, specifically in the modulation of Th1- or Th2-cell polarization. It should be noted, however, that the analysis of H₂R-deficient mice yields conflicting results, probably because of the variability of the disease models studied (Neumann et al. 2014). The human histamine H₂R (hH₂R) has been pharmacologically characterized in both human cells and in the Sf9 cell expression system (Seifert et al. 2013). Neutrophils are specifically well suited for the analysis of hH₂R pharmacology, because they are primary cells that can be easily isolated from human blood in large numbers. The hH₂R inhibits superoxide anion production induced by chemotactic peptides in neutrophils (Burde et al. 1989, 1990; Reher et al. 2012a) and eosinophils (Reher et al. 2012a). Moreover, H₂R activation induces functional differentiation of HL-60 promyelocytes (Klinker et al. 1996). Furthermore, it is discussed that decreased hH₂R function may contribute to inflammation in bronchial asthma (Seifert et al. 2013).

2.2.2 Characterization of the hH₂R in Sf9 Cell Membranes

The hH₂R was extensively characterized in Sf9 cells with regard to ligand pharmacology, and activation of G proteins. Moreover, the pharmacological differences between the hH₂R and its guinea pig orthologue (gpH₂R) were addressed by mutational and molecular modeling studies. An overview of the most important results is provided in Table 2.

Functional expression of the human hH₂R in Sf9 cells requires G α_s proteins as intracellular coupling partners. Indeed, Sf9 cells express endogenous G α_s proteins and activation of Sf9 cell G α_s has been reported for mammalian GPCRs, e.g. the bradykinin B2 receptor (Shukla et al. 2006), the LH/CG receptor (Narayan et al. 1996), or the histamine H₂R (Kühn et al. 1996). Mostly, however, the interaction of mammalian GPCRs with Sf9 cell G α_s shows only low productivity, which is most likely due to rapid dissociation of the activated G α_s subunit from the plasma membrane. Redistribution of stimulated G α_s proteins has been investigated in more detail in S49 lymphoma cells treated with the β -AR agonist isoproterenol (Ransnäs et al. 1989).

Table 2 Overview on the pharmacological characterization of the human histamine H₂R in the Sf9 cell expression system

Expressed proteins	Most important new findings	Reference
hH ₂ R-Gsα ₅ gpH ₂ R-Gsα ₅	Some H ₁ R agonists distinguish between H ₂ R species isoforms (steady-state GTPase assay)	Seifert et al. (2003)
hH ₂ R-Gsα _L hH ₂ R-Gsα _S	<i>Comparison of hH₂R-Gsα_L and hH₂R-Gsα_S:</i> <ul style="list-style-type: none"> • Similar expression level and [³H]tiotidine binding • B_{\max} from ligand-regulated [³⁵S]GTPγS binding $\gg B_{\max}$ from [³H]tiotidine binding → large part of fusion proteins not radiolabeled • GDP/GTPγS exchange velocity: hH₂R-G_{sαL} > hH₂R-G_{sαS} • Similar constitutive activity; comparable pharmacological properties of partial/inverse agonists 	Wenzel-Seifert et al. (2001)
hH ₂ R hH ₂ R-Gsα ₅	<ul style="list-style-type: none"> • Only hH₂R: no agonist-induced signal in steady-state GTPase assays, not even with GAIP • AC activation by hH₂R (Sf9 Gα_s) and by hH₂R-Gsα₅ • No activation of insect cell or co-expressed mammalian Gα_q by hH₂R in Sf9 cells • Mammalian Gα_q most likely inactive in Sf9 cells 	Houston et al. (2002)
hH ₂ R-Gsα ₅ gpH ₂ R-Gsα ₅	<ul style="list-style-type: none"> • Affinity of large guanidine-type agonists in [³H]tiotidine binding: hH₂R-Gsα₅ < gpH₂R-Gsα₅ • Disruption of guanidine-type agonist high-affinity binding by GTPγS more effective at hH₂R-Gsα₅ than at gpH₂R-Gsα₅ • Potencies and efficacies of guanidines in steady-state GTPase assays: gpH₂R-Gsα₅ > hH₂R-Gsα₅ 	Kelley et al. (2001)
hH ₂ R-Gsα ₅ gpH ₂ R-Gsα ₅ hH ₂ R-A271D-Gsα ₅ NgpChH ₂ R-Gsα ₅ ^a NhCgpH ₂ R-Gsα ₅ ^b	<ul style="list-style-type: none"> • Higher (more “gpH₂R-like”) potencies of guanidines in steady-state GTPase assays at hH₂R-A271D-Gsα₅ and NhCgpH₂R-Gsα₅ than at hH₂R-Gsα₅ • Efficacies of guanidine-type agonists at hH₂R-Gsα₅, hH₂R-A271D-Gsα₅, NgpChH₂R-Gsα₅ and NhCgpH₂R-Gsα₅ are lower than at gpH₂R-Gsα₅ • Potency and efficacy are independent H₂R properties 	
hH ₂ R-Gsα ₅ gpH ₂ R-Gsα ₅ hH ₂ R-C17Y-Gsα ₅ hH ₂ R-C17Y-A271D-Gsα ₅	<ul style="list-style-type: none"> • Potencies and efficacies of guanidines in steady-state GTPase assays with hH₂R-C17Y-A271D-Gsα₅: higher than at hH₂R-Gsα₅, but lower than at gpH₂R-Gsα₅ → Tyr-17/Asp-271 interaction not solely responsible for h/gp species differences • Possibly stabilization of ligand-specific receptor conformations • hH₂R-C17Y-Gsα₅: basal AC activity and agonist-induced steady-state GTPase activity reduced (impaired G protein coupling or degradation of Gsα₅?) 	Preuss et al. (2007b)

(continued)

Table 2 (continued)

Expressed proteins	Most important new findings	Reference
hH ₂ R-gpE2-Gsα _S ^c gpH ₂ R-hE2-Gsα _S ^d	Pharmacology of guanidines in steady-state GTPase assays not significantly changed by the mutations → interaction of the mutated residues with the guanidine-binding pocket unlikely	Preuss et al. (2007c)
hH ₂ R-K173A-Gsα _S hH ₂ R-K175A-Gsα _S	<ul style="list-style-type: none"> • Neither Lys-173- nor Lys-175 influence agonist binding in the hH₂R • Significantly lower histamine-induced steady-state GTPase signals of hH₂R-K173A-Gsα_S or hH₂R-K175A-Gsα_S → Lys173 and Lys175 important for Gsα_S activation? 	

^aSequence from N-terminus to TM3 from gpH₂R and sequence from TM4 to C-terminus from hH₂R

^bSequence from N-terminus to TM3 from hH₂R and sequence from TM4 to C-terminus from gpH₂R

^cFour e2 amino acids of hH₂R exchanged by the corresponding residues of gpH₂R

^dFour e2 amino acids of gpH₂R exchanged by the corresponding residues of hH₂R

Fusion of a GPCR to Gα_s keeps the G protein at the cell membrane and largely enhances G protein activation. This approach was used for the human histamine H₂R, which was expressed in Sf9 cells as a fusion protein with the long (G_{sαL}) or short (G_{sαS}) splice variant of Gα_s (Wenzel-Seifert et al. 2001). Both fusion proteins were expressed at a similar level in Sf9 cell membranes and the affinity of the radiolabeled H₂R agonist [³H]tiotidine (tritiated 1-cyano-3-[2-[[2-(diaminomethylideneamino)-1,3-thiazol-4-yl]methylsulfanyl]ethyl]-2-methyl-guanidine) was comparable (~ 32 nM) for hH₂R-Gsα_L and hH₂R-Gsα_S (Wenzel-Seifert et al. 2001). Unexpectedly, the B_{max} values of ligand-regulated [³⁵S]GTPγS binding for hH₂R-Gsα_L or hH₂R-Gsα_S exceeded the B_{max} value from [³H]tiotidine binding by ~tenfold, which suggests that a large subpopulation of fusion proteins is not labeled by the radioligand (Wenzel-Seifert et al. 2001).

G_{sαL} exhibits lower GDP affinity than Gsα_S, and therefore, the β₂AR-G_{sαL} fusion protein shows higher constitutive activity than β₂AR-Gsα_S (Seifert et al. 1998b). Similarly, the hH₂R-Gsα_L fusion protein exhibited a faster GDP/GTPγS exchange than hH₂R-Gsα_S. Surprisingly, however, unlike the corresponding β₂AR fusion proteins, hH₂R-Gsα_L and hH₂R-Gsα_S showed similar constitutive activity and comparable pharmacological properties of partial agonists and inverse agonists in steady-state GTPase and [³⁵S]GTPγS binding assays (Wenzel-Seifert et al. 2001). This illustrates that the GDP affinity of G proteins does not influence the constitutive activity of all GPCRs to the same extent (Wenzel-Seifert et al. 2001).

It has been reported that the rH₂R couples to insect cell Gα_q and increases intracellular Ca²⁺ in Sf9 cells (Kühn et al. 1996). However, this effect could not be confirmed and was also not observed with hH₂R or gpH₂R (Houston et al. 2002). Moreover, co-expressed GAIP did not unmask a potential interaction of hH₂R with insect cell Gα_q (steady-state GTPase assays) although this approach was successful

with hH₁R (Houston et al. 2002). The hH₂R did not even activate mammalian G α_q co-expressed in Sf9 cells or fused to the hH₂R (Ca²⁺ assays, high-affinity agonist binding and [³⁵S]GTP γ S binding) (Houston et al. 2002). Surprisingly, not even the hH₁R was able to activate co-expressed mammalian G α_q in Sf9 cells. Thus, mammalian G α_q was probably inactive in Sf9 cells, despite high expression levels, and therefore, Sf9 cells are not suited to investigate the interaction of GPCRs with mammalian G α_q (Houston et al. 2002).

When only hH₂R was expressed in Sf9 cells, no ternary complex formation with insect cell G α_s was observed in high-affinity agonist binding with [³H]tiotidine (effect of GTP γ S on histamine competition curve) and in [³⁵S]GTP γ S binding (characterization of the stimulatory effect of histamine). Surprisingly, however, AC assays clearly indicated hH₂R-mediated activation of insect cell G α_s . Thus, AC assays probably exhibit higher sensitivity than [³H]tiotidine high-affinity agonist binding or [³⁵S]GTP γ S binding and detect even very low insect cell G α_s stimulation (Houston et al. 2002). Co-expression of hH₂R with mammalian G α_s resulted in efficient G protein interaction (high-affinity agonist binding, [³⁵S]GTP γ S binding, AC assays). A further increase in interaction efficiency was observed for the hH₂R-G α_s fusion protein (Houston et al. 2002).

The fusion protein approach was also used for the pharmacological comparison of hH₂R and gpH₂R (Kelley et al. 2001). In [³H]tiotidine radioligand binding assays, the hH₂R-G α_s fusion protein expressed in Sf9 cells bound large guanidine-type agonists with lower affinity than gpH₂R-G α_s . Moreover, GTP γ S disrupted high-affinity binding of guanidine-type agonists at hH₂R-G α_s more efficiently than at gpH₂R-G α_s . This indicates that the guanidine-stabilized conformation of gpH₂R interacts more tightly with the tethered G protein than the corresponding conformation of hH₂R (Kelley et al. 2001). In steady-state GTPase assays, the potencies and efficacies of guanidines were also higher with gpH₂R-G α_s than with hH₂R-G α_s . However, the species isoforms did not differ in case of small agonists or antagonists (Kelley et al. 2001).

Based on molecular modeling data (bovine rhodopsin-based alignment), it was hypothesized that the high potency of guanidine-type agonists at gpH₂R is caused by the non-conserved Asp-271 in TM7 (Ala-271 in hH₂R). This hypothesis was tested by expressing the mutant hH₂R-A271D-G α_s as well as the chimeras NgpChH₂R-G α_s (N-terminus – TM3 from gpH₂R and TM4-C-terminus from hH₂R, containing Ala-271) and NhCgpH₂R-G α_s (N-terminus – TM3 from hH₂R, and TM4-C-terminus from gpH₂R, containing Asp-271) in Sf9 cell membranes (Kelley et al. 2001). In fact, steady-state GTPase assay data clearly showed increased potency of guanidines at both hH₂R-A271D-G α_s and NhCgpH₂R-G α_s , confirming the importance of Asp-271 in the gpH₂R for guanidine binding. Unexpectedly, the efficacies of guanidine-type agonists at hH₂R-G α_s and NgpChH₂R-G α_s as well as the more “gpH₂R-like” constructs hH₂R-A271D-G α_s and NhCgpH₂R-G α_s were lower than at gpH₂R. This demonstrates that potency and efficacy are independent properties of the H₂R. The modeling and experimental data suggest that an interaction between TM1 (Tyr-17) and TM7 (Asp-271) is important for the stabilization of the guanidine-induced agonistic conformation of the gpH₂R and therefore for guanidine efficacy.

This interaction is absent in hH₂R and in the other constructs analyzed by Kelley et al. (2001).

The hypothesis that a Tyr-17/Asp-271 interaction in the gpH₂R molecule stabilizes an active receptor conformation and increases efficacy of guanidine-type agonists was tested by characterizing the mutant fusion proteins hH₂R-C17Y-Gsα₅ and hH₂R-C17Y-A271D-Gsα₅ (Preuss et al. 2007b). As expected, the potencies and efficacies of guanidines in the steady-state GTPase assay were higher at the hH₂R-C17Y-A271D-Gsα₅ double mutant as compared to the wild-type hH₂R-Gsα₅ fusion protein, but they were still below the values determined for wild-type gpH₂R-Gsα₅. Thus, the Tyr-17/Asp-271 interaction is probably not solely responsible for the different pharmacology of hH₂R and gpH₂R (Preuss et al. 2007b). Moreover, the data suggest the stabilization of ligand-specific receptor conformations by agonists and inverse agonists in wild-type and mutant hH₂R-Gsα₅ fusion proteins (Preuss et al. 2007b).

The results from the analysis of the hH₂R-C17Y-Gsα₅ single mutant support the notion that an H-bond between Tyr-17 and Asp-271 stabilizes an active receptor conformation (Preuss et al. 2007b). The hH₂R-C17Y-Gsα₅ fusion protein exhibits lower basal AC and decreased agonist-induced GTPase activities (Preuss et al. 2007b), indicating impaired G protein coupling. One possible explanation may be degradation of the hH₂R-C17Y-Gsα₅ fusion protein in the Sf9 cells. This is suggested by the apparent molecular mass of 40 kDa instead of the expected ~80 kDa in Western blots (Preuss et al. 2007b).

In bovine rhodopsin (Palczewski et al. 2000) as well as in various aminergic GPCRs, e.g. dopamine D₂R (Shi and Javitch 2002), adenosine A_{2a}R (Kim et al. 1996), or muscarinic M₃ receptor (Scarselli et al. 2007), residues in the second extracellular loop, ECL2, probably contribute to ligand binding. Thus, it was hypothesized that differences in e2 may also determine the distinct pharmacology of hH₂R-Gsα₅ and gpH₂R-Gsα₅ (Preuss et al. 2007c). This hypothesis was addressed by generating mutant fusion proteins with the four e2 amino acids of hH₂R exchanged by the corresponding residues of gpH₂R (hH₂R-gpE2-Gsα₅) and vice versa (gpH₂R-hE2-Gsα₅). Steady-state GTPase assays, however, revealed that this exchange of ECL2 did not significantly alter the pharmacology of the receptors. Thus, the mutated residues most likely do not interact with the guanidine-binding pocket (Preuss et al. 2007c).

In both hH₂R and gpH₂R, Cys-174 probably forms a disulfide bond with Cys-91 in TM3 and is framed by two lysines in position 173 and 175 (Preuss et al. 2007c). A homology model of the hH₂R predicted that these two lysines are located close to the binding site of guanidine-type agonists and are involved in agonist binding (Preuss et al. 2007c). Thus, the two mutated fusion proteins hH₂R-K173A-Gsα₅ and hH₂R-K175A-Gsα₅ were expressed in Sf9 cells and analyzed in steady-state GTPase activity assays. The results, however, indicate that these mutations were ineffective at altering potency or efficacy of small as well as bulky H₂R agonists (Preuss et al. 2007c). Interestingly, the effect of histamine on steady-state GTPase activity of both hH₂R-K173A-Gsα₅ and hH₂R-K175A-Gsα₅ was reduced, which

suggests that the lysines in positions 173 and 175 increase the efficiency of hH₂R-coupling to G α_s (Preuss et al. 2007c).

2.3 The Histamine H₃ Receptor

2.3.1 General Information About the hH₃R

The G $\alpha_{i/o}$ -coupled histamine H₃R is mainly expressed on neurons and acts as a presynaptic auto- and heteroreceptor. It inhibits the release of histamine (Arrang et al. 1983, 1985), but also of other neurotransmitters such as acetylcholine, noradrenaline, dopamine, or glutamate (Haas et al. 2008). Additionally, there is increasing evidence that H₃R is expressed postsynaptically (Ellenbroek and Ghiabi 2014), where it regulates, e.g. dopamine D₁R signaling (Ferrada et al. 2008; Brabant et al. 2009). Knockout mouse models demonstrate that the H₃R regulates numerous behaviors like locomotor activity, pain perception, food intake, memory, circadian rhythm, cognition, and anxiety (Schneider et al. 2014b). Moreover, H₃R-deficiency reduces addictive behavior in mouse models of ethanol consumption, which is probably due to the reward-inhibiting function of an increased histamine release (Vanhanen et al. 2013; Schneider et al. 2014b). This renders the H₃R an interesting target for the treatment of alcohol addiction (Nuutinen et al. 2012). Despite the decade-long research on H₃R pharmacology, only the inverse H₃R agonist pitolisant is currently used as an orphan drug to treat narcoleptic patients (Dauvilliers et al. 2013). Mouse models suggest that, in contrast to the other three histamine receptor subtypes, the H₃R does not seem to play a major role in immunological processes and inflammation (Neumann et al. 2014).

2.3.2 Characterization of the hH₃R in Sf9 Cell Membranes

There is no standard human cell culture model available that endogenously expresses hH₃R. Thus, expression and characterization of hH₃R and its species orthologues in the Sf9 insect cell system is of major importance (Schnell et al. 2010a, b; Schnell and Seifert 2010; Seifert et al. 2013; Strasser et al. 2013). Sf9 cells do not express endogenous G α_i -like protein that could interact with the corresponding mammalian GPCRs. It is, therefore, required to co-express the receptor of interest with mammalian G α_i and G $\beta\gamma$ subunits. This, however, provides the unique opportunity to freely combine G α_i -coupled receptors with any G $\alpha_{i/o}$ isoform, allowing the characterization of G α_i isoform specificity of GPCRs. As described in the following sections, the pharmacology of the hH₃R was extensively characterized in Sf9 cells. An overview of the most important results is provided in Table 3.

Specificity of the hH₃R for G $\alpha_{i/o}$ Isoforms and Investigation of Protean Agonism

The hH₃R was co-expressed in Sf9 cells with G $\beta_1\gamma_2$ and G α_{i1} , G α_{i2} , G α_{i3} , or G α_o . All hH₃R/G protein combinations could be readily expressed in Sf9 cells, and a semiquantitative analysis of expression levels by Western blot (purified G α_{i2} and

Table 3 Overview on the pharmacological characterization of the human histamine H₃R in the Sf9 cell expression system

Expressed proteins	Most important new findings	Reference
hH ₃ R	No relevant stimulation of insect cell G proteins	
hH ₃ R + G α_{i1} , G α_{i2} , G α_{i3} or G α_o + G $\beta_1\gamma_2$	<ul style="list-style-type: none"> • hH₃R/Gα coupling ratio between 1:2 and 1:11 • Steady-state GTPase assay: high constitutive activity of hH₃R (comparable to hH₄R) • [³H]NAMH binding and steady-state GTPase assays: pharmacological properties independent of the type of co-expressed G$\alpha_{i/o}$ protein • No protean agonism of proxyfan 	Schnell et al. (2010a)
hH ₃ R-G α_{i2} or hH ₃ R-G α_o + G $\beta_1\gamma_2$	<ul style="list-style-type: none"> • Similar pharmacological properties of hH₃R-Gα_{i2} and hH₃R-Gα_o (steady-state GTPase assays) → hH₃R pharmacology independent of G$\alpha_{i/o}$ isoform • No protean agonism of proxyfan 	
rH ₃ R + G α_{i1} , G α_{i2} , G α_{i3} or G α_o + G $\beta_1\gamma_2$	<ul style="list-style-type: none"> • rH₃R/G$\alpha_{i/o}$ coupling stoichiometry similar to hH₃R • High constitutive activity with all G$\alpha_{i/o}$ subunits • Pharmacological properties independent of co-expressed G$\alpha_{i/o}$ isoform (similar to hH₃R) • No protean agonism of proxyfan 	
rH ₃ R or hH ₃ R + G α_{i2} + G $\beta_1\gamma_2$	<p><i>[³H]NAMH binding and/or steady-state GTPase assays:</i></p> <ul style="list-style-type: none"> • No species selectivity of histamine, Nα-methylhistamine, (R)-α-methylhistamine, imetit, and clobenpropit • Striking species selectivity of imoproxifan: nearly full agonist at hH₃R, but inverse agonist at rH₃R • Imoproxifan: pEC₅₀ > pK_i (hH₃R and rH₃R) → conformations with low partial/inverse agonist affinity, but efficient Gα interaction? 	Schnell et al. (2010b)
hH ₃ R + G α_{i2} + G $\beta_1\gamma_2$ Influence of ions on hH ₃ R properties	<p><i>[³H]NAMH radioligand binding:</i></p> <p>Increase in radioligand B_{max} and no significant reduction of binding affinity by 100 mM of NaCl</p> <p><i>Effect of NaCl (100 mM) in steady-state GTPase assays:</i></p> <ul style="list-style-type: none"> • Increase in efficacy and reduction of potency of histamine • Reduction of efficacy and increase in potency of thioperamide → stabilization of hH₃R inactive state by NaCl <p><i>Comparison of various cations and anions:</i></p> <p>Rank order of efficacy at inhibiting hH₃R constitutive activity: Li⁺ ~ Na⁺ ~ K⁺ < Cl⁻ < Br⁻ < I⁻</p>	Schnell and Seifert (2010)
hH ₃ R + G α_{i1} , G α_{i2} , G α_{i3} or G α_o + G $\beta_1\gamma_2$	<i>NaCl effect on hH₃R basal activity:</i> strongest NaCl-mediated reduction of constitutive activity in the presence of G α_{i3}	

(continued)

Table 3 (continued)

Expressed proteins	Most important new findings	Reference
hH ₃ R-D2.50N + G α ₁₂ + G β ₁ γ ₂	<ul style="list-style-type: none"> • Reduction of binding sites and lower [³H]NAMH affinity (absence of NaCl) • Constitutive activity (steady-state GTPase assays) completely eliminated • Stimulatory effect of histamine still NaCl-sensitive 	Schnell and Seifert (2010)
hH ₃ R-D2.50N + G α ₁₁ , G α ₁₂ , G α ₁₃ or G α _o + G β ₁ γ ₂	Surprising G protein selectivity of hH ₃ R-D2.50N mutation: no interaction with G α ₁₃ , but activation of G α ₁₁ , G α ₁₂ and G α _{o1}	

G α _o as reference) yielded receptor-to-G protein ratios between 1:50 and 1:100 (Schnell et al. 2010a). The receptor expression levels determined by Western blot were confirmed by radioligand saturation binding assays with the antagonist [³H]JNJ-7753707 ((4-Fluorophenyl)(1-methyl-2-[[1-(1-methylethyl)piperidin-4-yl]methoxy]-1*H*-imidazol-5-yl)methanone). By contrast, quantitation of the total number of activated G α _{*i/o*} proteins in [³⁵S]GTP γ S binding assays revealed a much lower amount of [³⁵S]GTP γ S binding sites as compared to the Western blot results, yielding hH₃R/G α _{*i*} isoform coupling ratios between 1:2 (hH₃R/G α ₁₁) and 1:11 (hH₃R/G α _o) (Schnell et al. 2010a).

Potencies and efficacies of the physiological agonist histamine and the inverse agonist thioperamide (*N*-Cyclohexyl-4-(imidazol-4-yl)-1-piperidinecarbothioamide) were determined in steady-state GTPase assays for all hH₃R/G α _{*i/o*} combinations (Schnell et al. 2010a). When hH₃R was expressed in Sf9 cell membranes without any mammalian G protein, the signals induced by histamine and thioperamide were only small, indicating that hH₃R-mediated stimulation of insect cell G proteins was virtually absent (Schnell et al. 2010a). A comparison of all five expression systems (hH₃R alone and combined with G α ₁₁, G α ₁₂, G α ₁₃, or G α _o) revealed that the relative stimulatory signal of histamine and the relative inhibitory signal of thioperamide were comparable, indicating that the constitutive activity of hH₃R does not depend on the type of co-expressed G α _{*i/o*} protein (Schnell et al. 2010a). Overall, the constitutive activity of the hH₃R was similar to the basal activity of the hH₄R (Schneider et al. 2009) (see following section).

Steady-state GTPase experiments were also performed with various hH₃R standard ligands in all hH₃R/G α _{*i/o*} co-expression systems. *N* ^{α} -methylhistamine (NAMH) and (R)- α -methylhistamine (RAMH) turned out to be full agonists under all conditions and imetit almost reached full efficacy. Proxifyfan (4-[3-(Phenylmethoxy)propyl]-1*H*-imidazole) and impentamine (4-(5-Aminopentyl)imidazole) were partial agonists with comparable efficacy under all conditions. Ciproxifan (cyclopropyl-(4-(3-(1*H*-imidazol-4-yl)propyloxy)phenyl) ketone), clobenpropit (*N*-(4-Chlorobenzyl)-*S*-[3-(4(5)-imidazolyl)propyl]isothioureia), and thioperamide exhibited inverse agonism in all systems, but efficacies were significantly different between the various G α _{*i/o*} proteins. Nevertheless, the rank orders of potency and efficacy of the ligands remained unaltered. Taken together, these experiments again confirm the notion that the hH₃R

exhibits similar pharmacological properties independently of the co-expressed $G\alpha_{i/o}$ isoforms (Schnell et al. 2010a).

As mentioned above, the hH_3R/G protein ratios ranged between 1:2 and 1:11, indicating that it is difficult to exactly control the expression levels of receptor and G proteins. Thus, the fusion protein approach was used to ensure a 1:1 coupling ratio of hH_3R and $G\alpha$ subunit. The hH_3R was fused to $G\alpha_{i2}$ and $G\alpha_o$, because these two $G\alpha_{i/o}$ isoforms exhibit the lowest structural similarity. The pharmacological properties of the standard ligands histamine, imetit, proxyfan, clobenpropit, and thioperamide were similar in steady-state GTPase assays with $hH_3R-G\alpha_{i2}$ and $hH_3R-G\alpha_o$. This indicates again that the hH_3R pharmacology is largely independent of the type of co-expressed or fused $G\alpha$ subunit (Schnell et al. 2010a).

Previously published studies about hH_3R pharmacology had reported that, depending on the expression system and the functional readout, proxyfan can be a full, a partial, or even an inverse agonist (Gbahou et al. 2003; Krueger et al. 2005). This was explained by the phenomenon of “protean agonism,” which is the ability of a ligand to induce GPCR conformations with lower G protein-coupling efficiency than the agonist-stimulated or constitutively active receptor (Gbahou et al. 2003). It has been hypothesized that protean agonism of proxyfan is due to functional selectivity, i.e. G protein coupling of the proxyfan-bound hH_3R differentiates between various $G\alpha_{i/o}$ isoforms. The data reported by Schnell et al. (2010a), however, strongly suggest that neither proxyfan nor any other of the tested hH_3R ligands exhibits this kind of functional selectivity, at least when the hH_3R is co-expressed with or fused to various $G\alpha_{i/o}$ isoforms in Sf9 cell membranes. One reason for this discrepancy could be the influence of different types of $G\beta\gamma$ subunits, which was not systematically investigated in Sf9 cells, because in the experiments performed by Schnell et al. (2010a) all $hH_3R/G\alpha_{i/o}$ combinations were uniformly co-expressed with $G\beta_1\gamma_2$. Moreover, specific combinations of various $G\alpha_{i/o}$ isoforms or cross-talk between signaling pathways could have influenced the results reported by Gbahou et al. (2003) and Krueger et al. (2005).

Species Differences Between Human and Rat Histamine H_3R

As discussed in the preceding section, the study of Gbahou et al. (2003) suggested that proxyfan shows protean agonism, which, however, was not confirmed in the Sf9 cell system (Schnell et al. 2010a). One of the reasons for this discrepancy could be a pharmacological difference in H_3R isoforms. Gbahou et al. (2003) used rat H_3R (rH_3R), while the experiments of Schnell et al. (2010a) were performed with hH_3R . To test this hypothesis, both species isoforms were directly compared in the Sf9 cell expression system (Schnell et al. 2010b).

Similar to the human isoform (Schnell et al. 2010a), the rH_3R was also co-expressed with $G\beta_1\gamma_2$ and the $G\alpha_{i/o}$ isoforms $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_o$. A quantitation of rH_3R binding sites by radioligand binding with [3H]JNJ-7753707 and of receptor-coupled $G\alpha$ subunits by [^{35}S]GTP γ S binding revealed a rH_3R/G protein stoichiometry between 1:2 and 1:7 (Schnell et al. 2010b), which is comparable to the properties of the corresponding hH_3R membranes (Schnell et al. 2010a). Moreover, similar to the hH_3R , the rH_3R showed similar high constitutive activity with each of the four $G\alpha_{i/o}$

subunits as indicated by comparable relative effects of the agonist histamine and the inverse agonist thioperamide (Schnell et al. 2010b). The independence of rH₃R pharmacology of the co-expressed G $\alpha_{i/o}$ type was confirmed by steady-state GTPase experiments.

Several H₃R standard ligands were characterized at rH₃R (+ G α_{i2} G $\beta_{1\gamma_2}$) and hH₃R (+ G α_{i2} G $\beta_{1\gamma_2}$) in [³H]NAMH radioligand binding assays. The affinities of histamine, N $^{\alpha}$ -methylhistamine, (R)- α -methylhistamine, imetit, proxifan, and clobenpropit did not differ between species isoforms, while the affinities of impentamine, imoproxifan, ciproxifan, and thioperamide were increased at the rH₃R (Schnell et al. 2010b). The radioligand binding results were largely confirmed on the functional level by steady-state GTPase experiments. Histamine, N $^{\alpha}$ -methylhistamine, RAMH, imetit, and clobenpropit did not show species selectivity. Impentamine, however, was more potent at rH₃R than at hH₃R. Additionally, ciproxifan and thioperamide exhibited higher potency but less efficacy at rH₃R as compared to hH₃R (Schnell et al. 2010b). The hypothesis that the protean agonism of proxyfan reported by Gbahou et al. (2003) was characteristic for the rat H₃R orthologue had to be rejected, because proxyfan acted as a strong partial agonist at rH₃R expressed in Sf9 cells, independently of the co-expressed G protein (Schnell et al. 2010b).

A striking difference between hH₃R and rH₃R was observed for the H₃R ligand imoproxifan, which acted as a nearly full agonist at the hH₃R, but exhibited inverse agonism at the rat orthologue (Schnell et al. 2010b). To explain this switch in quality of action, molecular modelling studies were performed by docking imoproxifan into the binding site of the active hH₃R and the inactive rH₃R. The simulations revealed different electrostatic surfaces between TM V and TM III. While the hH₃R shows a positive surface potential in this region (NH moiety of Trp^{6,48}), the corresponding part of the rH₃R is slightly negatively charged (OH moiety of Thr^{6,52}), which results in different orientations of the ligand at both receptors. Moreover, hH₃R differs from rH₃R in amino acid position 3.37. Thr^{3,37} of the hH₃R interacts with Glu^{5,46}, making Glu^{5,46} pointing away from the binding pocket, which creates a binding site for the imoproxifan methyl moiety (Schnell et al. 2010b). By contrast, an alanine in position 3.37 of the rH₃R precludes any electrostatic interaction between Glu^{5,46} and position 3.37.

Ala^{3,40} of hH₃R is replaced by the bulkier Val^{3,40} in rH₃R. Thus, the imoproxifan oxime moiety points downward towards Ala^{3,40} in hH₃R and stabilizes Trp^{6,48} in its horizontal conformation via a hydrogen bond. By contrast, the oxime moiety is directed upwards in rH₃R and interacts with Thr^{6,52}, while the methyl group of imoproxifan fits into a pocket between Val^{3,40} and Trp^{6,48}. This stabilizes Trp^{6,48} of rH₃R in its vertical conformation. According to the rotamer toggle switch mechanism of GPCR activation (Shi et al. 2002), the horizontal conformation of Trp^{6,48} corresponds to the active state, while the vertical conformation stabilizes the inactive receptor state. Thus, this model explains the different quality of action of imoproxifan at hH₃R and rH₃R (Schnell et al. 2010b).

Interestingly, in case of imoproxifan, a comparison of steady-state GTPase assay and [³H]NAMH radioligand binding data revealed that the pEC₅₀ values at hH₃R

and rH₃R were significantly higher than the corresponding pK_i values. This suggests that both hH₃R and rH₃R can adopt conformations with low affinity to partial/inverse agonists that nevertheless exhibit efficient G protein interaction (Schnell et al. 2010b).

Influence of Monovalent Ions on hH₃R Function

According to the (simplifying) two-state model of receptor activation (Fig. 4), GPCRs can adopt an active or an inactive conformation (Leff 1995). The equilibrium between both receptor states is shifted to the active side by (partial) agonists and/or interaction with G proteins. The inactive state, however, is stabilized by (partial) inverse agonists (Schneider et al. 2010b; Sato et al. 2016). The degree of constitutive activity depends on the intrinsic tendency of the receptor protein to occur in the active state. It is well established that ions are able to modulate GPCR function (Strasser et al. 2015). Specifically, sodium represents an allosteric stabilizer of the inactive receptor conformation and inhibits constitutive activity, which was, e.g., demonstrated for chemoattractant receptors (Seifert and Wenzel-Seifert 2001, 2003).

As discussed above, the hH₃R exhibits high constitutive activity. Thus, hH₃R represents an interesting model for the detailed investigation of the activity-modulating effects of ions. The hH₃R was co-expressed with G α_{i2} and G $\beta_1\gamma_2$ in Sf9 cells and the influence of 100 mM of NaCl on [³H]NAMH high-affinity agonist binding and on GTP hydrolysis in the steady-state GTPase assay was investigated. Unexpectedly, in contrast to the data reported for other G $\alpha_{i/o}$ -coupled receptors like FPR1 (Seifert and Wenzel-Seifert 2003), the affinity of the hH₃R to the radioligand

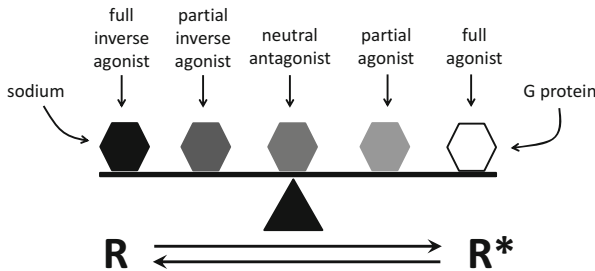


Fig. 4 Two-state model of receptor activation and factors stabilizing the active (R*) and inactive (R) receptor conformation. Every GPCR population exists in an equilibrium of active and inactive receptor conformations. Full agonists produce a maximum shift towards the active side, while inverse agonists cause a maximum stabilization of the inactive GPCR conformation. Partial agonists and partial inverse agonists induce only an incomplete shift towards either side. Neutral antagonists bind to all receptor states with the same affinity and therefore do not change the equilibrium. G proteins stabilize the active conformation, while sodium ions usually uncouple GPCRs from their G proteins by shifting the equilibrium towards the inactive side. It should be noted that, despite its usefulness, the two-state model is very simplistic and does not account for the numerous distinct ligand- and G protein-specific receptor conformations occurring in reality. Adapted from Schneider and Seifert (2010a)

was not significantly reduced by NaCl. Moreover, most surprisingly, the B_{\max} value was even increased by NaCl. The NaCl resistance of the hH₃R in the [³H]NAMH radioligand binding assays is not fully explained yet, but may be caused by the extremely high constitutive activity of the hH₃R (Schnell and Seifert 2010).

The resistance of the hH₃R to the effect of NaCl in radioligand binding was not reflected by the data from steady-state GTPase experiments. In the presence of 100 mM of NaCl, the efficacy of histamine (full agonist) was increased and the pEC₅₀ value of histamine was reduced from 8.01 to 7.53. By contrast, the pIC₅₀ value of thioperamide (inverse agonist) was increased from 7.15 to 7.43 by NaCl, while the efficacy of thioperamide was reduced. This clearly indicates that NaCl stabilizes the inactive state of the hH₃R and reduces the constitutive activity of the system, which agrees with the predictions of the two-state model system of receptor activation (Schnell and Seifert 2010).

Since NaCl does not only contain sodium cations but also chloride anions, it is not clear if the effect of NaCl on hH₃R constitutive activity is mediated by Na⁺, by Cl⁻ or by both ions. To address this question, a profile of the effects of various monovalent cations (Li⁺, Na⁺, and K⁺) as well as of different anions (Cl⁻, Br⁻, and I⁻) was determined in steady-state GTPase assays with membranes expressing hH₃R plus Gα₁₂ and Gβ₁γ₂. The rank order of efficacy was Li⁺ ~ Na⁺ ~ K⁺ < Cl⁻ < Br⁻ < I⁻. This indicates a direct proportionality between anion radii and reduction of basal hH₃R activity and shows that anions contribute more to the salt-induced reduction of constitutive activity than cations. Moreover, the different efficacies of the anions exclude the possibility that an increased osmolality may be responsible for the effect on constitutive activity (Schnell and Seifert 2010). Similar results had been previously obtained with the hβ₂AR-Gsα_L fusion protein, and it had been hypothesized that anions may enhance GDP affinity to the G protein, reducing the ability of the receptor to promote GDP dissociation (Seifert 2001). Interestingly, a comparison of the NaCl effect on hH₃R basal activity in membranes co-expressing Gβ₁γ₂ and various Gα_{i/o} subunits (Gα₁₁, Gα₁₂, Gα₁₃ or Gα_{i/o}) revealed the strongest NaCl-mediated reduction of constitutive activity in the presence of Gα₁₃ (Schnell and Seifert 2010).

It is generally assumed that the highly conserved Asp^{2.50} acts as a Na⁺ binding site in GPCRs (Horstman et al. 1990; Wittmann et al. 2014). Thus, the functional consequences of a charge-neutralizing mutation from Asp^{2.50} to Asn^{2.50} in the hH₃R protein were investigated. In the absence of sodium, the D2.50N mutant (co-expressed with Gα₁₂ and Gβ₁γ₂) exhibited a reduced number of [³H]NAMH binding sites and an affinity reduction of [³H]NAMH by about 90% as compared to the wild-type hH₃R (Schnell and Seifert 2010). Constitutive activity in steady-state GTPase assays was completely eliminated by the D2.50N mutation (co-expressed with Gα₁₂ and Gβ₁γ₂) and consequently, neither thioperamide nor NaCl further inhibited basal activity. Interestingly, however, the stimulatory effect of histamine at the D2.50N mutant was highly sensitive to NaCl and was completely eliminated at NaCl concentrations > 90 mM. Most surprisingly, the D2.50N mutation introduced G protein selectivity, as the mutant did not productively interact any more with Gα₁₃, but still activated Gα₁₁, Gα₁₂, and Gα_{o1}. Thus, Asp^{2.50} seems to play a decisive role in the hH₃R/Gα₁₃-interaction (Schnell and Seifert 2010). In

summary, the characterization of the hH₃R in the Sf9 cell expression system by Schnell and Seifert (2010) revealed that G α_{i3} interacts with hH₃R in a very distinct manner as compared to the other tested G $\alpha_{i/o}$ isoforms (stronger NaCl effect on activity of wild-type hH₃R and complete inactivity of the hH₃R-D2.50N mutant). Interestingly, the D2.50N mutant was not completely NaCl-insensitive, which indicates that the interaction between ions and hH₃R is more complex and cannot be explained by a single interaction site (Schnell and Seifert 2010).

In contrast to the hH₃R, the structurally similar hH₄R (see Sect. 2.4) exhibits completely NaCl-resistant constitutive activity (Schneider et al. 2009). A potential explanation for this discrepancy was recently offered by Wittmann et al. (2014). A comparison of various human aminergic GPCRs revealed that in the majority of receptors, glycine is the most abundant (80%) amino acid in the sodium binding channel between the ligand binding site and the sodium binding region (Wittmann et al. 2014). This is, however, not the case for hH₃R and hH₄R. Moreover, in hH₄R the glutamine in position 7.42 disrupts a water chain, which is extending from Asp^{3.32} (orthosteric binding site) to Asp^{2.50} (allosteric binding site). This might kinetically prevent sodium from binding to the allosteric binding site (Wittmann et al. 2014).

2.4 The Histamine H₄ Receptor

The fourth histamine receptor couples to PTX-sensitive G α_i proteins, specifically to G α_{i2} and shows high constitutive activity (Schneider et al. 2009). The H₄R is a chemotactic receptor mainly expressed on hematopoietic cells, specifically on eosinophils (O'Reilly et al. 2002; Buckland et al. 2003; Reher et al. 2012b). Human eosinophils belong to the best characterized primary cells endogenously expressing hH₄R, but it is difficult to isolate this rare cell type in sufficiently high purity and numbers from healthy volunteers (Seifert et al. 2013). Moreover, H₄R is expressed on mast cells (Hofstra et al. 2003; Jemima et al. 2014) as well as dendritic cells (Gutzmer et al. 2005; Damaj et al. 2007; Bäumer et al. 2008; Gschwandtner et al. 2011) and expression on natural killer cells has been reported, too (Damaj et al. 2007). The presence of the H₄R on monocytes is discussed controversially (Damaj et al. 2007; Gschwandtner et al. 2013; Werner et al. 2014). Data from a comprehensive analysis of hH₄R expression on various myeloid cell types have been published very recently (Capelo et al. 2016). H₄R knockout mouse models suggest that this receptor plays a role in the pathophysiology of itch, experimental asthma and EAE (Neumann et al. 2014).

The H₄R represents an interesting target for anti-inflammatory drugs. For example, the H₄R regulates eosinophilic inflammation in a mouse model of ovalbumin-induced allergic asthma (Hartwig et al. 2015). Moreover, the hH₄R seems to be a key player in pruritus during inflammatory reactions (Bell et al. 2004; Dunford et al. 2007; Rossbach et al. 2011). However, studies with mouse models should be interpreted with caution, because H₄R pharmacology strongly differs between various species (Strasser et al. 2013). For example, the "prototypical" hH₄R antagonist JNJ7777120 (1-[(5-Chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine) is an inverse

agonist at the hH₄R, but a partial agonist at the rat, mouse, and canine orthologues (Schnell et al. 2011; Strasser et al. 2013). Another caveat is H₄R-induced G protein-independent β -arrestin signaling. Although JNJ-777120 is an inverse H₄R agonist with regard to G protein activation, it exhibits agonistic effects on H₄R-dependent β -arrestin signaling (Rosethorne and Charlton 2011; Seifert et al. 2011; Nijmeijer et al. 2013). Recently, the H₄R antagonist JNJ 39758979 ((R)-4-(3-amino-pyrrolidin-1-yl)-6-isopropyl-pyrimidin-2-ylamine) was shown to be safe and efficacious at reducing histamine-induced pruritus in a phase 1 clinical study (Kollmeier et al. 2014).

2.4.1 Successful Reconstitution of Functional Human Histamine H₄R (hH₄R) in Sf9 Cells

The N-terminally FLAG-tagged and C-terminally His-tagged wild-type hH₄R was co-expressed with G α _{i2} and G β ₁ γ ₂ in Sf9 cells. Binding studies with [³H]histamine revealed a K_D value of ~10 nM (Schneider et al. 2009), which fits well to the literature range (5–20 nM). Steady-state GTPase and [³⁵S]GTP γ S binding experiments confirmed the high constitutive activity of the hH₄R, which was effectively inhibited by the inverse agonist thioperamide (Schneider et al. 2009). Surprisingly, thioperamide was not able to suppress [³⁵S]GTP γ S binding in the co-expression system (hH₄R + G α _{i2} + G β ₁ γ ₂) to the level of control membranes expressing only G α _{i2} and G β ₁ γ ₂ (Schneider et al. 2009). This strongly indicates that thioperamide is only a partial H₄R inverse agonist and not, as originally suggested in the literature (Lim et al. 2005), a full inverse agonist. The Sf9 cell system provides a “clean” background devoid of mammalian G α _i proteins and their cognate GPCRs. Thus, expression of mammalian G proteins without GPCRs in Sf9 cells provides a valid control for baseline G α activity and for the maximum possible effect of a full inverse agonist. In the following, the most important results from the pharmacological characterization of the hH₄R in Sf9 cell membranes are discussed. An overview of the most important results is provided in Table 4.

2.4.2 G Protein-Independent High-Affinity-State of the hH₄R

According to the ternary complex model (De Lean et al. 1980), a GPCR shows its highest agonist affinity, when it is part of the ternary complex (Sect. 1.2.1, Fig. 2). Ternary complex formation, however, is prevented in the presence of GTP γ S which binds to the G α subunit like GTP (Gilman 1987), but cannot be hydrolyzed. Thus, GTP γ S disrupts the G protein cycle, resulting in the accumulation of uncoupled inactive GPCRs with reduced agonist affinity. Surprisingly the hH₄R shows an active state which is completely independent of G proteins (Schneider et al. 2009). This is supported by the following four observations: First, high-affinity [³H]histamine binding (K_D and B_{max}) to membranes expressing hH₄R, G α _{i2}, and G β ₁ γ ₂ was retained in the presence of GTP γ S. Second, [³H]histamine binding affinity was almost identical in the hH₄R/G α _{i2}/G β ₁ γ ₂ co-expression system and in Sf9 cell membranes expressing hH₄R in the absence of mammalian G proteins. Third, the K_i values of the inverse hH₄R agonists thioperamide and JNJ-777120 were unaltered in membranes expressing only hH₄R, although the two-state model of receptor activation (Fig. 4) suggests that inverse agonist affinity increases, when

Table 4 Overview on the pharmacological characterization of the human histamine H₄R in the Sf9 cell expression system

Expressed proteins	Most important new findings	Reference
hH ₄ R	<ul style="list-style-type: none"> • GTPγS-insensitive high-affinity agonist binding • No activation of insect cell G proteins 	Schneider et al. (2009)
hH ₄ R + Gα ₁₁ , Gα ₁₂ , Gα ₁₃ or Gα ₆ + Gβ ₁ γ ₂	<ul style="list-style-type: none"> • hH₄R most efficiently activates Gα₁₂ • Least efficient interaction with Gα₆ 	
hH ₄ R + Gα ₁₂ + Gβ ₁ γ ₂	<ul style="list-style-type: none"> • GTPγS-insensitive high-affinity agonist binding • Very high sodium-insensitive constitutive activity • Structural instability • Catalytic signaling 	
hH ₄ R-Gα ₁₂ + Gβ ₁ γ ₂	<ul style="list-style-type: none"> • Compared to co-expression system (hH₄R + Gα₁₂ + Gβ₁γ₂): • Increased expression level • Unaltered histamine affinity • More efficient hH₄R/Gα₁₂ interaction • Increased constitutive activity • Linear signaling 	
hH ₄ R-A6.30E + Gα ₁₂ + Gβ ₁ γ ₂	<p><i>Compared to wild-type (hH₄R + Gα₁₂ + Gβ₁γ₂):</i></p> <ul style="list-style-type: none"> • Slight (non-significant) reduction of constitutive activity and G protein coupling efficiency • Unaltered K_D of histamine • G protein-independent high-affinity binding retained 	Schneider et al. (2010a)
hH ₄ R-R3.50A + Gα ₁₂ + Gβ ₁ γ ₂	<p><i>Compared to wild-type (hH₄R + Gα₁₂ + Gβ₁γ₂):</i></p> <ul style="list-style-type: none"> • G protein coupling eliminated • Affinity of thioperamide increased • Affinity of histamine reduced 	
hH ₄ R + RGS4 + Gα ₁₂ + Gβ ₁ γ ₂	<p><i>Compared to wild-type co-expression system (+ Gα₁₂Gβ₁γ₂):</i></p> <ul style="list-style-type: none"> • No significant change of histamine effect and baseline steady-state GTPase activity • Significant increase of thioperamide inverse agonistic effect 	Schneider and Seifert (2009)
hH ₄ R-RGS4 + Gα ₁₂ + Gβ ₁ γ ₂	<p><i>Compared to wild-type co-expression system (+ Gα₁₂Gβ₁γ₂):</i></p> <ul style="list-style-type: none"> • Significant increase of baseline steady-state GTPase activity and of thioperamide inverse agonistic effect • Significantly increased EC₅₀-values of histamine and JNJ-777120 (~twofold) • Significantly increased apparent K_M value of Gα₁₂ intrinsic GTPase activity in the presence of histamine 	

(continued)

Table 4 (continued)

Expressed proteins	Most important new findings	Reference
hH ₄ R + GAIP + G α_{i2} + G $\beta_1\gamma_2$	<p><i>Compared to wild-type co-expression system (+ Gα_{i2}G$\beta_1\gamma_2$):</i></p> <ul style="list-style-type: none"> • No significant change of signal range and baseline activity in steady-state GTPase assays • Significantly increased apparent K_M value of Gα_{i2} intrinsic GTPase activity in the presence of histamine 	
hH ₄ R-GAIP + G α_{i2} + G $\beta_1\gamma_2$	<p><i>Compared to wild-type co-expression system (+ Gα_{i2}G$\beta_1\gamma_2$):</i></p> <ul style="list-style-type: none"> • Basically identical pharmacological properties • Significantly increased relative histamine- and thioperamide-induced signals in steady-state GTPase assays 	
hH ₄ R-GAIP + G α_{i1} , G α_{i2} , G α_{i3} or G α_o + G $\beta_1\gamma_2$	Identical G protein selectivity of hH ₄ R-GAIP and hH ₄ R → G protein coupling is mainly determined by the GPCR, but not by the RGS protein	
hH ₄ R-F169V+S179A or hH ₄ R-F169V+S179M hH ₄ R-F169V hH ₄ R-S179A hH ₄ R-S179M + G α_{i2} + G $\beta_1\gamma_2$	<p><i>Compared to wild-type hH₄R:</i> constitutive activity not affected by the S179A or S179M single mutations. Constitutive activity slightly reduced in F169V single mutant; stronger reduction in double mutants. S179A single mutant: increased potency and affinity of JNJ-777120</p>	Wifling et al. (2015b)
mH ₄ R-V171F mH ₄ R-V171F+M181S + G α_{i2} + G $\beta_1\gamma_2$	No constitutive activity of wild-type mH ₄ R and mH ₄ R-V171F mutant, but weak constitutive activity of mH ₄ R-V171F+M181S double mutant	
hH ₄ R-F168A + G α_{i2} + G $\beta_1\gamma_2$	Total loss of hH ₄ R constitutive activity.	Wifling et al. (2015a)

the receptor is not coupling to G proteins and assumes an inactive state. Finally, steady-state GTPase assays with membranes co-expressing hH₄R, G α_{i2} and G $\beta_1\gamma_2$ revealed that the constitutive activity of the hH₄R is insensitive to sodium ions. According to the standard two-state model of receptor activation depicted in Fig. 4, however, it is expected that Na⁺ stabilizes the inactive state of a GPCR. This has been shown previously, e.g. for FPR-26 (Wenzel-Seifert et al. 1998; Seifert and Wenzel-Seifert 2001) or the α_2 -adrenoceptor (Tian and Deth 2000).

2.4.3 Analysis of hH₄R-G Protein Coupling

Analysis of hH₄R activation in the steady-state GTPase assay in membranes co-expressing hH₄R with G $\beta_1\gamma_2$ and a specific G α subunit (G α_{i1} , G α_{i2} , G α_{i3} or

$G\alpha_o$) revealed that $G\alpha_{i2}$ was most effectively stimulated by the hH_4R . By contrast, the hH_4R hardly activated $G\alpha_o$ proteins (Schneider et al. 2009). Since $G\alpha_o$ is the main G protein subtype in the brain, this result suggests that the hH_4R is not of major importance in the CNS. We have seriously questioned the widespread but largely unfounded notion of functional hH_4R expression on neurons (Schneider and Seifert 2016).

The stoichiometry of the receptor-G protein interaction can be calculated by dividing the total number of receptor-regulated G proteins (from GTP γ S binding assays) by the number of receptors per cell (B_{max} from radioligand binding or from Western blot). When co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 cell membranes, the hH_4R catalytically activates up to five $G\alpha_{i2}$ subunits simultaneously (Schneider et al. 2009). The affinity of [35 S]GTP γ S to the $G\alpha$ subunit (K_D value) reflects efficiency of G protein activation. The inverse agonistic character of thioperamide was confirmed in [35 S]GTP γ S assays with membranes co-expressing hH_4R , $G\alpha_{i2}$ and $G\beta_1\gamma_2$. While the [35 S]GTP γ S K_D value was 3.4 nM in the presence of histamine, it was about threefold increased by thioperamide (Schneider et al. 2009), indicating reduced [35 S]GTP γ S affinity of the $G\alpha$ subunit due to uncoupling from the hH_4R .

2.4.4 Conformational Instability of hH_4R

As demonstrated for the constitutively active mutant of the β_2 -adrenoreceptor (β_2AR_{CAM}) (Gether et al. 1997), constitutive activity of a GPCR increases conformational flexibility and favors denaturation. By contrast, ligand binding reduces conformational flexibility and stabilizes the receptor. Thus, addition of ligands to a cell culture expressing β_2AR_{CAM} increased the B_{max} value of this receptor (Gether et al. 1997). This effect was caused by both agonists and inverse agonists, suggesting that it is the switch between different activation states rather than the nature of the activation state, which destabilizes the receptor.

The high constitutive activity of the hH_4R prompted us to investigate its conformational stability and the stabilizing effect of ligands. In fact, addition of histamine (10 μ M) or thioperamide (1 μ M) to Sf9 cells co-expressing hH_4R , $G\alpha_{i2}$ and $G\beta_1\gamma_2$ significantly increased the B_{max} value in histamine high-affinity agonist binding assays (Schneider et al. 2009). Interestingly, this effect was not visible in immunoblots, indicating that histamine and thioperamide mainly support the correct folding of hH_4R in the cell membrane, but not during intracellular protein synthesis. This was confirmed in experiments, where denaturation of hH_4R (co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$) was induced by incubation of the membranes at 37°C. After 120 min, almost 70% of the histamine binding sites in the ligand-free control were lost, but only 35% in the presence of histamine. Most surprisingly, however, thioperamide increased the B_{max} by 30–40%, suggesting that it did not only prevent hH_4R denaturation, but even re-folded a priori misfolded receptors. This intriguing “refolding” effect of the inverse agonist thioperamide was confirmed in a two-step assay, during which the receptor was first denatured and then incubated with thioperamide.

2.4.5 Characterization of the hH₄R-G α _{i2} Fusion Protein

To analyze the interaction of the hH₄R with G α _{i2}, the C-terminus of the receptor was fused to the N-terminus of the G protein by using a His₆ linker (Fig. 3). The hH₄R-G α _{i2} protein co-expressed with G β ₁ γ ₂ in Sf9 cell membranes exhibited linear signaling with a coupling factor of ~1 in [³⁵S]GTP γ S binding assays and a turnover number of ~1 in steady-state GTPase assays. Thus, hH₄R exclusively activates the tethered mammalian G protein but not the insect cell G proteins (Schneider et al. 2009). This was additionally supported by the lack of [³⁵S]GTP γ S binding in membranes expressing non-fused hH₄R in the absence of mammalian G proteins (Schneider et al. 2009). The K_D value of [³⁵S]GTP γ S in the presence of the full hH₄R agonist histamine or the inverse agonist thioperamide in membranes co-expressing hH₄R-G α _{i2} and G β ₁ γ ₂ was significantly reduced as compared to the coexpression system, indicating enhanced efficiency of G protein activation (Schneider et al. 2009). A higher GTP affinity of G α _{i2} in the fusion protein was also reflected by a significantly decreased K_M value in the presence of histamine in steady-state GTPase assays. Moreover, a slight increase of constitutive activity in steady-state GTPase assays additionally demonstrates the increased efficiency of G protein activation in the fusion protein system (Schneider et al. 2009). Interestingly, the B_{\max} value of the hH₄R-G α _{i2} fusion protein in immunoblots and [³H]histamine binding assays was increased as compared to the non-fused receptor (Schneider et al. 2009). This suggests a chaperone-like stabilizing effect of G α _{i2}, favoring membrane insertion of the receptor protein. Incubation of the cell culture with histamine or thioperamide did not further enhance the B_{\max} value of the fusion protein in [³H]histamine binding (Schneider et al. 2009), suggesting that the fusion of hH₄R to G α _{i2} induces already the maximum possible number of correctly folded receptors. An overview of the most important features of the hH₄R-G α _i fusion protein in comparison to the co-expression system (hH₄R + G α _{i2} + G β ₁ γ ₂) is provided in Table 4.

2.4.6 Role of Glycosylation for hH₄R Expression and Function

Western blotting of hH₄R-expressing Sf9 cell membranes revealed two bands at 43 and 46 kDa. Incubation of the baculovirus-infected Sf9 cell culture with the glycosylation inhibitor tunicamycin removed the 46 kDa band, indicating that this is most likely a glycosylated H₄R species (Schneider et al. 2009). Although the total protein amount on the Western blot was comparable for both untreated and tunicamycin-treated H₄R protein (2.5–3 pmol/mg as assessed by using FLAG- β ₂AR standard membranes with known receptor expression levels), the B_{\max} value in [³H]histamine binding was reduced by 75% after tunicamycin treatment. Nevertheless, the K_D value of [³H]histamine remained unchanged (Schneider et al. 2009). Thus, hH₄R deglycosylation does not significantly affect the [³H]histamine binding site of functional hH₄R, although it significantly reduces the amount of correctly folded receptor protein.

The activation of G α _{i2} proteins by deglycosylated hH₄R was investigated in [³⁵S]GTP γ S saturation binding and steady-state GTPase assays. Even in the presence of histamine, the deglycosylated hH₄R in the tunicamycin-treated membranes

activated $G\alpha_{i2}$ less efficiently than the glycosylated H_4R (increased K_D value of [^{35}S]GTP γ S) (Schneider et al. 2009). Thus, proper glycosylation of hH_4R seems to be a prerequisite for efficient G protein coupling. By contrast, determination of the K_M value of GTP at the $G\alpha_{i2}$ subunit in steady-state GTPase assays only revealed a non-significant trend towards an increased K_M -value in the tunicamycin-treated membranes (Schneider et al. 2009).

In [^{35}S]GTP γ S binding assays, deglycosylation reduced the constitutive activity of H_4R coexpressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ from 70 to 40% (Schneider et al. 2009). Neither the coupling factor from [^{35}S]GTP γ S binding assays nor the turnover number from steady-state GTPase assays changed significantly, when hH_4R was deglycosylated (Schneider et al. 2009). This suggests that deglycosylation of hH_4R reduces efficacy of $G\alpha$ activation without affecting the total number of activated G proteins.

2.4.7 Reasons for the High Constitutive Activity of hH_4R

The inactive state of GPCRs is established by intramolecular interactions that conformationally restrain the receptor. Data obtained from the rhodopsin molecule have led to the assumption that the so-called ionic lock is highly important for the inactivation of GPCRs (Palczewski et al. 2000; Vogel et al. 2008). The ionic lock is a salt bridge between a highly conserved glutamate in position 6.30 of TM6 and the arginine of the DRY motif located on the bottom of TM3 (position 3.50). The importance of the ionic lock for the regulation of odorant GPCR activity has been shown recently (de March et al. 2015). However, some receptors do not form an ionic lock, despite the presence of the required amino acids. This has been reported, e.g. for the human β_2AR (Cherezov et al. 2007; Rasmussen et al. 2007; Rosenbaum et al. 2007) or the human A_{2A} adenosine receptor (Jaakola et al. 2008), both of which show considerable constitutive activity.

The hH_4R is the only histamine receptor with an alanine in position 6.30, which precludes ionic lock formation (Schneider et al. 2010a) and possibly explains the observed high G protein-independent activity of the hH_4R (Schneider et al. 2009). To test this hypothesis, the TM6 part of the potential ionic lock was reconstituted by introducing the A6.30E mutation, and the resulting mutant was analyzed in the Sf9 cell expression system. Immunoblots and [3H]histamine saturation binding indicated comparable expression levels of the mutant and the wild-type hH_4R . Unexpectedly, the pharmacological properties of hH_4R -A6.30E (co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$) in radioligand binding, steady-state GTPase assay and [^{35}S]GTP γ S binding assays were basically unaltered as compared to the wild-type hH_4R (Schneider et al. 2010a). The replacement of alanine 6.30 by glutamate resulted in a slight but non-significant reduction of coupling factor ([^{35}S]GTP γ S binding), turnover number (steady-state GTPase assay) and constitutive activity ([^{35}S]GTP γ S binding and steady-state GTPase assay). This indicates that the ionic lock interaction was either not fully reconstituted or not sufficient to stabilize the inactive conformation of hH_4R (Schneider et al. 2010a). An overview of the most important features of the hH_4R -A6.30E mutation in comparison to the wild-type hH_4R is provided in Table 4.

Molecular modeling studies revealed potential interactions that may stabilize the active conformation despite the presence of the reconstituted ionic lock. The hH₄R active state was modeled in complex with the C terminus of G α_{i2} by using the crystal structures of the turkey β_1 AR (Warne et al. 2008) and the human adenosine A_{2A} receptor (Jaakola et al. 2008) as templates. This revealed an additional salt bridge between D5.69 at the N-terminus of the second cytoplasmic loop (CL3) and R6.31, which may stabilize an active receptor conformation (Schneider et al. 2010a). Since D5.69 is nearly unique among the GPCRs for biogenic amines, this salt bridge may be at least partly responsible for the high constitutive activity of hH₄R and should be analyzed in future studies.

Recently, the reasons for the high constitutive activity of hH₄R were further elucidated (Wifling et al. 2015a, b). These studies made use of the large pharmacological differences between human and rodent H₄R (Schnell et al. 2011; Strasser et al. 2013). For example, constitutive activity of mH₄R and rH₄R is strongly reduced as compared to hH₄R (Schnell et al. 2011) and the inverse hH₄R agonist JNJ7777120 exhibits partial agonism at mH₄R and rH₄R. Moreover, the potency of the agonist histamine is lower for the rodent orthologues as compared to hH₄R (Schnell et al. 2011). Mutational studies indicate that position 169 of the second extracellular loop is an important determinant of the distinct agonist binding properties of human and mouse H₄R (Lim et al. 2008). The F169 of the hH₄R is replaced by a V169 in the mH₄R. Thus, Wifling et al. (2015b) performed a detailed analysis of the “mouse-like” hH₄R-F169V mutant in the Sf9 cell system. In fact, hH₄R-F169V exhibited decreased constitutive activity as compared to wild-type hH₄R, resulting in an increased agonistic effect of histamine. Moreover, histamine binding affinity as well as the inverse agonistic effect of thioperamide was reduced (Wifling et al. 2015b). The second key amino acid identified by Wifling et al. (2015b) was S179, which is replaced by methionine in the mH₄R and by alanine in the rH₄R. The double mutants hH₄R-F169V+S179A and hH₄R-F169V+S179M showed an even stronger reduction of constitutive activity as compared to the hH₄R-F169V single mutant (Wifling et al. 2015b). These results suggest that the constitutively active state of hH₄R at least partly depends on hydrophobic interactions between the extracellular domains of TM 5, 6, and 7 and ECL2. A hydrogen bond between S179 and T323 additionally stabilizes the agonist-free active state of the hH₄R (Wifling et al. 2015b).

These mutations, however, did not completely eliminate the constitutive activity of hH₄R. A total loss of constitutive activity was only achieved by introducing the F168A mutation (Wifling et al. 2015a). This indicates that – despite the strong reduction of constitutive activity in the hH₄R-F169V mutation – the adjacent amino acid in the FF motif, F168, is the key residue responsible for the high constitutive activity of hH₄R (Wifling et al. 2015a). An FF motif in ECL2 is also present in other GPCRs, e.g. β_2 AR, hH₃R and M₂R, suggesting a similar role of the ECL2 conformation on constitutive activity of these receptors.

2.4.8 The Role of the DRY Motif in G Protein Activation by the Human hH₄R

The arginine R3.50 of the DRY motif at the bottom of TM3 stabilizes the inactive receptor state by forming a salt bridge with the adjacent D/E3.49 residue (Nygaard et al. 2009). Therefore, we analyzed the effect of the hH₄R-R3.50A mutation on constitutive activity and ligand binding in membranes co-expressing hH₄R-R3.50A, G α_{i2} and G $\beta_1\gamma_2$. Surprisingly, the R3.50A exchange totally eliminated G protein coupling as indicated by the complete absence of receptor-regulated steady-state GTPase activity (Schneider et al. 2010a). Moreover, the hH₄R-R3.50A mutant adopted an inactive state with reduced affinity of the agonist histamine and increased affinity of the inverse agonist thioperamide (Schneider et al. 2010a). However, introduction of the R3.50A mutation reduced histamine affinity only by 50% and did not affect B_{\max} . This suggests that the hH₄R-R3.50A mutant still adopts a “residual” G protein-independent high-affinity state.

To explain the total loss of G protein coupling of the hH₄R-R3.50A mutant, molecular modelling studies were performed using the active-state of the hH₄R in complex with the C-terminus of G α_{i2} . This analysis revealed that R3.50 of the hH₄R may interact with the backbone oxygens of C352 and G353 in the G α_{i2} C-terminus (Schneider et al. 2010a). This supports the adoption of the G α_{i2} conformation, which is required for interaction with TM6 of the receptor. Thus, the R3.50A mutation hampers G protein recognition by hH₄R. Nevertheless, the hH₄R-R3.50A mutant is still able to form the salt bridge between D5.69 and R6.31, which stabilizes an active state. This could explain why hH₄R-R3.50A still exhibits relatively high histamine affinity (Schneider et al. 2010a). However, the effect of mutations in the E/DRY motif is not disrupting G protein coupling in all GPCRs. Rovati et al. (2007) described two phenotypes P1 and P2 that are produced by mutations of the E/D3.49- or the R3.50-residue. While in P1-type receptors high-affinity agonist binding and G protein coupling are retained after mutating position R3.50, P2-type receptors show a disrupted receptor-G protein interaction and reduced agonist binding affinity (Rovati et al. 2007). Accordingly, the hH₄R belongs to the group of P2-type GPCRs. An overview of the most important features of the hH₄R-R3.50A mutation in comparison to the wild-type hH₄R is provided in Table 4.

2.4.9 Pharmacological Characterization of hH₄R Ligands

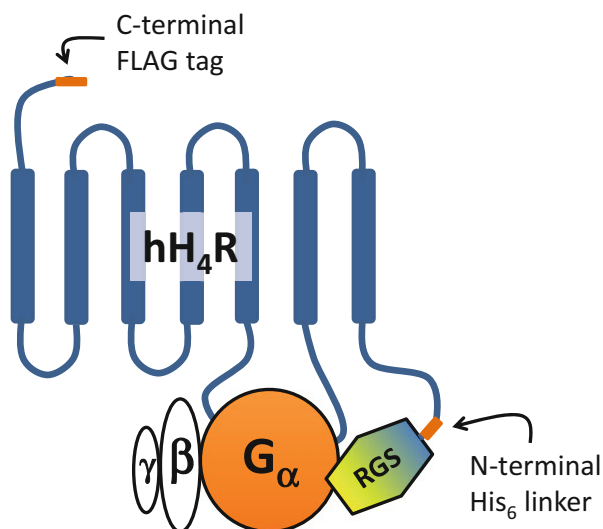
As explained above, co-expression of the hH₄R and its cognate mammalian G proteins in Sf9 cells results in high constitutive activity (Schneider et al. 2009). This reduces the maximum available signal range, yielding a very low signal-to-noise ratio. Even in the presence of 100 mM of NaCl, the full agonist histamine produced only a signal intensity of ~30% (related to baseline) (Schneider and Seifert 2009). The expression of an hH₄R-G α_{i2} fusion protein did not improve the signal-to-noise ratio, but resulted in even higher constitutive activity and reduced relative intensity of histamine-induced signals (Schneider et al. 2009). Thus, the properties of the hH₄R/G protein co-expression system and the hH₄R-G α_{i2} fusion protein are rather unfavorable for the characterization of hH₄R ligands.

This prompted us to perform a closer investigation of the effects of regulators of G protein signaling (RGS proteins). A common feature of RGS proteins is the 120 amino acid RGS domain, which interacts with $G\alpha$ subunits and increases their intrinsic GTPase activity (Willars 2006). RGS proteins are classified in eight subfamilies that differ from each other by protein size and the presence of additional functional domains. They regulate the activity of $G\alpha_{i/o}$ - or $G\alpha_q$ proteins, but no RGS protein-mediated activation of $G\alpha_s$ has been reported to date. Due to their mechanism of action, RGS proteins should enhance signal intensity in steady-state GTPase assays. In fact, fusion of the α_2AR C-terminus to the RGS4 N-terminus significantly increased α_2AR -mediated stimulation of GTPase activity (Bahia et al. 2003).

For the experiments with the hH_4R , the two RGS proteins RGS4 and GAIP ($G\alpha$ -interacting protein; also known as RGS19) were selected. RGS4 and GAIP both exhibit a simple protein structure without additional functional domains. Therefore, only activation of $G\alpha_i$ GTPase activity is expected. Both RGS proteins were fused to the hH_4R via a His_6 linker (Fig. 5), very similar to the previously described hH_4R - $G\alpha_{i2}$ fusion protein approach (Fig. 3). The hH_4R -RGS fusion proteins were co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 cell membranes. The corresponding co-expression system was characterized by infecting Sf9 cells with baculoviruses encoding hH_4R , $G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS4 or GAIP.

Both RGS4 and GAIP, irrespective of whether they were co-expressed or fused to hH_4R , increased the apparent K_M value of $G\alpha_{i2}$ in the presence of histamine in steady-state GTPase assays. This effect reached significance for the co-expressed GAIP and the hH_4R -RGS4 fusion protein (Schneider and Seifert 2009). By contrast, there was no effect of RGS proteins on the K_M value in the presence of the inverse agonist thioperamide (Schneider and Seifert 2009). This suggests that GPCR-mediated activation of the G protein is a prerequisite for the RGS protein effect.

Fig. 5 Schematic depiction of the hH_4R -RGS fusion protein. The C-terminus of the hH_4R is fused to the N-terminus of the RGS protein by a hexahistidine linker. This brings the RGS protein into close proximity to the heterotrimeric G protein. Adapted from Schneider and Seifert (2010c)



Compared to the RGS4-free co-expression system ($\text{hH}_4\text{R} + \text{G}\alpha_{i2} + \text{G}\beta_1\gamma_2$), both the quadruple expression system ($\text{hH}_4\text{R} + \text{G}\alpha_{i2} + \text{G}\beta_1\gamma_2 + \text{RGS4}$) and the fusion protein system ($\text{hH}_4\text{R-RGS4} + \text{G}\alpha_{i2} + \text{G}\beta_1\gamma_2$) yielded a significantly increased relative steady-state GTPase signal of the inverse agonist thioperamide, while the histamine-induced signal remained unaffected (Schneider and Seifert 2009). The only major difference between co-expressed and hH_4R -attached RGS4 was an increased baseline steady-state GTPase activity in the $\text{hH}_4\text{R-RGS4}$ fusion protein system, but an unaltered baseline, when RGS4 was co-expressed (Schneider and Seifert 2009).

Co-expression of GAIP with hH_4R , $\text{G}\alpha_{i2}$ and $\text{G}\beta_1\gamma_2$ had no significant effect on baseline activity or thioperamide- and histamine-induced signals in steady-state GTPase assays. However, when GAIP was fused to hH_4R , the histamine-induced relative signal in steady-state GTPase assays was significantly increased by ~69% and the thioperamide-induced signal was enhanced by ~45%. The baseline activity of the GAIP- hH_4R fusion protein system, however, remained unaffected (Schneider and Seifert 2009). Thus, in contrast to $\text{hH}_4\text{R-RGS4}$, the $\text{hH}_4\text{R-GAIP}$ fusion protein (co-expressed with $\text{G}\alpha_{i2}$ and $\text{G}\beta_1\gamma_2$) enhanced the absolute histamine-induced signal without changing baseline activity. Therefore, the relative stimulatory effect of histamine was increased (Schneider and Seifert 2009).

The different behavior of RGS4 and GAIP in the fusion proteins is surprising, because both RGS proteins have a similar RGS domain and no additional functionalities. Possibly, the differences are caused by distinct G protein affinities of these RGS proteins. According to the UniProtKB database entry P49795, GAIP binds to $\text{G}\alpha_i$ proteins in the rank order $\text{G}\alpha_{i3} > \text{G}\alpha_{i1} > \text{G}\alpha_o \gg \text{G}\alpha_z/\text{G}\alpha_{i2}$. Thus, among the $\text{G}\alpha_i$ isoforms, $\text{G}\alpha_{i2}$ is the one with the lowest affinity to GAIP. This means that the effect of GAIP may only become visible, when the number of activated $\text{G}\alpha_{i2}$ subunits exceeds a certain threshold. While under basal conditions the number of activated $\text{G}\alpha_{i2}$ subunits is too low for a visible $\text{hH}_4\text{R-GAIP}$ -mediated effect, stimulation by histamine increases the number of active $\text{G}\alpha_{i2}$ to a level, where the GAIP-mediated effect becomes visible. By contrast, RGS4 may exhibit a higher $\text{G}\alpha_{i2}$ affinity than GAIP and therefore show already an effect under basal conditions. This hypothesis, however, should be tested by a side-by-side comparison of the $\text{G}\alpha_{i2}$ protein affinity of RGS4 and GAIP.

Co-expression of the $\text{hH}_4\text{R-GAIP}$ fusion protein with $\text{G}\alpha_{i2}$ and $\text{G}\beta_1\gamma_2$ produces a system with improved signal-to-noise ratio as compared to the standard co-expression system ($\text{hH}_4\text{R} + \text{G}\alpha_{i2} + \text{G}\beta_1\gamma_2$). A comparison of $\text{hH}_4\text{R-GAIP}$ and wild-type hH_4R (both co-expressed $\text{G}\alpha_{i2}$ and $\text{G}\beta_1\gamma_2$) in steady-state GTPase assays revealed comparable pharmacological properties. First, potency and efficacy of selected hH_4R standard ligands were unaltered. Second, similar to wild-type hH_4R , the $\text{hH}_4\text{R-GAIP}$ fusion protein exhibited sodium chloride-insensitive constitutive activity (Schneider and Seifert 2009). Third, the $\text{hH}_4\text{R-GAIP}$ fusion protein showed an unchanged G protein selectivity profile as compared to the unmodified hH_4R protein (Schneider et al. 2009; Schneider and Seifert 2009). The unaltered G protein profile is surprising, because GAIP shows distinct affinities to different $\text{G}\alpha_i$ isoforms, which should theoretically influence the interaction between $\text{hH}_4\text{R-GAIP}$

and the G protein. The results, however, indicate that the G-protein-specificity of the hH₄R-GAIP fusion protein is governed by the properties of the receptor rather than by the RGS protein part. In summary, the hH₄R-GAIP fusion protein (co-expressed with G α_{i2} and G $\beta_1\gamma_2$) can fully replace the standard co-expression system (hH₄R + G α_{i2} + G $\beta_1\gamma_2$) in steady-state GTPase assays and allows the functional characterization of new hH₄R ligands with higher sensitivity and signal-to-noise ratio. The hH₄R-GAIP fusion protein approach was successfully used to evaluate a new class of N^G-acylated imidazolylpropyl-guanidine-derived hH₄R agonists (Ghorai et al. 2008; Igel et al. 2009b) or of cyanoguanidine-related hH₄R agonists (Igel et al. 2009a; Geyer et al. 2016). An overview of the most important features of the various H₄R/RGS fusion protein and co-expression approaches in comparison to the “standard” co-expression system (hH₄R + G α_{i2} + G $\beta_1\gamma_2$) is provided in Table 4.

2.4.10 Structure-Activity Relationships of hH₄R Inverse Agonists

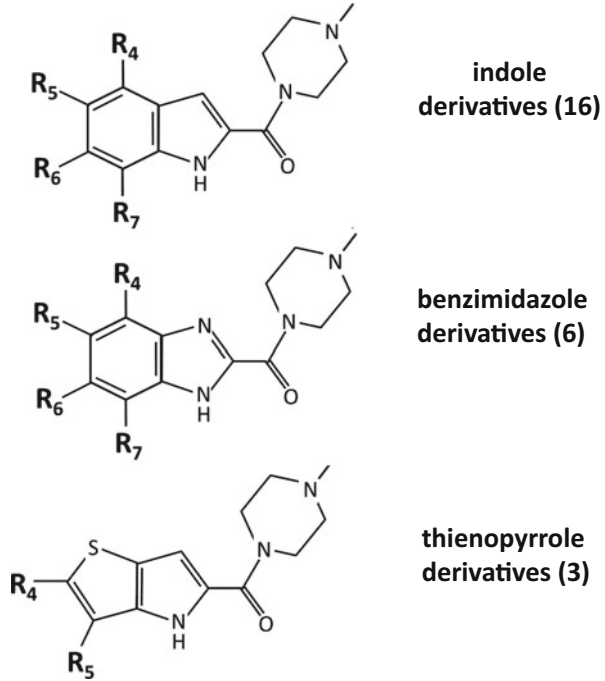
The high constitutive activity of hH₄R significantly reduces the signal-to-noise ratio in steady-state GTPase assays and reduces the sensitivity of agonist assays. However, this feature becomes an advantage, when inverse agonists are characterized. The hH₄R may maintain its constitutive activity under physiological conditions, because it is resistant to high sodium concentrations. As hypothesized by Schneider et al. (2009), on the one side, inverse agonists could be therapeutically advantageous in case of pathophysiologically increased constitutive H₄R activity, because they may exert a stronger anti-pruritic effect than neutral antagonists. On the other side, the re-folding of misfolded H₄R protein observed with the inverse agonist thioperamide (Schneider et al. 2009) (Sect. 2.4.4) may be a general effect of inverse H₄R agonists. Thus, inverse agonist-mediated upregulation of intact H₄R protein may result in rebound effects after drug discontinuation (Schneider et al. 2009). Although these hypotheses were not proven yet under physiological conditions, they illustrate the potential importance of characterizing inverse H₄R agonism during drug development. Therefore, structure-activity relationships for hH₄R inverse agonism should be established.

A series of 25 previously described (Venable et al. 2005) H₄R ligands (indoles, benzimidazoles, and thienopyrroles; Fig. 6) structurally derived from the prototypical H₄R antagonist JNJ7777120 (Thurmond et al. 2004) was characterized in [³H]histamine binding assays and steady-state GTPase assays using membranes expressing hH₄R + G α_{i2} + G $\beta_1\gamma_2$. The steady-state GTPase assays were performed in the absence of sodium chloride to obtain maximum constitutive activity.

The steady-state GTPase assay data reveal that most of the compounds were inverse agonists with a lower efficacy than thioperamide. Only three of the 25 compounds (~12%) were neutral antagonists (Schneider et al. 2010b). This confirms a previous analysis of literature data on 380 antagonists binding to 73 GPCRs. Only 15% of these compounds were neutral antagonists (Kenakin 2004). Thus, neutral antagonism seems to be a rare phenomenon.

In general, the pK_b values from steady-state GTPase assays in the presence of histamine fit very well to the pK_i values from [³H]histamine binding. In a subset of

Fig. 6 Scaffold structure of three classes of H₄R antagonists/inverse agonists. The numbers in brackets indicate the number of compounds tested



compounds, the pEC₅₀ values determined in the absence of histamine were significantly lower than the pK_i and/or pK_b values. Such discrepancies have been reported before for inverse agonists, e.g. at the hH₄R (Smits et al. 2008) or the β₂AR (Chidiac et al. 1994). Maybe, this subset of hH₄R antagonists discriminates between the agonist-free constitutively active receptor and the histamine-activated receptor state (Schneider et al. 2010b). These observations confirm the insufficiency of the two-state model of receptor activation and point to the existence of ligand-specific receptor states.

The potential binding mode of inverse hH₄R agonists of the indole series was analyzed by molecular dynamics simulations with the completely unsubstituted indole compounds (R₄₋₇ = H; Fig. 6). The positively charged piperazine amino group interacts electrostatically with the highly conserved Asp^{3.32}. Moreover, both the carbonyl moiety and the indole NH of the ligand establish an interaction with the side chain of the uncharged Glu^{5.46}. The indole moiety of the ligand shows a hydrophobic interaction with the indole part of Trp^{6.48} (Schneider et al. 2010b). Trp^{6.48} is a key player in the so-called rotamer toggle switch mechanism of receptor activation, which had been previously postulated for the β₂AR (Shi et al. 2002). The stabilization of Trp^{6.48} in its vertical conformation by the indole-derived ligand is a typical feature of the inactive receptor conformation and may explain the inverse agonism of such compounds. The benzimidazole-related structures bind in a similar way, but, in contrast to the indole-derived compounds, they form two tautomers with distinct binding modes (Schneider et al. 2010b).

Replacement of the R5/R7 hydrogen of the indole derivatives by the more space-filling chlorine increases H₄R binding affinity. Molecular dynamics simulations suggest that two small binding pockets in the H₄R protein may be filled by these chlorine residues, which increases the ligand-receptor contact area (Schneider et al. 2010b). Substitution of R5 by -OCH₃ reduces binding affinity, suggesting that larger substituents may be unfavorable. However, there is no significant correlation between molar volume and affinity of a series of indole compounds, suggesting that the volume of R5 may not be the only descriptor that influences binding affinity (Schneider et al. 2010b). By contrast, the size of R5 correlates excellently with the inverse agonistic efficacy of a subset of eight indole-derived compounds with varying R5 substituents. A calculation of the descriptors logP, molar refractivity, molar volume, polarizability, refraction index and polar surface area revealed that inverse agonistic efficacy solely depended on molar volume, but not on the other factors. The inverse agonistic efficacy of these compounds was inversely correlated to the molar volume of the substituent R5 (Schneider et al. 2010b).

In summary, despite the limited number of compounds and substitution patterns available, in this study the first structure-activity relationships for inverse H₄R agonism were identified. It was, however, not possible to predict all changes in binding mode and receptor conformation that result from small structural alterations of the ligand. Moreover, a general model that applies to structurally distinct classes of hH₄R inverse agonists could not be established yet. In the future, the hH₄R should be co-crystallized with various inverse agonists to elucidate the exact binding mode of these compounds. Although this would be a very ambitious project, the numerous crystallized ligand-receptor complexes published in the recent years (Cherezov et al. 2007; Rasmussen et al. 2007, 2011; Shonberg et al. 2015) demonstrate that this is not impossible.

3 Summary and Outlook

In this chapter, the results from the characterization of all four histamine receptor subtypes in the Sf9 insect cell system were summarized. On the one hand, it might be argued that insect cells do not represent physiological conditions as well as primary cells. On the other hand, it is difficult to isolate primary cells in sufficiently high numbers. Moreover, a side-by-side comparison of receptor isoforms or species orthologues in a defined environment is virtually impossible in primary cells. Since cells from different tissues have to be used, cell type-specific properties like crosstalk with other receptors or special features of the signaling pathways can lead to heterogeneous results, even for the same receptor isoform. Also, for some receptors like H₃R, no suitable primary cell system is available (Seifert et al. 2013).

Thus, for a comparison of the intrinsic properties of GPCR isoforms, e.g. G protein affinity/selectivity or constitutive activity, Sf9 cells represent a superior option. As explained in this chapter, Sf9 cells do not contain background GPCR activity and do not produce endogenous agonists activating mammalian GPCRs. Moreover, Sf9 cells allow the co-expression of defined mammalian Gα_s or Gα_i

protein subunits on a “clean” signaling background. This was demonstrated by the analysis of the hH₂R interaction with long and short Gα_s splice variants or by in-depth studies of hH₃R/hH₄R Gα_i isoform specificity and ion sensitivity. Table 5 shows numerous aspects of histamine receptor pharmacology addressed by using the Sf9 insect cell expression system.

The ligand binding studies and the G protein activation assays discussed in this chapter were all performed with radiolabeled reagents. Radioactivity-based assays, however, are increasingly hampered by legal overregulation and growing waste disposal costs. In this situation, fluorescence-based GPCR ligand binding and G protein activation assays could represent interesting alternatives. Unfortunately, many histamine receptor ligands are rather small molecules and easily lose binding affinity when coupled to a bulky fluorophore. Nevertheless, some progress has been made during the past years. For example, a cyanine dye-labeled aminopotentidine derivative exhibited nanomolar hH₂R potency (Xie et al. 2006c). Moreover, fluorescent pyrylium- or cyanine-labeled dimeric carbamoylguanidines were synthesized, but these compounds failed in binding assays due to intracellular accumulation and the resulting high fluorescence background (Kagermeier et al. 2015). A high-affinity fluorescent H₁R antagonist was obtained by labeling mepyramine with a BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-derived dye (Rose et al. 2012). Fluorescent hH₃R-selective ligands were developed by using the chalcone partial structure (Tomasch et al. 2012). Moreover, a compound named “Bodilisant,” which has been reported recently, is a BODIPY-labeled non-imidazole ligand with nanomolar hH₃R affinity (Tomasch et al. 2013). Some progress has also been made in the field of fluorescence-based G protein activation assays. For example, a europium-labeled non-hydrolysable GDP derivative can replace [³⁵S]GTPγS in GTPγS binding assays (Koval et al. 2010). This enables a time-resolved fluorescence-based assay that is, e.g., suited for the functional characterization of hH₃R ligands (Singh et al. 2012).

The functional assays described in this chapter focused on the determination of GPCR-mediated G protein activation (steady-state GTPase and [³⁵S]GTPγS binding assays). However, GPCRs can additionally activate G protein-independent signaling mechanisms, most importantly through β-arrestin recruitment (Lefkowitz and Shenoy 2005; Shukla et al. 2014). The hH₄R ligand JNJ-7777120, which acts as an inverse hH₄R agonist in G protein activation assays (Schneider et al. 2009), unexpectedly turned out to be an agonist with regard to hH₄R-mediated β-arrestin recruitment (Rosethorne and Charlton 2011). This phenomenon is also known as “biased signaling” or “functional selectivity” and has important implications for drug development (Seifert et al. 2011; Nijmeijer et al. 2013). In future studies, biased signaling of hH₁R, hH₂R, or hH₃R and functional selectivity of the corresponding ligands should be investigated in more detail.

However, the most important, but also most ambitious, goal in future studies would be the crystallization of all four histamine receptor subtypes. To date, only the crystal structure of the hH₁R has been resolved (Shimamura et al. 2011). The crystal structures of the histamine receptors are required to answer several still unresolved questions. For example, exact knowledge of the hH₄R conformation could help to explain, why this receptor shows such a high constitutive activity

Table 5 Various aspects of histamine receptor pharmacology and medicinal chemistry investigated in the Sf9 insect cell expression system

Aspect	Receptor subtype	Example	Reference
Species-specificity of receptor pharmacology	H ₁ R	<ul style="list-style-type: none"> • Efficacy/potency of some agonistic bulky 2-phenylhistamines and histaprodifens: gpH₁R > hH₁R • Potency of several arpromidine-type H₁R antagonists: gpH₁R > hH₁R 	Seifert et al. (2003)
	H ₂ R	<ul style="list-style-type: none"> • Affinity of large guanidine-type agonists in [³H]tiotidine binding: hH₂R-Gsα₅ < gpH₂R-Gsα₅ • GTPγS-sensitivity of high-affinity agonist binding: hH₂R-Gsα₅ > gpH₂R-Gsα₅ • potencies and efficacies of guanidines (steady-state GTPase): gpH₂R-Gsα₅ > hH₂R-Gsα₅ 	Kelley et al. (2001)
	H ₃ R	Imoproxifan: nearly full agonist at hH ₃ R, but inverse agonist at rH ₃ R (steady-state GTPase)	Schnell et al. (2010b)
	H ₄ R	Constitutive activity of mH ₄ R and rH ₄ R < hH ₄ R; inverse hH ₄ R agonist JNJ7777120 is a partial agonist at cH ₄ R, mH ₄ R and rH ₄ R; histamine potency at cH ₄ R, mH ₄ R and rH ₄ R < hH ₄ R	Schnell et al. (2011)
Studies with chimeric receptor proteins	H ₁ R	N-terminus and ECL2 of hH ₁ R replaced by guinea-pig sequences (h(gpNgpE2)H ₁ R): higher maximum G _q -activation and lower histamine potency as compared to hH ₁ R or h(gpE2)H ₁ R; extracellular surface of the H ₁ R influences ligand binding, recognition and guiding into the binding pocket	Strasser et al. (2008b) and Wittmann et al. (2011)
	H ₂ R	Comparison of hH ₂ R-A271D-Gsα ₅ , NhCgpH ₂ R-Gsα ₅ , NgpChH ₂ R-Gsα ₅ , hH ₂ R-Gsα ₅ and gpH ₂ R-Gsα ₅ to investigate the causes for the pharmacological differences between hH ₂ R and gpH ₂ R with regard to large guanidine-type agonists	Kelley et al. (2001)

(continued)

Table 5 (continued)

Aspect	Receptor subtype	Example	Reference
Importance of individual amino acids for defined receptor functions	H ₁ R	hH ₁ R-F153L and hH ₁ R-I433V: reduced expression, function and [³ H]mepyramine affinity, altered electrophoretic mobility; hH ₁ R-F153L/I433V double mutant: excellent expression, but only partial change of pharmacological properties (compared to wild-type hH ₁ R)	Seifert et al. (2003)
	H ₂ R	hH ₂ R-K173A-Gsα ₅ or hH ₂ R-K175A-Gsα ₅ : reduced histamine-induced steady-state GTPase signals	Preuss et al. (2007c)
	H ₃ R	hH ₃ R-D2.50N: interaction with Gα _{i3} disrupted, but still activation of Gα _{i1} , Gα _{i2} and Gα _{o1}	Schnell and Seifert (2010)
	H ₄ R	hH ₄ R-F168A: Contribution of ECL2 to ligand binding and constitutive activity	Wifling et al. (2015a)
hH ₄ R-R3.50A: DRY motif important for G protein coupling		Schneider et al. (2010a)	
Medicinal chemistry, SARs for ligands	H ₂ R	Replacement of the imidazolyl moiety in the imidazolylpropylguanidine structure by an aminothiazol moiety increases selectivity for H ₂ R over H ₁ R, H ₃ R and H ₄ R	Kraus et al. (2009)
	H ₄ R	N ^G -acylation increases hH ₄ R affinity and efficacy of the partial H ₃ R/H ₄ R agonist 3-(1 <i>H</i> -Imidazol-4-yl)propylguanidine (SK&F 91486) and reduces efficacy at hH ₃ R → increase of hH ₄ R selectivity	Igel et al. (2009b)
		5-Methyl substitution of imbutamine increases selectivity for hH ₄ R over hH ₃ R	Geyer et al. (2014)
Factors reducing constitutive activity	H ₁ R	Weak inverse agonism of some antidepressants or antipsychotics (e.g., chlorpromazine)	Appl et al. (2012)
	H ₂ R	hH ₂ R-Gsα ₅ or hH ₂ R-Gsα ₁ : weak inverse agonism of ranitidine and famotidine (efficacy higher at hH ₂ R-Gsα ₁ than at hH ₂ R-Gsα ₅)	Wenzel-Seifert et al. (2001)
	H ₃ R	Inverse agonist: thioperamide; inhibition of hH ₃ R constitutive activity by anions or cations:	Schnell and Seifert (2010)

(continued)

Table 5 (continued)

Aspect	Receptor subtype	Example	Reference
		$\text{Li}^+ \sim \text{Na}^+ \sim \text{K}^+ < \text{Cl}^- < \text{Br}^- < \text{I}^-$; strongest effect of sodium in the presence of $\text{G}\alpha_{i3}$	
	H ₄ R	(Partial) inverse agonist: thioperamide; insensitivity of constitutive activity to sodium	Schneider et al. (2009)
Stoichiometry of receptor/G protein coupling	H ₃ R	Coupling ratio in co-expression system: 1:2 ($\text{G}\alpha_{i1}$), 1:6 ($\text{G}\alpha_{i2}$); 1:3 ($\text{G}\alpha_{i3}$); 1:11 ($\text{G}\alpha_{o1}$)	Schnell et al. (2010a)
	H ₄ R	Coupling ratio in hH ₄ R/ $\text{G}\alpha_{i2}$ co-expression system: ~1:5; hH ₄ R- $\text{G}\alpha_{i2}$ fusion protein: ~1:1	Schneider et al. (2009)
Selectivity for closely related G proteins	H ₂ R	hH ₂ R-Gs α fusion protein: similar apparent constitutive activity with Gs α_L and Gs α_S	Wenzel-Seifert et al. (2001)
	H ₃ R	Interaction of hH ₃ R with $\text{G}\alpha_{i1}$, $\text{G}\alpha_{i2}$, $\text{G}\alpha_{i3}$, and $\text{G}\alpha_{i01}$ (co-expression): no pharmacological differences	Schnell et al. (2010a)
	H ₄ R	Co-expression system, comparison of hH ₄ R coupling to $\text{G}\alpha_{i1-3}$ and $\text{G}\alpha_o$: most efficient activation of $\text{G}\alpha_{i2}$, least efficient interaction with $\text{G}\alpha_o$	Schneider et al. (2009)
Role of N-terminal glycosylation for receptor function	H ₄ R	Tunicamycin (glycosylation inhibitor): no effect on total hH ₄ R protein expression and on [³ H]histamine affinity, but reduction of B_{max} value → N-terminal glycosylation important for correct folding; less efficient activation of $\text{G}\alpha_{i2}$ by deglycosylated hH ₄ R	Schneider et al. (2009)
Function of RGS proteins in receptor/G protein coupling	H ₁ R	Co-expression of hH ₁ R with RGS4 or GAIP unmasks a productive interaction with insect cell $\text{G}\alpha_q$	Houston et al. (2002)
	H ₄ R	hH ₄ R-RGS4 fusion protein: increase of baseline steady-state GTPase activity and of thioperamide inverse agonistic effect (compared to hH ₄ R); hH ₄ R-GAIP: pharmacological properties unchanged (compared to hH ₄ R), but significantly increased relative histamine- and thioperamide-induced GTPase signals	Schneider and Seifert (2009)

(continued)

Table 5 (continued)

Aspect	Receptor subtype	Example	Reference
Analysis of the G protein cycle	H ₂ R	Different ternary complex stabilization of hH ₂ R-Gα _s or gpH ₂ R-Gα _s by N ^G -acylated imidazolylpropylguanidines (AIPGs) as compared to the corresponding guanidines	Xie et al. (2006a)
	H ₄ R	GTPγS binding: GTPγS affinity of Gα _{i2} (co-expressed with hH ₄ R and Gβ ₁ γ ₂) was significantly reduced by the inverse agonist thioperamide; fusion of Gα _{i2} to hH ₄ R increases Gα _{i2} affinity	Schneider et al. (2009)
	H ₄ R	GTP hydrolysis: a GTP turnover number of ~4 was determined for hH ₄ R (+Gβ ₁ γ ₂ , +Gα _{i2}); the influence of regulators of G protein signaling (co-expressed or fused to hH ₄ R) on the K _M value of the Gα _{i2} GTPase activity was determined	Schneider et al. (2009) and Schneider and Seifert (2009)
	H ₂ R	Effector activation: Comparison of AC activity (basal, with GTP and with GTP/histamine) in membranes expressing h, gp, r or cH ₂ R (alone or combined with Gsα _s or fused to Gsα _s) → highest basal and GTP-induced AC activity detected for cH ₂ R	Preuss et al. (2007a)
Probing of receptor models	H ₄ R	Two-state model: high tendency of the hH ₄ R to occur in the active state; no stabilization of the inactive state by sodium; equilibrium shifted to the inactive side by thioperamide and other inverse agonists	Schneider et al. (2009)
	H ₃ R, H ₄ R	Protean agonism: Steady-state GTPase assays with membranes co-expressing hH ₃ R with Gβ ₁ γ ₂ plus Gα _{i1} , Gα _{i2} , Gα _{i3} , or Gα _{o1} as well as with membranes co-expressing hH ₃ R-Gα _{i2} or hH ₃ R-Gα _{o1} did not confirm the previously reported protean agonism of proxyfan	Schnell et al. (2010a)
		Comparison of H ₄ R mutants/species orthologues with different constitutive activities: JNJ-7777120 is a protean agonist	Wifling et al. (2015b)

(continued)

Table 5 (continued)

Aspect	Receptor subtype	Example	Reference
	H ₁ R, H ₂ R	Ligand-specific conformations: hH ₂ R, gpH ₂ R or rH ₂ R: AIPGs stabilize different active conformations as compared to guanidines. hH ₁ R: Unlike guanidines, AIPGs stabilize a partially active state	Xie et al. (2006a)
Comparison of receptors coupled to the same G protein type	β ₂ AR/ hH ₂ R (Gα _s)	Higher constitutive activity of β ₂ AR-Gsα _L as compared to β ₂ AR-Gsα _s , but comparable constitutive activity of hH ₂ R-Gsα _L and hH ₂ R-Gsα _s → GDP affinity of G proteins does not influence the constitutive activity of all GPCRs	β₂AR: Seifert et al. (1998b) hH₂R: Wenzel-Seifert et al. (2001)
	hH ₃ R/ hH ₄ R (Gα _{i2})	Steady-state GTPase assays: unlike hH ₄ R, the hH ₃ R exhibits NaCl-sensitive constitutive activity	hH₃R: Schnell and Seifert (2010) hH₄R: Schneider et al. (2009)
	hH ₄ R/ FPR1 (Gα _{i2})	hH ₄ R: high NaCl-resistant constitutive activity, no effect of GTPγS on high-affinity agonist ([³ H]histamine) binding; FPR1: high NaCl-sensitive constitutive activity, dramatic reduction of high-affinity agonist ([³ H]fMLF) binding by GTPγS	hH₄R: Schneider et al. (2009) FPR1: Wenzel-Seifert et al. (1998)

(Schneider et al. 2009; Wifling et al. 2015a). Moreover, a crystal structure of the hH₃R may provide important information about the hH₃R-G protein interaction interface and possibly answer the question, why the hH₃R discriminates between Gα_{i3} and other Gα_{i/o} isoforms (Schnell and Seifert 2010). Furthermore, an hH₃R crystal may lead to the identification of the anion binding sites responsible for the monovalent anion-mediated reduction of constitutive hH₃R activity (Schnell and Seifert 2010). Finally, the knowledge of H_xR crystal structures could lead to the development of compounds that alter H_xR function as allosteric modulators. The concept of GPCR modulation by allosteric ligands is well established, and such ligands have been identified, e.g. for dopamine, muscarinic, adenosine, or chemokine receptors (Christopoulos 2014). By contrast, to the best of our knowledge, to date nothing is known about allosteric modulation of histamine receptors.

As a prerequisite for the preparation of H_xR crystals, high amounts of receptor protein have to be expressed, e.g. in Sf9 cells. After purification and solubilization,

the physical properties of the receptors can be investigated, e.g. with fluorescence-based methods. Such studies have been previously performed with the β_2 AR (Gether et al. 1995; Kobilka 1995; Neumann et al. 2002) and were important steps towards the final goal of receptor crystallization (Cherezov et al. 2007; Rasmussen et al. 2007, 2011).

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