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Histamine H_4 receptor–RGS fusion proteins expressed in Sf9 insect cells: A sensitive and reliable approach for the functional characterization of histamine H_4 receptor ligands

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ABSTRACT

The human histamine H_4 receptor (hH_4R), co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 cells, is highly constitutively active. In the steady-state GTPase assay, the full agonist histamine (HA) induces only a relatively small signal (~20–30%), resulting in a low signal-to background ratio. In order to improve this system for ligand screening purposes, the effects of the regulators of G-protein signaling (RGS) RGS4 and RGS19 (GAIP) were investigated. RGS4 and GAIP were fused to the C-terminus of hH_4R or co-expressed with non-fused hH_4R, always combined with $G\alpha_{i2}$ and $G\beta_1\gamma_2$. The non-fused RGS proteins did not significantly increase the relative effect of HA. With the hH_4R–RGS4 fusion protein the absolute GTPase activities, but not the relative HA-induced signal were increased. Fusion of hH_4R with GAIP caused a selective increase of the HA signal, resulting in an enhanced signal-to-noise ratio. A detailed characterization of the hH_4R–GAIP fusion protein (co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$) and a comparison with the data obtained for the non-fused hH_4R (co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$) led to the following results: (i) the relative agonist- and inverse agonist-induced signals at hH_4R–GAIP are markedly increased. (ii) Compared to the wild-type hH_4R, standard ligands show unaltered potencies and efficacies at hH_4R–GAIP. (iii) Like hH_4R, hH_4R–GAIP shows high and NaCl-resistant constitutive activity. (iv) hH_4R–GAIP shows the same G-protein selectivity profile as the non-fused hH_4R. Collectively, hH_4R–GAIP provides a sensitive test system for the characterization of hH_4R ligands and can replace the non-fused hH_4R in steady-state GTPase assays.

1. Introduction

Histamine exerts its physiological effects *via* binding at four different receptor subtypes. The H_1-receptor mediates e.g. the increase of vascular permeability and NO production associated with inflammatory and allergic reactions [1]. The H_2-receptor regulates gastric acid secretion and shows a positive inotropic effect on the heart [1]. The presynaptic H_3-receptor negatively modulates neurotransmitter release in the CNS [1]. The fourth histamine (HA) receptor was first pharmacologically characterized on human eosinophils [2] and was later identified as a GPCR with 390 amino acids [3], sharing 43% overall homology with the H_3-receptor [4].

The human histamine H_4 receptor (hH_4R) is expressed e.g. in spleen and bone marrow [5,6] and mediates HA-induced chemotaxis, e.g. of eosinophils [7] and mast cells [8], suggesting a role in inflammatory and immunological processes. Recently, the hH_4R was also detected in the brain and may be involved in the regulation of central neurotransmission [9].

In animal models, H_4R antagonists were effective in the treatment of itch [10], colitis [11] or allergic airway inflammation [12]. Since pruritus, colitis or asthma still lack a curative or at least an optimized alleviating therapy, it is vitally important to investigate the potential of hH_4R antagonists for the treatment of these widespread diseases. Thus, reliable test systems are required to characterize compounds that could serve as potential candidates for new hH_4R-antagonizing anti-inflammatory drugs. To obtain a most reliable readout of receptor activation or inhibition, it is necessary to determine the functional signal as proximal to the receptor activation event as possible. Assays that determine a signal more downstream from receptor activation (e.g. adenylyl cyclase or reporter gene assays), may suffer from unclear and complicated stoichiometry of the involved proteins or from interfering side-processes in the signal transduction cascade. For example, it is reported for S49 cells that G-proteins exist in stoichiometric excess compared to the effector adenylyl cyclase

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(AC), which limits the agonist-induced stimulation of AC activity [13]. This may hamper the determination of small efficacy differences between different partial agonists in AC assays. Moreover, as reported for the hH_4R antagonist JNJ-7777120, cAMP reporter gene assays can eliminate the effects of partial inverse agonists, which, in contrast, are still detectable by steady-state GTPase assays [14].

The steady-state GTPase assay with receptors and G-proteins expressed in baculovirus-infected Sf9 cells provides a reliable and sensitive test system with a very proximal readout. In general, the steady-state GTPase assay, when used as readout for $G\alpha_i$-coupled receptors, shows a higher sensitivity than cAMP accumulation or AC assays [15]. When GPCR–$G\alpha$ fusion proteins are used, steady-state GTPase assays can be performed with a defined 1:1 stoichiometry of receptor and G-protein [16].

Steady-state GTPase assays with Sf9 cell membranes were successfully employed for the investigation of the formyl peptide receptor clone 26 [17], the chemokine receptor CXCR4 [18] or the cannabinoid receptor subtypes CB_1 and CB_2 [19]. Recently, we also reported on the characterization of the hH_4R in Sf9 cells [14]. However, the hH_4R system showed a very weak relative agonist-induced signal (20–30%). This resulted in a low signal-to-noise ratio. Fusion of the hH_4R to $G\alpha_{i2}$ did not improve the relative intensity of the agonist-induced signal, since it resulted in an increase of the constitutive activity in steady-state GTPase assays [14].

An interesting possibility to increase signal intensity in steady-state GTPase assays is the co-expression of regulators of G-protein signaling (RGS). RGS proteins form a large group of proteins that are classified in eight subfamilies, showing high structural diversity [20]. A common feature of all RGS proteins is the RGS-domain, which consists of 120 amino acids and is of central importance for binding $G\alpha$ subunits and accelerating their GTPase activity [20]. It has also been reported that the effect of RGS4 on the GTPase activity induced by the α_{2A} adrenoceptor was enhanced by fusing the C-terminus of the GPCR to the N-terminus of the RGS protein. Despite the covalent binding of the RGS protein to the receptor, there was no interference with receptor-mediated activation of the G-protein [21].

In this paper we report on the co-expression of the RGS proteins RGS4 and RGS19 (GAIP) with hH_4R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 cells by performing quadruple infections with genetically modified baculoviruses. Moreover, we adopted the approach from Ref. [21] to the H_4R and generated fusion proteins with RGS4 and GAIP that were co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$.

RGS4 belongs to the R4 sub-family of RGS proteins and accelerates the GTPase activity of $G\alpha_i$ [22,23] and $G\alpha_q$ [24] proteins. Two conserved cysteines in the RGS4 N-terminus act as potential palmitoylation sites [25]. GAIP (RGS19) belongs to the RZ sub-family and interacts with members of the $G\alpha_i$ class, but not with $G\alpha_q$ [26]. Membrane-bound GAIP is highly palmitoylated in its cysteine string region, containing eight cysteines [26].

We chose these two RGS proteins, because they belong to the structurally simplest sub-families and do not possess additional functional domains. Their stimulating effect on GPCR-activated $G\alpha_q$ and $G\alpha_i$ proteins was previously demonstrated for the human H_1R [27] and the chemokine receptor CXCR4 [18]. Thus, RGS4 and GAIP should be promising candidates for enhancing the GTPase activity in the co-expression system of the hH_4R with $G\alpha_{i2}$ and $G\beta_1\gamma_2$.

2. Materials and methods

2.1. Materials

The pcDNA 3.1 plasmids containing the sequences encoding RGS4 and GAIP were obtained from the UMR cDNA Resource Center at the University of Missouri-Rolla (Rolla, MO, USA). The DNA primers for PCR were synthesized by MWG Biotech (Ebersberg, Germany). The *Pfu* polymerase was obtained from Stratagene (La Jolla, CA, USA). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Recombinant baculovirus encoding the unmodified versions of the $G\beta_1\gamma_2$ subunits was a kind gift of Dr. P. Gierschik (Department of Pharmacology and Toxicology, University of Ulm, Germany). Recombinant baculoviruses for $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ were donated by Dr. A.G. Gilman (Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX, USA) and the baculovirus encoding rat $G\alpha_o$ was generously provided by Dr. J.C. Garrison (University of Virginia, Charlottesville, VA). Baculoviruses for mammalian RGS4 and GAIP (N-terminally His-tagged) were kindly donated by Dr. E. Ross (Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX, USA).

The anti-$G\alpha_o$ antibody was purchased from Calbiochem (San Diego, CA, USA); the M1 anti-FLAG antibody was obtained from Sigma (St. Louis, MO, USA). The antibody recognizing the $G\alpha_i$ subunits (anti-$G\alpha_{common}$) was generously provided by Dr. B. Nürnberg (Institute for Pharmacology, University of Tübingen, Germany). The antibodies selective for RGS4 and GAIP were purchased from Santa Cruz (Santa Cruz, CA, USA). The H_4R antagonist 1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methyl-piperazine (JNJ-7777120) was kindly provided by Dr. Robin Thurmond (Department of Immunology, Johnson & Johnson Pharmaceutical R&D, San Diego, CA, USA). Immepip, imetit, iodophenpropit, R-α-methylhistamine, 5-methylhistamine and THIO were obtained from Tocris (Avonmouth, Bristol, UK). HA was purchased from Sigma (St. Louis, MO, USA). The 10 mM stock solution of JNJ-7777120 was prepared in dry Me_2SO, the stock solutions (10 mM) and dilutions of all other H_4R agonists and antagonists described in this paper were prepared in distilled water.

[^{3}H]HA (specific activities 14–18 Ci/mmol) and [^{3}H]dihydroalprenolol (97.4 Ci/mmol) were obtained from PerkinElmer (Boston, MA, USA). [γ-^{32}P]GTP was purchased from PerkinElmer or was prepared in our laboratory using GDP and [^{32}P] (orthophosphoric acid, 150 mCi/ml, obtained from PerkinElmer) according to a previously described enzymatic labeling procedure [28]. $MgCl_2$ was purchased from Merck (Darmstadt, Germany) and Tris base was obtained from usb (Cleveland, OH, USA). Radioactive samples were counted in a PerkinElmer Tricarb 2800TR liquid scintillation analyzer.

2.2. Construction of pVL-1392 plasmids encoding FLAG-hH_4R-His_6, FLAG-hH_4R-His_6-$G\alpha_{i2}$, FLAG-hH_4R-His_6-RGS4 and FLAG-hH_4R-His_6-GAIP

The generation of FLAG-hH_4R-His_6 as well as of FLAG-hH_4R-His_6-$G\alpha_{i2}$ was previously described [14]. The hexahistidine tagged C-terminus of the histamine H_4 receptor was fused to the N-terminus of RGS4 or GAIP by overlap extension PCR using *Pfu* polymerase as follows.

For preparation of the FLAG-hH_4R-His_6-RGS4 fusion protein, two fusion primers were synthesized. The sense primer s6H-RGS4 (5′-CAC CAT CAT CAC CAT CAC ATG TGC AAA GGG CTT GC-3′) contained an 18 bp sequence encoding a hexahistidine tag followed by the first 17 bp of the RGS4 cDNA. The antisense primer a6H consisted only of the 18 bp sequence encoding the hexahistidine tag (5′-GTG ATG GTG ATG ATG GTG-3′). In PCR 1a the sequence between the sEcoRI-hH_4 primer (5′-GCC ATC ACA TCA TTC TTG GAA TTC GTG ATC CCA GTC-3′) and the a6H fusion primer was amplified using the pGEM-3Z-SF-hH_4R-His_6 plasmid as template. In PCR 1b the RGS4 sequence between the s6H-RGS4 fusion primer and the antisense primer aRGS4-XbaI (5′-TCT AGA CTC GAG TTA GGC ACA CTG AGG GAC C-3′) was