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Heterologous expression of rat epitope-tagged histamine H_2 receptors in insect Sf9 cells

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1 Rat histamine H_2 receptors were epitope-tagged with six histidine residues at the C-terminus to allow immunological detection of the receptor. Recombinant baculoviruses containing the epitope-tagged H_2 receptor were prepared and were used to infect insect Sf9 cells.

2 The His-tagged H_2 receptors expressed in insect Sf9 cells showed typical H_2 receptor characteristics as determined with [^{125}I]-aminopotentidine (APT) binding studies.

3 In Sf9 cells expressing the His-tagged H_2 receptor histamine was able to stimulate cyclic AMP production 9 fold ($EC_{50} = 2.1 \pm 0.1$ μM) by use of the endogenous signalling pathway. The classical antagonists cimetidine, ranitidine and tiotidine inhibited histamine induced cyclic AMP production with K_i values of 0.60 ± 0.43 μM, 0.25 ± 0.15 μM and 28 ± 7 nM, respectively (mean $\pm$ s.e.mean, $n = 3$).

4 The expression of the His-tagged H_2 receptors in infected Sf9 cells reached functional levels of 6.6 ± 0.6 pmol mg^{-1} protein (mean $\pm$ s.e.mean, $n = 3$) after 3 days of infection. This represents about 2×10^6 copies of receptor/cell. Preincubation of the cells with 0.03 mM cholesterol-β-cyclodextrin complex resulted in an increase of [^{125}I]-APT binding up to $169 \pm 5\%$ (mean $\pm$ s.e.mean, $n = 3$).

5 The addition of 0.03 mM cholesterol-β-cyclodextrin complex did not affect histamine-induced cyclic AMP production. The EC_{50} value of histamine was 3.1 ± 1.7 μM in the absence of cholesterol-β-cyclodextrin complex and 11.1 ± 5.5 μM in the presence of cholesterol-β-cyclodextrin complex (mean $\pm$ s.e.mean, $n = 3$). Also, the amount of cyclic AMP produced in the presence of 100 μM histamine was identical, 85 ± 18 pmol/10^6 cells in the absence and 81 ± 11 pmol/10^6 cells in the presence of 0.03 mM cholesterol-β-cyclodextrin complex (mean $\pm$ s.e.mean, $n = 3$).

6 Immunofluorescence studies with an antibody against the His-tag revealed that the majority of the His-tagged H_2 receptors was localized inside the insect Sf9 cells, although plasma membrane labelling could be identified as well.

7 These experiments demonstrate the successful expression of His-tagged histamine H_2 receptors in insect Sf9 cells. The H_2 receptors couple functionally to the insect cell adenylate cyclase. However, our studies with cholesterol complementation and with immunofluorescent detection of the His-tag reveal that only a limited amount of H_2 receptor protein is functional. These functional receptors are targeted to the plasma membrane.

Keywords: Sf9 cells; histamine H_2 receptors; epitope tagging; cyclic AMP production; [^{125}I]-aminopotentidine binding; cholesterol; glycosylation

Introduction

The histamine H_2 receptor belongs to the large family of G protein-coupled receptors (GPCRs) (Leurs *et al*., 1995). Using parietal cells mRNA and degenerate primers based on the structural homology of various GPCRs, Gantz *et al.* cloned the cDNA encoding the canine H_2 receptor. (Gantz *et al*., 1991b). The rat (Ruat *et al*., 1991), human (Gantz *et al*., 1991a) and guinea-pig (Traiffort *et al*., 1995) homologues followed rapidly. Heterologous expression of the various H_2 receptor cDNAs has allowed detailed studies of signal transduction (Traiffort *et al*., 1992; Leurs *et al*., 1994; Harteneck *et al*., 1995; Kühn *et al*., 1996) and receptor regulation (Smit *et al*., 1995; 1996a,b) and has provided new information on the molecular properties of the H_2 receptor protein (Leurs *et al*., 1995).

Following the successful expression of bovine rhodospin (Janssen *et al*., 1988; 1991), in recent years several GPCRs have been heterologously expressed in insect Sf9 cells. Many of these GPCRs belong to the class of biogenic amine receptors. The β_2-adrenoceptor was the first GPCR for biogenic amines that was expressed in Sf9 cells (Mouillac *et al*., 1992). Soon after, several other biogenic amine receptors such as α_{2A}- and α_{2B}-adrenoceptors (Jansson *et al*., 1995), M_2- and M_5-muscarinic (Hu *et al*., 1994; Heitz *et al*., 1995), D_1 and D_2 dopamine- (Javich *et al*., 1994; Ng *et al*., 1994) and 5-HT_{1A}, 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$ and 5-HT_{1E} 5-hydroxytryptamine receptors (Mulheron *et al*., 1994; Parker *et al*., 1994; Butkerait *et al*., 1995) were expressed in this system. The high expression level of GPCRs in insect cells allows an easy molecular and pharmacological analysis of the receptor protein; Robeva *et al*. (1996) demonstrated, for example, the successful expression of the adenosine A_{2A} receptor in insect Sf9 cells, whereas high level expression of this receptor in mammalian cells had failed. In addition, second messenger systems such as adenylate cyclase (Mouillac *et al*., 1992; Chidiac *et al*., 1993; Ng *et al*., 1994; 1995; Mulheron *et al*., 1994; Butkerait *et al*., 1995; Jansson *et al*., 1995) and phospholipase C (Hu *et al*., 1994; Alblas *et al*., 1995) are present in Sf9 cells and couple in most cases functionally to expressed mammalian GPCRs. Moreover, Nebigil *et al*. (1995) have been able to study 5-HT_{1A} receptor desensitization and phosphorylation in intact Sf9 cells, whereas GPCRs expressed in Sf9 cell membranes have been shown to be suitable substrates for *in vitro* GRK (G protein-coupled receptor kinases)-mediated phosphorylation (Pei *et al*., 1994). As mentioned, expression levels of these receptors are usually high in Sf9 cells and, in combination with the relative ease of culturing large volumes in spinner flasks, this has led to the purification of β_2-adrenoceptors (Kobilka, 1995; Kwatra *et al*.,

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1995), adenosine A_1 receptors (Robeva *et al*., 1996) and rhodospin (De Caluwé *et al*., 1993; Janssen *et al*., 1995) from Sf9 cells.

In the present study, we demonstrate the functional expression of the rat histamine H_2 receptor in Sf9 cells. An epitope tag of six histidine residues was introduced at the C-terminus of the receptor allowing the verification of H_2 receptor expression by Western blotting and immunofluorescence microscopy (Janssen *et al*., 1995). The expressed H_2 receptors were characterized pharmacologically by [^{125}I]-iodoaminopotentidine binding studies and the measurement of cyclic AMP production. The expression of the H_2 receptor in Sf9 cells has been demonstrated previously (Harteneck *et al*., 1995; Kühn *et al*., 1996), but in those studies no pharmacological or molecular characterization of the expressed H_2 receptor protein was given.

Methods

Construction of epitope-tagged H_2 receptors

An *Eco*RI restriction site was introduced at the 3′ end of the rat H_2 receptor cDNA (Ruat *et al*., 1991) at nucleotide position 1045 by replacement of a C by a T by use of the pAlter system (Promega) with a 5′-phosphorylated oligonucleotide (5′-GGG GTG TGT *GAA* TTC TGT CCC ACT C, Eurogentec, Seraing, Belgium) containing an *Eco*RI site (italic). The mutant receptor cDNA was digested with *Bsm*I and *Eco*RI. The *Bsm*I/*Eco*RI fragment was isolated and ligated together with a linker, encoding the C-terminal H_2 receptor sequence, a six histidine epitope-tag (underscored), a stopcodon (bold), a 3′ *Bgl*II site (italic) and a 5′ sequence that eliminated the *Eco*RI site again (T was replaced by a C), into a pSP73 plasmid that contained the rat H_2 receptor cDNA from which a *Bsm*I/*Bgl*II fragment had been removed. The linker consisted of two complementary oligonucleotides (Eurogentec, Seraing, Belgium): 5′-AA TTG ACA CAC CCC CAG GGA AAC CCA ATC AGG CAC CAT CAC CAT CAC CAC **TAA** AGG TTG TA and 5′-C TGT GTG GGG GTC CCT TTG GGT TAG TCC GTG GTA GTG GTA GTG GTG **ATT** TCC AAC *ATC TAG*. The resulting pSP73 vector containing the His-tagged H_2 receptor cDNA was digested with *Hind*III, blunt ended and digested with *Bgl*II. The receptor cDNA was isolated and ligated into pVL1393 that had been cut with *Sma*I and *Bam*HI. To construct an H_2 receptor with a Flag- and a His-tag the pSP73 vector containing the His-tagged H_2 receptor was digested with *Kpn*I and *Bgl*II. In addition, a pSP73 vector containing the H_2 receptor with a Flag-tag (Smit *et al*., 1995) was digested with *Hind*III, blunt ended and digested with *KpnI*. Both fragments were ligated via a triple ligation into pVL1393 that had been cut with *SmaI* and *BamHI*.

Cell culturing

IPLB-Sf9 cells were cultured as monolayers in culture flasks (Costar, Badhoevedorp, The Netherlands) at 27°C in TNM-FH medium with 10% foetal calf serum, 5 mg ml^{-1} bovine serum albumin, 50 μg ml^{-1} streptomycin and 50 iu ml^{-1} of penicillin or as suspension cultures in spinner bottles in the same medium with the addition of 0.1% pluronic F-68 from Sigma.

Infection of Sf9 cells

Sf9 cells were transfected with 0.25 μg linearized Baculogold virus (Pharmingen, San Diego, U.S.A.) in combination with 3.7 μg pVL1393 containing the epitope-tagged H_2 receptor cDNA by use of the calcium phosphate precipitation method. Sf9 cells were grown to 70% confluency in 25 cm^2 culture flasks. DNA was mixed and left at room temperature for 5 min. Subsequently, 1 ml transfection buffer (25 mM HEPES, 125 mM $CaCl_2$, 140 mM NaCl, pH 7.10) was added to the DNA. TNM-FH medium was replaced by Grace's medium with 10% FCS, and without antibiotics. The DNA was added dropwise to the cells. After 4 h the cells were washed and cultured with TNM-FH with 10% FCS and antibiotics. After 6 days the supernatant was collected and a plaque assay was performed. Individual plaques were isolated and used to infect Sf9 cells to increase the virus titre. After two rounds of infection, the virus titre was determined with a plaque assay.

Sf9 cells were infected with a multiplicity of infection (MOI) of 0.3 unless indicated otherwise. For [^{125}I]-aminopotentidine (APT) binding experiments the cells were harvested 3 days post infection (d.p.i.), unless indicated otherwise. For adenosine 3′:5′-cyclic monophosphate (cyclic AMP) generation assays and confocal laser immunofluorescence microscopy cells were harvested at 2 d.p.i.

[^{125}I]-APT binding

Radiolabelled iodoaminopotentidine was synthesized as described previously (Ruat *et al*., 1990; Leurs *et al*., 1994). Cell homogenates were incubated in 50 mM Na_2HPO_4/KH_2PO_4 buffer pH 7.4 with 0.4 nM [^{125}I]-APT at 30°C for 90 min. Non-specific binding was determined in the presence of 1 μM tiotidine. The incubation was terminated by rapid dilution with 3 ml of ice-cold 20 mM Na_2HPO_4/KH_2PO_4 buffer (pH 7.4) supplemented with 0.1% ovalbumin. Bound radioligand was subsequently separated from non-bound with rapid filtration over GF/C filters pretreated with 0.3% PEI by use of a Brandell cell harvester. Filters were washed twice with 3 ml buffer and retained radioactivity was determined in an LKB-γ-counter at an efficiency of 63%.

Cyclic AMP generation

Infected Sf9 cells were centrifuged and washed twice with isoosmotic NaCl (140 mM NaCl, 5 mM KCl, 5 mM glucose, 20 mM Tris, pH 7.4). Cells were resuspended in isoosmotic NaCl to a final concentration of 10^6 cells ml^{-1}. Cells (150 μl) were incubated with 500 μM isobutylmethylxanthine (IBMX) and varying concentrations of histamine or forskolin in a total volume of 200 μl of isoosmotic NaCl for 30 min at 27°C. The incubation was terminated by freezing the cells at −20°C. After thawing, the cells were incubated at 95°C for 3 min. The cyclic AMP production was measured as described below.

Cyclic AMP determination

Formation of cyclic AMP was determined with a radioligand assay by use of protein kinase A (PKA). PKA was isolated from bovine adrenals as described previously (Smit *et al*., 1996b). Samples (50 μl) were incubated on ice for 150 min in a total volume of 225 μl isoosmotic NaCl containing 30,000 d.p.m. [^{3}H]-cyclic AMP and 100 μl PKA. A calibration curve was made with 0.25–16 pmol cyclic AMP/sample. Incubation was terminated by addition of 3 ml ice-cold 50 mM Tris pH 7.4 buffer followed by rapid filtration over GF/B filters with a Brandell cell harvester. Filters were counted in a β-counter at least 4 h after addition of 4 ml scintillation fluid.

Confocal laser microscopy

Sf9 cells were grown on cover slips (∅ 16 mm) at a density of 200,000 cells/cover slip and were infected for 2 days with an MOI of 10. After washing with PBS (3 × 5 min) the cells were fixed with 1% paraformaldehyde in 0.1 M Na_2HPO_4/KH_2PO_4 buffer pH 7.4 for 1 h. Next, the cells were dehydrated with methanol for 5 min at −20°C. Methanol was removed and the cells were air-dried. Cells were permeabilized with PBS containing 0.05% Tween-20, 1% gelatin and 2% FCS for 30 min at room temperature, thereafter the cells were incubated for 1 h with a polyclonal antibody. This antibody was raised against a conjugate of an 8 × His peptide with rabbit serum albumin and has been described in Vissers & DeGrip (1996).

Following washing with 0.05% Tween-20 in PBS the cells were incubated for 2 h with a rhodamine-conjugated swine-anti-rabbit antibody. Subsequently, all procedures were carried out in the dark. After being washed with PBS, the cells were washed with H_2O and were dried in 100% methanol. Subsequently, 10 μl mowiol was added to the cells, an object glass was attached, and the samples were stored at -20°C in the dark. Fluorescence was determined at 568 nm after excitation with 488 nm by an argon laser with a Biorad MRC1000 confocal microscope, the images were averaged over 5 scans.

Western blot

After centrifugation for 5 min at 3500 r.p.m. the cell pellets were taken up in sample buffer (2% sodium dodecyl sulphate (SDS), 0.04 M dithioerythrol (DTE) and 0.015% bromophenol blue in 0.5 M Tris pH 6.8). Samples were run on a 12% SDS-PAGE gel at 100 V for the stacking gel (5% acrylamide) and 200 V for the running gel. Protein was blotted onto nitrocellulose for 1 h at 100 V in ice-cold blot buffer (25 mM Tris, 0.2 M glycine in 20% methanol) with a Miniprotean system (Biorad). Blots were incubated for 20 min with 4% BSA in PBS at room temperature, followed by an overnight incubation at room temperature or a 2 h incubation at 37°C with either the rabbit polyclonal antibody against the histidine tag or the antibody against the Flag tag. Blots were washed 3×10 min with PBS followed by a 1 h incubation with a secondary swine-anti-rabbit antibody (histidine tag) or rabbit-anti-mouse antibody (Flag tag). Blots were washed again with PBS (3×10 min) and were incubated for 1 h with soluble complexes of peroxidase conjugated anti-peroxide antibodies from rabbit (histidine tag) or from mouse (Flag tag). After being washed with PBS (3×10 min) the presence of peroxidase activity was identified with 4-chloro-1-naphtol in 5 ml methanol with 15 μl 30% H_2O_2 in 25 ml phosphate-buffered saline (PBS).

Data analysis

K_i values of the H_2 receptor antagonists in the cyclic AMP experiments were calculated by use of the modified Cheng-Prusoff equation as described by Leff & Dougall (1993).

$$K_i = \frac{IC_{50}}{(2 + ([A]/[EC_{50}])^{n_H})^{1/n_H} - 1}$$

with [A] as the agonist concentration used and n_H is the Hill coefficient of the agonist dose-response curve. The Hill coefficient of the histamine dose-response curve was 0.99 ± 0.22 and this value was used in the calculations.

Chemicals

Histamine dihydrochloride, cyclic AMP, cholesterol-β-cyclodextrin complex, egg ovalbumin, forskolin, gelatin, IBMX and pluronic F-68 were obtained from Sigma (St. Louis, U.S.A.). Tunicamycin was from Boehringer Mannheim (Almere, The Netherlands). The mouse anti-Flag M2 monoclonal antibody was obtained from International Biotechnology Inc. Secondary antibodies were obtained from DAKO, Glostrup, Denmark. [^{3}H]-cyclic AMP (40 Ci mmol^{-1}) was from Amersham. Gifts of cimetidine (Smith Kline Beecham, U.K.), ranitidine dihydrochloride (Glaxo, U.K.) and tiotidine (ICI, U.K.) were greatly appreciated.

Results

After the successful generation of recombinant baculovirus containing the epitope-tagged rat H_2 receptor, insect Sf9 cells were infected to study the properties of the expressed H_2 receptor protein. First of all two parameters, days post infection (d.p.i.) and multiplicity of infection (MOI), were optimized to achieve maximal H_2 receptor protein expression levels in Sf9 cells. In Figure 1a and b a characteristic experiment showing the effect of both parameters on the expression levels of His-tagged H_2 receptors is presented. Receptor levels were verified by radioligand binding of the H_2 antagonist [^{125}I]-APT. In addition, the level of receptor expression as a function of d.p.i. was monitored on a Western blot stained with an antibody against the His-tag (Figure 2a). Figure 2b shows a Western blot stained with an antibody against the Flag-tag. The immunoreactive band at approximately 36 kD on these blots represents the H_2 receptor. In mock-infected Sf9 cells, cells that were infected with control baculovirus without the receptor sequence, this immunoreactive band is absent (lane 6). Also at 1, 2 and 4–6 d.p.i. this immunoreactive band was absent in mock-infected cells (data not shown). In time there seems to be a strong increase in aggregated receptor (immunopositive bands at >100 kD). Both the radioligand binding experiments and the Western blot experiments show maximal receptor expression levels at 3–4 d.p.i. Optimal expression levels at 3–4 d.p.i. were obtained with an MOI of 0.3–1.0.

In addition, the effect of tunicamycin (a glycosylation inhibitor) on the expression of His-tagged H_2 receptors was determined. Tunicamycin was added, immediately after infection with recombinant baculovirus, at a concentration of 3 μg ml^{-1}. As can be seen in Figure 2a neither the size, nor the intensity of the immunoreactive band at 36 kD was altered upon tunicamycin treatment. Yet, the amount of specific labelling with [^{125}I]-APT was decreased to $59.5 \pm 3.2\%$ (mean $\pm$ s.e.mean, $n=3$) of non-treated cells. In HEK-293 cells expressing rat H_2 receptors (Smit *et al*., 1995) incubation with 3 μg ml^{-1} tunicamycin also resulted in a decrease in receptor labelling to approximately 10% (9.4% and 10.8%, $n=2$).

The His-tagged H_2 receptors on Sf9 cells were pharmacologically characterized after infection of the cells under the

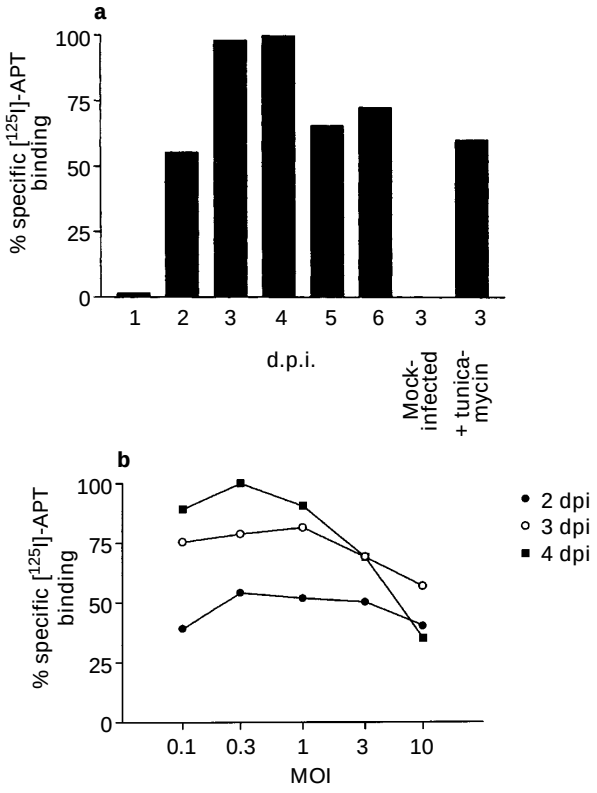

Figure 1 Effect of d.p.i. (a) and both MOI and d.p.i. (b) on the expression level of rat H_2 receptors on Sf9 cells. Experiments are single measurements representing three and two independent experiments, respectively. The maximum expression level was 2.7 pmol mg^{-1} protein. Tunicamycin was used at a concentration of 3 μg ml^{-1}.

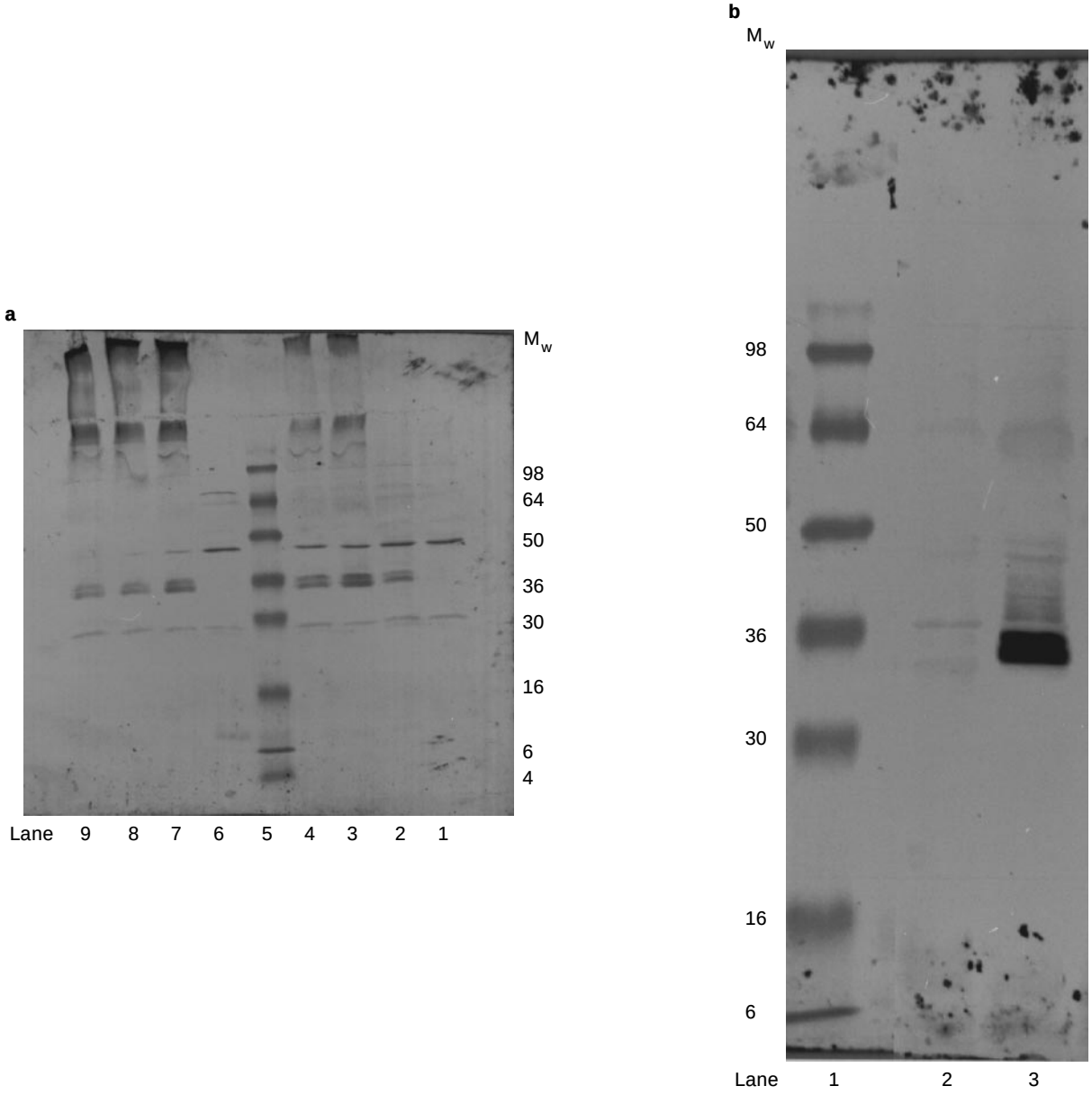

Figure 2 Western blots of Sf9 cells infected with H_2 (His)6 × baculovirus (MOI = 0.3) and stained as described in Methods. Blot (a) was stained with the antibody against the His-tag. Lanes 1–3 and 7–9 represent 1–6 d.p.i., respectively. The effect of 3 μg ml^{-1} tunicamycine at 3 d.p.i. is shown in lane 4. Molecular weight calibration is shown in lane 5. Lane 6 represents mock-infected cells at 3 d.p.i. Blot (b) was stained with an antibody against the Flag-tag. Lane 1 represents the molecular weight calibration, lane 2 the mock infected cells at 3 d.p.i. and lane 3 cells infected with H_2 (His)6 × baculovirus (MOI = 0.3).

above-mentioned optimal conditions (3 d.p.i., MOI 0.3). Both the endogenous ligand histamine and the H_2 antagonists tiotidine, cimetidine, ranitidine, metiamide and iodoaminopotentidine were studied for their ability to displace the binding of [^{125}I]-APT. The rat H_2 receptor expressed in CHO cells was used for comparison (Table 1). Iodoaminopotentidine was the most potent antagonist of the series tested with a K_i of 0.63 ± 0.08 nM (mean ± s.e.mean, $n = 3$), followed by tiotidine, ranitidine, cimetidine and metiamide. We were unable to discriminate between high and low affinity sites for histamine and upon addition of guanosine 5′-*O*-(γ-thio-triphosphate) (GTPγS) no shift of the displacement curve was observed (Table 1).

Although the radioligand binding studies did not reveal an interaction of the H_2 receptor with G proteins, we investigated the ability of the His-tagged rat H_2 receptors to stimulate adenylate cyclase via endogenous G proteins. In Figure 3 the dose-dependent production of cyclic AMP after stimulation of the insect Sf9 cells with histamine is shown. Histamine induced cyclic AMP production (9 fold over basal) with an EC_{50} value of 2.1 ± 0.1 μM (mean ± s.e.mean, $n = 3$). No histamine-induced cyclic AMP production was obtained in Sf9 cells infected with wild-type baculovirus (Figure 3). Basal cyclic AMP levels in mock-infected Sf9 cells, 5.4 ± 2.5 pmol/10^6 cells (mean ± s.e.mean, $n = 3$), did not differ significantly from basal cyclic AMP levels in Sf9 cells expressing His-tagged H_2 receptors, 13.3 ± 6.0 pmol/10^6 cells (mean ± s.e.mean, $n = 3$). The histamine-induced cyclic AMP production was dose-dependently inhibited by the H_2 antagonists cimetidine, ranitidine and tiotidine (Figure 4). Of these three antagonists tiotidine was the most potent with a K_i value of 28 ± 7 nM, followed by ranitidine, $K_i = 0.25 \pm 0.15$ μM and cimetidine $K_i = 0.60 \pm 0.43$ μM (mean ± s.e.mean, $n = 3$).

Besides ligand binding and second messenger responses we also studied the localization of the rat H_2 receptors with im-

munofluorescent confocal laser microscopy. A representative picture is shown in Figure 5a. Using the antibody against the His-tag, some plasma membrane staining was observed, but the majority of the H_2 receptors was localized in intracellular compartments. As a control, Sf9 cells infected with wild type baculoviruses are shown in Figure 5b; no specific immunofluorescence was detected in these cells.

Recently, cholesterol has been shown to increase the number of high affinity binding sites of oxytocin receptors on Sf9 cells as determined by [^{3}H]-oxytocin binding (Gimpl *et al*., 1995). Moreover, the coupling between oxytocin receptors and endogenous G proteins was enhanced as approximately 20% of the low-affinity binding sites was converted into high affinity binding sites. In analogy, we incubated Sf9 cells expressing H_2 receptors with the water-soluble β-cyclodextrin cholesterol complex and binding of [^{125}I]-APT was determined (Figure 6). Indeed, a dose-dependent increase in the number of functional H_2 receptors was obtained up to a maximum of $169 \pm 5\%$ ($100\% = 6.6 \pm 0.6$ pmol mg^{-1} protein) at a concentration of 0.03 mM cholesterol-β-cyclodextrin complex (mean $\pm$ s.e.mean, $n = 3$).

In addition, the effect of cholesterol-β-cyclodextrin complex on the histamine-induced cyclic AMP production was determined. Sf9 cells expressing the His-tagged H_2 receptor were incubated 10 min at 30°C with 0.03 mM cholesterol-β-cyclodextrin complex before activation with histamine. As expected, the H_2 receptor level was increased to $244 \pm 61\%$ upon incubation with 0.03 mM cholesterol-β-cyclodextrin complex (mean $\pm$ s.e.mean, $n = 3$, $P < 0.05$). Yet, no significant shift of the dose-response curve of histamine was obtained after incubation with the cholesterol-β-cyclodextrin complex. The EC_{50} values of histamine in the absence and presence of 0.03 mM cholesterol-β-cyclodextrin complex were 3.1 ± 1.7 μM and 11.1 ± 5.5 μM, respectively (mean $\pm$ s.e.mean, $n = 3$). Also, the maximal response to histamine was not altered upon addition of 0.03 mM cholesterol-β-cyclodextrin complex. In the

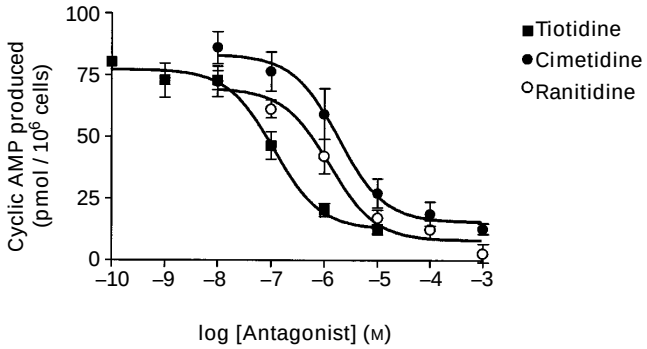

Figure 4 Dose-dependent inhibition of histamine (10 μM)-induced cyclic AMP production of Sf9 cells expressing His-tagged rat H_2 receptors. Inhibition curves for tiotidine, ranitidine and cimetidine are shown. Data are mean of three independent experiments and vertical lines show s.e.mean.

Table 1 Displacement of [^{125}I]-APT binding to His-tagged rat H_2 receptors by histamine and various H_2 receptor antagonists

Ligand	*Sf9*	*CHO*
Histamine	163 ± 8 μM (4)	3.5 μM (39%); 240 μM (61%)
Histamine + GTPγS	165 ± 8 μM (4)	180 μM (100%)
Tiotidine	34 ± 6 nM (3)	18 nM
Iodoaminopotentidine	0.63 ± 0.08 nM (3)	0.43 nM
Cimetidine	1.4 ± 0.8 μM (4)	0.8 μM
Metiamide	1.6 ± 0.8 μM (4)	0.6 μM
Ranitidine	0.67 ± 0.02 μM (4)	0.2 μM

Values are K_i values except for the histamine values obtained in CHO cells, which indicate % displacement. Data from Sf9 cells are mean ± s.e.mean, data from CHO cells are from Traiffort *et al*. (1992) and Smit *et al*. (1996a).

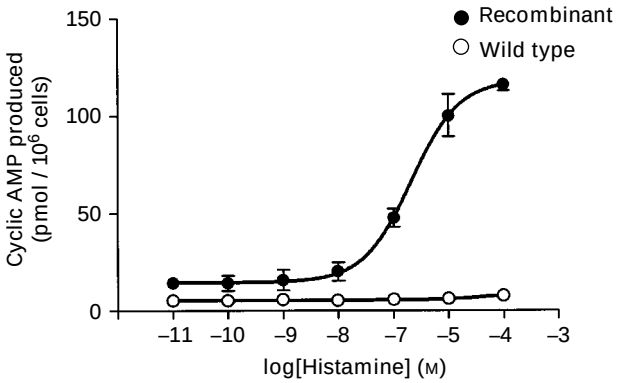

Figure 3 Histamine-induced accumulation of cyclic AMP in Sf9 cells infected with recombinant (expressing the rat H_2 receptor) or wild type baculoviruses. Data are mean of three independent experiments and vertical lines show s.e.mean.

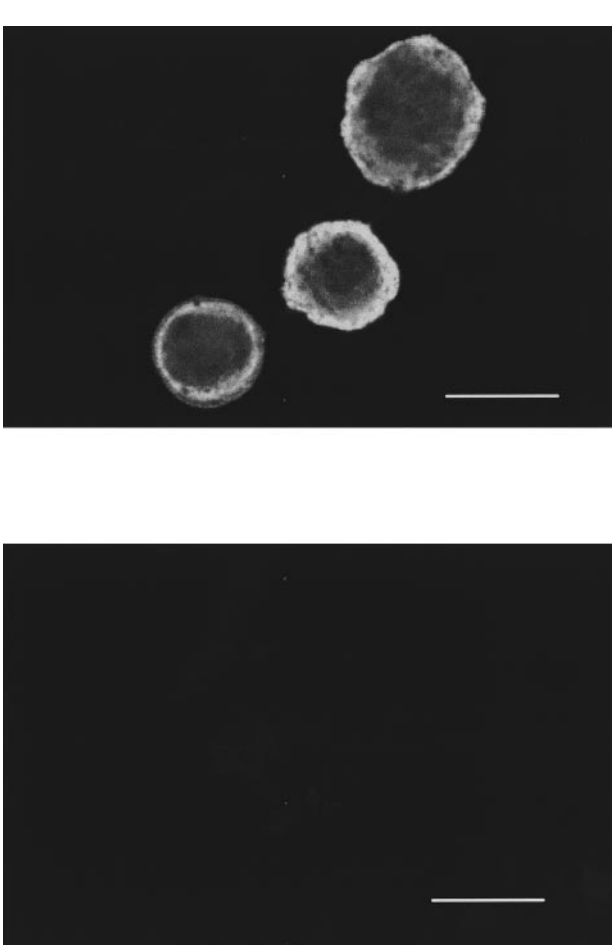

Figure 5 Immunofluorescence staining of permeabilized Sf9 cells at 2 d.p.i. after infection with either recombinant His-tagged rat H_2 receptors (a) or wild type baculovirus (b). Cells were labelled with 100× diluted specific anti-His antibody. Both scale bars are equivalent to 20 μm.

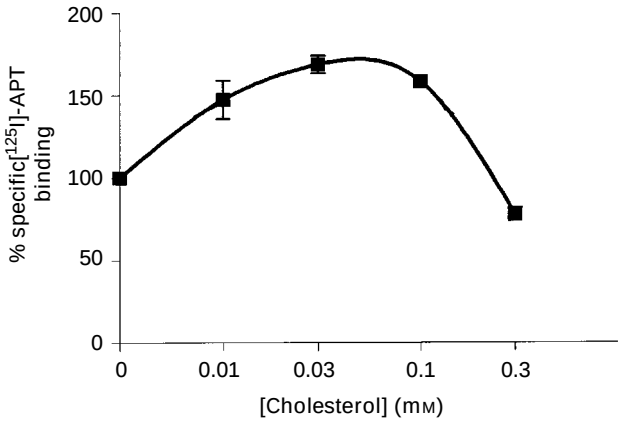

Figure 6 Effect of increasing concentrations of cholesterol-β-cyclodextrin complex on [^{125}I]-APT binding to His-tagged rat H_2 receptors expressed on Sf9 cells as a percentage of [^{125}I]-APT binding in the absence of cholesterol-β-cyclodextrin complex. The amount of receptor protein expressed in the absence of cholesterol-β-cyclodextrin complex was 6.6 ± 0.6 pmol mg^{-1} protein ($n=3$).

absence and presence of 0.03 mM cholesterol-β-cyclodextrin complex 100 μM histamine induced the production of 85 ± 18 pmol cyclic AMP/10^6 cells and 81 ± 11 pmol cyclic AMP/10^6 cells, respectively.

Discussion

In the present study, we describe the pharmacological and molecular characterization of an His-tagged H_2 receptor, heterologously expressed in insect Sf9 cells. After the generation of a recombinant baculovirus encoding the His-tagged H_2 receptor infection of Sf9 cells was optimized. The highest expression levels of the 36 kD band, without major aggregation of proteins (see Figure 2a), were obtained at 3 d.p.i. and MOI = 0.3. Moreover, the level of H_2 receptor expression as determined with [^{125}I]-APT binding closely reflected the expression levels as shown on the Western blot (Figure 1a and 2a).

The molecular weight of the immunoreactive band on the Western blot is somewhat lower as would be expected from the calculated molecular mass of the non-glycosylated H_2 receptor protein ($M_W = 40$ kD). Similar observations have been made for 5-HT and dopamine receptors after expression in Sf9 cells (Parker *et al*., 1994; Javitch *et al*., 1994; Ng *et al*., 1994; 1995; Butkerait *et al*., 1995; Kwatra *et al*., 1995; Nebigil *et al*., 1995). We excluded the possibility that this discrepancy between experimental and theoretical molecular weights was a result of H_2 receptor degradation. Experiments with a recombinant baculovirus encoding the rat H_2 receptor with both a C-terminal poly-histidine tag and an N-terminal Flag-epitope revealed that antibodies against both epitopes recognized the same bands on the Western blot, confirming that this band indeed represents the full-length H_2 receptor (Figure 2b). Conflicting results concerning the molecular weight of the H_2 receptor have also been obtained in other systems. Ruat *et al*. (1990) identified proteins in guinea-pig brain by use of the photoaffinity probe [^{125}I]-iodoazidopotentidine with molecular weights of 87, 59, 51 and 32 kDa. Only the labelling of the 59 and 32 kDa bands could be prevented upon incubation with H_2 antagonists (Ruat *et al*., 1990). In line with these findings were the results of Smit *et al*. (1995) with Flag-tagged H_2 receptors expressed in HEK-293 cells, yielding major bands at 57 and 31 kDa upon Western blot-analysis. Both authors claimed the 57 kDa protein to represent the glycosylated H_2 receptor, whereas the 31–32 kDa band was considered to represent a proteolytic degradation product. Finally, Fukushima *et al*. (1995) detected in Cos-7 cells, expressing the canine H_2 receptor, a high molecular weight band of ca 75 kDa, by use of an anti-peptide antibody. Upon treatment with the glycosylation inhibitor tunicamycin a single low molecular weight band of less than 32.5 kD was recognized by the antibody. The authors argued that limitations of SDS–PAGE electrophoresis were responsible for this lower molecular weight of the non-glycosylated canine H_2 receptor.

In view of the low molecular weight (36 kDa) observed in the present study we assumed that in the Sf9 cells the majority of the H_2 receptor protein is not glycosylated. This assumption is strengthened by the fact that treatment with tunicamycin did not significantly affect the results of the Western blot experiments. Previously, Fukushima *et al*. (1995) showed, by use of site-directed mutagenesis studies, that in Cos-7 cells glycosylation is not required for ligand binding or second messenger responses of the canine H_2 receptor. Unfortunately, Fukushima did not present H_2 receptor expression levels for the wild-type or mutant receptors. Our [^{125}I]-APT binding experiments with tunicamycin-treated cells showed that glycosylation is very important for functional H_2 receptor expression both in the insect Sf9 cells and in mammalian HEK-293 cells. These data suggest that the major 36 kD immunoreactive protein on the Western blot does not represent the protein that is labelled by [^{125}I]-APT. This finding is in good correspondence with the results of our immunofluorescent confocal laser microscopic images of the infected Sf9 cells. The majority of the immunoreactivity was localized in intracellular stores. Similar data were recently obtained for dopamine D_{2S} receptor expression in Sf9 cells (Grünewald *et al*., 1996). Upon treatment with tunicamycin these authors obtained a reduction in functional D_2 receptor levels, about 40% of ligand binding remained, whereas the pattern and the intensity of bands on the immunoblots was not affected. Moreover, Guan *et al*. (1992) have shown that in Sf9 cells complete glycosylation of the β_2-adrenoceptor was a reliable indicator of a proper membrane translocation of the GPCR. Our data therefore suggest that we obtained high level expression of the H_2 receptor in the Sf9 cells, but that the majority of the receptor protein is neither properly glycosylated nor targeted to the plasma membrane and consequently not functional.

Nevertheless, the relatively small amount of receptor protein in the plasma membrane represents pmol mg^{-1} protein quantities of functional H_2 receptor protein. Displacement studies with [^{125}]-APT revealed that the ligand binding characteristics of the His-tagged rat H_2 receptors expressed in Sf9 cells were similar to the ones we obtained previously in CHO cells (Traiffort *et al*., 1992; Smit *et al*., 1996a). Moreover, in Sf9 cells H_2 receptors couple to an endogenous G_s protein and are able to activate an endogenous adenylate cyclase. These findings confirm previous studies with β_2-adrenoceptors and dopamine D_1 receptors (Mouillac *et al*., 1992; Chidiac *et al*., 1993; Ng *et al*., 1994). In our radioligand binding studies we were unable to observe high and low affinity sites of histamine as an indication of G protein coupling of the H_2 receptor. Moreover, we were not able to shift the displacement curve of histamine by the addition of guanosine 5′-*O*-(γ-thio-triphosphate) (GTPγS). These findings can be explained by the relatively high receptor expression level that we obtained (6 pmol mg^{-1} protein). For the G_i protein-coupled dopamine D_2 receptor Boundy *et al*. (1996) could only demonstate a GTPγS-induced shift of the displacement curve of dopamine binding to the D_2 dopamine receptor, when expression levels were <1 pmol mg^{-1} protein. Apparently, at higher expression levels the endogenous G_s proteins of Sf9 cells are a limiting factor. This suggestion also explains the relatively weak activity of histamine at inducing cyclic AMP production in infected Sf9 cells ($EC_{50} = 3.1 \pm 1.7$ μM). In eukaryotic cells, expressing high levels of G_s protein, similar H_2 receptor expression levels are always accompanied by a high potency of histamine at inducing cyclic AMP production (Traiffort *et al*., 1992; Smit *et al*., 1996a).

Our results with the cholesterol-β-cyclodextrin complex are in good agreement with our conclusions on the relatively low plasma membrane level of the H_2 receptor and the level of G_s

protein being a limiting factor for H_2 receptor signal transduction. We suggest that cholesterol levels in the plasma membrane of Sf9 cells are suboptimal for functional expression of H_2 receptors. Indeed, the cholesterol levels of plasma membranes of insect Sf9 cells are lower than those of mammalian cells (Gimpl *et al*., 1995). Moreover, Gimpl *et al*. (1995) have demonstrated that incubation with a cholesterol-β-cyclodextrin complex results in higher plasma membrane cholesterol levels. Whether the incorporation of cholesterol leads to more [^{125}I]-APT binding sites because of an improved accessibility of plasma membrane receptors for the radioligand or whether part of the intracellular receptor protein is transferred to the plasma membrane is still unclear. The addition of cholesterol-β-cyclodextrin complex did not alter the histamine-induced cyclic AMP production in the Sf9 cells expressing His-tagged H_2 receptors. Apparently, despite the increase in the number of receptors, the amount of G_s expressed by the insect cells is insufficient to allow a more efficient coupling of the receptor to adenylate cyclase. In contrast, Gimpl *et al*. (1995) were able to shift 20% of the low affinity binding sites of [^{3}H]-oxytocin to high affinity upon incubation with cholesterol-β-cyclodextrin complex (in the absence of cholesterol-β-cyclodextrin complex only low affinity binding sites were present). This difference may be explained by the lower expression levels, 1.66 pmol mg^{-1} protein (as determined with the agonist [^{3}H]-oxytocin) for the oxytocin receptor, versus 11 pmol mg^{-1} protein for the His-tagged H_2 receptor in cholesterol-supplemented Sf9 cell membranes. As observed by Boundy *et al*. (1996), G_i-protein levels become limiting at receptor levels substantially larger than 1 pmol mg^{-1} protein.

In conclusion, the data presented demonstrate high-level expression of His-tagged rat H_2 receptors in Sf9 cells. As indicated by Western blots and immunofluorescent confocal laser microscopy studies only a portion of the expressed H_2 receptor protein is functionally targeted to the plasma membrane. This is probably due to incomplete or absent receptor glycosylation and/or suboptimal cholesterol levels in the plasma membrane of Sf9 cells.

Nevertheless, the ligand binding characteristics of the functionally expressed His-tagged H_2 receptors were similar to the characteristics of H_2 receptors expressed in mammalian cells. Moreover, in the Sf9 cells the H_2 receptor couples to endogenously expressed G_s proteins to stimulate an endogenously expressed adenylate cyclase. The successful expression of the His-tagged H_2 receptor in insect Sf9 cells should allow the future purification of the H_2 receptor from these cells for more detailed studies of structure-function relationships. For that purpose, further increase of the percentage of functional receptors is one of our primary targets.

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