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Recombinant Semliki Forest virus for over-expression and pharmacological characterisation of the histamine H_2 receptor in mammalian cells

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Abstract

We describe the use of recombinant Semliki Forest virus (SFV) vectors for efficient expression of the rat histamine H_2 (rH_2) receptor in COS-7 (African green monkey kidney cells) cells. Recombinant SFV-infected COS-7 cells express the histamine rH_2 receptor in a time-dependent fashion with a maximum expression level of 50 pmol mg^{-1} after 40 h. SFV-mediated histamine rH_2 receptor expression shows similar pharmacological properties as the receptor expressed transiently or stably in mammalian cells. In addition, we demonstrate the pharmacological and functional characterisation of the $D^{115}N$ mutated histamine rH_2 receptor. It has been shown that the $D^{115}N$ mutation renders the receptor constitutively active and structurally unstable. The rapid onset of and high maximal expression levels obtained from SFV-infected COS-7 cells enabled us to characterise this mutant receptor. We prove that recombinant SFV vectors are powerful tools for heterologous expression of G-protein-coupled receptors and that one can achieve both the high-level gene expression described for baculovirus-infected insect cells and the use of mammalian cells as hosts.

Keywords: Histamine H_2 receptor; Semliki Forest virus; [^{125}I]APT; cAMP determination; Ranitidine; Cimetidine; Histamine; COS-7 cell

1. Introduction

Expression of cloned G-protein coupled receptor sequences is currently one of the central technologies in the pharmacological characterisation of these seven transmembrane receptors. In general, transfection of mammalian cells using chemical carriers is the most widespread approach to obtain heterologous expression of cDNAs. So far, G-protein coupled receptor expression in yeast (Arkinstall et al., 1995; Sudbery, 1996; Pausch, 1997), bacteria (Freissmuth et al., 1991; Bertin et al., 1992; Munch et al., 1995; Stanasila et al., 1999; Furukawa and Haga, 2000), insect cells (George et al., 1989; Mouillac et al., 1992; Mills et al., 1993; Ng et al., 1993; Oker-Blom et al., 1993; Parker et al., 1994; Butkerait et al., 1995; Gimpl et al., 1995; Jansson et al., 1995; Kusui et al., 1995; Schreurs et al., 1995; Boundy et al., 1996; Obermeier et al., 1996; Beukers et al., 1997; Massotte et al., 1997; Wehmeyer and Schulz, 1997; Ohtaki et al., 1998; Creemers et al., 1999; Aztiria et al., 2000) and mammalian cells has proven to be useful for either radioligand binding studies and/or the delineation of signal transduction mechanisms. The use of non-mammalian expression systems has some disadvantages as these cells lack, e.g. the typical post-translational modification mechanisms, which can be important for proper G-protein coupled receptor folding and function. Insect cells are often used for baculovirus-driven protein over-expression, but are known to have a different protein glycosylation pattern than mammalian cells (Altmann et al., 1999). Moreover, yeast, bacteria and insect cells are only of limited value for the study of the coupling of mammalian G-protein coupled receptors to their cognate signal transduction pathways. Despite these limitations non-mammalian cells have been used quite frequently as

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expression systems, because they allow considerable scale-up. Especially, the baculovirus system has been commonly used for the production of seven transmembrane domain receptors (Creemers et al., 1999; Juntunen et al., 1999).

Heterologous gene expression in mammalian cells has often been impeded by poor transfection efficiency, relatively low expression levels, relatively high costs of large-scale protein production and the limited host cell range for most expression systems. Yet, with the recent developments in viral expression technology these disadvantages have largely been overcome. Currently, viral expression systems are highly efficient tools for protein expression in higher eukaryotic cells. The Semliki Forest (SFV) and Sindbis virus, members of the family of Togaviridae, are the best-studied alphaviruses and have both been adopted for heterologous gene expression (Xiong et al., 1989; Liljestrom and Garoff, 1991). These systems are highly successful, as their self-amplifying viral genomes can take over the host cell translation machinery for high-level expression of recombinant proteins (Lundstrom, 1999).

The histamine H_2 receptor is a typical seven transmembrane domain containing G-protein coupled receptor, which predominantly couples to the G_s class of G-proteins, (Leurs et al., 1995). Previously, we showed that the histamine H_2 receptor can be functionally expressed in insect Sf9 cells at a level of 10 pmol mg^{-1} protein (Beukers et al., 1997). Although this receptor expression is substantially higher compared to expression observed in native tissue, the usefulness for receptor purification is questionable. Because substantially higher levels of recombinant G-protein coupled receptors have been obtained for other G-protein coupled receptors in Sf9 cells (Ravet et al., 1993; Robeva et al., 1996; Ohtaki et al., 1998), we reasoned that the relatively low expression level of the histamine H_2 receptor is due to its inherent high level of constitutive activity and/or structural instability (Alewijnse et al., 1998, 2000). Membrane expression of constitutively active mutant histamine H_2 receptors was, e.g. strongly reduced or even completely absent in mammalian cells (Alewijnse et al., 2000). High level expression and purification of wildtype and constitutive active mutant histamine H_2 receptors did therefore not seem feasible using standard expression technology.

In this study we show that infection of mammalian cells with recombinant SFV can (partly) overcome the problem of structural instability. This infection technology offers a simple, effective way to achieve high-level expression of both the rat histamine H_2 receptor and the constitutive active $D^{115}N$ mutant histamine H_2 receptor in mammalian cells. The obtained expression levels are substantially higher than observed in insect cells, offering a new potential for histamine H_2 receptor purification. Furthermore, we demonstrate that the SFV expression technology offers the opportunity to perform signal transduction studies with (mutant) histamine H_2 receptors as early as 4 h after infection of COS-7 cells.

2. Materials and methods

2.1. Construction of pSFVgen2–rH_2 expression vector

Plasmid handling was done by standard laboratory procedure (Maniatis et al., 1982). Full length rat histamine H_2 receptor cDNA was subcloned into the *Bam*HI and *Xho*I sites of the polylinker of pSFVgen2 vector.

2.2. Cell culture

Baby hamster kidney cells (BHK-21) were grown in a 1:1 mixture of F12-MEM (Modified Eagles Medium) and Iscove's modified Dulbecco's medium (Gibco-BRL) containing 10 % foetal calf serum and 5 mM glutamine (Gibco-BRL). SV40 immortalised African green monkey kidney cells (COS-7) were grown in DMEM (Dulbecco's Modified Eagles Medium) supplemented with 5% foetal calf serum, 5 mM glutamine and 0.1% penicillin/streptomycin. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3. Generation of recombinant SFV particles

pSFVgen2–rH_2 and pSFV–helper2 plasmids (Lundstrom et al., 1997) were linearised with *Sap*I and *Spe*I, respectively, and were purified by phenol extraction prior to in vitro transcription. RNA was synthesised in vitro driven from the bacterial SP6 promoter. 2.5 μg of linearised plasmids were transcribed with 7 U SP6 RNA polymerase (Amersham Pharmacia Biotech) in a buffer containing 40 mM HEPES (pH 7.4), 6 mM MgOAc, 2 mM spermidine, 1 mM of ATP, CTP and UTP, 0.5 mM GTP, 1 mM m7G(5′)ppp(5′)G (CAP; Amersham Pharmacia Biotech), 1.5 U RNAsin (Roche) and 5 mM dithiothreitol in a final volume of 50 μl for 1 h at 37 °C. BHK-21 cells from a semiconfluent 168 cm^2 flask were detached with Versene/Trypsine solution. Cells were collected by centrifugation for 5 min at 1500 rpm and washed twice with 10 ml phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH = 7.4). Cells were resuspended in 2.4 ml PBS prior to electroporation with the in vitro synthesised RNA. 400 μl of BHK-21 cell suspension was transferred to a Biorad Genepulser cuvette (Biorad, 165–2086) together with 50 μl of transcribed pSFV2gen–rH_2 RNA and 25 μl of transcribed pSFV–helper2 RNA. Cells were electroporated twice in a Biorad Genepulser (settings: 25 μF, 1500 V, ∞Ω). Following electroporation cells were immediately resuspended in growth medium and seeded in 25-cm^2 flasks. 24 h after electroporation, the medium from the cells was collected, passed through a 0.22-μm filter. Viral particles were stored at −80 °C.

2.4. Infection of mammalian cells

Prior to infection the recombinant virus was activated by chymotrypsin treatment. 500 μg Chymotrypsin (Roche Biochemicals) was added per 10 ml of virus suspension and incubated for 15 min at room temperature. Subsequently, the chymotrypsin was inactivated by the addition of 250 μl aprotinin (10 mg/ml, Sigma). Infection of mammalian cells with the activated viral particles was similar for all used cell lines. Cells were grown to 80% confluency, the medium was aspirated and cells were washed once with PBS. Diluted viral suspensions were added in a small volume, just enough to cover the cells. In most experiments, the original viral suspension was diluted 10-fold. This did not result in a significant loss of expression. Cells were incubated with the virus suspension at 37 °C for 1 h, after which appropriate culture medium was added and the cells were cultured for 4–40 h.

2.5. [^{125}I]APT binding

To determine the histamine H_2 receptor expression, cells were washed once with cold PBS, detached with a cell scraper and recovered by a 5-min centrifugation at $500 \times g$. Cells were homogenised in ice-cold 50 mM Na_2/K phosphate buffer (pH = 7.4) with a Polytron homogeniser (5 s, maximal speed) and used for radioligand binding studies. The determination of histamine H_2 receptor binding with the histamine H_2 receptor antagonist [^{125}I]iodoaminopotentidine ([^{125}I]APT) was performed as essentially described by Leurs et al. (1994). Briefly, triplicate assays were performed in polyethylene tubes in 400 μl of 50 mM Na_2/K phosphate buffer (pH = 7.4) containing gelatine (0.1%), 0.5 nM [^{125}I]APT, 0.1–1 μg of membrane proteins. Non-specific binding was determined in the presence of 1 μM tiotidine. After 90 min at 30 °C the incubation was terminated by rapid dilution with 3 ml ice-cold 20 mM Na_2/K phosphate buffer (pH = 7.4) supplemented with 0.1% chicken egg albumin, and rapid filtration with a Brandel cell harvester (Semat, UK) through polyethyleneimine (0.3%)-treated Whatman GF/C glass fibre filters was performed. Filters were washed twice with 3 ml buffer and radioactivity retained on the filters was counted with a LKB-γ-counter at an efficiency of 63%. Radioligand binding data was analysed using Graphpad Prism version 2.01.

2.6. Cyclic AMP generation

For the measurements of cAMP levels, COS-7 cells were seeded in 24-well plates and allowed to attach prior to infection with recombinant SFV. Cells were grown for the indicated time. For the actual cAMP determination transfected cells were incubated for 10 min at 37 °C with the appropriate drugs in DMEM supplemented with 25 mM HEPES (pH = 7.4 at 37 °C) and 300 μM of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Aspiration of the medium and addition of 200 μl ice-cold HCl terminated the incubation. Cells were disrupted by sonication (5 s, 50 W, Labsonic 1510, Braun-Melsungen, Germany) and the homogenate was neutralised with 1 N NaOH. The amount of cAMP present in samples was determined using a competitive protein kinase A binding assay according to Nordstedt and Fredholm (1990), with some minor modifications (Smit et al., 1996b). cAMP data were analysed using Assayzap and Graphpad Prism version 2.01 software packages. Protein concentrations were determined according to Bradford using bovine serum albumin as a standard (Bradford, 1976).

2.7. Chemicals

Histamine dihydrochloride, isobutylmethylxanthine (IBMX), forskolin, cyclic AMP (cAMP), gelatine, polyethyleneimine and chicken egg albumin were obtained from Sigma (USA). [2,8-^{3}H]cAMP (40 Ci/mmol) was obtained from Amersham. Aminopotentidine was from a laboratory stock. Gifts of cimetidine and burimamide (SmithKline Beecham, UK), ranitidine dihydrochloride (Glaxo Wellcome, UK), tiotidine (Imperial Chemical Industries, UK) are greatly acknowledged.

3. Results

3.1. Histamine H_2 receptor overexpression

After the successful cloning of histamine H_2 receptor cDNA into the pSFVgen2 plasmid (Lundstrom et al., 1997), viral particles were generated and COS-7 cells were infected. Since in contrast to the baculovirus the SFV is a non-replicative virus, viral titers can only be estimated indirectly. In order to get an impression of the titer BHK cells were infected with serial dilutions of the SFV–rH_2 stock. Typically, SFV-infected BHK cells stop growing and show visible morphological changes compared to control cells and microscopical examinations can give an approximate estimation of SFV-titers. Generally, the titers obtained for SFV–rH2 were in the range of 10^8–10^9 infectious particles per milliliter. Serial dilutions of SFV–rH_2 were subjected to infection of COS-7 cells followed by radioligand binding assays. No effect was noticed on the expression level of the rat histamine H_2 receptor up to a 10-fold dilution. After a 100-fold dilution of the SFV–rH_2 stock the expression of the rat histamine H_2 receptor was still detectable, although at a considerably reduced level (results not shown). To maximally benefit from the recombinant SFV–rH_2 all infections were carried out at a 10-fold dilution of the original viral stock.

In order to analyse the pharmacological properties of the SFV-mediated histamine rH_2 expression, all studies were carried out in COS-7 cells, since most of our his-

tamine H_2 receptor studies in the past were carried out with this cell line. Affinity of the histamine H_2 receptor radioligand [^{125}I]APT was determined by saturation binding experiments. The K_D-value for [^{125}I]APT for the histamine H_2 receptor expressed in COS-7 cells infected with recombinant SFV–rH_2 was 0.5 ± 0.11 nM (mean $\pm$ S.E.M., $n = 3$). This value is in good agreement with the K_D values obtained previously for [^{125}I]APT in cell homogenates of COS-7 cells transfected with pcDNA$_3$rH_2 (0.85 nM) and the observed values in native tissue (Ruat et al., 1990; Traiffort et al., 1992).

The time-course of SFV–rH_2 histamine H_2 expression in COS-7 cells is shown in Fig. 1. As early as 4 h post-infection the expression of the histamine H_2 receptor was 700 fmol mg^{-1} protein and the expression reached a maximum at 40 h post-infection (50.9 pmol mg^{-1} protein). Extending the time after infection did not further increase the expression levels of the histamine H_2 receptor and at 60 h post-infection the cells started to die. Treatment of the cells with 100 μM ranitidine, an inverse agonist for the histamine H_2 receptor (Smit et al., 1996a), led to a significant increase in receptor density. At 40 h post-infection the ranitidine treatment increased the histamine H_2 receptor density by 1.5-fold up to a maximum of 80 pmol mg^{-1} (see Fig. 1, hatched bars). This effect on the expression levels of the histamine H_2 receptors is probably due to the inhibition of the constitutive activity of the receptor by the inverse agonist ranitidine. A similar treatment of the cells with low (1 μM) and high (1 mM) concentrations of histamine did not effect the receptor density (results not shown). This indicates that ligand-dependent receptor stabilisation (Alewijnse et al., 1998, 2000) plays a minor role in the ranitidine produced up-regulation of the histamine H_2 receptor.

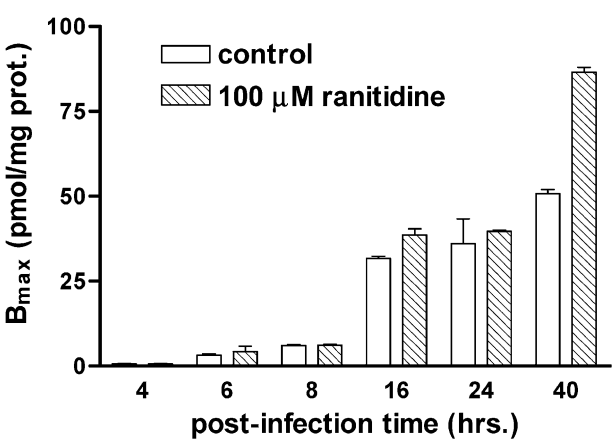

Fig. 1. Expression of rat histamine H_2 receptor at various times after SFV–rH_2 infection. COS-7 cells were infected with recombinant SFV–rH_2 as indicated in Materials and methods. Cells were allowed to express the receptor for the indicated time; harvested and expression levels in cell homogenates were determined by [^{125}I]APT radioligand binding. B_{max} was determined after treatment with or without ranitidine for the total post-infection time. Data represent the mean $\pm$ S.E.M. of three independent experiments.

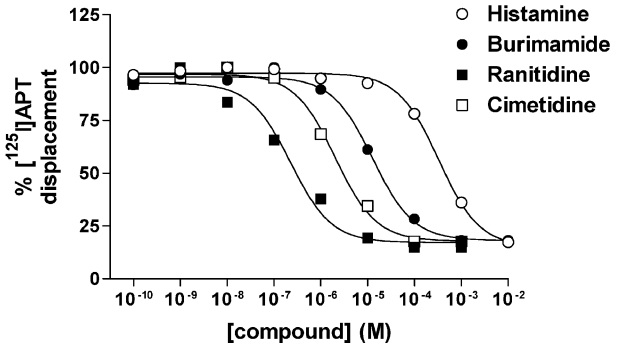

Fig. 2. Typical displacement of [^{125}I]APT binding by histamine, burimamide, cimetidine and ranitidine. COS-7 cells were infected with recombinant SFV–rH_2 and were allowed to express the receptor for 16 h. Cells were harvested, resuspended in Na/K phosphate buffer and homogenised by ultrasonic pulse. Radioligand binding and displacement were carried out as described in Materials and methods. Receptor density in this particular experiment was 30 pmol mg^{-1} protein.

Both the agonist histamine as well as the antagonist burimamide and the inverse agonists ranitidine and cimetidine were studied for their ability to displace the radioligand [^{125}I]APT. Typical displacement curves are shown in Fig. 2. The K_i values obtained in these experiments are in good agreement with the ones observed in COS-7 cells transfected according the DEAE–dextran method (Table 1).

3.2. Histamine H_2 receptor signalling

Since radioligand binding does not reveal a functional interaction between the receptor and the Gα_s subunit, we investigated the ability of the SFV–rH_2 expressed histamine H_2 receptor to stimulate adenylate cyclase via endogenous G-proteins. We studied the effect of increasing post-infection time (increasing receptor densities) on the functionality of the receptor by measuring both histamine-stimulated cAMP generation as well as basal cAMP levels.

Table 1
Displacement of [^{125}I]APT binding to rat histamine H_2 receptors by various ligands

Ligand	pK_I		
	SFV–rH_2 infected COS-7	DEAE–dextran transfected COS-7	SFV–H_2D^{115}N infected COS-7
Histamine	3.6 $\pm$ 0.10	3.5 $\pm$ 0.04	4.6* $\pm$ 0.3
Ranitidine	6.8 $\pm$ 0.11	7.1 $\pm$ 0.16	7.2 $\pm$ 0.1
Burimamide	5.2 $\pm$ 0.05	5.2 $\pm$ 0.02	5.2 $\pm$ 0.4
Cimetidine	6.0 $\pm$ 0.05	6.1 $\pm$ 0.04	N.D.

Displacement studies were done on cell homogenates prepared from COS-7 cells infected with SFV–rH_2 or SFV–rH_2–D^{115}N for 16 h or COS-7 cells transfected with pcDNA3–rH_2 according the DEAE–dextran method and prepared 48 h after transfection. Data represent the mean $\pm$ S.E.M. of three independent experiments.

* Indicates significant difference, $p < 0.05$.

In Fig. 3 the maximal effects of the endogenous agonist histamine and the inverse agonist ranitidine on the generation of cAMP are presented. As early as 4 h after infection an 18-fold histamine-induced stimulation of cAMP (3974 $\pm$ 50.9 pmol cAMP mg^{-1}) over basal level (213.4 $\pm$ 41.7 pmol cAMP mg^{-1}) was observed. After 6 h, a 20-fold stimulation by histamine was reached. However, with increasing post-infection time the fold-stimulation by histamine decreased. This is probably due to the fact that the basal cAMP levels continued to increase (Fig. 3B), whereas the maximal stimulation by histamine did not further increase. From 4 to 16 h post-infection, the basal cAMP generated by the histamine H_2 receptor increased 4.5-fold.

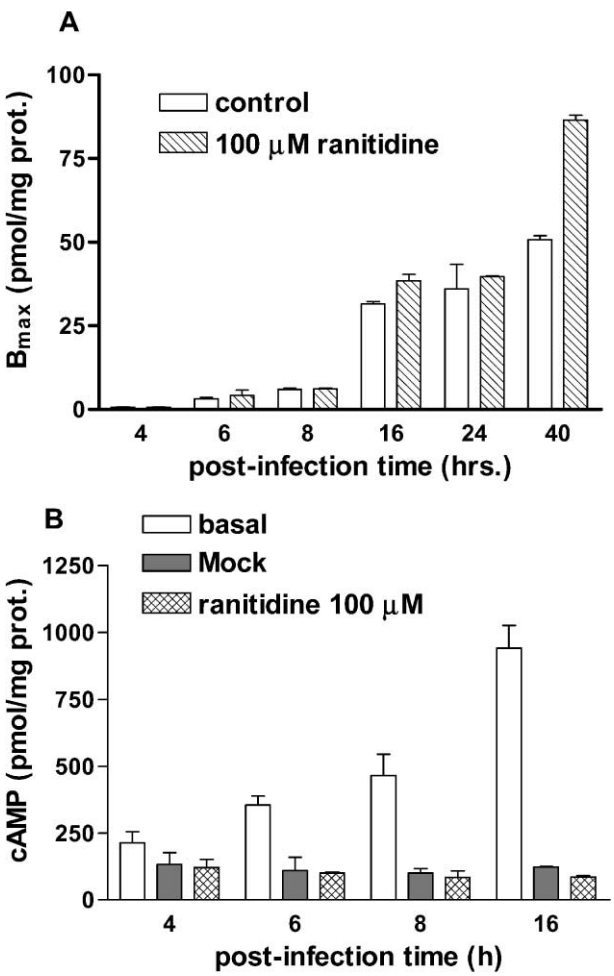

Fig. 3. Effects of histamine and ranitidine on the cAMP levels in SFV–rH_2 infected COS-7 cells. Panel A shows the levels of cAMP generated after a 10-min stimulation with 100 μM histamine in comparison to the basal cAMP levels and the levels measured in mock infected cells. Cells were assayed for cAMP generation at various time points after infection. The effect of prolonged infection time on the basal cAMP level can be seen more clearly in panel B. The inverse agonist ranitidine (100 μM) reduces the increased basal levels to the level of cAMP present in mock infected cells. cAMP measurements were done as described in Materials and methods. Both graphs represent the mean $\pm$ S.E.M. of three independent experiments.

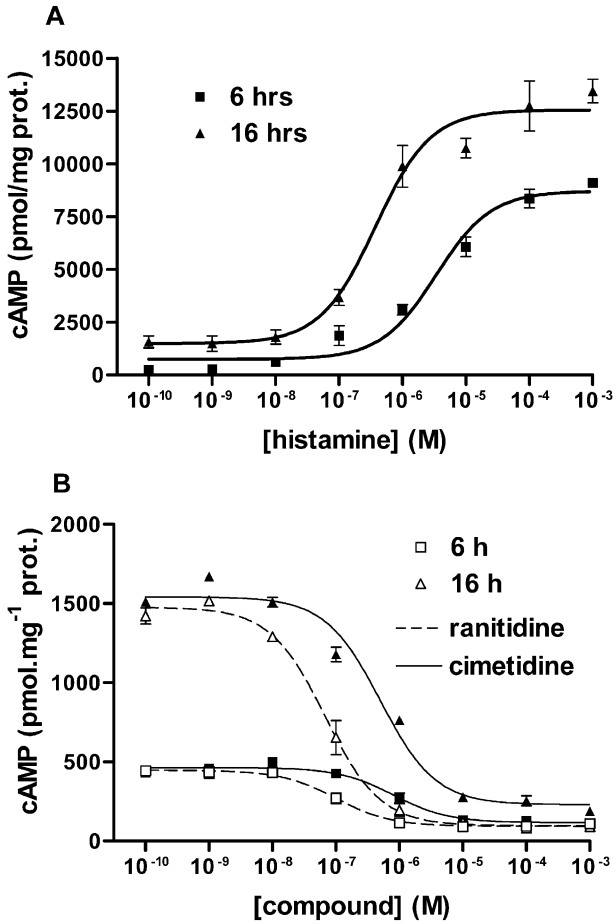

Fig. 4. Dose–response curves of histamine (A), and the inverse agonists ranitidine and cimetidine (B) of cAMP production in COS-7 expressing the histamine H_2 receptor. SFV–rH_2 infected COS-7 cells were allowed to express the histamine H_2 receptor for 6 h (■) or 16 h (▲). In panel B ranitidine curves are represented by a dotted line, cimetidine curves by a solid line. The corresponding pEC_{50} are given in the text in Results. Both graphs represent the mean $\pm$ S.E.M. of three independent experiments.

Already at 4 h post-infection the basal cAMP level was increased by over 1.5-fold as compared to mock-infected COS-7 cells. Incubation with 100-μM ranitidine reduced the basal cAMP to the same level as in mock-infected cells. 16 h post-infection, the basal cAMP level was 8-fold increased over that in mock infected cells. This increased basal cAMP level can again completely be inhibited by the inverse agonist ranitidine (Fig. 3B).

For the ligands presented in Table 1 cAMP dose–response curves were generated at 6 and 16 h post-infection (Fig. 4). Although the maximal cAMP production by histamine differs by only 1.5-fold (9089 $\pm$ 108.9 and 13445 $\pm$ 555.0 pmol mg^{-1} protein, respectively) the pEC_{50} values for histamine differ by 10-fold (Fig. 4A). Dose–response curves for the inverse agonists ranitidine and cimetidine (Fig. 4B) do not display the difference in pEC_{50} values as seen for the agonist dose–response curves. The various pEC_{50} values are summarised in Table 2.

Table 2
pEC_{50} values histamine and two histamine H_2 receptor inverse agonists as determined for the cAMP production in infected COS-7 cells

Ligand	pEC_{50}	
	6 h	16 h
Histamine	5.5 ± 0.15	6.4 ± 0.04
Ranitidine	7.0 ± 0.13	7.2 ± 0.13
Cimetidine	6.2 ± 0.19	6.5 ± 0.12

COS-7 cells were infected with recombinant SFV–rH_2 and were allowed to express the receptor for 6 and 16 h before cAMP determinations were carried out as described in Materials and methods. Data represent mean ± S.E.M. of three independent experiments.

3.3. Analysis of the $D^{115}N$ histamine H_2 receptor mutant

The high level expression of the histamine H_2 receptor obtained shortly after SFV–rH_2 infection raised the question whether the SFV system could be used to study constitutively active receptor mutants. These so-called constitutive active mutant receptors show very often low expression levels. It has been shown that mutations within the conserved DRY amino acid motif on the border of the transmembrane domain 3 and the intracellular domain 2 often leads to constitutive activity (Scheer et al., 1996; Rasmussen et al., 1999; Alewijnse et al., 2000). Previously, we showed that the $D^{115}N$ histamine H_2 receptor mutant was constitutively active but also expressed at hardly detectable levels (Alewijnse et al., 1998, 2000). To test our hypothesis that the SFV expression system is an effective method to study constitutively active receptor mutants, we cloned the $rH_2D^{115}N$ cDNA into the pSFV-gen2 plasmid and generated recombinant SFV particles. Expression levels for both the wildtype and the $D^{115}N$ mutant histamine H_2 receptor were determined by [^{125}I]APT radioligand binding studies. Saturation binding experiments showed no significant difference in the K_D values for the wildtype and the $rH_2D^{115}N$ mutant receptor (0.35 ± 0.1 and 0.5 ± 0.1 nM, $n = 3$ respectively). However, the B_{max} values showed that the $D^{115}N$ receptor had a significantly lower B_{max} value at all time points measured when compared to the wildtype receptor (inset Fig. 5). [^{125}I]APT displacement studies indicated a small increase in affinity for ranitidine (from 158 to 50 nM). However, for the agonist histamine a 10-fold increase in affinity was observed (from 251 to 25 μM). No biphasic [^{125}I]APT displacement curves were observed for histamine at the wildtype or the $D^{115}N$ mutant receptor.

To analyse the nature of the $D^{115}N$ histamine H_2 receptor mutant, cAMP experiments were conducted. Table 3 shows the average pEC_{50} values and maximal amounts (E_{max}) of cAMP generated by histamine in comparison to the wildtype receptor. At 6 h post-infection the maximum histamine response for the $D^{115}N$ mutant is 1.3-fold higher compared to the wildtype receptor. Moreover, the potency of histamine is increased by over 50-fold on the $D^{115}N$ mutant as compared to the wildtype receptor. The effect is even more pronounced when basal cAMP levels are compared. At 6 h post-infection, the basal level of the $D^{115}N$ mutant is 3.3-fold increased over that of the wildtype

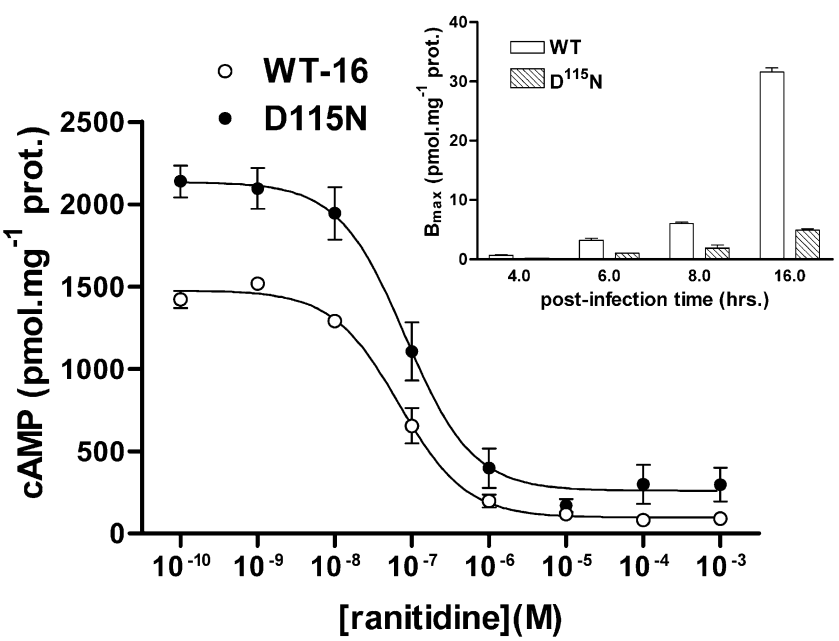

Fig. 5. Dose response curve of ranitidine on the wildtype and mutant $D^{115}N$ histamine H_2 receptor. COS-7 cells were infected with recombinant SFV–rH_2 as indicated in Materials and methods. Graphs show cAMP dose–response curves for ranitidine measured 16 h after infection of the cells with both wildtype and $D^{115}N$ SFV–rH_2. Inset shows the expression levels of both the wildtype (open bars) and the mutant $D^{115}N$ (solid bars) histamine H_2 receptor measured at various time points after infection. Both graphs represent the mean ± S.E.M. of three independent experiments.

Table 3
Basal and maximal histamine cAMP response from by the wildtype and $D^{115}N$ histamine H_2 receptor

Hours	Wildtype			$D^{115}N$		
	Basal cAMP (pmol mg^{-1})	E_{max}	pEC_{50}	Basal cAMP (pmol mg^{-1})	E_{max}	pEC_{50}
6	249 ± 14.5	9089 ± 108.9	5.5 ± 0.15	835 ± 36.0	11,335 ± 317	7.12 ± 0.05
16	1563 ± 287	13,445 ± 550	6.4 ± 0.04	1956 ± 161.1	15,470 ± 316	6.9 ± 0.06

COS-7 cells were infected with either wildtype or $D^{115}N$ SFV–rH_2. Cells were allowed to express the receptor for the indicated amount of time. Receptor densities for the respective time points can be obtained from Fig. 5. All values are mean ± S.E.M. of three independent experiments.

receptor. At 16 h, the basal level of the $D^{115}N$ mutant is still 1.3-fold higher compared to the wildtype receptor. At 16 h post-infection the difference in maximal stimulation is reduced to 1.2-fold in favour of the $D^{115}N$ mutant. Treatment of the $D^{115}N$ histamine H_2 receptor with ranitidine reduced the cAMP levels to approximately the basal cAMP level of mock-transfected cells. This observation is similar to what can be seen for the wildtype receptor (Fig. 5).

4. Discussion

In the present study we describe the pharmacological characterisation of the rat histamine H_2 receptor heterologously expressed from recombinant SFV vectors. After the successful generation of recombinant SFV–rH_2 particles, the infection of COS-7 cells was optimised with respect to receptor density. Maximal expression levels were reached at 40 h post-infection. Increasing the post infection time did not further increase the expression level. This is not unexpected since it is well documented that SFV-infections efficiently inhibit the endogenous gene expression in host cells and eventually kill the cells within 48–72 h (Lundstrom et al., 1994). Viral stocks could be diluted 10-fold without affecting the expression level of the histamine H_2 receptor. The expression level of the histamine H_2 receptor was approximately 10-fold higher at 40 h post-infection than measured from DEAE–dextran transfected COS-7 cells at 48 h post-transfection. As compared to baculovirus infected Sf9 cells the expression levels in SFV-infected COS-7 cells were 5-fold higher (50 and 10 pmol mg^{-1}, respectively) (Beukers et al., 1997). The advantage over the baculovirus system is not only the obtained expression levels but also the fact that mammalian cells rather than insect cells are used, which ensures proper glycosylation of the expressed protein. As can be seen from Table 4, SFV can be used to infect a broad range of commonly used mammalian cell lines (see also review by Lundstrom, 1999). Moreover, primary cell cultures like hippocampal neurons can be efficiently infected by SFV particles (Ulmanen et al., 1997). Although the histamine H_2 receptor expression varies from one cell line to another, in general the expression levels exceed those obtained from any other mammalian transfection system.

Displacement studies with [^{125}I]APT showed that the ligand binding characteristics of the endogenous agonist histamine, the inverse agonists ranitidine and cimetidine and antagonist burimamide are similar to what was observed in DEAE-transfected COS-7 cells. The affinities are also in good agreement with the data from baculovirus-infected Sf9 cells and stably transfected CHO (Chinese Hamster Ovary Cells) cells (Traiffort et al., 1992; Smit et al., 1996b; Beukers et al., 1997). Moreover, the histamine H_2 receptors expressed in COS-7 cells from recombinant SFV vectors couple to endogenous $G\alpha_s$ subunits and activate adenylate cyclase. In our radioligand binding studies, we were unable to observe clear biphasic displacement curve for the endogenous agonist histamine as an indication of G-protein coupling. Apparently at high histamine H_2 receptor expression levels, the G-protein becomes the limiting factor. This has indeed been demonstrated for SFV-mediated expression of the α_{1b}-adrenergic receptor where the limited expression of endogenous G-proteins was compensated by co-infection of SFV vectors expressing $G\alpha_q$ to achieve stronger functional responses (Scheer et al., 1999). In our study we also obtained data indicative for a limited amount of G-proteins with respect to the over-expressed histamine H_2 receptors. With increasing levels of histamine H_2 receptor expression, the maximal histamine induced cAMP production did not further increase. Yet, the dose–response curve for histamine was significantly leftward shifted. Similarly, the maximal histamine response of the constitutive active mutant $D^{115}N$ histamine H_2 receptor did not exceed the maximal his-

Table 4
Expression levels of the rat histamine H_2 receptor in various mammalian cell lines after infection with a recombinant SFV. Determined by [^{125}I]APT radioligand binding

Cell line	H_2 receptor (pmol mg^{-1} protein)
CHO	12.4 ± 2
BHK-21	12.2 ± 2.6
C6 glioma	38.0 ± 2.9
HEK-293	22.0 ± 3.6
COS-7	31.6 ± 0.7

Expression levels of histamine H_2 receptor in various cell lines were determined 24 h post-infection. The values represent mean ± S.E.M. of at least three independent experiments.

tamine effect at the wildtype histamine H_2 receptor, expressed at high levels. Again, the pEC_{50} value for histamine at the $D^{115}N$ histamine H_2 receptor was significantly higher compared to the value for histamine at the wildtype receptor.

Using the SFV expression the previously reported constitutive activity of the wildtype histamine H_2 receptor was easily detected (Smit et al., 1996a; Alewijnse et al., 1998). The basal cAMP levels increased progressively upon increased post-infection time. Moreover, the increase in basal activity was effectively inhibited by the inverse agonists cimetidine and ranitidine. In contrast to the histamine effects, the effectiveness of the inverse agonists did not depend on the post-infection time.

The SFV system also offers an excellent tool to study highly constitutively active receptors or constitutive active mutant receptors that usually express at very low levels using conventional transfection methods and are therefore difficult to analyse as, e.g. the $D^{115}N$ mutant of the histamine H_2 receptor. This constitutive active mutant histamine H_2 receptor (Alewijnse et al., 1998, 2000) has a mutation in the highly conserved DRY motif on the boundary of transmembrane domain 3 and the second intracellular loop. This mutated aspartate is highly conserved within the G-protein coupled receptor family and is suggested to play an important role in receptor activation (Cohen et al., 1993; Scheer et al., 1996; Morin et al., 1998; Rasmussen et al., 1999; Alewijnse et al., 2000). Mutation of the aspartate in the histamine H_2 receptor results in higher levels of constitutive activity and structural instability (Alewijnse et al., 2000). Pharmacological and biochemical studies of such constitutive active, structurally unstable G-protein coupled receptors is hampered by the expression levels of these mutants. In HEK-293 (Human Embryonic Kidney) cells expressing the histamine $H_2D^{115}N$ mutant after Ca^{2+}-phosphate transfections, the B_{max} is only 246 fmol mg^{-1} protein against 2.5 pmol mg^{-1} protein for the wildtype receptor (Alewijnse et al., 2000). In this study we show that the histamine $H_2D^{115}N$ mutant receptor transiently expressed in COS-7 cells using a recombinant SFV reaches expression levels of 4.9 ± 0.3 pmol mg^{-1} protein 16 h post-infection. The results from the pharmacological characterisation of this mutant receptor are in good agreement with the data obtained previously (Alewijnse et al., 2000).

Despite the relative low expression levels, the histamine $H_2D^{115}N$ receptor shows all characteristics of a constitutive active mutant receptor, i.e. increased constitutive basal signalling, higher agonist affinity and increased agonist responses (Leurs et al., 1998). This last feature is specifically seen at early time points (6 h) when the maximal histamine effects at the wildtype receptor are not yet saturated. At this time point both the pEC_{50} value and the maximal response for histamine is significantly higher for the histamine $H_2D^{115}N$ mutant, even though the wildtype receptor is expressed at significantly higher levels.

In conclusion, recombinant SFV offers an excellent way for the pharmacological characterisation of the histamine H_2 receptor. Infection of COS-7 cells gives rise to membrane preparations with a high histamine H_2 receptor content offering a good starting point for receptor purification. The pace at which receptors are expressed makes the system an excellent tool for studies of constitutive active and/or structurally unstable receptor mutants, which with traditional expression techniques are expressed at very low or undetectable levels. Furthermore, the use of recombinant SFV drastically reduces the time required for functional studies (from 48 to 6 h). Since the SFV system is simple in its application, it is very well suitable for the simultaneous analysis of several receptor mutants. In addition, the broad host cell range of SFV enables the study of receptors in cell lines or primary cells that are usually difficult or impossible to transfect. The SFV can even be used in vivo (Lundstrom et al., 1998). Finally, we have shown that expression levels of the histamine H_2 receptor when expressed by recombinant SFV exceed the levels that are obtained by other methods. This observation makes the system a valuable tool for the large-scale production of histamine H_2 receptors with the aim to purify the receptor for structural studies.

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