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Large-scale overproduction, functional purification and ligand affinities of the His-tagged human histamine H1 receptor

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This report describes an efficient strategy for amplified functional purification of the human H1 receptor after heterologous expression in Sf9 cells. The cDNA encoding a C-terminally histidine-tagged (10xHis) human histamine H1 receptor was used to generate recombinant baculovirus in a *Spodoptera frugiperda*-derived cell line (IPLB-Sf9). As judged from its ligand affinity profile, functional receptor could be expressed at high levels (30–40 pmol per 10^6 cells). Rapid proteolysis in the cell culture led to limited fragmentation, without loss of ligand binding, but could be efficiently suppressed by including the protease inhibitor leupeptin during cell culture and all subsequent manipulations. Effective solubilization of functional receptor with optimal recovery and stability required the use of dodecylmaltoside as a detergent in the presence of a high concentration of NaCl and of a suitable inverse agonist. Efficient purification of solubilized receptor could be achieved by affinity chromatography over nickel(II) nitrilotriacetic acid resin. Functional membrane reconstitution of purified H1 receptor was accomplished in mixed soybean lipids (asolectin). The final proteoliposomic H1 receptor preparation has a purity greater than 90% on a protein basis and displays a ligand binding affinity profile very similar to the untagged receptor expressed in COS-7 cells. In conclusion, we are able to produce pharmacologically viable H1 receptor in a stable membrane environment allowing economic large-batch operation. This opens the way to detailed studies of structure–function relationships of this medically and biologically important receptor protein by 3D-crystallography, FT-IR spectroscopy and solid-state NMR spectroscopy.

Keywords: functional reconstitution; G-protein coupled receptor; histamine H1 receptor; ligand affinity; overproduction.

Biomembranes mediate many functions of the cell including its communication with the outside environment through membrane-bound proteins, such as receptors, transporters and channels [1]. Obtaining a detailed insight into structure, dynamics and mechanism of these membrane proteins is essential to enable progress in medical and biological sciences [2].

The G-protein coupled receptor (GPCR) family employs heterotrimeric guanine-nucleotide binding proteins (G-proteins) for signal transduction and in the active state triggers a variety of intracellular signal transduction cascades. This family represents one of the largest and functionally most differentiated gene families in our genome [3,4]. GPCRs mediate a large variety of signaling processes such as visual and olfactory perception, hormone action, neurotransmission, growth and differentiation control. GPCRs therefore represent major therapeutic targets.

Histamine has one of the broadest spectra among signaling molecules in the human body, ranging from involvement in mast cell activation, acid secretion in the stomach, up to circadian physiology [5,6]. Currently four subtypes of histamine receptors have been identified (H1, H2, H3 and H4) that all belong to the opsin subclass or class A of the GPCR family. Their estimated molecular masses range from 45 to 60 kDa and the subtypes can be distinguished on the basis of their differential sensitivity to specific ligands [7–10]. The histamine H1 receptor mediates many of the histamine-induced symptoms of allergic reactions by coupling to different signaling pathways. Consequently, during the past 20 years H1 receptor antagonists have become one of the most prescribed drug families in Western countries [11] to relieve the symptoms of allergic reactions.

Histamine receptors have been investigated predominantly from a pharmacological point of view [12–15]. One of the problems that have considerably slowed down the progress in structural and mechanistic characterization of GPCRs in general is their low native abundance. The use of heterologous mammalian overexpression systems allowed

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; DDM, *N*-dodecyl-β-D-maltoside; dpi, days post infection; FBS, fetal bovine serum; GPCR, G-protein coupled receptor; HOM-β-cyclodextrin, heptakis-2, 6-di-*O*-methyl-β-cyclodextrin; NG, *N*-nonyl-β-D-glucoside; IMAC, immobilized metal-affinity chromatography; PEA, 2-pyridylethylamine.

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the production of several GPCRs at levels 10–100-fold those observed in native cells or tissues. This level is insufficient, however, to enable thorough mechanistic and structural studies at a molecular level, as these require mg quantities of functional purified receptor.

To obtain sufficient amounts of purified H1 receptor for structural and mechanistic studies by state-of-the-art biophysical techniques, we have designed procedures for overproduction, purification and reconstitution. We exploit the baculovirus/insect cell system, that has been successfully used for overproduction of a variety of GPCRs in Sf9 cells [16–21], an ovarian cell line derived from *Spodoptera frugiperda*, which is able to perform most eukaryotic post-translational modifications, such as phosphorylation, fatty acid acylation, disulfide bond formation and glycosylation [22–25]. The insect cell system has the advantage that large-scale suspension cultures can be grown using commercially available protein-free media. A C-terminal polyhistidine tag was added to the H1 receptor construct (H1−10xHis), which allows a single-step purification of the recombinant receptor via metal-affinity chromatography, so that even large batches can be completed within 2 days. By optimizing solubilization and purification conditions, highly purified H1 receptor preparations (≥ 90%) were achieved with an excellent recovery of up to 70%. Subsequently, purified H1 receptor was reconstituted into asolectin proteoliposomes by a single-step detergent extraction procedure [26]. The ligand-binding affinity profile of the final purified preparation is similar to that of the original H1−10xHis receptor expressed in Sf9 cell membranes, and matches the affinity profile of the untagged H1 receptor expressed in COS-7 cells.

Experimental procedures

Materials

N-Dodecyl-β-D-maltoside (DDM) and *N*-nonyl-β-D-glucoside (NG) were obtained from Anatrace (Maumee, OH, USA). 3-[(3-Cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), heptakis-2, 6-di-*O*-methyl-β-cyclodextrin (HOM-β-cyclodextrin), histamine dihydrochloride, Pluronic F-68, asolectin, leupeptin and TNM-FH insect medium were from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). Insect-Xpress medium was from Cambrex (Walkersville, MD, USA). Nickel(II)/nitrilotriacetic acid resin was obtained from Qiagen (Hilden, Germany). Penicillin/streptomycin was from Gibco-BRL (Breda, the Netherlands) and fetal bovine serum (FBS) from Greiner B.V. (Alphen aan den Rijn, the Netherlands). Rabbit anti-(His-tag) polyclonal antibody was used as a primary antibody and has been described before [27]. The goat anti-rabbit peroxidase (GARPO) secondary antibody was obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Mepyramine (pyrilamine maleate) and tripelennamine hydrochloride were obtained from RBI (Natick, MA, USA). [^{3}H]Mepyramine (20 Ci·mmol^{-1}) was purchased from NEN, Boston, MA, USA. 2-Pyridylethylamine (PEA) was taken from our own stock. Gifts of (*R*)- and (*S*)-cetirizine hydrochloride (UCB Pharma, Belgium), mianserine hydrochloride (Organon NV, the Netherlands), pcDEF3 (J. Langer, Robert Wood Johnson Medical School, Piscataway, NJ, USA), pBacPAK9 encoding the human histamine H1 receptor (J E. Leysen, Janssen Pharmaceutica N.V., Beerse, Belgium) and of the cDNA encoding the human histamine H1 receptor (H. Fukui, University of Tokushima, Japan) are gratefully acknowledged.

Buffer solutions

Buffer A: 7 mM Pipes [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] 10 mM EDTA, 5 mM DTE, and 5 μM leupeptin (pH 6.5). Buffer B: 20 mM Bis/Tris propane, 1 M NaCl, 1 mM histidine, 5 μM leupeptin, 2 μM tripelennamine, and 20% (w/v) glycerol (pH 7.2). Buffer C: 20 mM Bis/Tris propane, 20 mM DDM, 1 M NaCl, 1 mM histidine, 5 μM leupeptin, 2 μM tripelennamine, and 20% (w/v) glycerol (pH 7.6). Buffer D: 20 mM Bis/Tris propane, 20 mM imidazole, 20 mM DDM, 1 M NaCl, 5 μM leupeptin, 1 mM histidine, 2 μM tripelennamine, and 20% (w/v) glycerol (pH 7.6). Buffer E: 20 mM Bis/Tris propane, 125 mM imidazole, 20 mM DDM, 1 M NaCl, 5 μM leupeptin, 2 μM tripelennamine, and 20% (w/v) glycerol (pH 7.6). Buffer F: mix four parts of 50 mM Na2HPO4 with approximately one part of 50 mM KH_2PO_4. Check pH continuously and add 50 mM KH_2PO_4 until a pH of 7.4 is obtained.

Construction and generation of recombinant baculovirus

The pBacPAK9 vector (BD Biosciences Clontech, Palo Alto, CA, USA) containing the cDNA encoding the human H1 receptor was generously provided by J. E. Leysen, Janssen Pharmaceutica N.V., Beerse, Belgium. This construct was digested with *Xho*I and *Eco*RI. The vector (*Eco*RI-*Eco*RI) fragment and the H1 receptor (*Eco*RI-*Xho*I) fragment were isolated. The vector fragment was ligated with a primer cassette (Eurogentec), encoding the C-terminal H1 receptor sequence and a 10xHis-tag with a 5′- and 3′-*Xho*I overhang. The vector with the primer cassette was ligated with the *Eco*RI/*Xho*I fragment of the H1 receptor. The resulting transfer vector pBacPAK9-H1His containing the 10xHis-tagged human H1 receptor cDNA was used to generate recombinant baculovirus in the *S. frugiperda*-derived Sf9 cell line (IPLB-Sf9, ATCC: CRL-1711). For this purpose, the Baculogold recombination system (BD Biosciences Clontech, Palo Alto, CA, USA) was employed according to the manufacturer's instructions to insert the cDNA under control of the strong AcMNPV polyhedrin promoter. A monolayer of Sf9 cells was used for the generation and amplification of the recombinant baculovirus (pBac-H1His10) [15]. The sequence of the His-tagged H1 receptor insert was verified by cycle sequencing of baculovirus DNA isolated from Sf9 cell nuclei [28]. The virus titer was determined using a plaque assay as described previously [28].

Sf9 cell culture

Sf9 cells were cultured as monolayers at 27 °C in tissue culture flasks in complete TNM-FH insect medium supplemented with 10% (v/v) FBS and with penicillin and streptomycin at 50 units·mL^{-1} and 50 mg·mL^{-1}, respectively. Under these conditions, the cell doubling time was

typically 20–24 h. For small-scale cultures (100–400 mL), 5×10^5 attached cells were transferred from the culture flask to 500 mL spinner bottles (Bellco, Vineland, NY, USA). Large-scale cultures of 5 or 10 L were grown in a bioreactor (Applikon, Schiedam, the Netherlands). The culture conditions were: temperature 27 °C, partial oxygen pressure 50%, overlay aeration (air) 10% (v/v)·min^{-1}, sparger (O_2) maximum 0.005 (v min^{-1}.v) (computer controlled), impeller (marine) 80 r.p.m. Suspension cultures in spinner bottles or bioreactors were maintained in culture media containing 0.1% Pluronic F-68.

Infection of Sf9 cells

Cells were infected with pBac-H1His10 at a multiplicity of infection (MOI) of 0.1. Infected Sf9 cells were maintained in complete TNM-FH medium with the addition of 0.1% Pluronic F-68 and 5 mM leupeptin. Total cell counts were made with a hemocytometer: an experimental error of $\approx 10\%$ is therefore to be expected. Production tests were routinely performed at different days post infection (dpi) using dot blot assays.

Cell culture and transfection of COS-7 cells

COS-7 African green monkey kidney cells (ATTC # CRL-1651) were maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere in DMEM medium containing 2 mM L-glutamine, 50 IU·mL^{-1} penicillin, 50 mg·mL^{-1} streptomycin and 5% (v/v) FBS. COS-7 cells were transiently transfected with a plasmid containing the human H1 receptor cDNA under control of the CMV promoter (pcDEF3hH1) using the DEAE-dextran method [29].

Solubilization and affinity purification of His-tagged H1 receptor

Five days post infection, Sf9 cells were collected by centrifugation for 10 min at 3000 ***g*** and 4 °C. The cell pellet was resuspended to a density of 10^8 cells·mL^{-1} in buffer A (volume = V). Cells were subsequently homogenized at 4 °C using a tight-fitting Potter–Elvehjem tube. The cell homogenate was centrifuged for 20 min at 40 000 ***g*** and 4 °C and the pellet was resuspended into half the original volume (0.5× V) using buffer B. After an incubation of 15 min at room temperature to saturate the receptor with inverse agonist, the suspension was centrifuged for 15 min with 40 000 ***g*** at 4 °C. Although the resulting pellet may be stored at −80 °C at this stage, in our hands the purification is more effective if we proceed with the next steps immediately.

The cell pellet was resuspended in a volume of 1× V of buffer B and DDM and 2-mercaptoethanol were then added to obtain final concentrations of 20 and 5 mM, respectively, and mixed properly to get a homogeneous suspension. After incubation by rotation at 4 °C for 1 h, the insoluble material was removed by centrifugation for 60 min at 80 000 ***g*** (4 °C). The amount of solubilized H1 receptor in the supernatant was routinely estimated by a dot-blot assay [30]. Functional levels were determined by radioligand-binding assays on selected samples.

The supernatant was then incubated with a 0.1× V of nitrilotriacetic acid resin that had been equilibrated in buffer C. Binding of the H1 receptor to the nitrilotriacetic acid resin was accomplished by overnight incubation under constant rotation at 4 °C. The resin was then collected in a small calibrated syringe tube fitted with a frit and subsequently washed with a volume of 1× V buffer C and a volume of 1× V buffer D. Finally, H1 receptor was eluted with 0.5× V of buffer E. The collected fractions were monitored for H1 receptor using comparative dot-blot assays, and stored at −80 °C for further processing.

Membrane reconstitution

H1 receptor-containing fractions were pooled and reconstituted into the natural lipid preparation asolectin using the cyclodextrin extraction procedure [26]. Asolectin, a crude soybean lipid extract containing a mixture of several lipids [31], was found to be very suitable to sustain functional properties of the H1 receptor. The transition temperature of membranes prepared from asolectin is below 0 °C, thus allowing functional analysis at low temperature [31].

Approximately 0.6 μmol of phospholipid corresponding to about 0.5 mg of asolectin was dissolved in 10% DDM, and subsequently diluted 10-fold using 50 mM phosphate-buffered saline ($NaCl/P_i$), filtered using a 0.2 μm (Millipore) filter, and stored in aliquots at −80 °C. For reconstitution of the purified H1 receptor a molar lipid to protein ratio of about 100 : 1 was used. The required volume of asolectin solution was mixed with purified receptor at 0 °C, and β-cyclodextrin was added to yield a final concentration of 15 mM. After 30 min incubation at 0 °C a second amount of β-cyclodextrin was added to yield a final concentration of 30 mM. H1 proteoliposomes were subsequently separated from cyclodextrin complexes by sucrose density centrifugation [26].

A sucrose step density gradient was prepared with equal volumes of 15, 20 and 45% (w/w) steps in buffer E. The H1 proteoliposome preparation was loaded at the top of the sucrose gradient at up to 8 nmol of receptor per milliliter of gradient and centrifuged overnight (200 000 ***g*** at 4 °C). Fractions of 1 mL were collected from top to bottom of the centrifuge tube without disturbing the gradient and tested for the presence of H1 receptor using the dot-blot assay. H1 receptor containing proteoliposomes were typically present just above the 45% (w/w) layer in the sucrose gradient. Removal of the sucrose in the H1 receptor proteoliposome fraction by dilution with five volumes of Milli-Q water, and subsequent centrifugation for 30 min (80 000 ***g*** at 4 °C) yielded a visible precipitate, which was subsequently stored as a pellet at −80 °C for future studies. Protein was determined using the Bradford assay (Bio-Rad, Melville, NY, USA) according to the manufacturer's instructions using bovine rhodopsin for calibration [19].

Gel electrophoresis and Western blotting

Sf9 cells expressing the human (10xHis) H1 receptor were collected and centrifuged for 5 min at 2000 ***g***. Cell pellets were taken up in an SDS/PAGE sample buffer (2% sodium dodecyl sulfate (SDS), 0.04 M dithioerythrol (DTE) and 0.015% bromophenol blue in 0.5 M Tris, final pH 6.8). Samples were run on a 12% SDS/PAGE gel at 100 V for the 5% acrylamide stacking gel and 200 V for the running

gel. Protein staining was performed using Coomassie blue or silver staining (Pierce Chemical Co., Etten-Leur, the Netherlands). For immunodetection proteins were blotted onto a nitrocellulose membrane (1 h at 100 V) in ice-cold blot buffer (25 mM Tris and 0.2 M glycine in 20% methanol) using a MiniProtean system (Bio-Rad, Melville, NY, USA). Blots were subsequently immunoassayed for the presence of His-tagged receptor (see below).

Dot blot assay

Dot blotting was used as a rapid and convenient method for detection of 10xHis-tagged proteins in crude lysates or solutions. Nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech, Buckinghamshire, UK) was soaked in distilled water for 10 min and subsequently soaked for 10 min in NaCl/P_i and left to dry at room temperature. Protein samples were diluted in NaCl/P_i to yield a final protein concentration between 1 and 100 ng·mL^{-1}. Samples (1–2 μL of diluted protein) were applied directly onto the membrane. A purified bacterial reaction center preparation (kindly provided by Alia, Leiden University, the Netherlands) was taken as a negative control. Dot blots were subsequently assayed for the presence of immunoreactive proteins (see below). For semiquantitative analysis, a two-fold dilution series was applied for every sample and a concentration range of His-tagged rhodopsin (0.03–1.00 pmol) was used for calibration (generously provided by P. Bovee, University of Nijmegen Medical School, the Netherlands).

Immunodetection of H1–10xHis receptor

Western or dot blots were incubated for 20 min with 5% bovine serum albumin and 0.1% Tween-20 in NaCl/P_i at room temperature, followed by overnight incubation at room temperature or 2 h incubation at 37 °C with primary antibody (rabbit polyclonal anti-(His-tag) Ig [27]). Blots were washed three times 10 min with NaCl/P_i followed by 1-h incubation with secondary antibody [goat anti-(rabbit peroxidase), GARPO]. Antisera were used at a dilution in NaCl/P_i of 1 : 20 000 for the primary antibody and 1 : 100 000 for the secondary antibody. After washing with NaCl/P_i (3 × 10 min), peroxidase activity was assayed by the SuperSignal Kit for horseradish peroxidase (Pierce) and the resulting chemiluminescence was recorded on Hyperfilm ECL (Eastman Kodak Company, Rochester, NY, USA).

Radioligand binding assays

For radioligand binding studies infected Sf9 cells or transfected COS-7 cells were harvested at 5 dpi and 48 h, respectively, and homogenized in ice-cold buffer F. Aliquots of cell homogenates corresponding to 2000–3000 cells were diluted to 400 μL with buffer F and incubated for 30 min at 25 °C with 1 nM [^{3}H]mepyramine. Non-specific binding was determined in the presence of 1 mM mianserin. The reaction was stopped by rapid dilution with 3 mL ice-cold 50 mM Na_2/K phosphate buffer (pH 7.4). Non-bound radioactivity was removed by filtration through Whatman GF/C filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 mL buffer and radioactivity retained on the filters was measured by liquid scintillation counting. Binding data were evaluated by a nonlinear, least squares curve-fitting procedure using GRAPHPAD PRISM® (GRAPHPAD Software, Inc., San Diego, CA, USA). Total binding was below 10%, and ligand affinities were calculated according to Swillens [32] by using a global fitting procedure to determine total and nonspecific binding at the same time. In saturation binding experiments the experimentally determined nonspecific binding was used to estimate nonspecific binding under total binding conditions.

Results

Production conditions

As the production level of a given receptor is difficult to predict, we performed an initial screening on two types of histamine receptors, the human H1 and the rat H2 receptor. Both subtypes were extended with a 10xHis tag and expressed under identical conditions in Sf9 cells. Functional expression was monitored by radioligand binding assays and reached 3–6 pmol/10^6 cells for the H2 receptor, similar to a previous report [33], and at least 20 pmol/10^6 cells for the H1 receptor (data not shown). Ligand affinities of the Sf9 cell expressed His-tagged H1 receptor were similar to those of the untagged receptor expressed in COS-7 cells (Tables 1 and 2). Hence we

Table 1. K_d and pK_i values of mepyramine for the human H1 receptor. Receptors were transiently expressed in COS-7 cells and Sf9 cells. Reconstituted receptor was purified from Sf9 cells. pK_i values are averages with standard error from three independent experiments carried out in triplicate.

H1 receptor preparation	K_d (nM)	pK_i	Reference
COS-7	1.6	8.68 ± 0.01	[43,44]
10xHis (Sf9 membranes)	3.6	8.4 ± 0.10	Present work
10xHis (reconstituted)	1.9	8.7 ± 0.10	Present work

Table 2. Ligand affinity profile of H1 receptor preparations. Ligand affinities were obtained by displacement analysis of [^{3}H]mepyramine binding. pK_i values are averages with standard error from three independent experiments carried out in triplicate. Preparations represent 10xHis-tagged human H1 receptor in either Sf9 cell membranes or following subsequent purification and reconstitution, and untagged human H1 receptor in COS-7 cell membranes. Membrane preparation, purification, reconstitution, and competition experiments were carried out as outlined in the Experimental procedures.

Ligand	Sf9 membranes	Reconstituted H1 receptor	COS-7 cells [44]
Agonists			
Histamine	5.1 ± 0.1	4.7 ± 0.3	4.2 ± 0.1
PEA	4.4 ± 0.1	3.7 ± 0.4	3.8 ± 0.1
Inverse agonists			
Mepyramine	8.4 ± 0.1	8.7 ± 0.1	8.7 ± 0.1
(*R*)-Cetirizine	8.5 ± 0.1	8.3 ± 0.1	7.7 ± 0.1
(*S*)-Cetirizine	6.9 ± 0.2	6.7 ± 0.2	6.8 ± 0.1

decided to select the H1 receptor for large-scale production and purification studies. For rhodopsin, good production levels were obtained in a protein-free medium (Insect Xpress) [19]. Therefore we compared H1 receptor production levels in Insect Xpress and in the established standard serum-supplemented TNM-FH medium. In our hands, high production levels were obtained with TNM-FH medium supplemented with 10% FBS. In TNM-FH medium Sf9 cells had a doubling time of about 20 h, compared to about 24 h in Insect-Xpress medium, and could reach densities of $(8–9) \times 10^6$ cells·mL^{-1}, compared to $(6–7) \times 10^6$ cells·mL^{-1} in Insect Xpress medium. Although as judged by radioligand binding assays functional expression levels of recombinant H1 receptor did vary two- to three-fold between different productions, they were always up to two-fold higher in serum-supplemented TNM-FH medium (results not shown). Because of the potentially high cell densities and the good cellular production levels, resulting in optimal volumetric production levels, serum-supplemented TNM-FH medium was used for all subsequent H1 receptor production. Scaling up of our insect cell suspension cultures in TNM-FH medium from 100 mL spinner bottle to 10-l bioreactor was achieved without significant loss in production level.

Over a large number of experiments, we obtained production levels of functional His-tagged H1 receptor in Sf9 cells in the range of 30–60 pmol per 10^6 cells (18–35×10^6 copies·cell^{-1}) as estimated from radioligand binding assays. Estimation by dot blot was more variable and usually indicated higher production levels. This probably is due to some cross-reactivity of the His-tag antibody with endogenous proteins as well as to the presence of misfolded or otherwise nonfunctional receptor [27]. It has been previously reported that the MOI and the time-point of infection in the cellular growth cycle are important parameters in determining volumetric production levels and optimal time of harvesting [19]. Final levels did not vary significantly when cells were infected in their early mid-exponential growth phase for an MOI of 0.1, 1 and 10, but usually lagged behind at a MOI of 0.01. As a lower MOI requires less viral inoculate, which is a strong advantage for large-scale cultures, a number of parameters (the total cell number, cellular production yield and volumetric production yield) were examined in more detail for a MOI of 0.1 to optimize the production levels. Production levels leveled at 4–5 dpi, when cell viability had not yet suffered much. Therefore cells were routinely harvested at 5 dpi. During these studies we followed the expression by immunoblotting. Unfortunately extensive fragmentation of the H1 receptors occurred after 2 dpi (Fig. 1, lanes 2–4). Remarkably, this degradation is accompanied by only limited reduction in ligand binding capacity (not shown). As it was reported that Sf9 cells very well tolerate the presence of effective levels of protease inhibitors [34,35] protease inhibitors were included in the cell culture at various production stages. We observed that the fragmentation could be nearly completely suppressed by adding 5 μM leupeptin at 0 dpi (Fig. 1, lanes 6–9) with only limited reduction in production level. We have observed that adding similar concentrations of leupeptin to cultures up to 10 L suppresses degradation without significant effects on cell growth.

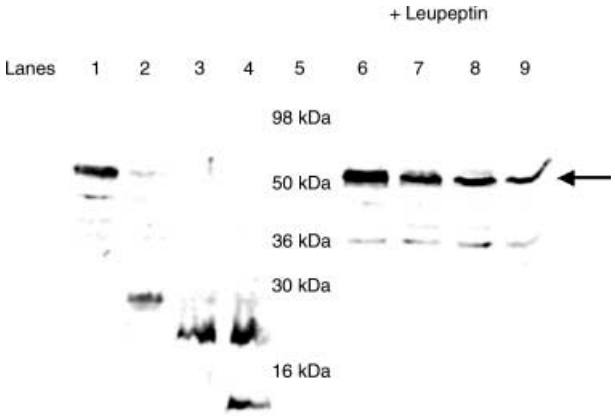

Fig. 1. Proteolysis of H1 receptor in Sf9 cells can be suppressed by addition of leupeptin. Expression of H1−10xHis receptor in Sf9 cells is followed by SDS/PAGE and immunoblotting with anti-(His-tag) serum. The expression of H1 receptor was triggered by baculovirus infection at a MOI of 0.1 and samples were taken at 3, 4, 5 and 6 dpi in the absence (lanes 1–4) or presence (lanes 6–9) of 5 μM leupeptin in the culture medium. The molecular mass calibration is shown in lane 5. The intact His-tagged H1 receptor (arrow) migrates with an apparent mass of 55 ± 5 kDa.

Scaling-up

The culture conditions yielding optimal volumetric production of recombinant H1 receptor in 100 mL spinner cultures could be directly scaled up to 10-L bioreactor level, maintaining production yields (5–7 mg of functional receptor per liter). Thanks to the low MOI employed (0.1), a viral stock obtained from a standard culture flask of 75 cm^2, corresponding to 10 mL of culture, will usually yield enough virus to infect several 10-L bioreactor cultures. We also did a first test with a new disposable type of plastic bioreactor (cellbag; Wave Biotech AG, Tagelswangen, Switzerland). This innovative design claims better mixing and oxygen transfer and lower shear stress, and offers a broad range of culture volumes (from 2–200 L). Preliminary experiments with the cellbag technology gave approximately 50 mg of functional receptor from a 10 L culture, making this approach promising as an alternative for the classical nondisposable glass or steel bioreactor set-up, which is quite time-consuming in maintenance and preparation for sterilization.

Functional solubilization of H1 receptor

Much effort was put into finding optimal conditions to solubilize recombinant human H1 receptor from the insect cell membranes for subsequent binding to the metal-affinity matrix. Careful optimization of the detergent and the buffer composition was critical for obtaining maximum solubilization efficiency as well as optimal stability of functional receptors in micellar solution. First tests showed that detergent solubilized receptor was quite unstable and lost all activity within 2–3 days at 4 °C. Similar to the situation in visual pigments [28] we could significantly stabilize the H1 receptor by addition of the high-affinity inverse agonist, mepyramine [32]. As the binding assay had to be intrinsically modified for detergent-solubilized preparations and is time-

consuming anyway, we exploited this ligand stabilization further by using radiolabeled [^{3}H]mepyramine. In this way functional receptor could be easily identified and traced by scintillation counting. This dual effect of both stabilizing and estimating functional receptors greatly facilitated the optimization of conditions for solubilization, purification and reconstitution.

For solubilization a range of detergents was tested at 20 mM concentration. Most exhibited either poor solubilization efficiency (< 20%) or induced rapid inactivation of solubilized receptor. This is evident from low levels of radiolabel retained on the nickel matrix. More extensive screening was then performed with a smaller panel (DDM, Digitonin, Triton-X100, NG, C12E10 and CHAPS) in various combinations and concentrations. This panel represents different classes of detergents that were reported to preserve a relatively good thermal stability of membrane proteins [28,32] or held some promise in the first test. From this panel better than 20% solubilization efficiencies of functional receptor could only be achieved with NG and CHAPS (30–35%) and with DDM (40–50%). Combinations of detergents did not help to improve the solubilization efficiency significantly. In further studies with DDM a large variety of additives was tested and the solubilization efficiency could be raised to 70–90% by including 1 M NaCl. However in this high-ionic strength medium the solubilized receptor is not very stable and over 50% was lost during further purification. While the pH had little effect on H1 receptor stability in the range 6.5–7.8, addition of glycerol to 20% (w/v) had a significant stabilizing effect (buffer C). When a low density of cell membrane suspension was used in buffer C, the extraction of functional H1 receptor was nearly quantitative with sufficient stability to survive subsequent purification.

The low dissociation rate (K_{off}) of mepyramine [36] is convenient for protocol development. On the other hand, removal or exchange of H1 receptor bound mepyramine from the final proteoliposomal preparation (see below) is difficult at temperatures below 20 °C. Therefore, saturation with an alternative, more readily releasable ligand was exploited for routine production. The low-affinity agonist histamine was observed to reduce the stability of the solubilized receptor, as indicated by a marked decrease in recovery of functional receptor upon purification. On the other hand, the high affinity inverse agonist tripelennamine behaved quite similar to mepyramine. In agreement with its higher Koff [37], tripelennamine could be more easily washed away from the final preparation. Thus, the entire procedure was performed in the presence of 2 μM tripelennamine, which is sufficient to fully saturate the receptor ($K_d \approx 4.2$ nM) [37].

Purification and reconstitution of the H1–10xHis receptor

Extending the H1 receptor with a 10xHis-tag aimed at rapid single-step affinity purification by immobilized metal-affinity chromatography (IMAC). In several small-scale trials, super flow nitrilotriacetic acid resin (Qiagen) gave best results with the H1–10xHis receptor solubilized in buffer C. The pH of buffer C was raised to pH 7.6 to optimize binding of the His-tagged receptor to the matrix. One millimolar histidine was included in this buffer to suppress low-affinity binding. However, we were not able to properly elute bound receptor with high concentrations of histidine. Hence we resorted to imidazole, which proved to be more effective. The receptor started to elute at imidazole concentrations between 100 and 125 mM (Fig. 2). As most of the low-affinity contamination could be removed by washing with 20 mM imidazole (buffer D) we routinely used 125 mM imidazole for rapid and complete elution of the H1 receptor (buffer E). As estimated from SDS/PAGE analysis, the purity of the H1 receptor after IMAC purification ranges between 75 and 95% (e.g. Fig. 3, lane 5).

The fractions eluted with 125 mM imidazole were stored at −80 °C and screened for H1 receptor by dot blotting. Those with a positive response were processed for reconstitution within 1–2 days. Asolectin was added to the combined purified receptor fractions in a molar lipid to receptor ratio of about 100 : 1. This is within the natural lipid to protein range of cellular membranes and with this ratio full functionality of recombinant rhodopsin has been demonstrated [27,28]. Subsequently, the proteoliposomes containing reconstituted receptor can be separated from nonreconstituted receptor and from cyclodextrin-detergent complexes in sucrose step-density gradients as described [26]. A proteoliposome fraction just above the 45% sucrose layers would indicate proper reconstitution [26]. The major receptor fraction indeed was

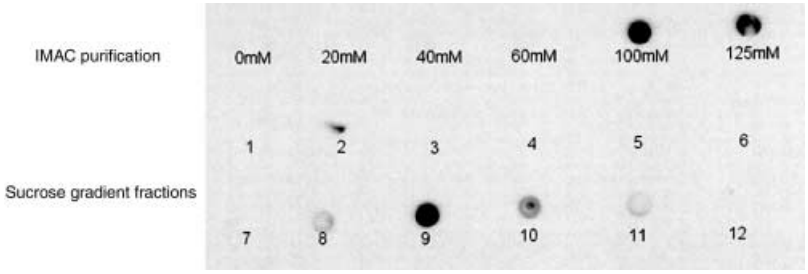

Fig. 2. Dot blot screening of H1 receptor during purification and reconstitution. Fractions in the top row indicated by imidazole concentration represent a typical IMAC-purification. H1 receptor starts to elute at 100 mM imidazole. Numbers in the bottom rows represent fractions collected from top to bottom of a sucrose step-gradient isolation of reconstituted receptor. Fraction 9 corresponds to the fraction just above the 45% layer. A small contamination by an unidentified fluorescent object is seen on fraction 2.

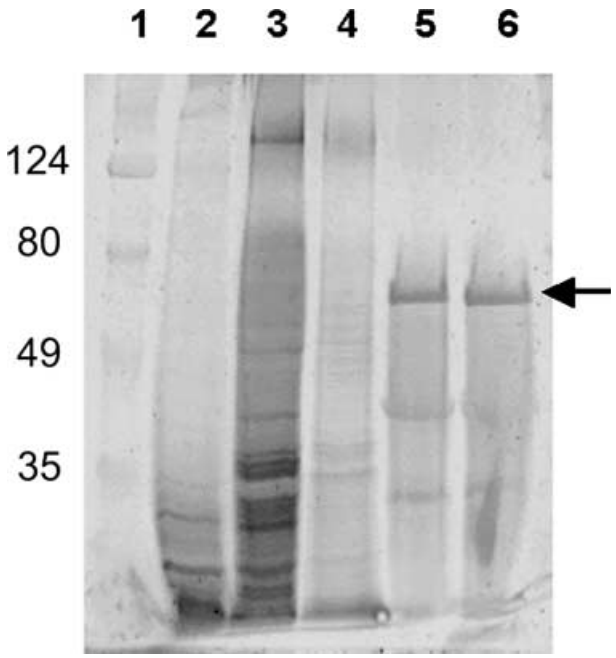

Fig. 3. Purification of the H1 receptor assayed by 12% PAGE and silver staining. Molecular mass markers (Bio-Rad, Veenendaal, the Netherlands) are shown in lane 1. Whole Sf9 cell lysate is shown in lane 2. The crude DDM extract of infected insect cells is shown in lane 3, throughput IMAC column wash with 0 mM imidazole is depicted in lane 4, while purified and reconstituted receptor preparations are shown in lanes 5 and 6, respectively. The position of the intact His-tagged H1 receptor is indicated by the arrow. The quantity of the remaining minor contaminating bands in the purified receptor varied between preparations. Their identity is unclear. Most likely they do not represent proteolytic fragments of the receptor, as neither reacts with the anti-(His-tag) serum (Fig. 5).

Table 3. Recovery of protein and functional H1 receptor at several stages during purification. Data are given per liter of culture volume and represent averages of three experiments with standard error.

Preparation	mg protein	% functional receptor (mg·mg protein^{-1})	Recovery of functional receptor (%)
Sf9 cells	1430 ± 210	0.4 ± 0.1	100
Solubilized Sf9 membranes	380 ± 90	1.4 ± 0.3	94 ± 10
Purified reconstituted receptor	4.0 ± 0.6	85 ± 9	58 ± 11

collected at this position (Fig. 2), with a recovery of at least 90%. The reconstitution procedure also further increases the purity of the preparation to at least 90% on a protein basis (Fig. 3, lane 6). This purity is sufficient for functional and biophysical studies, but for crystallization studies further purification will be required, e.g. by ligand-affinity chromatography [16,17]. Global results on functionality and recovery of the H1 receptor are collected in Table 3.

Ligand affinity profile of H1–10xHis receptor preparations

The amount of correctly folded receptor was determined by its affinity to bind the inverse agonist mepyramine. Saturation binding assays were performed with [^{3}H]mepyramine, using an excess of mianserin to estimate nonspecific binding. Fig. 4A,B show representative saturation binding curves for Sf9 membranes and reconstituted H1 receptors, respectively. The corresponding K_d (nM) and pK_i values from three independent assays are given in Table 1. Although the variation is somewhat larger for the Sf9 membranes, the K_ds of both preparations are very close, and also in good agreement with results reported for expression in COS-7 cells [29,38]. We therefore used [^{3}H]mepyramine in competition experiments in order to determine K_i values for various H1 ligands. Fig. 4C,D show representative displacement curves for Sf9 cell membranes and reconstituted H1 receptor, respectively. The corresponding pK_i values are given in Table 2. They are well in line with data obtained from untagged H1 receptors expressed in COS-7 cells [37,39].

Discussion

In spite of their widespread physiological relevance, relatively little is known about structure and receptor–ligand interactions of the histamine receptors. Elucidation of the structure and dynamics of membrane proteins is a challenging task essential for proper understanding of the functioning of fundamental biological processes at the atomic level. The majority of membrane proteins are only found in very small quantities in native membranes. They have to be overexpressed in a functional state, solubilized for purification and reconstituted into a lipid environment. There are few examples of highly overproduced eukaryotic membrane proteins, and it is difficult to establish general rules for the successful functional overproduction of a desired membrane protein [16]. Biophysical studies that can provide detailed structural and functional information require mg amounts of purified protein. There are several reports claiming a high level production of functional GPCRs in insect cells in the range of 2–4 mg·mL^{-1} after infection with the corresponding recombinant baculovirus [19,23–25,33,40,41]. Recent advances include further development of the system for production of multisubunit protein complexes and coexpression of protein-modifying enzymes to improve heterologous protein production [16]. Thus, this system should be able to support efficient and economic production of functional GPCRs in sufficient quantities to allow structural and mechanistic studies.

Functional expression of the H1 receptor in baculovirus/insect cells

Functional GPCR production levels vary widely in heterologous systems and depend on a variety of factors including culture medium, growth phase, affinity tag, sequence motifs, etc. [19,27], in a complex manner. Functional expression of H1 receptor was achieved with proper ligand affinity at levels of several tens of millions of copies per cell, at least 1000-fold higher than in native tissue. This level is high also

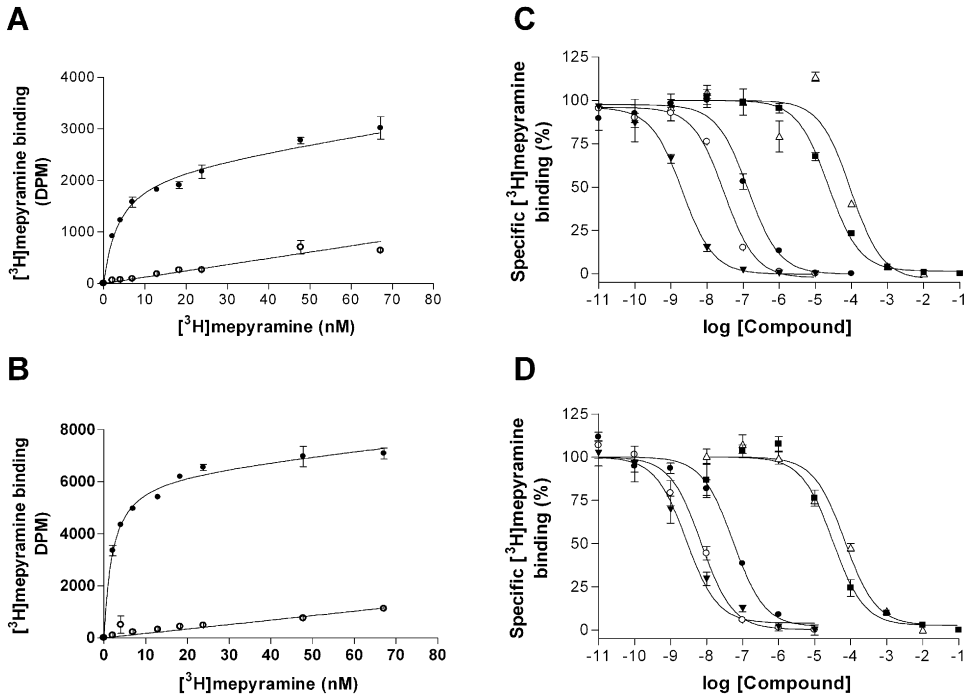

Fig. 4. Typical ligand affinity analysis of H1 receptor preparations. Preparations represent His-tagged H1 receptor in Sf9 cell membranes (A and C) and the corresponding receptor after purification and reconstitution (B and D). (A) and (B) show saturation radioligand binding experiments using the Sf9 cell membrane fraction and reconstituted H1 receptors, respectively, that in each set yielded a single high affinity binding site for [^{3}H]mepyramine with pK_d values of 8.82, and 8.65, respectively. ● represents total binding; ○ represents experimentally determined nonspecific binding. (C) and (D) show radioligand displacement studies using either Sf9 cell membranes or reconstituted H1 receptors, respectively, for histamine (■), PEA (△), mepyramine (▼), (*R*)-cetirizine (○) and (*S*)-cetirizine (●). Representative curves are shown, with standard error in triplicate experiments.

compared to previous expression work on GPCRs [16,17,19]. Without precautionary measures, however, ongoing proteolytic fragmentation of the receptor was observed, initially without loss of ligand binding capacity. It has been reported before that limited fragmentation of GPCRs can occur without loss of ligand binding capacity, if the seven transmembrane segments harboring the binding site can functionally interact without connecting loops [42,43, (W. J. DeGrip, P. J. G. M. VanBreugel and P. H. M. Bovee-Geurts, unpublished data)]. This can explain our observation for the H1 receptor, where most likely the long third intracellular i3-loop is vulnerable to proteolytic attack [18,29,44]. If the i3-loop is cleaved close to its N-terminal as well as its C-terminal end, His-tagged fragments with an approximate size of ≈ 30 and ≈ 20 kDa are generated, which could explain the smaller fragments detected upon PAGE analysis (Fig. 1). This limited proteolysis can be suppressed by a single protease inhibitor, leupeptin, which is able to penetrate the cell to inhibit intracellular protease activity [38].

Using the procedures as described in the experimental section, we obtained excellent production levels of functional 10xHis-tagged human histamine H1 receptor up to 7 mg·L^{-1} of cell culture. Binding assays performed for a variety of ligands on isolated Sf9 cell membranes containing this receptor show similar affinities compared to the untagged receptor expressed in COS-7 cells (Tables 1 and 2) [37]. Hence we are confident that the C-terminal His-tag does not affect ligand binding. This is in line with observations for other receptors [17,20,27]. According to SDS/PAGE the recombinant receptor migrates with an apparent mass of 55 ± 5 kDa. This corresponds well with the mass calculated from its amino acid composition (55.7 kDa). Membrane proteins often show relatively high levels of SDS binding, and consequently migrate faster in SDS/PAGE than expected on the basis of their mass [16,27,45]. This suggests that the His-tag reduces the migration rate, similar to rhodopsin [27]. On the other hand, glycosylation can also reduce the migration rate [28]. The N-terminal sequence of the H1 receptor indeed contains two N-linked glycosylation consensus sites, but in preliminary studies no binding to concanavalin A was observed, indicating that the recombinant receptor is not *N*-glycosylated (V. R. P. Ratnala, P. H. M. Bovee-Geurts & W. J. DeGrip, unpublished data).

Purification

Selection of the proper detergent, and searching for appropriate stabilizing components played an essential role in obtaining solubilized functional H1 receptor. Solubilization is a critical step in membrane protein purification. On the one hand it is essential for purification, while on the other hand it destabilizes the receptor, resulting in a time-dependent loss of functional properties. The kinetics of this loss of activity depends on the type of detergent and

on buffer composition [26]. Solubilization of functional GPCRs has in many cases been achieved with the very mild detergent digitonin [26,46–48]. However, impurities and batch variations make this natural product unsuitable for reproducible purification and reconstitution. For the solubilization of the H1 receptor we have obtained best results with dodecylmaltoside, a mild detergent that is commercially available in high purity and also yields good results with other membrane proteins [16,19,49,50]. Even mild detergents do not always guarantee optimal solubilization, stabilization and purification of GPCRs, however. To improve the performance of dodecylmaltoside, variation of ionic strength and pH, and addition of glycerol, lipid, and ligands were investigated [16,19,27,51]. Solubilization of the H1 receptor in a micellar dodecylmaltose solution increased to 70–90% at higher ionic strength. This may reflect denser detergent packing that mimics the lipid bilayer in a better way than at low ionic strength [50]. For rhodopsin, the presence of an inverse agonist has been shown to stabilize the protein upon solubilization using a wide variety of detergents as evident from a large decrease in the rate of denaturation [49]. A similar effect has been reported for the histamine H2 receptor [52,53]. The H1 receptor is indeed stabilized by the inverse agonists mepyramine and tripelennamine while the agonist histamine destabilizes the receptor. Probably the inverse agonists reduce the dynamics and flexibility of the protein, while an agonist has the opposite effect, making the receptor more vulnerable to detergent destabilization. The presence of inverse agonist and a 20% level (w/v) of the renowned protein stabilizing agent glycerol, rendered the H1 receptor sufficiently stable in micellar solution to allow purification with good recovery.

Binding of the C-terminal 10xHis–H1 receptor to the nitrilotriacetic acid resin was not very prominent at the pH of solubilization (7.2) as recovery was less than 20% in a variety of conditions. Raising the pH to 7.6 improved binding considerably, however, yielding recoveries of 60–80%, also when performed batchwise. This is in line with a neutral pK ≈ 7 of the pseudo-aromatic ring of histidine residues in proteins, where an increase in pH will decrease the net positive charge and increase the affinity for cations. Purity and integrity of the purified H1 receptor were monitored by SDS/PAGE and immunoblotting. Protein stained gels (Coomassie blue or silver staining) revealed a major band at 55 $\pm$ 5 kDa that is detected by the anti-His-tag antibody and therefore represents the intact receptor (Figs 3 and 5). This accounts for 80–95% of the total protein.

Reconstitution into proteoliposomes

Reconstitution of membrane proteins into liposomes offers the only possibility to study these proteins in a stable environment, mimicking their native membrane. Best results with respect to recovery and integrity were obtained when the H1 receptor was reconstituted in a lipid matrix of asolectin, and we selected asolectin as the standard lipid source for reconstitution. This natural lipid source has a good variety in lipid species, quite well mimicking mammalian cellular membranes [31,54,55]. As shown before [27], reconstitution also removes protein contamination resulting in receptor purities exceeding 90% (Fig. 3). Overall recovery

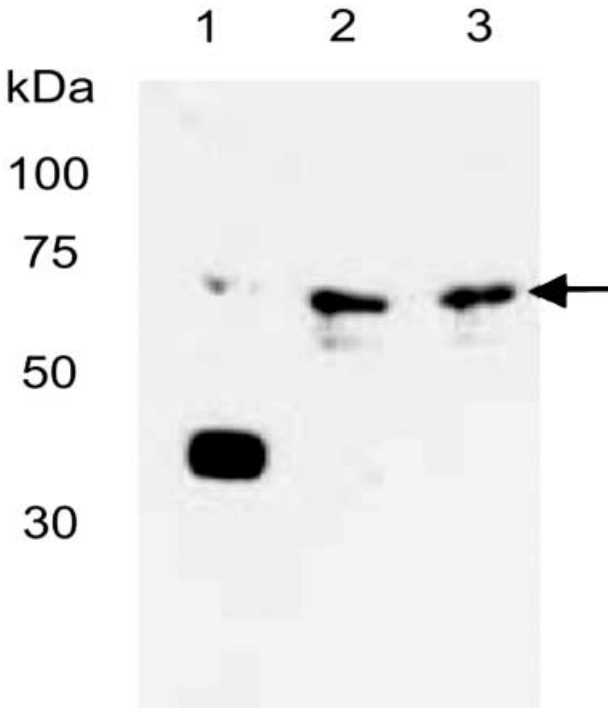

Fig. 5. Immunoblot analysis of purified H1 receptor preparations. Purified and reconstituted H1 receptor preparations are shown in lanes 2 and 3, respectively. Lane 1 shows His-tagged rhodopsin as a positive control. Samples were subjected to SDS/PAGE (12% gel), followed by immunoblotting with anti-(His-tag) serum as the primary antibody and GARPO as secondary antibody. The position of the intact His-tagged H1 receptor is indicated by the arrow.

of functional reconstituted receptor through purification and reconstitution ranges between 50 and 70% (Table 3). Using these conditions, scale-up to bioreactor level generates tens of mg of purified receptor, sufficient for structural studies.

In this study, our main goal was to produce highly purified functional H1 receptor, reconstituted in lipid bilayers. The ligand-binding profile of the reconstituted H1 receptor was very similar to that of the untagged H1 receptor expressed in COS-7 cells. Our data also show that the purified receptor has retained the chiral selectivity for (*R*)- over (*S*)-cetirizine that has been previously demonstrated in COS-7 cells [37]. Interestingly, the binding data suggest a somewhat higher affinity for agonists in the Sf9 membrane preparation compared to the reconstituted H1 receptor. Because in particular agonist affinities may significantly depend on interactions or the microenvironment of GPCR, a tentative explanation may be that the lower fluidity of the Sf9 cell membrane positively affects agonist affinity. This explanation needs to be verified by changing the composition of the lipid used for reconstitution. Overall, the ligand binding data unequivocally demonstrate that we were able to successfully purify and reconstitute the H1 receptor in a stable form, with full preservation of ligand-binding integrity. So far, we have been able to maintain reconstituted receptor in the frozen state at −80 °C for up to 8 months without loss of activity.

Conclusions

This is the first report on baculovirus-mediated production of human H1 receptor in insect cells. With this approach we could produce up to 40 pmol/10^6 cells, corresponding to 4–7 mg·L^{-1} of functional human H1 receptor. This represents an at least three-fold improvement compared to data available for other expression systems. The batch procedure exploited provides good recoveries during purification and reconstitution of the receptor (50–70%) and is easily amenable to scale-up. The milligram quantities of purified H1 receptor that are required to perform structural and mechanistic studies with state of the art biophysical technology in crystallography, SS-NMR and FT-IR can now be provided. One of the most exciting prospects is likely to arise from structural studies aimed at better understanding how small ligands interact with this receptor, enabling structure-based tailored design of drug candidates. The H1 receptor has an important role in many physiological and pathological processes and a better understanding of its structure and of its ligand interaction pattern will be highly relevant for future pharmacological intervention. A first pharmacologically important outcome of our work is the availability of ligand affinities for the pure receptor without any interference by 'putative cellular modulators'.

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