Functional Overexpression and Characterization of Human Bradykinin Subtype 2 Receptor in Insect Cells Using the Baculovirus System

Arun Kumar Shukla,[1]* Winfried Haase,[2] Christoph Reinhart,[1] and Hartmut Michel[1]*

[1]Department of Molecular Membrane Biology, Max Planck Institute of Biophysics, Max-von-Laue Street 3, 60438 Frankfurt/M, Germany
[2]Department of Structural Biology, Max Planck Institute of Biophysics, Max-von-Laue Street 3, 60438 Frankfurt/M, Germany

Abstract   Bradykinin exerts its actions via binding to B_1 and B_2 receptors (B_1R and B_2R), which are members of G protein-coupled receptor superfamily. B_2R is constitutively expressed in a variety of cells such as endothelial cells, vascular smooth muscle cells, and cardiomyocytes and it is an important drug target for the treatment of cardiovascular disorders. During this study, the human B_2R was functionally overexpressed in insect cells using the baculovirus expression system. The maximum expression level in Sf9 cells under optimized condition was 10 pmol/mg. This corresponds to approximately 0.25 mg active receptor per liter culture. The recombinant receptor showed high affinity for its endogenous ligand bradykinin, similar to the B_2R expressed in native tissues. Functional coupling of the recombinant receptor to the endogenous $G\alpha_s$ protein was demonstrated via cAMP release assay upon agonist stimulation. Confocal laser scanning microscopy and immunogold-labeling experiment revealed that the recombinant B_2R was mainly localized intracellularly and only a minor fraction of the recombinant receptor reached the plasma membrane. To our knowledge, this is the first report of high level expression of recombinant B_2R in insect cells and provides a way for large scale production and structural characterization of this receptor. J. Cell. Biochem. 99: 868–877, 2006. © 2006 Wiley-Liss, Inc.

Key words: GPCR; bradykinin receptor; overexpression; insect cells; glycosylation; cAMP

Bradykinin is a potent vasoactive peptide that is generated from kininogen by the action of kallikrein, either directly or via the intermediate kallidin [Imamura et al., 2004]. In vivo, bradykinin is rapidly degraded by kininases (metalloproteases) such as metallopeptidases, aminopeptidase P, and carboxypeptidases M and N [Dendorfer et al., 1997; Kuoppala et al., 2000]. In view of its short half-life, it is generally assumed that bradykinin is synthesized at tissue sites in order to have local effects [Nolly et al., 1994]. Two bradykinin receptor subtypes, B_1R and B_2R have been identified. These two receptors are members of G protein-coupled receptor superfamily [Prado et al., 2002]. Activation of B_2R plays crucial roles in inflammation, pain, tissue injury, and cardioprotective mechanisms [Farmer and Burch, 1992; Hall, 1992]. These effects are mediated by synthesis and release of vasorelaxant, anti-hypertrophic and anti-atherosclerotic mediators such as nitric oxide, prostaglandins, and plasminogen activators. The regulatory role of B_2R makes it an important drug target in the prevention and treatment of cardiovascular disorders (e.g., hypertension, ischaemic heart disease, ventricular hypertrophy, and congestive heart failure) as well as in pain and inflammation [Liebmann, 2004].

GPCRs, in general, are important drug targets for the pharmaceutical industry due to their

diverse roles in various cellular and physiological processes [Wise et al., 2002]. However, very little biophysical and structural information is currently available on these receptors. Structural studies on GPCRs and membrane proteins in general, require milligram amounts of functional protein. Except rhodopsin, most GPCRs are present only in small quantities in native tissues (100–300 fmol/mg), making it unsuitable for structural characterization. Therefore, heterologous expression of recombinant receptors is necessary. Baculovirus-mediated expression in insect cells is an efficient heterologous expression system and several GPCRs have been successfully overproduced and characterized using this system [Massotte, 2003; Sarramegna et al., 2003]. In most cases, the recombinant receptors produced in insect cells exhibit identical ligand-binding properties as in native tissues and mammalian cell lines.

In this study, we report high-level expression of the full-length N-terminally and C-terminally tagged human B_2R in insect cells using the baculovirus expression system. The production level has been optimized with regard to expression time, different cell lines and multiplicity of infection (MOI). The cellular localization of the tagged B_2R was investigated using confocal laser scanning microscopy and immunogold staining. The recombinant receptor was expressed at high level and it exhibits similar ligand-binding properties as the B_2R expressed in native tissues. Cyclic AMP release assay revealed that the recombinant receptor induces G-protein activation upon agonist stimulation.

MATERIALS AND METHODS

Materials

[^{3}H]bradykinin (68–90 Ci/mmol) was purchased from NEN Life Sciences Products (Boston, MA). The cell culture medium, L-glutamine, and gentamycin were from ccPRO (Neustadt, Germany) and Invitrogen (Carlsbad, CA). Bradykinin was obtained from Bachem (Torrance, CA). BCA Protein Assay Kit was purchased from Pierce (Rockford, IL). Complete protease inhibitor tablets were obtained from Roche Diagnostics (Mannheim, Germany). Restriction endonucleases, PNGase F and Endo H were purchased from MBI-fermentas (St. Leon-Rot, Germany). Immobilon-P polyvinylidene difluoride (PVDF) membranes were from Millipore (Bedford, MA). Modified baculovirus DNA (BaculoGold) was obtained from Pharmingen (San Diego, CA). Anti-flag M2 antibody, alkaline phosphate-coupled streptavidin, alkaline phosphatase-coupled secondary antibody and tunicamycin were from Sigma (St. Louis, MO). cAMP kit was purchased from Assay Designs (Michigan).

Construction of Recombinant Baculovirus

Three different expression constructs of the B_2R were created (Fig. 1). The constructs encode B_2R alone (pVLB$_2$R), B_2R with Flag and His$_{10}$ tags at the N-terminus and biotinylation domain of *Propionobacterium shermanii* transcarboxylase at the C-terminus (pVLMelFlagHis$_{10}$B$_2$RBio) or B_2R with eGFP at the C-terminus (pVL MelFlagHis$_{10}$B$_2$R-eGFP). The prepromelittin signal peptide from honeybee was used for proper targeting of the recombinant receptor. In all constructs, the polyhedrin promoter (PHP) drives the expression of recombinant receptor. A PCR reaction was performed with primers B$_2$R_Fw (5′-CGGGATCC-CCTCAATGTCACCTTGCAAGGGCCC-3′) and B$_2$R_Rv (5′-CGGAATTCCTGTCTGCTCCCTG-CCCAGTCC-3′), using pcDNA3-B$_2$R as a template. The resulting DNA fragment was digested with *BamH*I and *EcoR*I enzymes and
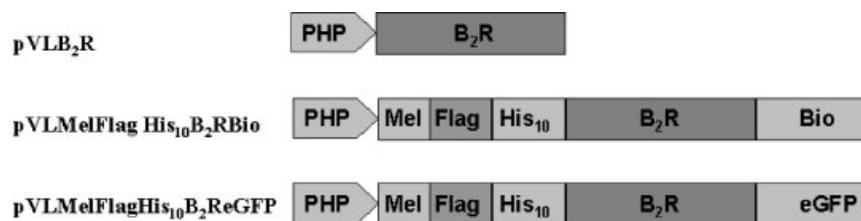
Fig. 1. Schematic representation of various recombinant baculovirus transfer vectors for heterologous production of the human B_2R in insect cells. PHP, polyhedrin promoter; Mel, coding sequence for the prepromelittin signal sequence; Flag, coding region for the Flag epitope; B_2R, coding region for the human B_2R; His$_{10}$, Histidine 10 tag; Bio, biotinylation domain of *Propionobacterium shermanii* transcarboxylase; eGFP, enhanced green fluorescent protein.

ligated into appropriately digested expression vectors. The constructs were verified by DNA sequencing.

Recombinant baculovirus were obtained by cotransfection of 0.1 µg of linearized BaculoGold DNA (Pharmingen) and 10 µg of recombinant baculovirus transfer vector in Sf9 cells, using cationic liposomes (Lipofectin) according to the manufacturer's specifications (Life Technologies, Inc.). The screening and selection of recombinant baculovirus was performed as described in detail previously [O'Reilly et al., 1992]. Afterwards, for each receptor construct, recombinant baculovirus was amplified and the virus titer was determined by end-point dilution [Reed and Muench, 1938].

Insect Cell Culture, Infection, and Membrane Preparation

Sf9 and Sf21 cells (*Spodoptera frugiperda* ovarian cells) were propagated in TNM-FH medium (Grace's basal medium supplemented with lactalbumin hydrolysate and yeastolate), supplemented with 5% (v/v) fetal bovine serum and 50 mg/ml gentamycin. High Five cells (derived from *Trichoplusia ni* egg cell homogenates) were propagated in Express Five medium (Invitrogen). Suspension cultures were grown in Erlenmeyer flasks at 27°C with shaking at 110 rpm. Cells were grown to a density of $2.0–2.5 \times 10^6$ cells/ml and then infected with recombinant baculovirus. For expression optimization, cells were infected at different multiplicity of infection (MOI) and harvested at indicated time intervals. During large-scale cultures for ligand binding and immunoblot analysis, cells were infected at MOI of 10 and harvested 96-h post-infection. For preparation of membranes, cells were harvested, washed in cold breaking buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, and Complete protease inhibitor cocktail) and lysed by nitrogen cavitation (Parr Instruments, Moline, IL) [Broekman, 1992]. The crude lysate was centrifuged (3,000g, 10 min, 4°C) in order to remove the nuclei and cell debris. Subsequently, the membranes were pelleted by centrifugation (100,000g, 1 h, 4°C). The membrane pellet was resuspended in storage buffer (breaking buffer supplemented with 5% glycerol) at a protein concentration of 5–10 mg/ml, flash-frozen in liquid nitrogen, and stored at −70°C until used. The protein concentration was determined using the bicincho-

ninic acid reagent (Pierce) and BSA as standard.

[³H] Bradykinin-Binding Assay

For [³H]bradykinin-binding assay, membranes were diluted in binding buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, and Complete protease inhibitor cocktail). For single point measurements, membranes (5 µg of total protein per assay point) were incubated with 25 nM [³H]bradykinin for 45–60 min at 4°C. For saturation-binding analysis, different concentration of [³H]bradykinin (0.1–25 nM) were used. Non-specific interactions were determined in the presence of 1 µM bradykinin. After incubation, the reaction was terminated by rapid filtration through GF/C glass-fiber filters, which were presoaked in 0.3% (v/v) polyethyleneimine. Filters were quickly washed four times with ice-cold binding buffer and subsequently transferred to counting vials. Radioactivity was measured using a Wallac microbeta counter. Dissociation constant (K_d) and maximum expression level (B_{max}) was calculated with the "KaleidaGraph" software by non-linear regression using a single site model.

Immunoblot Analysis and Deglycosylation

For immunoblot analysis, proteins were separated by 10% SDS–PAGE and subsequently transferred to a PVDF membrane as described previously [Towbin et al., 1979]. PVDF membranes were blocked with 5% (w/v) non-fat dry milk powder in TBST buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% Tween-20) for 1 h at room temperature. The anti-Flag M2 antibody and alkaline phosphate-coupled streptavidin was used according to the manufacturer's protocol (Sigma). Blots were developed in 10 ml of alkaline phosphatase buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl$_2$) containing 66 µl of BCIP (5-bromo-4-chloro-3-indolyl phosphate-p-toluidinium salt, stock solution 50 mg/ml in dimethylformamide), and 33 µl of NBT (nitro blue tetrazolium chloride, stock solution 50 mg/ml in 70% dimethylformamide).

For in vivo inhibition of glycosylation, tunicamycin was added in the culture medium (36 h before harvesting the cells) at a final concentration of 10 µg/ml. Subsequently, membranes were prepared and subjected to immunoblot analysis. Alternatively, enzymatic deglycosylation was performed by incubating 50 µg of

membranes (5 mg/ml) with 1–2 U of PNGase F or Endo H at 37°C for 2 h. Subsequently, immunoblot analysis was performed, as described above.

cAMP Assay

Sf9 cells were grown in 6-well plates (0.5×10^6 cells per well) and infected with recombinant baculovirus. Seventy-two hours post-infection, cells were incubated in serum-free medium for 1 h and increasing dose of bradykinin (1 pM– 1 μM) was added to the cells (except control cells). The plate was incubated for 1 h at 37°C, and the cells were subsequently lysed with a prewarmed detergent solution (0.03% digitonin in 20% DMSO). Accumulation of cAMP in response to bradykinin was determined by using cAMP kit (Assay Designs) according to the manufacturer's instructions (non-acetylation protocol). Absorbance was measured with a Power WaveX microplate reader (BIO-TEK instruments, Vermont).

Confocal Microscopy

Cells were grown on poly-L-lysine coated cover slips in 24 well plates and infected with pVLMelFlagHis$_{10}$B$_2$R-eGFP virus. Seventy-two hours after infection, the cells were washed twice in PBS and then fixed with 4% (v/v) paraformaldehyde for 15 min at room temperature. Subsequently, the cells were washed three times with PBS, three times with water and then mounted onto glass slides using gel mount medium (Vectashield). B$_2$R-eGFP fusion protein was visualized using a Leica confocal laser-scanning microscope (488 nm).

Immunogold Staining

Seventy-two hours after infection, Sf9 cells expressing pVLMelFlagHis$_{10}$B$_2$Rbio fusion protein were fixed with 4% (v/v) paraformaldehyde for 2 h at room temperature. Subsequently, the cells were washed and treated with 2% (w/v) glycine in order to block unreacted aldehyde groups. For post-embedding immunostaining, glycine-treated cells were dehydrated with ethanol, embedded in LRWhite resin, and samples were polymerized at 58°C. Immunogold staining was performed on thin sections using anti-Flag M2 antibody (1:300 dilution, w/v). A gold-coupled goat anti-mouse antibody (Sigma) (1:60 dilution, w/v) was used as a secondary antibody. For pre-embedding immu-

nostaining, fixed and glycine treated cells were incubated first with 1% (w/v) BSA, then with 0.1% (w/v) BSA and then with the anti-Flag M2 antibody (1:100 dilution, w/v) at 4°C. Afterwards, the cells were washed twice and then incubated with the secondary goat anti-mouse antibody (diluted 1:20) coupled to 10 nm gold particles for 90 min at 4°C. Subsequently, the cells were washed and then fixed with 1% (v/v) glutardialdehyde for 30 min and subsequently postfixed with 1% (w/v) OsO$_4$ in 100 mM sodium cacodylate buffer. Further, the cells were treated with 2% (w/v) uranyl acetate, dehydrated with ethanol, embedded in Spurr's resin [Spurr, 1969], and polymerized at 70°C. Thin sections were analyzed by electron microscopy (EM 208S, Philips, the Netherlands).

RESULTS

Generation of Different Constructs for Production of Recombinant B$_2$R in Insect Cells

For production of the human B$_2$R in insect cells, three recombinant baculovirus expression vectors were constructed. These vectors are schematically depicted in Figure 1. For reasons of comparison, the transfer vector pVLB$_2$R contained the *B$_2$R* gene without any modifications. It has been reported previously that fusion of the prepromelittin signal sequence enhances the expression of some GPCRs, such as oxytocin receptor [Gimpl et al., 1995], the 5HT$_{5A}$ serotonin receptor [Lenhard et al., 1996] and the D$_{2S}$ dopamine receptor [Grunewald et al., 1996], in baculovirus infected Sf9 cells. Therefore, to improve the yield of the heterologously produced receptor, two B$_2$R expression vectors containing in-frame fusion to this signal sequence were created. In these vectors, Flag and His$_{10}$ tag were fused at the N-terminus of B$_2$R to allow immunodetection and purification of the recombinant receptor. At the C-terminus of B$_2$R, either the biotinylation domain of *Propionibacterium shermanii* transcarboxylase [Cronan, 1990], or enhanced green fluorescent protein (eGFP) was fused. The biotinylation domain can be used for immunodetection and eGFP fusion is an efficient tool for localization analysis of the recombinant receptor.

Expression Optimization of the Recombinant B$_2$R

Sf9 cells infected with recombinant baculovirus bearing the different expression vectors

revealed specific binding of [^{3}H]bradykinin. No specific binding was observed in non-infected cells or cells infected with wild-type baculovirus. Sf9 cells infected with the expression vector pVLMelFlagHis$_{10}$B$_2$RBio displayed maximum expression of the recombinant B$_2$R. Therefore, this vector was used for expression optimization with regard to time of expression, type of cell line and MOI.

[^{3}H]bradykinin binding was detected as early as 24 h after infection and it reached the maximum level at 96-h post-infection (in Sf9 and Sf21 cells, Fig. 2A,C). In High Five cells, the maximum expression of B$_2$R was observed at 72-h post-infection (Fig. 2B). Effect of MOI on B$_2$R expression was only moderate in Sf9 and Sf21 cells but more significant in High Five cells. Sf9 cells exhibited maximum expression of B$_2$R (10 pmol/mg, i.e., ~0.25 mg recombinant receptor per liter culture) among the three different cell lines, which were tested. For further characterization of the recombinant B$_2$R, Sf9 cells were infected at MOI of 10 and harvested 96-h post-infection.

Saturation-Binding Analysis of the Recombinant B$_2$R

Sf9 cells were infected with each of the three vectors shown in Figure 1, membranes were prepared 96-h post-infection and saturation-binding analysis was performed using [^{3}H]bradykinin. As shown in Figure 3, the ligand binding was saturable and a single binding site was observed. The B$_{max}$ (maximum expression) and K$_d$ (equilibrium dissociation constant)

values for the three vectors are summarized in Table I. [^{3}H]bradykinin exhibited high-affinity binding (K$_d$ = 0.39–1.42 nM) to the recombinant receptor. These K$_d$ values are in good agreement with that of B$_2$R in mammalian cells [Naraba et al., 1999; Shukla et al., 2006] and in native tissue [Sawutz et al., 1992]. The B$_{max}$ values of the three vectors were significantly different and the best expression level was observed for the vector pVLMelFlagHis$_{10}$ B$_2$RBio.

Immunoblot Analysis of Recombinant B$_2$R

In order to further characterize and to monitor the integrity of the recombinant receptor, membranes from Sf9 cells expressing B$_2$R were analyzed by Western blot. As depicted in Figure 4A (lane 2), the anti-Flag M2 antibody recognized two bands of ~55 and ~35 kDa, respectively. The 55-kDa band corresponded well with the size of recombinant B$_2$R fusion protein as calculated from the amino acid sequence (51.7 kDa). The 35-kDa band probably represented a proteolytic degradation product. However, the immunoblot with alkaline phosphate-coupled streptavidin, which should recognize the bio tag, revealed a single band of ~55 kDa (Fig. 4B, lane 2) and no degradation product was observed on the Western blot. This result suggested that the 35-kDa band on anti-Flag blot was the N-terminal fragment of the recombinant receptor. Staining of the recombinant receptor by alkaline phosphate-coupled streptavidin also revealed that in vivo biotinylation
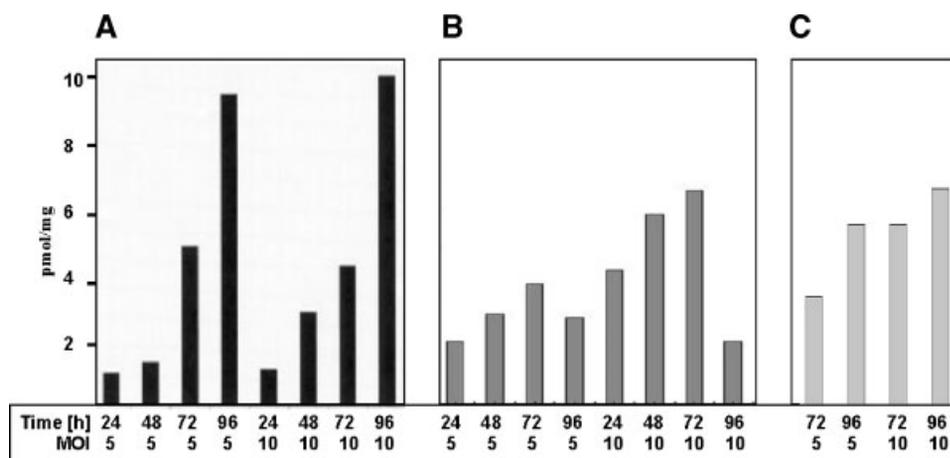
Fig. 2. Effect of cell type, time, and MOI on expression of the recombinant B$_2$R (pVLMelFlagHis$_{10}$B$_2$Rbio). **A**: Sf9 cells; (**B**) High Five cells; and (**C**) Sf21 cells. Expression time and MOI is shown at the bottom of the figure. Each data point represents the average of two independent experiments performed in triplicates.
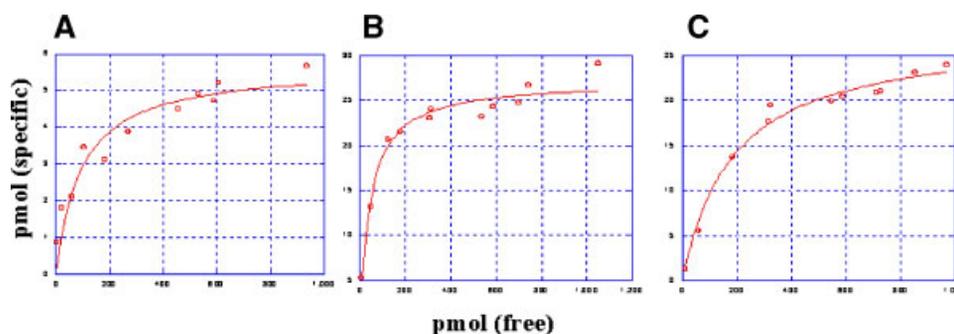
Fig. 3. Saturation-binding analysis using [^{3}H]bradykinin on membranes prepared from the Sf9 cells expressing pVLB$_2$R (**A**); pVLMelFlagHis$_{10}$B$_2$Rbio (**B**); and pVLMelFlagHis$_{10}$B$_2$R-eGFP (**C**). The values are average of two independent experiments. The curves were generated in KaleidaGraph software using one site model.

of the *Propionobacterium shermanii* transcarboxylase domain fused to the receptor took place. Membranes from non-infected Sf9 cells did not show any significant signal on the Western blot (lane 1 in Fig. 4A and B).

Glycosylation Analysis

B$_2$R contains three putative *N*-linked glycosylation sites (i.e., N^3, N^{12}, and N^{180}). N-linked glycosylation is generally of high-mannose type in insect cells. In order to determine the glycosylation state of the recombinant receptor, either tunicamycin was added to the culture medium during expression or enzymatic deglycosylation using PNGaseF and EndoH was performed on membranes. As shown in Figure 5, the size of 55-kDa band was reduced in response to enzymatic deglycosylation (lane 2 and 3) or tunicamycin treatment (lane 4). Thus, the 55-kDa band represented glycosylated form of the recombinant receptor. Additionally, deglycosylation also reduced the size of 35-kDa proteolytic cleavage product supporting the assumption that it was N-terminal fragment of the recombinant receptor. Growth of Sf9 cells expressing B$_2$R in the presence of tunicamycin reduced the [^{3}H]bradykinin binding (B$_{max}$) to

approximately 60% of that detected in non-treated cells (data not shown).

cAMP Release Assay

Activation of endogenous Gα_s protein by the recombinant B$_2$R was examined by bradykinin-induced cyclic AMP release. In response to bradykinin stimulation, a concentration-dependent increase in cAMP accumulation was observed in Sf9 cells expressing the recombinant B$_2$R (Fig. 6). An EC$_{50}$ value of 0.5 ± 0.2 nM

TABLE I. B$_{max}$ and K$_d$ Values (Calculated by Non-Linear Regression Using a Single Site Model) for the Three Different Constructs in Sf9 Cells

Construct	B$_{max}$ (pmol/mg)	K$_d$ (nM)
pVLB$_2$R	1.5 ± 0.2	0.39 ± 0.04
pVLMelFlagHis$_{10}$B$_2$Rbio	10 ± 0.6	0.42 ± 0.06
pVLMelFlagHis$_{10}$B$_2$ReGFP	2.3 ± 0.3	1.42 ± 0.1

The values are average of two independent experiments, each performed in triplicates.
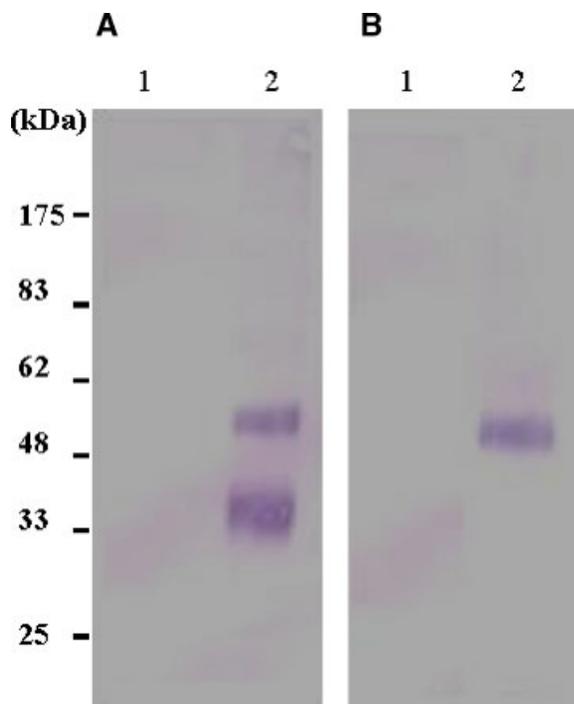
Fig. 4. Immunoblot analysis of membranes from Sf9 cell expressing the recombinant B$_2$R. Anti-Flag M2 antibody (**A**) and alkaline phosphate coupled streptavidin (**B**) were used. **Lane 1**, membranes from non-infected Sf9 cells; **lane 2**, 10 µg membranes from Sf9 cells expressing the recombinant B$_2$R.
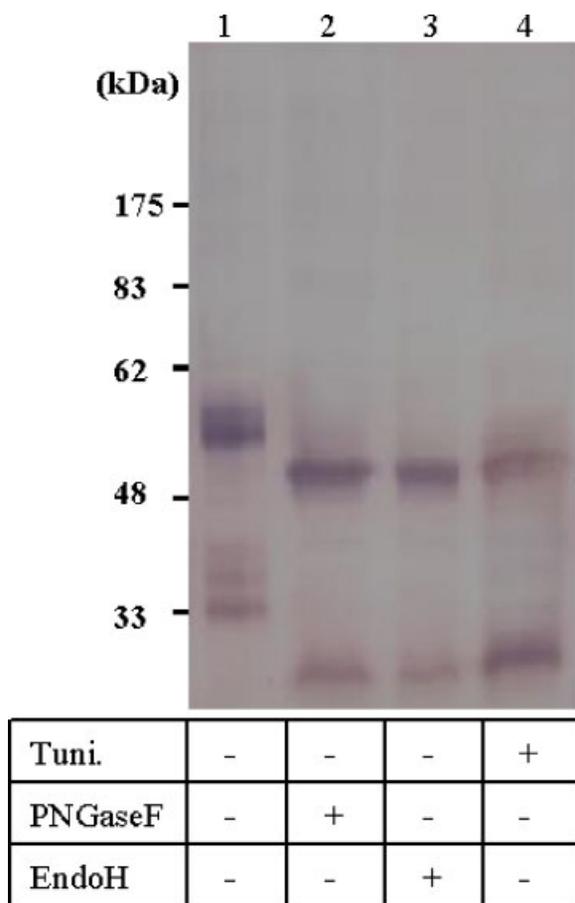
Fig. 5. Glycosylation analysis of the recombinant B_2R. Ten micrograms of membranes treated with PNGaseF and EndoH (**lane 2** and **3**, respectively) or 10 µg of membranes from tunicamycin treated Sf9 cells (**lane 4**). Immunodetection was performed with anti-Flag M2 antibody.

Fig. 6. Bradykinin-stimulated accumulation of cAMP in Sf9 cells expressing the recombinant B_2R. Shown is the ability of increasing concentrations of bradykinin to stimulate cAMP accumulation. Values are expressed as the means ± SEM of three independent experiments.

was observed which is in good agreement with that in native tissues. It represented the ability of the recombinant receptor to couple to endogenous $G\alpha_s$ protein in Sf9 cells. No cAMP release was observed in response to HOE-140, a B_2R antagonist (data not shown). Also, bradykinin stimulation did not lead to any significant increase in cAMP level in non-infected Sf9 cells.

Receptor Localization

To investigate the cellular localization of the recombinant B_2R, confocal microscopy and immunogold labeling experiment was performed. For confocal microscopy, Sf9 and High Five cells expressing pVLMelFlagHis$_{10}$B$_2$R-eGFP fusion protein were used. As shown in Figure 7, the recombinant B_2R was mainly localized in the perinuclear membranes (probably retained in the endoplasmic reticulum).

Only a small fraction of the recombinant receptors reached the plasma membrane. Pre- and post-embedding immunogold staining experiments on Sf9 cells expressing pVLMel-FlagHis$_{10}$B$_2$RBio fusion protein also revealed intracellular localization of the recombinant B_2R (Fig. 8). Only occasionally, gold particles were found at the plasma membrane. No immunogold staining was observed in non-infected Sf9 cells.

DISCUSSION

G protein-coupled receptors represent the largest group of surface receptors and include many clinically important drug targets. Due to their low-level expression in native tissues, these molecules are difficult to isolate in large quantities for structural characterization.
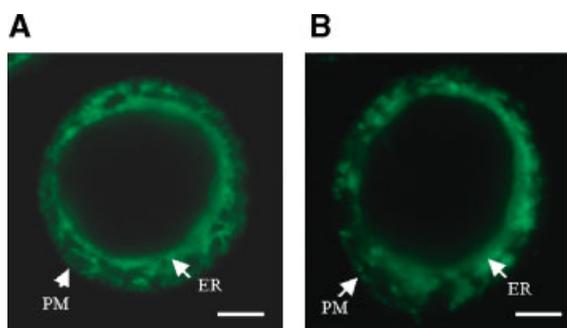
Fig. 7. Confocal laser-scanning microscopy of Sf9 cells (**A**) and High Five cells (**B**) expressing the B_2R-eGFP fusion protein using FITC filter (488 nm). The scale bar represents 1 µm. PM is plasma membrane and ER is endoplasmic reticulum.
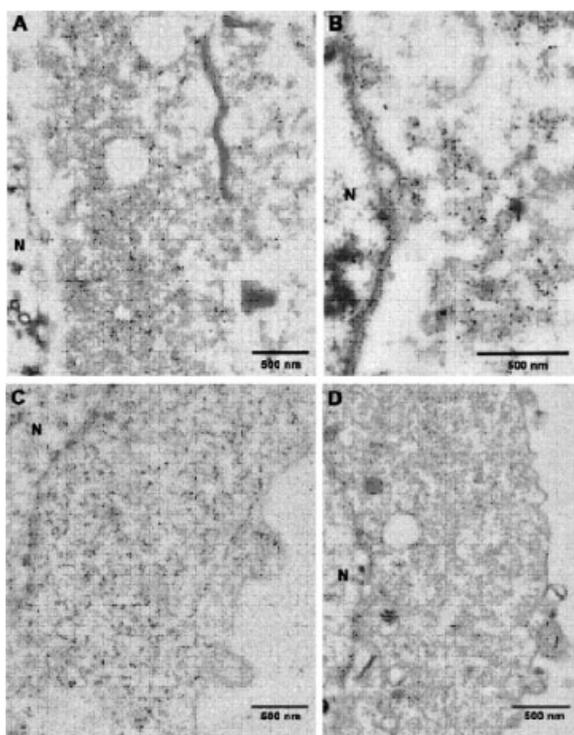
Fig. 8. Immunogold labeling of Sf9 cells expressing the recombinant B_2R. Pre-embedding labeling (**A**) and post-embedding labeling (**B**) was performed using anti-Flag M2 antibody and gold-coupled goat anti-mouse secondary antibody. Non-infected cells were used as controls for pre- (**C**) and post-embedding (**D**) labeling. N indicates nucleus.

However, structural studies are required to understand the molecular basis of ligand binding and to facilitate rational drug design. Therefore, heterologous expression of recombinant GPCRs is necessary for structural studies. Baculovirus-mediated expression in insect cells offer the possibility of expressing recombinant GPCRs in large quantities and they perform post-translational modifications similar to the mammalian cells. Several GPCRs have already been produced in this system [Massotte, 2003; Sarramegna et al., 2003]. B_2R, a member of the GPCR superfamily, is a promising therapeutic target in the treatment of asthma, arthritis, colitis, tissue injury, pain, cardiovascular diseases, and lung cancer. We have recently reported expression and characterization of recombinant B_2R in mammalian cells [Shukla et al., 2006]. In this study, we attempted to overexpress the recombinant B_2R in insect cells. Previous attempts to overexpress recombinant B_2R in insect cells using baculovirus system resulted in either low [Akermoun et al., 2005] or moderate [Blaukat et al., 1999] expression level. In this study, we

have demonstrated that high levels of functional B_2R can be expressed in Sf9 cells using the baculovirus expression system. The construct containing N-terminal Flag and His_{10} tag and a C-terminal bio tag, exhibited expression level of up to 10 pmol/mg (i.e., $\sim$0.25 mg functional receptor/liter culture). A positive effect of prepromelittin signal sequence on the expression of recombinant B_2R was observed. Similar effect of this signal sequence on the expression of oxytocin receptor [Gimpl et al., 1995], $5HT_{5A}$ serotonin receptor [Lenhard et al., 1996], and D_{2S} dopamine receptor [Grunewald et al., 1996] has been reported earlier. Additionally, fusion of the biotinylation domain of *Propionibacterium shermanii* transcarboxylase at the C-terminus of B_2R also resulted in a significant increase in the expression level of B_2R. A previous study also indicates a positive effect of biotinylation domain on the expression of human ET(B) endothelin receptor in the methylotrophic yeast *Pichia pastoris* [Schiller et al., 2000]. In addition to facilitate the detection of the recombinant receptor (by immunoblot), the affinity tags used here provide a possibility to purify the recombinant B_2R after detergent solubilization. The His_{10} tag and the biotinylation domain can be used for affinity purification using Ni-NTA and monomeric avidin matrix, respectively. Flag tag fused at the N-terminus of the recombinant receptor can also be used for purification on anti-Flag antibody matrix. Saturation-binding analysis using [^{3}H]bradykinin revealed that the recombinant B_2R binds to its endogenous ligand bradykinin with high affinity (K_d, 0.39–1.2 nM). The K_d value of the recombinant B_2R obtained here is similar to that of B_2R in native tissues. This result indicated that the tags and fusions introduced at the N- and C-terminus of B_2R, had no detrimental effect on its ligand-binding properties. Immunoblot analysis revealed two major bands of $\sim$55 and $\sim$35 kDa size. The band of 55 kDa represented the full-length B_2R fusion protein and indicated that complete in vivo processing of the prepromelittin signal sequence took place. The band of 35 kDa probably resulted from partial proteolytic degradation of the recombinant B_2R. Similar proteolysis has been reported previously for the muscarinic M_2 acetylcholine receptor [Heitz et al., 1997], the human beta 2-adrenergic receptor [Hampe et al., 2000], the human histamine H_1 receptor [Ratnala et al., 2004],

and human $CCXR_1$ chemokine receptor [Maeda et al., 2004] in Sf9 cells. The long expression time may result in proteolytic cleavage of recombinant GPCRs expressed in insect cells. Rapid proteolysis of human histamine H_1 receptor in Sf9 cells could be efficiently suppressed by including the protease inhibitor leupeptin during cell culture [Ratnala et al., 2004]. However, addition of leupeptin in cell culture medium did not prevent the proteolysis of recombinant B_2R (data not shown). In vitro enzymatic deglycosylation on membranes using PNGaseF and EndoH resulted in reduced size of the recombinant B_2R band on immunoblot. This suggested that the recombinant B_2R produced in Sf9 cells was glycosylated. Similar results were obtained during in vivo deglycosylation using tunicamycin in cell-culture medium. However, [^{3}H]bradykinin binding demonstrated that the expression level of recombinant receptor was significantly decreased in presence of tunicamycin. Similar effect of tunicamycin on expression level of human $CXCR_1$ chemokine receptor has been reported earlier [Maeda et al., 2004]. The B_2R is generally described to signal through $G\alpha_q$ but, this receptor also interacts with $G\alpha_i$, $G\alpha_s$, and $G\alpha_{12/13}$ [reviewed in Leeb-Lundberg et al., 2005]. A previous study reports that activation of endogenous B_2R in human epidermoid cell line A431 results in accumulation of cAMP [Liebmann et al., 1996]. In this study, bradykinin stimulated increase in cAMP level was observed in Sf9 cells expressing the recombinant B_2R. This directly demonstrated the functional coupling of the recombinant B_2R to the endogenous $G\alpha_s$ in Sf9 cells. Confocal laser scanning microscopy of Sf9 and High Five cells expressing B_2R-eGFP fusion protein revealed that the recombinant receptor was mainly localized in perinuclear membranes. These membranes probably represent endoplasmic reticulum. Only a little fluorescence was observed in the plasma membrane. Endoplasmic retention of the recombinant receptor was further confirmed by sucrose density gradient centrifugation (data not shown). Similar results were obtained by the pre- and post-embedding immunogold-labeling experiment of Sf9 cells expressing the recombinant B_2R. The gold labeling was predominantly found in perinuclear membranes and gold particles were only occasionally present in the plasma membrane. However, the intracellularly retained recombinant B_2R binds to [^{3}H]bradykinin with high affinity and showed functional coupling to endogenous G protein. It is likely that the G protein coupling was observed only due to the mature receptors (those reaching the plasma membrane). Intracellular retention of overexpressed human D_2S dopamine receptor [Grunewald et al., 1996], $CXCR_1$ chemokine receptor [Maeda et al., 2004], and rat M_3 muscarinic acetylcholine receptor [Vasudevan et al., 1995] in insect cells has been reported earlier. High-level overexpression of recombinant GPCRs often leads to intracellular accumulation of the recombinant receptors probably due to the saturation of trafficking machinery of the host cell.

In conclusion, this study established for the first time, baculovirus mediated high-level expression of the recombinant B_2R in insect cells. The recombinant receptor was biochemically and pharmacologically characterized and its characteristics were similar to that of the native receptor. Thus, the baculovirus-mediated expression provides a suitable system for the large scale production and structural characterization of B_2R.

REFERENCES

Akermoun M, Koglin M, Zvalova-Iooss D, Folschweiller N, Dowell SJ, Gearing KL. 2005. Characterization of 16 human G protein-coupled receptors expressed in baculovirus-infected insect cells. Protein Expr Purif 44:65–74.

Blaukat A, Herzer K, Schroeder C, Bachmann M, Nash N, Muller-Esterl W. 1999. Overexpression and functional characterization of kinin receptors reveal subtype-specific phosphorylation. Biochemistry 38:1300–1309.

Broekman MJ. 1992. Homogenization by nitrogen cavitation technique applied to platelet subcellular fractionation. Methods Enzymol 215:21–32.

Cronan JE. 1990. Biotination of proteins in vivo. A post-translational modification to label, purify, and study proteins. J Biol Chem 265:10327–10333.

Dendorfer A, Wellhoner P, Braun A, Roscher AA, Dominiak P. 1997. Synthesis of kininogen and degradation of

bradykinin by PC12 cells. Br J Pharmacol 122:158515–158592.

Farmer SG, Burch RM. 1992. Biochemical and molecular pharmacology of kinin receptors. Annu Rev Pharmacol Toxicol 32:511–536.

Gimpl G, Klein U, Reilander H, Fahrenholz F. 1995. Expression of the human oxytocin receptor in baculovirus-infected insect cells: High-affinity binding is induced by a cholesterolcyclodextrin complex. Biochemistry 34:13794–13801.

Grunewald S, Reilander H, Michel H. 1996. In vivo reconstitution of dopamine D2S receptor-mediated G protein activation in baculovirus-infected insect cells: Preferred coupling to Gi1 versus Gi2. Biochemistry 35: 15162–15173.

Hall JM. 1992. Bradykinin receptors:pharmacological properties and biological roles. Pharmacol Ther 56:131–190.

Hampe W, Voss RH, Haase W, Boege F, Michel H, Reilander H. 2000. Engineering of a proteolytically stable human beta 2-adrenergic receptor/maltose-binding protein fusion and production of the chimeric protein in *Escherichia coli* and baculovirus-infected insect cells. J Biotechnol 77:219–234.

Heitz F, Nay C, Guenet C. 1997. Expression of functional human muscarinic M2 receptors in different insect cell lines. J Recept Signal Transduct Res 17:305–317.

Imamura T, Potempa J, Travis J. 2004. Activation of the kallikrein-kinin system and release of new kinins through alternative cleavage of kininogens by microbial and human cell proteinases. Biol Chem 385:989–996.

Kuoppala A, Lindstedt KA, Saarinen J, Kovanen PT, Kokkonen JO. 2000. Inactivation of bradykinin by angiotensin-converting enzyme and by carboxypeptidase N in human plasma. Am J Physiol Heart Circ Physiol 278:1069–1074.

Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL. 2005. Internati-onal union of pharmacology. XLV. Classification of the kinin receptor family: From molecular mechanisms to pathophysiological consequences. Pharmacol Rev 57:27–77.

Lenhard T, Maul G, Haase W, Reilander H. 1996. A new set of versatile vectors for the heterologous expression of foreign genes using the baculovirus system. Gene 169: 1871–1890.

Liebmann C. 2004. G protein-coupled receptors and their signaling pathways: Classical therapeutical targets susceptible to novel therapeutic concepts. Curr Pharm Des 10:1937–1958.

Liebmann C, Graness A, Ludwig B, Adomeit A, Boehmer A, Boehmer FD, Nurnberg B, Wetzker R. 1996. Dual bradykinin B2 receptor signalling in A431 human epidermoid carcinoma cells: Activation of protein kinase C is counteracted by a GS-mediated stimulation of the cyclic AMP pathway. Biochem J 313:109–118.

Maeda Y, Kuroki R, Haase W, Michel H, Reilander H. 2004. Comparative analysis of high-affinity ligand binding and G protein coupling of the human CXCR1 chemokine receptor and of a CXCR1-Galpha fusion protein after heterologous production in baculovirus-infected insect cells. Eur J Biochem 271:1677–1689.

Massotte D. 2003. G protein-coupled receptor overexpression with the baculovirus-insect cell system: A tool for structural and functional studies. Biochim Biophys Acta 17:77–89.

Naraba H, Ueno A, Yoshimura M, Kosugi Y, Oh-ishi S. 1999. Examination of signal transduction pathway of stimulated B1 and B2 kinin receptors; MAP kinase pathway to AP-1 translocation in HEK 293 cells. Immunopharmacology 45:35–38.

Nolly H, Carbini LA, Scicli G, Carretero OA, Scicli AG. 1994. A local kallikrein-kinin system is present in rat hearts. Hypertension 23:919–923.

O'Reilly DR, Miller LK, Luckow WA. 1992. Baculovirus expression vectors: A laboratory manual. New York: W. H. Freeman.

Prado GN, Taylor L, Zhou X, Ricupero D, Mierke DF, Polgar P. 2002. Mechanisms regulating the expression, self-maintenance, and signaling-function of the bradykinin B2 and B1 receptors. J Cell Physiol 193:275–286.

Ratnala VR, Swarts HG, VanOostrum J, Leurs R, DeGroot HJ, Bakker RA, DeGrip WJ. 2004. Large-scale overproduction, functional purification and ligand affinities of the His-tagged human histamine H1 receptor. Eur J Biochem 271:2636–2646.

Reed LJ, Muench H. 1938. A simple method of estimating fifty percent endpoints. Am J Hygiene 27:493–497.

Sarramegna V, Talmont F, Demange P, Milon A. 2003. Heterologous expression of G-protein-coupled receptors: Comparison of expression systems from the standpoint of large-scale production and purification. Cell Mol Life Sci 60:1529–1546.

Sawutz DG, Faunce DM, Houck WT, Haycock D. 1992. Characterization of bradykinin B2 receptors on human IMR-90 lung fibroblasts: Stimulation of $45Ca^{2+}$ efflux by D-Phe substituted bradykinin analogues. Eur J Pharmacol 227:309–315.

Schiller H, Haase W, Molsberger E, Janssen P, Michel H, Reilander H. 2000. The human ET(B) endothelin receptor heterologously produced in the methylotrophic yeast Pichia pastoris shows high-affinity binding and induction of stacked membranes. Receptors Channels 7:93–107.

Shukla AK, Haase W, Reinhart C, Michel H. 2006. Biochemical and pharmacological characterization of the human bradykinin subtype 2 receptor produced in mammalian cells using the Semliki Forest virus system. Biol Chem (In Press).

Spurr AR. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:43–50.

Vasudevan S, Hulme EC, Bach M, Haase W, Pavia J, Reilander H. 1995. Characterization of the rat m3 muscarinic acetylcholine receptor produced in insect cells infected with recombinant baculovirus. Eur J Biochem 15:466–475.

Wise A, Gearing K, Rees S. 2002. Target validation of G-protein coupled receptors. Drug Disc Today 7:235–246.