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Dimethylsulphoxide as a tool to increase functional expression of heterologously produced GPCRs in mammalian cells

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Abstract High-level overexpression of G protein-coupled receptors GPCRs in mammalian cells remains a difficult task inspite of newly developed virus based expression systems. Here, we show that the functional expression level of the recombinant bradykinin receptor (B_2R) in mammalian cells can be increased up to sixfold just by the addition of dimethylsulphoxide in the culture medium. Total expression level, cellular localization and binding affinity of the recombinant receptor for its endogenous ligand remains unaltered. The strategy presented here, with recombinant B_2R as a case example, is applicable to other GPCRs and provides a generic tool to improve the functional expression level of recombinant GPCRs in mammalian cells.
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Keywords: GPCR; BHK cells; Overexpression; DMSO; Localization

1. Introduction

G protein-coupled receptors (GPCRs) are integral membrane proteins with seven transmembrane helices and they play regulatory roles in many different cellular and physiological processes [1]. Approximately half of the currently available drugs target these receptors [2,3]. However, only little structural information is available on GPCRs and therefore, structure based drug design has not been possible. In recent years, a number of heterologous expression systems have been used for overexpression of recombinant GPCRs [4]. Out of these, mammalian cells represent the most suitable hosts for heterologous expression of human recombinant GPCRs as they provide natural like membrane environment. However, transient transfection results in low expression levels of recombinant GPCRs. To overcome this problem, the Semliki Forest virus based vectors have been developed to overexpress recombinant GPCRs in mammalian cells [5]. Although some GPCRs have been expressed at high levels using the SFV system, for others, the expression level remain either low or moderate [5,6]. Therefore, further attempts are required to improve the functional expression level of recombinant GPCRs in mammalian cells. Recently, a positive effect of dimethylsulphoxide on the expression level of recombinant GPCRs in *Pichia pastoris* was reported [7] and that led us to investigate if it is applicable to mammalian cells also.

Here, we report that dimethylsulphoxide (DMSO) exerts a similar positive effect in mammalian cells also and present a strategy to improve the functional expression level of low or moderately expressed GPCRs in mammalian cells. As a case example, we evaluated the effect of DMSO on the functional expression of the recombinant B_2R in baby hamster kidney cells (BHK cells). We also studied the effect of DMSO on total expression, binding affinity and localization of the recombinant receptor. In addition, effect of DMSO on the physical parameters of the host cells was also evaluated.

2. Materials and methods

2.1. Materials

[^{3}H]Bradykinin (60–90 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, USA) and DMSO was obtained from Sigma (St. Louis, MO, USA). The cell culture medium, glutamine, phosphate buffer saline (PBS), trypsin–EDTA, and penicillin–streptomycin solution were obtained from Cell Concepts GmbH (Umkirch, Germany) or Sigma (St. Louis, MO, USA). Fetal calf serum was from PAA laboratories (Colbe, Germany). α-Chymotrypsin was from ICN chemicals (CA, USA) and aprotinin from Roche Applied Science (Mannheim, Germany). Electroporation cuvettes were obtained from Bio-Rad laboratories (CA, USA). SP6 polymerase, transcription buffer and restriction enzymes were from MBI fermentas (St. Leon-Rot, Germany). Immobilon-P polyvinylidene difluoride (PVDF) membranes were from Millipore (Bedford, MA, USA). Anti-his antibody and alkaline phosphatase-coupled secondary antibody were obtained from Sigma (St. Louis, MO, USA).

2.2. Cell culture and expression of recombinant receptor

Monolayer culture of baby hamster kidney cells (BHK-21) were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and 0.1% streptomycin–penicillin. BHK cells in suspension were cultured using Glasgow minimal essential medium supplemented with 5% FCS, 2 mM glutamine, 10 mM tryptose phosphate broth and 0.1% streptomycin–penicillin. Cells were maintained in a humified atmosphere at 37 °C with 5% CO_2. Generation of virus stocks and infection of cells have been described previously [8]. Different concentrations (v/v) of DMSO were added to the cells at the time of infection.

2.3. Membrane preparation and [^{3}H]bradykinin binding assay

Membrane preparation and radioligand binding assay was performed as published earlier [8]. In brief, the cells were harvested 24 h post-infection, resuspended 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 (500–800 psi) (Parr

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Abbreviations: GPCR, G protein-coupled receptor; DMSO, dimethylsulphoxide; BHK cells, baby hamster kidney cells

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Instruments, Moline, IL, USA) [9]. The crude lysate was centrifuged ($3000 \times g$, 10 min, 4 °C) to remove the nuclei and cell debris and the membranes were pelleted by ultracentrifugation ($100000 \times g$, 1 h, 4 °C).

[^{3}H]Bradykinin binding assay was performed on membranes (5 μg of total protein per assay point) using binding buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA and complete protease inhibitor cocktail) at 4 °C for 40–45 min. 10 nM [^{3}H]bradykinin was used for single point measurements and 0.1–25 nM [^{3}H]bradykinin was used for saturation binding analysis. Following incubation, the reaction was terminated by rapid filtration through GF/C glass–fiber filters, presoaked in 0.3% (v/v) polyethyleneimine. Filters were washed four times with ice cold binding buffer and radioactivity was measured by liquid scintillation counting.

2.4. Immunoblot analysis and confocal microscopy

Western blot and confocal imaging was essentially carried out as described earlier [8]. Briefly, proteins were separated using 10–12% SDS–PAGE and then electrophoretically transferred to a PVDF membrane. The PVDF membranes were incubated in 5% (w/v) non-fat dried milk powder in TBST buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% Tween-20) for 1 h at room temperature. Subsequently, the proteins were visualized using the anti-his antibody and the alkaline phosphate-conjugated secondary antibody according to the manufacturer's protocol (Sigma).

For localization analysis, the BHK cells expressing the B_2R–eGFP fusion protein were 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 the gel mount medium (Sigma). Cells were analyzed using a Leica confocal laser-scanning microscope (488 nm for eGFP and 550 nm for Cy3).

2.5. Analysis of physical parameters of DMSO treated cells

BHK cells were incubated with different concentrations of DMSO and after 24 h, analyzed by CEDEX Automated Cell Culture Analyzer (Innovatis AG, Bielefeld, Germany) for cell number, cell viability, cell compactness, cell diameter and cell aggregation rate.

3. Results and discussion

3.1. Effect of DMSO on functional expression and binding affinity of the recombinant B_2R

We have recently reported expression and characterization of the human B_2R in BHK cells using the Semliki Forest virus vectors [8]. An expression level of 10 ± 1 pmol/mg was observed, which is only moderate from the point of view of large-scale production. In an attempt to increase it further, we evaluated the effect of DMSO on the expression level of B_2R in BHK cells. The cells were infected with recombinant B_2R construct and different amounts of DMSO were added in the culture medium. 24 h post-infection, membranes were prepared and [^{3}H]bradykinin binding was measured. As shown in Fig. 1, a significant increase in [^{3}H]bradykinin binding was observed upon addition of DMSO. Best expression level was obtained in the presence of 2% DMSO and further increase of DMSO concentration had adverse effects on B_2R expression level.

The expression level of B_2R in presence of 2% DMSO corresponds to 55 ± 5 pmol/mg (0.5 mg recombinant receptor per litre culture). This expression level of B_2R is maximum compared to any other expression system for this particular receptor and it is one of the best expression levels of GPCRs obtained using the Semliki Forest virus system [5].

In order to check if the addition of DMSO alters the affinity of the recombinant receptor for its cognate ligand, a saturation binding experiment was performed. Membranes prepared from BHK cells grown in absence or presence of 2% DMSO was used. As shown in Fig. 2, a K_d value of 0.15 nM was observed in the membranes prepared from DMSO treated cells, which is similar to that obtained in the membranes of untreated BHK cells (0.12 nM). Taken together, these results suggest that addition of DMSO improves functional expression of B_2R without having any adverse effect on the binding affinity of the receptor.

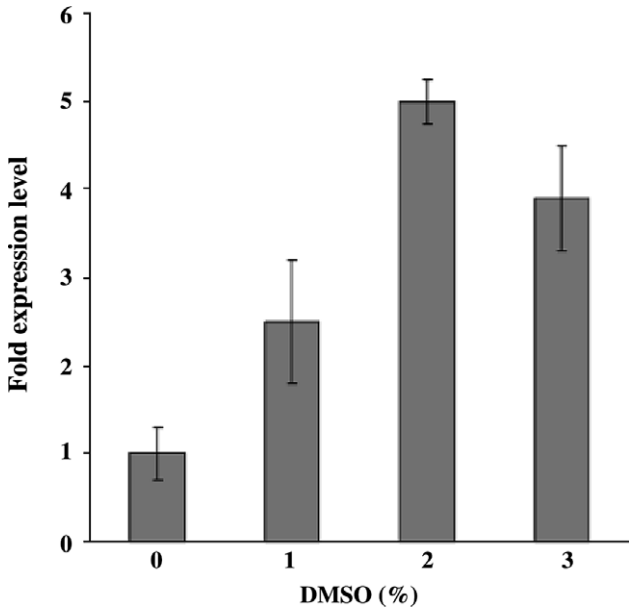

Fig. 1. Effect of DMSO on functional expression of recombinant B_2R. [^{3}H]Bradykinin binding analysis on membranes prepared from BHK cells expressing B_2R (with or without DMSO). The values represent an average of three independent experiments.

3.2. Total expression and localization analysis of the recombinant receptor

Next, we determined if addition of DMSO affects the total expression of recombinant B_2R as determined by Western blot analysis. Membranes were prepared from BHK cells expressing B_2R in absence or presence (1–2%) of DMSO and probed using anti-his antibody. As revealed in Fig. 3, no significant change was observed in the intensity of recombinant B_2R band on the Western blot, indicating that the total expression of recombinant receptor remains unchanged upon DMSO treatment.

We have previously reported that recombinant B_2R exhibits intracellular localization in BHK cells [8]. It is believed that

		B_{max} (pmol/mg)	K_d (nM)
DMSO (%)	0	10±1	0.12 ±0.02
	2	55±5	0.15 ±0.03

Fig. 2. Saturation binding analysis of recombinant B_2R. [^{3}H]Bradykinin binding was measured on membranes from BHK cells expressing B_2R with (2%) or without DMSO. The dissociation constant (K_d) and maximum expression level (B_{max}) were calculated with the KaleidaGraph software by non-linear regression using a single site model.

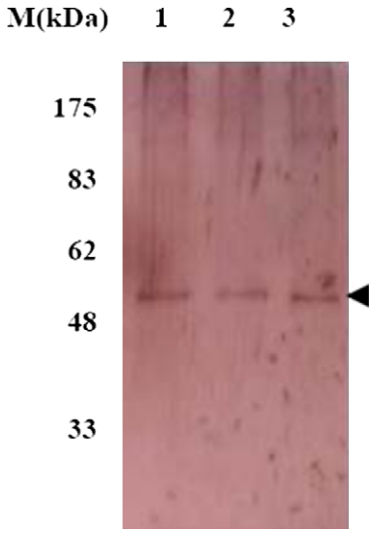

Fig. 3. Western blot analysis of the recombinant B_2R using anti-his antibody. Lane 1–3, 10 μg of membranes from BHK cells expressing B_2R in presence of different concentrations of DMSO (lane 1–3 = 0–2%).

overexpression of recombinant GPCRs saturates the trafficking machinery of the host cells leading to intracellular retention of the receptors. Colocalization of recombinant B_2R with calnexin (an ER marker) indicated that the recombinant receptor is mainly retained in the endoplasmic reticulum (Fig. 4A–C). In order to investigate if DMSO induced increase in functional expression results from altered trafficking of the recombinant B_2R, we used DMSO treated BHK cells (expressing B_2R–eGFP construct) for confocal microscopy. As shown in Fig. 4D–F, even in DMSO treated cells, the recombinant receptor was colocalized with calnexin, suggesting expression of B_2R in the endoplasmic reticulum, as in DMSO untreated cells (A–C).

These data suggest that increase in functional expression of B_2R upon DMSO treatment is not a result of increased protein synthesis or surface translocation of the recombinant receptor. Rather, it is more likely that DMSO affects the folding of the recombinant B_2R, resulting in an increased population of those receptors which can bind to its ligand. However, this possibility remains to be explored further.

3.3. Effect of DMSO on the physical parameters of BHK cells

Previous studies suggest that DMSO can induce morphological changes in mouse erythroleukemic cells [10] and it can also induce differentiation in neuroblastoma cells [11]. In contrast, it is also reported that DMSO can block differentiation of myoblasts, adipocytes and antibody producing plasma cells [12–15]. To address the effect of DMSO on physical parameters of BHK cells, if any, we compared the cell number, viability, compactness, aggregation and diameter of DMSO treated and untreated cells. As shown in Fig. 5A, the total cell number in DMSO treated samples was slightly decreased suggesting an inhibitory effect of DMSO on cell division. More importantly, the cell viability was reduced slightly up to 2% of DMSO, but further increase of DMSO resulted in severe cell death (Fig. 5B). This might explain the decrease in functional expression level of B_2R in presence of 3% DMSO (as observed in Fig. 1). Another striking alteration in response to DMSO addition was the cell aggregation behaviour. DMSO treated cells showed up to fivefold less aggregation rate compared to the untreated cells (Fig. 5C). The cell compactness was not much changed but a slight increase in the cell diameter was observed in DMSO treated cells (Fig. 5D–E).

We next analyzed the morphological changes in the BHK cells (adherent cultures) using differential interference contrast microscopy. It was observed that in presence of DMSO (up to 2%), the BHK cells were slightly elongated (Fig. 6A–C). Further increase in DMSO concentration resulted in rounded cells indicating cell death (data not shown).

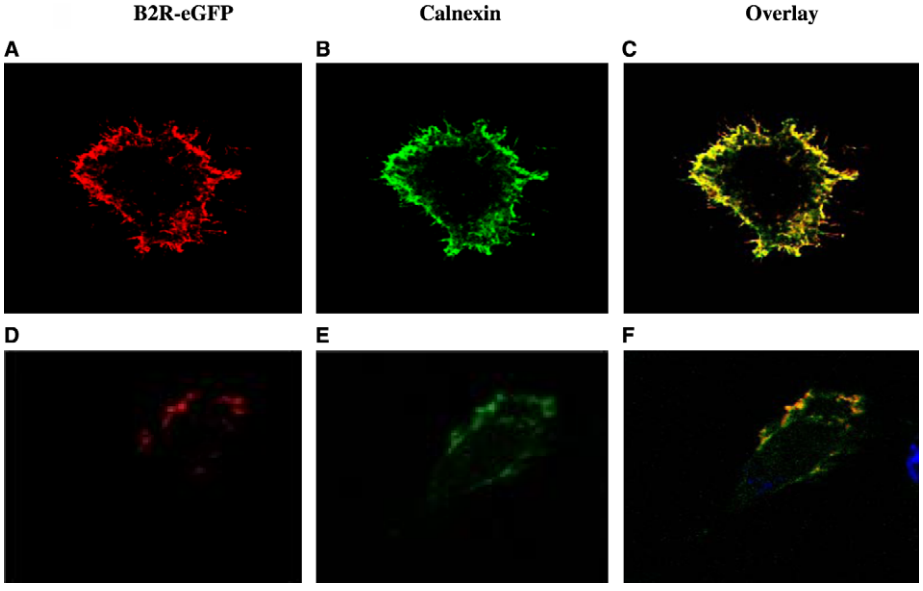

Fig. 4. Localization analysis of the recombinant B_2R. BHK cells were infected with B_2R–eGFP fusion construct and visualized by confocal laser-scanning microscope (488 nm for eGFP and 550 nm for Cy3).

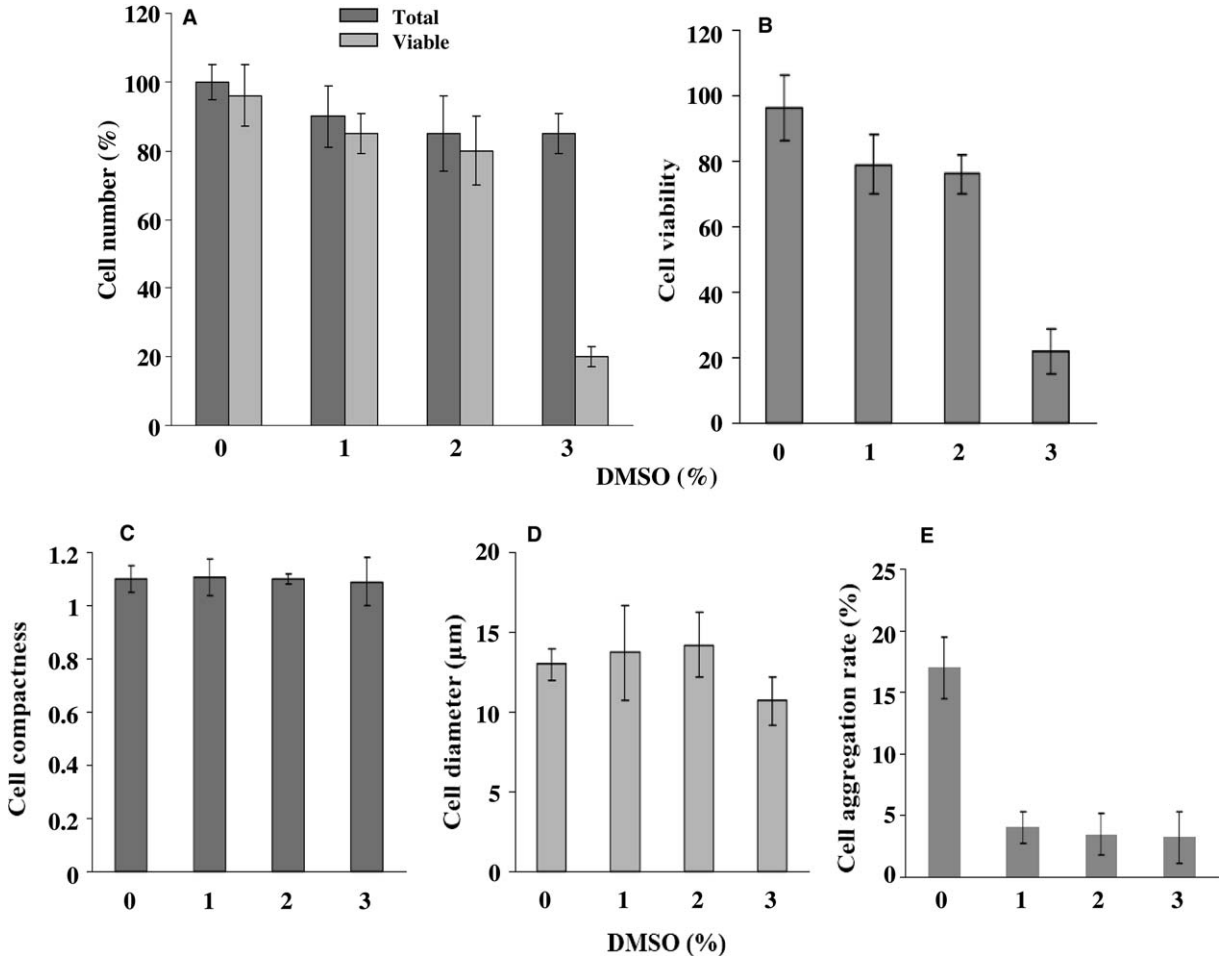

Fig. 5. Evaluation of the effect of DMSO on physical parameters of BHK cells. Cells were grown in presence or absence of DMSO and subsequently analysed by CEDEX automated cell culture analyzer. Values represent an average of three independent experiments.

3.4. Effect of DMSO on functional expression of other recombinant GPCRs

Next, we asked if the positive effect of DMSO is applicable to other GPCRs expressed in BHK cells. We found that the functional expression of recombinant human angiotensin II type 1 receptor and the human neuromedin subtype 2 receptor were also improved four to sixfold by addition of DMSO (Shukla et al., manuscript in preparation). This result indicates that use of DMSO might be a general strategy to increase the functional expression level of recombinant GPCRs in mammalian cells.

In *S. cerevisiae*, DMSO increases phospholipid biosynthesis via up regulation of corresponding genes and also leads to cell proliferation [16]. Additionally, DMSO also leads to decrease in cell number and viability when used at high concentrations [16]. In contrast, it has also been reported that DMSO inhibits

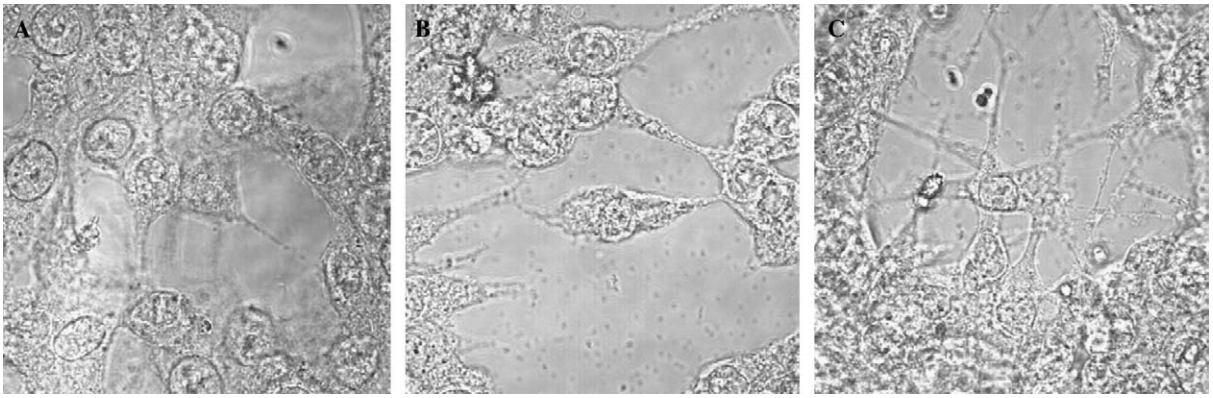

Fig. 6. Morphological changes in BHK cells upon addition of DMSO. Cells were treated with different concentrations of DMSO (A = 0%, B = 1%, C = 2%) and subsequently analysed by differential image contrast microscopy.

the genes involved in lipid biosynthesis but up regulates the genes involved in amino acid biosynthesis in *S. cerevisiae* [17]. Until now, no systematic study has been done to investigate the effect of DMSO on membrane composition in mammalian cells. The exact mechanism of DMSO induced increase in functional expression of GPCRs also remains to be studied in detail.

In conclusion, we present a general approach to improve functional expression of recombinant GPCRs in mammalian cells. This strategy can be tested for other GPCRs not only in the overexpression systems, but probably also in transient transfection studies.

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