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Uneven cellular expression of recombinant α_{2A}-adrenoceptors in transfected CHO cells results in loss of response in adenylyl cyclase inhibition

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Abstract

Two populations of Chinese hamster ovary (CHO) cells expressing similar numbers of recombinant human alpha2A-adrenergic receptors (α_{2A}-AR) showed different capacity to inhibit adenylyl cyclase (AC) activity. Cells transfected with an integrating vector exhibited agonist-dependent inhibition of forskolin-stimulated AC, whereas cells transfected with a non-integrating episomal vector showed no inhibition. Fluorescent microscopy and flow cytometry revealed a very uneven receptor distribution in the episomally transfected cell population. Monoclonal cell populations were expanded from this parent population. Most clones lacked significant amounts of receptors, while a few expressed receptors at high density; these exhibited efficient agonist-dependent inhibition of forskolin-stimulated AC activity. Thus, dense receptor expression in only a few cells is not sufficient to evoke a significant inhibitory response in a functional assay where AC is stimulated in all cells. Consequently, a false negative result was produced. Furthermore, the cell population transfected with an integrating vector showed loss of homogeneity with increasing passage number.

Keywords: Alpha2-adrenoceptor; cAMP; Expression vector; Functional assay; Recombinant receptor; Transfection

1. Introduction

Drug discovery aims to produce target-specific compounds for closely related receptor subtypes, which can best be studied using heterologous expression of cDNA or genes encoding these proteins [1]. Heterologous expression allows pharmacological analysis of each receptor subtype, as well as functional analysis of receptor coupling mechanisms and second messenger pathways in a controlled well-defined cellular environment [1,2].

GPCRs are generally of low natural abundance, and overexpression is usually a prerequisite to their structural and functional characterisation. Several well-characterised and controllable host cell environments are known, but mammalian host cell lines are commonly used for production of recombinant proteins of interest since these represent the closest alternative to the protein's native environment [3]. Heterologous protein production may then be achieved through stable, transient or semistable expression in these cell lines; the choice of a particular expression system depends on the nature and purpose of the study. Stable expression involves the integration of an expression vector into the host cell genome, and single cell clones are manually isolated after selection with an appropriate antibiotic. This approach has commonly been used for the pharmacological and functional characterisation of receptor subtypes [4]. Semistable transfection includes maintenance of the expression vector as an episomal element. Since all cells with

Abbreviations: α_2-AR, alpha2-adrenergic receptor; AC, adenylyl cyclase; cAMP, cyclic adenosine 3′,5′ monophosphate; CHO, Chinese hamster ovary; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IBMX, isobutylmethylxanthine; G protein, guanine nucleotide binding protein; GPCR, G protein-coupled receptor; HA, haemagglutinin; NA, noradrenaline; PTX, pertussis toxin

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antibiotic resistance are selected, the cell population is heterogeneous. Semistable transfections are often employed when, e.g., a series of mutated receptors needs to be investigated for the functional or structural importance of the mutation [5,6]. Transient transfections may be used to produce large amounts of receptor protein for use in receptor binding assays, when no functional coupling is required, or for protein isolation and structural analysis [1,7].

Recombinant receptor systems may be used in validated cell-based functional assays to discern receptor-coupled signalling mechanisms. The drug discovery process requires the discrimination of antagonists and full and partial agonists [1,8]. It is of great importance, however, that these functional cell-based assays are properly validated for the cell line used. Adham et al. [9] highlighted the importance of caution when interpreting functional data in transfected systems that often display large receptor reserves, as they showed that receptor reserve masks partial agonist activity.

Three genes encoding alpha2-adrenergic receptors (α_2-AR) have been cloned; these correspond to the pharmacologically defined α_2-AR subtypes α_{2A}, α_{2B} and α_{2C} in humans [10]. The α_2-ARs are GPCRs and mediate effects of the endogenous catecholamines adrenaline and noradrenaline (NA). They are primarily coupled to the inhibitory heterotrimeric guanine nucleotide binding protein ($G_{i/o}$ proteins), thereby inhibiting the activity of adenylyl cyclase (AC) [11]. Furthermore, Eason et al. [12] reported that recombinant α_2-ARs functionally couple to G_s proteins and stimulate AC activity, but only at high receptor density and high agonist concentrations.

The objective of the study was to examine the impact of heterogeneous or homogeneous receptor expression for the results and interpretation of a commonly used functional receptor assay, agonist-dependent inhibition of AC activity. We have used Chinese hamster ovary (CHO) cells as a model host cell type, transfected either stably or semistably with α_{2A}-ARs as a model case to highlight the importance of receptor distribution between cells and the need for an appropriate transfection technique when performing functional studies. We show here that there is uneven cellular expression of human α_{2A}-ARs after transfection with a non-integrating episomal expression vector used for semistable expression and that this results in a loss of functional response in an AC based second messenger assay. Furthermore, in a stably transfected cell population, with initially homogeneous expression, loss of homogeneity was observed with increasing passage number.

2. Materials and methods

2.1. Materials

[^{3}H]RX821002 (1,4-[6,7(*n*)-^{3}H]benzodioxan-2-methoxy-2yl)-2-imidazoline hydrochloride (54-60 Ci/mmol) was from Amersham Biosciences UK (Buckinghamshire, UK), fetal calf serum (FCS) was from Bioclear (Australia), MEM Alpha Medium was from Gibco BRL (UK), mouse monoclonal anti-HA antibody was from Roche (Indianapolis, IN), Hygromycin B® was from Roche (Pentzberg, Germany), Geneticin®, FITC (goat) anti-mouse-IgG, DMSO, BSA, forskolin, [−]-arterenol (noradrenaline), phentolamine, poly-L-lysine, pertussis toxin (PTX) and isobutylmethylxanthine (IBMX) were from Sigma-Aldrich (St. Louis, MO), Opti-Phase HiSafe3 and DELFIA cAMP kit 4003-0010 were from PerkinElmer Life and Analytical Sciences (Wallac Oy, Turku, Finland), Nonidet P40 was from Calbiochem (Novabiochem, La Jolla, CA) and Whatman glass fibre microfilters were from Whatman International (Maidstone, UK).

2.2. Methods

2.2.1. Construction of expression vectors and transfections

A semistable transfection construct expressing human α_{2A}-ARs [13] was produced by Marjamäki et al. [6] with the expression vector pREP4 (InVitrogen, NV Leek, Holland), which also contains a gene for hygromycin B resistance. A stable cell population expressing human α_{2A}-ARs was produced by Pohjanoksa et al. [14] with the expression vector pMAMneo (Clontech, Palo Alto, CA), containing a neomycin (G418) resistance gene. The HA-epitope was placed in front of the α_{2A}-AR coding sequence [15]. This epitope has earlier been shown not to affect ligand binding of murine α_2-ARs [16], or functional coupling of murine α_{2C}-ARs [17].

2.2.2. Cell culture

Adherent Chinese hamster ovary (CHO) cells (American Type Culture Collection, Rockville, MD) were cultured in α-MEM (MEM Alpha Medium) supplemented with 26 mM $NaHCO_3$, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% heat-inactivated FCS (referred to as growth medium). The semistable cell population was maintained in growth medium supplemented with Hygromycin B® (200 μg/ml), and the stable cell population was maintained in growth medium supplemented with Geneticin® (200 μg/ml). All cell cultures were grown in 37 °C and 5% CO_2. CHO cells (90% confluent) were harvested for experiments into phosphate buffered saline (PBS) with normal harvesting methods. Passages 6–17 of the stable cell population were used. For ligand binding studies, cell pellets were frozen prior to analysis. For measurements of cAMP production, cells were counted after harvesting and plated on a 96-well plate (~23 000 cells/well; for PTX treatment ~35 000 cells/well). For fluorescent microscopy, cells were plated on glass cover slips and fixed at ~50% confluence. For flow cytometry, 90% confluent cells were harvested and counted, and approximately 6×10^5 cells were used per sample.

2.2.3. Production of monoclonal cell populations

Monoclonal (stable) cell populations were produced from the semistable cell population (CHO H2α2 C10) [6]

according to two different methods. Diluted cell suspensions were plated on Petri dishes. Cells were kept in growth medium supplemented with Hygromycin B® (200 μg/ml). Single cell clones were marked after cell attachment, and cell colonies originating from a single cell clone were harvested 4 days later by detachment with trypsin and transfer of the colonies to a six-well plate. The second method included plating the diluted cells on a 96-well plate with approximately one cell per well. Wells containing only a single cell clone were subsequently expanded. Established monoclonal cell populations were maintained in growth medium supplemented with Hygromycin B® (200 μg/ml).

2.2.4. Ligand binding

All radioligand binding experiments were performed on CHO cell homogenates in 50 mM K^+ phosphate buffer as previously described [18]. Receptor densities were determined with saturation binding assays, using the α_2-antagonist radioligand [^{3}H]RX821002 (0.0625–8 nM). Nonspecific binding was determined by adding an excess of unlabelled phentolamine (10 μM, another α_2-antagonist). The cell pellet was suspended in 50 mM K^+ phosphate buffer and homogenised with an Ultra-Turrax homogeniser (Janke and Kunkel, Germany) for 3×10 s. An aliquot of the cell homogenate was taken for determination of total cellular protein content. The association of radioligand took place in a 30 min incubation at 25 °C, and was terminated through dilution with ice-cold buffer (TM buffer, 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4) and separation of bound radioactivity by filtration on Whatman glass fibre microfilters with a cell harvester (Brandel Cell Harvester M48R, Gathersburg, MD, at 6 °C) followed by two washes with ice-cold TM buffer. Radioactivity on the filters was determined by liquid scintillation counting (Wallac 1410 Liquid Scintillation Counter, PerkinElmer Life and Analytical Sciences, Wallac) in OptiPhase HiSafe3. The protein concentration of the cell homogenate was determined according to the method of Bradford [19] using bovine serum albumin as reference.

2.2.5. Measurement of intracellular cAMP levels

All measurements were carried out at least in three individual experiments performed in duplicate. Plated CHO cells were cultured overnight (20 h) in 37 °C and 5% CO_2 and subsequently incubated for 1 h in serum-free medium. For experiments on PTX pretreated cells, CHO cells were first cultured for 8 h in growth medium, and then treated with serum-free medium with PTX or without PTX (500 ng/ml) for 16 h. All cells were incubated with the phosphodiesterase inhibitor, IBMX (0.1 mM), for 10 min in 37 °C and 5% CO_2 and AC activity in all cells (except controls for basal cAMP levels) was subsequently stimulated with forskolin (5 μM). Increasing doses of noradrenaline (NA, [−]-arterenol, 1 nM–100 μM) were added to all cells (except control cells and cells for the determination of maximal forskolin-stimulated cAMP production). The plate was incubated for 1 h (37 °C and 5% CO_2), and the cells were subsequently lysed with a preheated detergent solution (0.03% digitonin in 19% DMSO). Concentration of cAMP was determined with a DELFIA cAMP kit according to the manufacturer's (PerkinElmer Life and Analytical Sciences, Wallac) instructions using an acetylation protocol. Fluorescence was measured with a VICTOR^2 1420 Multilabel Counter (Wallac). Inhibition of forskolin-stimulated cAMP accumulation was normalized to the effect of forskolin (5 μM). E_{max} was calculated as a mean±S.E. of the maximal inhibition/stimulation in the separate experiments.

2.2.6. Confocal fluorescence microscopy and flow cytometry

CHO cells were plated on poly-L-lysine-treated (0.1 mg/ml) glass cover slips, and fixed with 4% paraformaldehyde in PBS. The cells were subsequently washed three times with PBS and immunostained either directly or stored at 4 °C. Nonspecific binding was blocked by incubating the cells with blocking buffer (0.2% Nonidet P40, 5% nonfat dry milk in 50 mM Tris–HCl, pH 7.6). HA-tagged receptors were labelled with 7 μg/ml monoclonal anti-HA antibody in blocking buffer for 45 min. After incubation, the cells were washed three times with PBS, followed by a 5 min incubation with blocking buffer. The secondary antibody, Alexa Fluor 546 (1:500, Molecular Probes, Eugene, OR), was added, and the cells were incubated for another 45 min. After rinsing three times with PBS, the cells were mounted for fluorescence microscopy using anti-fade mounting medium (50% glycerol, 100 mg/ml 1,4-diazabicyclo-[2.2.2]octane and 0.05% sodium azide in PBS). As a negative control, non-transfected CHO cells were treated according to the same protocol. Cells were observed with a laser-scanning confocal microscope (Zeiss LSM 510 meta, Plan-Apochromat 63×/1.40 oil DIC objective). For flow cytometry analysis, approximately 6×10^5 CHO cells/sample were harvested into α-MEM supplemented with 30 mM HEPES and incubated for 1 h with 10 μg/ml monoclonal anti-HA-antibody in α-MEM+30 mM HEPES supplemented with 10% FCS on ice. A negative control sample for each cell population was incubated without the HA antibody. After two washes with α-MEM+30 mM HEPES+10% FCS, cells were labelled with FITC-conjugated goat anti-mouse IgG antibody (1:500) in α-MEM+30 mM HEPES+10% FCS for 30 min in the dark. After an additional wash, 10^4 cells/sample were analyzed on a FACScan flow cytometer with CellQuest 3.0.1 software for data acquisition and analysis (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

2.2.7. Analysis of data

The results were analysed using standard methods, using GraphPad Prism programs (GraphPad Software, San Diego, CA) and CellQuest 3.0.1 software. Statistical analyses were carried out with Student's *t*-test. *P* values smaller than 0.05 were considered to be statistically significant.

3. Results

3.1. Receptor density

Receptor densities of the transfected cell populations were determined with saturation binding assays in three individual experiments performed in duplicate. The results are presented in Table 1, and establish that the cell populations transfected to express human α_{2A}-ARs either stably or semistably had almost equal densities of receptors (1100 and 1200 fmol/mg total cellular protein, respectively). Saturation binding assays performed on the stable cell population several passages later showed a gradual decrease in receptor density with increasing passage number (from 1100 fmol/mg at passage 12 to 260 fmol/mg at passage 16), indicating a decrease in receptor expression with growing passage number. Single cell clones were isolated from the semistable cell population (CHO H2α_2 C10) and screened for receptor density (B_{max}). The results revealed that only a small number of the produced monoclonal cell populations expressed receptors at high density (24%) whereas most of the cell populations (76%) lacked significant amounts of receptors (data not shown). Four monoclonal cell populations (presented in Table 1) were chosen for further experiments, one that lacked a significant density of receptors (CHO H2α_2 C10 1), as well as three clones expressing α_{2A}-ARs at 3000 fmol/mg protein (CHO H2α_2 C10 15), at 5400 fmol/mg protein (CHO H2α_2 C10 3), and at 6100 fmol/mg protein (CHO H2α_2 C10 23).

3.2. Regulation of AC activity

Forskolin-stimulated AC activity was dose-dependently inhibited by NA in the cell population stably transfected with α_{2A}-ARs (represented by the −PTX experiments in Fig. 1A and Table 1). The extent of the inhibition was maximally 78% (E_{max}−78.2±0.5%, log EC_{50}−7.9±0.1). In contrast, in the semistably transfected cells, NA evoked at most 23% inhibition (not significant, P=0.11) of forskolin-stimulated AC activity, in spite of an identical α_{2A}-AR density with the stable cell population (Fig. 1B, Table 1). Three episomally transfected semistable cell populations were tested and all gave similar results (data not shown). In the monoclonal cell populations (expanded from single cells originating in the semistable cell population), dose-dependent inhibition (67–76%) of forskolin-stimulated AC activity was observed in those clones that expressed α_{2A}-ARs at high density (Fig. 2A, where E_{max}−75.8±0.3%, log EC_{50}−7.2±0.2), whereas a cell population lacking significant amounts of receptors did not show any significant inhibition (Fig. 2B).

A tendency to stimulation of AC activity was seen at high receptor density and at high agonist concentrations (Fig. 2A, −PTX). To examine the extent and coupling mechanisms of the stimulation of AC activity, cells were pretreated with pertussis toxin (PTX) to inactivate inhibitory G_i-proteins. This resulted in complete loss of AC inhibition by NA. In PTX-treated cells, concentration-dependent stimulation of forskolin-stimulated AC activity became evident at 100 nM NA (Figs. 1A and 2A and Table 1). In

Table 1
Saturation binding experiments and measurement of intracellular cAMP production in the stable, semistable and the four monoclonal cell populations expressing human α_{2A}-adrenoceptors

Cell population	Description	B_{max} (fmol/mg)	−PTX		+PTX	
			E_{max} (%)	logEC_{50} (M)	E_{max} (%)	logEC_{50} (M)
CHO C10R E47	Human α_{2A}-AR in pMAMneo (stable)	1100	−78.2±0.5	−7.9±0.1	304±12	−5.5±0.1
CHO H2α_2 C10	Semistable cell population (expressing human α_{2A}-AR in pREP4) from which monoclonal cell populations originate	1200±40	−23.1±5.8 (ns)	Na	51±5*	Na
CHO H2α_2 C10 1	Monoclonal cell population expressing human α_{2A}−AR in pREP4	Nd	−7.9±11.5 (ns)	Na	Na	Na
CHO H2α_2 C10 15	Monoclonal cell population expressing human α_{2A}-AR in pREP4	3000±196	−75.8±0.3	−7.2±0.2	572±131	−5.8±0.1
CHO H2α_2 C10 3	Monoclonal cell population expressing human α_{2A}-AR in pREP4	5400±134	−72.4±1.0	−8.1±0.2	510±122	−5.8±0.1
CHO H2α_2 C10 23	Monoclonal cell population expressing human α_{2A}-AR in pREP4	6100±210	−67.3±5.1	−7.3±0.2	574±87	−5.9±0.1

B_{max} stands for receptor density/mg total cellular protein. Measurement of intracellular cAMP production before (−PTX) and after PTX treatment (+PTX) (Figs. 1 and 2) gave E_{max} and logEC_{50} values for noradrenaline (NA). Negative values for E_{max} indicate percent inhibition and positive values percent stimulation, for +PTX the E_{max} was calculated as percent of further stimulation of 100% forskolin-stimulated AC activity. EC_{50} is the concentration of NA causing 50% of the maximal effect. Results are means±S.E.M. (log EC_{50}±SE) from three to five experiments performed in duplicate (except B_{max} for the stable cell population, where n=1).

Na=not applicable; curve could not be fitted due to small difference between maximal and minimal values.

Nd=not detectable (<25 fmol/mg protein).

ns=not significant.

* P value: 0.03.

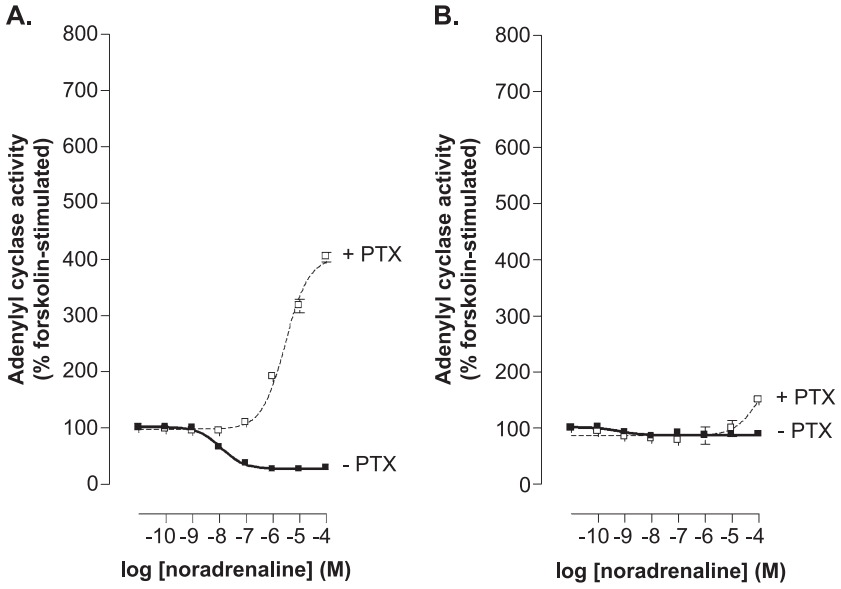

Fig. 1. Regulation of AC activity in CHO cells transfected to express human α_{2A}-adrenoceptors. (A) Forskolin-stimulated AC activity was efficiently inhibited by noradrenaline in stably transfected cells not pretreated with pertussis toxin (−PTX) (78.2±0.5% inhibition), and stimulated in cells pretreated with pertussis toxin (+PTX) (304±12% stimulation). (B) Only minor effects were observed in a semistable CHO cell population with an equal receptor density to that in A (23% inhibition; not significant, P=0.11; 51±5% stimulation). 100% AC activity is defined by the maximal forskolin-stimulated cAMP accumulation in each cell population over basal (in the absence of noradrenaline). Results are means±S.E.M. from three to five experiments performed in duplicate.

the semistable, heterogeneous CHO cell population, only 100 μM NA evoked some stimulation of AC activity after PTX treatment (Fig. 1B, where E_{max} 51±5, P-value 0.03), whereas the monoclonal cell population lacking significant amounts of receptors displayed no effect (Fig. 2B). In the absence of forskolin and after PTX treatment, NA also stimulated cAMP accumulation, but the effect was only ~6–20% of the maximal stimulation (~300–570%) obtained with forskolin and NA in the +PTX experiments, and started at 0.1–10 μM of NA (data not shown).

3.3. Confocal fluorescence microscopy and flow cytometry

Both confocal fluorescence microscopy (Fig. 3A) and flow cytometric analysis (Fig. 4A) of the semistable CHO cell population expressing recombinant human α_{2A}-ARs

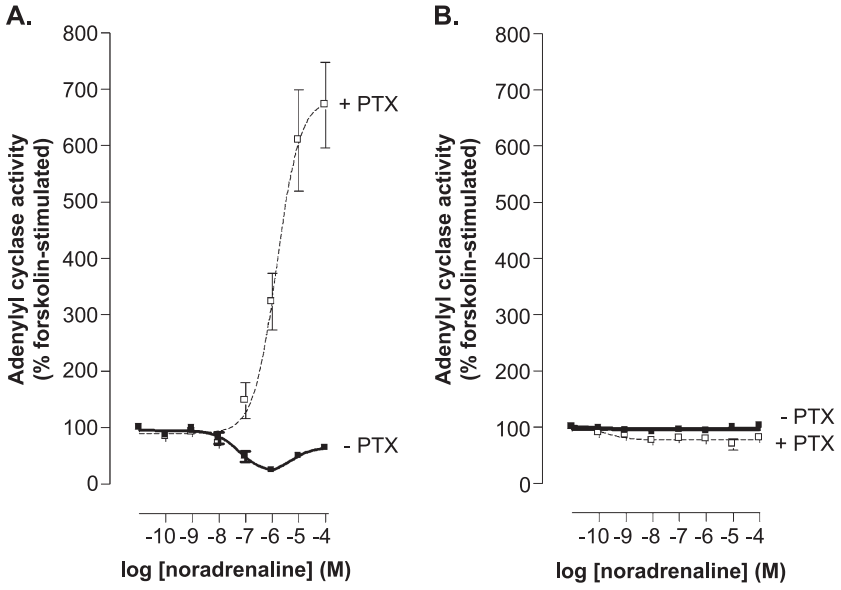

Fig. 2. Regulation of AC activity in monoclonal transfected CHO cell populations. (A) Efficient inhibition and stimulation of AC activity in a monoclonal CHO cell population expressing human α_{2A}-adrenoceptors at 3000 fmol/mg protein (75.8±0.3% inhibition, −PTX; 572±131% stimulation of +PTX). (B) No responses were observed in a monoclonal cell population lacking significant amounts of receptors. Results are means±S.E.M. from three experiments performed in duplicate.

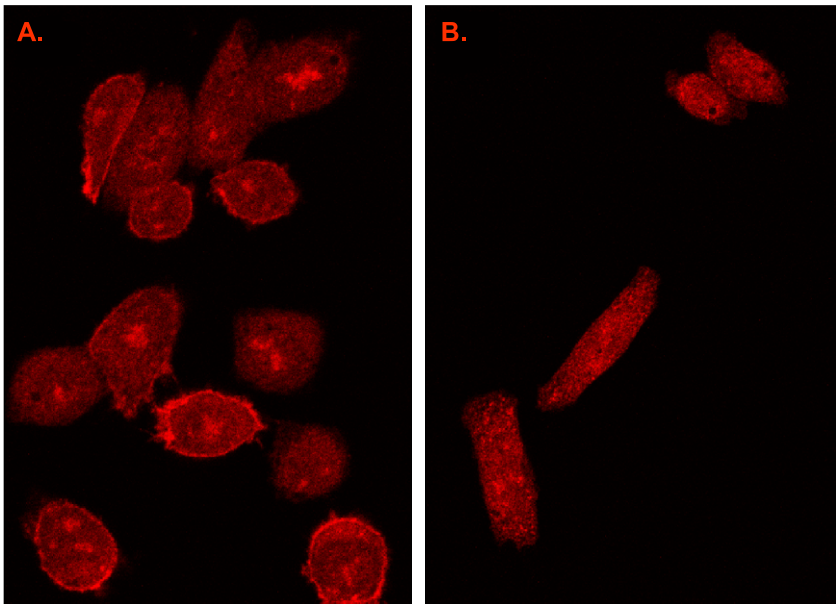

Fig. 3. (A) Confocal fluorescence microscopy of the semistable cell population with uneven cellular receptor expression. (B) Non-transfected CHO cells as negative controls. Cells were scanned with a confocal laser-scanning microscope. A and B are representative micrographs from three separate experiments, chosen to visualize receptor expression.

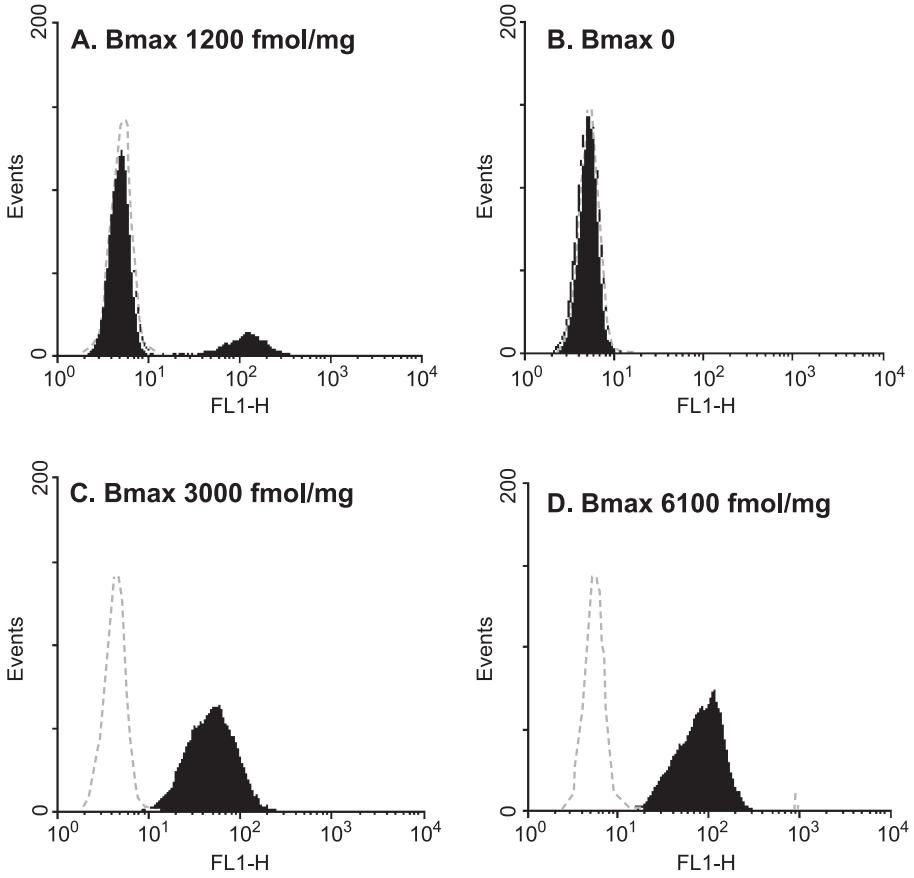

Fig. 4. (A) Flow cytometry analysis of the semistable cell population with uneven cellular receptor expression. (B) Monoclonal cell population lacking significant amounts of receptors. (C) Monoclonal cell population expressing α_{2A}-adrenoceptors at 3000 fmol/protein. (D) Monoclonal cell population expressing α_{2A}-adrenoceptors at 6100 fmol/protein. The dotted lines represent negative controls (no anti-HA antibody), and the filled peaks represent CHO cells labelled with anti-HA as primary antibody and FITC-conjugated goat anti-mouse IgG as secondary antibody. Note that FL1-H shows fluorescence intensity on a logarithmic scale. Results are representative of three individual experiments.

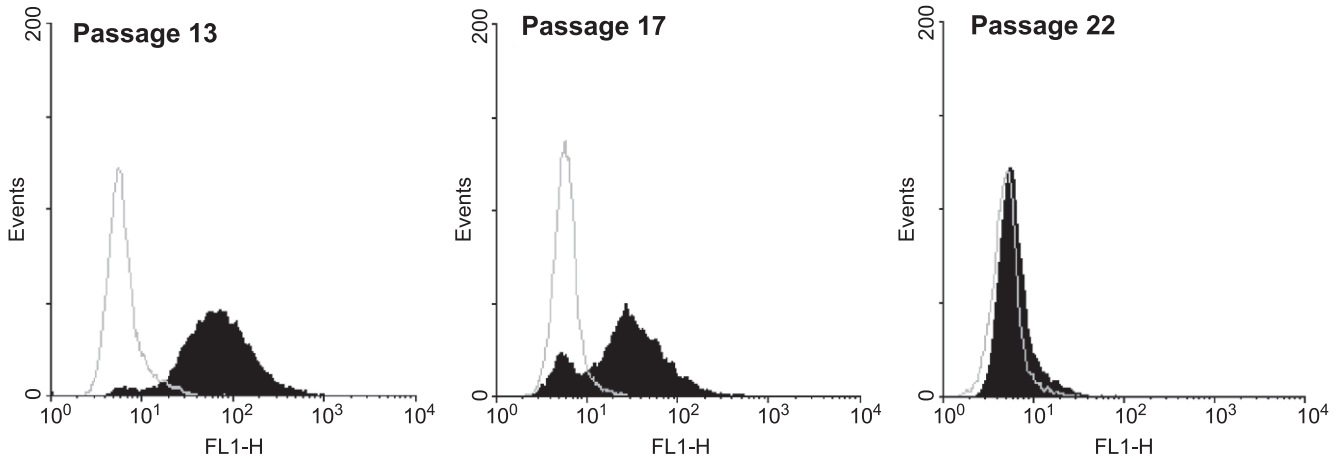

Fig. 5. Flow cytometry analysis of the stable cell population transfected with an integrating vector (pMAMneo). The dotted lines represent negative controls (no anti-HA antibody), and the filled peaks represent CHO cells labelled with anti-HA as primary antibody and FITC-conjugated goat anti-mouse IgG as secondary antibody. Results are representative of three individual experiments.

supported our view of heterogeneous expression with the episomal pREP4-based construct; some cells expressed receptors at high density, whereas most cells appeared to have no detectable expression at all (appearance similar to non-transfected CHO-K1 cells that served as negative controls, Fig. 3B). Flow cytometric analysis of the CHO cell population, transfected with the integrating pMAMneo-based vector and stably expressing human α_{2A}-AR, revealed that the receptor expression levels were attenuated as the passage number was increased (Fig. 5). The fluorescence intensity histogram was clearly bimodal at passage 17, and at passage 22, most cells fell within a low-intensity peak that was superimposed to the fluorescence intensity histogram of control cells incubated without the HA-antibody. For the monoclonal cell population lacking significant amounts of receptors, there was no difference in fluorescence intensity between control cells and immunolabelled cells (Fig. 4B). Flow cytometric analysis of the monoclonal cell populations expressing α_{2A}-AR at 3000 and 6100 fmol/mg protein, on the other hand, demonstrated a homogeneous expression pattern, even at high (>25) passage numbers. The intensity of fluorescence was proportional to receptor density (Fig. 4C and D).

4. Discussion

Recombinant receptors expressed in heterologous cellular systems are extensively used as models to understand receptor function and mechanisms of action. Functional cell-based assays are also widely employed to assess the pharmacological properties of the expressed receptor and their ligands [1,8].

In this study, two CHO cell populations containing similar numbers of recombinant human α_{2A}-ARs were found to yield dramatically different results in a functional cell-based assay based on measurement of intracellular cAMP accumulation. One of the cell populations, stably transfected with an integrating vector to express human α_{2A}-ARs, mediated dose-dependent inhibition of forskolin-stimulated AC activity, whereas the other cell population, transfected with a non-integrating episomal vector to express the same human α_{2A}-AR subtype, mediated no detectable AC inhibition (Fig. 1). We found that most cells in the latter cell population had undetectable amounts of α_{2A}-ARs, probably due to variable numbers of gene copies in different cells. Most cells apparently had enough copies of the expression vector to confer resistance to the selection antibiotic, hygromycin, but too few to yield detectable receptor expression. When AC activity was stimulated by forskolin in all cells, a false negative pharmacological test result was achieved, due to domination of cells expressing too few α_{2A}-AR gene copies.

The lack of α_{2A}-AR-mediated AC inhibition in the semistable cell population led us to isolate single cell clones from this cell population. These were thought to represent potentially stable homogeneous cell populations, since they originated from single cell clones and were produced according to InVitrogen's manual for the production of stable cell lines with pREP4. Receptor density determinations of these monoclonal cell populations supported our view of heterogeneous expression in the semistable cell population (from which these originated) and showed that most of the monoclonal cell populations lacked significant amounts of receptors and that a few monoclonal cell populations expressed α_{2A}-ARs at high receptor density.

When cells are transfected with non-integrating episomal vectors, variable amounts of plasmids are usually entered into different cells, which results in heterogeneous expression. The heterogeneity of the semistable cell population used in this study was visualized with flow cytometry and confocal fluorescence microscopy. In Fig. 4A, the flow cytometry results revealed a bimodal expression distribution for the semistable cell population, where most cells were located at a position identical to the position of the control

peak. Support to these results is also given in Fig. 3A, which shows that a few cells expressed receptors at high density, whereas most cells were comparable to the non-transfected control cells (Fig. 3B). The semistable cell population therefore seemed to mainly contain cells expressing no receptors at all, although obviously the hygromycin resistance gene was transferred during transfection as the cells survived in 200 μg/ml Hygromycin B®. The lack of receptor expression in the monoclonal cell populations lacking significant numbers of binding sites was supported by flow cytometry, where no difference in fluorescence intensity between control cells and immunolabelled cells was seen (Fig. 4B). After transfection with the integrating vector, on the other hand, there should have been a homogeneous expression pattern, as all cells originated from a single selected cell clone. This was the case in our study, but only until passage number 13. Flow cytometric results for the stably transfected cell population revealed that as the passage number was increased, expression levels were progressively attenuated (Fig. 5). This did not seem to be the case for the hygromycin-resistant monoclonal cell populations, where passage 28 still displayed homogeneous expression in flow cytometry (Fig. 4C and D). We also noticed that the AC inhibitory response to NA in the stably transfected cell population was blunted as the passage number exceeded 20. Consequently, all functional experiments for the stable cell population were performed on cells before passage 18. The reasons for the high stability of expression in the monoclonal (stable) cell populations containing the pREP4-plasmid compared to the relatively rapid loss of expression in the stable cell population containing the pMAMneo-based vector are presently unknown. The reasons could include a suboptimal G418 concentration used in the maintenance of the pMAMneo-based clonal cell line, dissociation of receptor expression from antibiotic resistance due to fragility of the vector, or some acquired mechanism of antibiotic resistance.

Four of the monoclonal episomally transfected cell populations were then tested for AC inhibition. NA induced inhibition of forskolin-stimulated AC activity in all monoclonal cell populations expressing high receptor numbers, and NA concentrations above 1 μM started to stimulate AC activity (Fig. 2A). This kind of biphasic response has previously been seen in CHO, PC12 and JEG-13 cells transfected with α_2-ARs [20–23], where low concentrations (<100 nM) of agonist have inhibited AC activity and concentrations in excess of 100 nM have stimulated AC activity. It has therefore been proposed that all α_2-AR subtypes can couple both physically and functionally to G_i and G_s proteins [12,22,24,25]. Our results are identical to the results of Eason et al. [12] and Fraser et al. [21] who also reported biphasic dose-response curves to NA and adrenaline in CHO cells. They not only demonstrated an increase of intracellular cAMP concentrations with increasing concentrations of α_2-agonists, but also that this increase was dependent on receptor density.

When G_i activity was abolished by PTX pretreatment, NA further increased forskolin-stimulated AC activity at concentrations above 100 nM, in accordance with results from Eason et al. [12] and Cotecchia et al. [26] (Figs. 1A and 2A). In the absence of forskolin and presence of PTX, NA stimulated AC activity at concentrations above 0.1–10 μM in the stable and monoclonal cell lines with high receptor density, but this stimulation was minimal compared to the effect seen when AC activity was co-stimulated by forskolin, indicating a need for pre-activated AC in order to see cAMP accumulation of the magnitude seen in this experiment. It is worth considering whether α_{2A}-AR coupling to stimulation of AC activity has physiological significance, as this phenomenon is somewhat inconsistently observed, related to the choice of host cell line, and seen only in transfected cell lines that express receptors at levels that are far higher than physiological [12]. Perhaps the use of primary cell cultures expressing α_2-ARs would provide more physiologically relevant results. On the other hand, the interference of other closely related receptor subtypes could result in problems that are bypassed in transfected cell lines.

For the functional characterisation of α_{2A}-ARs in heterologous cell systems, our results demonstrate that careful model validation is required, and suggest that a cell population expressing receptors in a homogeneous fashion needs to be employed for the assessment of the capacity of the investigated receptors to mediate inhibition of forskolin-stimulated AC activity. In conclusion, we have shown that there is heterogeneous expression of receptors after episomal transfection with a pREP4-based construct, a fact that most probably is applicable to all episomally transfected cell populations. Uneven receptor expression in the transfected cell population resulted in total loss of response in the functional assay based on AC inhibition, and consequently would have led to a false negative pharmacological test result and incorrect interpretation of the results. This would, e.g., be significant if mutated α_2-ARs were assessed for the functional importance of the mutation. Furthermore, it is important to note that also in the stable cell population transfected with an integrating vector, there was attenuation of expression levels and emergence of heterogeneity in the population as the passage number was increased. Thus, the test system should be continuously monitored in this respect during the conduct of extensive experiments. Flow cytometry is well suited for this purpose, if an extracellular epitope is available in the transfected cells.

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