Functional analysis of a human A_1 adenosine receptor/green fluorescent protein/$G_{i1}\alpha$ fusion protein following stable expression in CHO cells

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Abstract Fusion proteins between the human A_1 adenosine receptor and the pertussis toxin resistant (Cys351Gly) mutant of the G-protein α subunit $G_{i1}\alpha$ (A1/Gi), and between the human A_1 adenosine receptor, the *Aequorea victoria* green fluorescent protein (GFP) and Cys351Gly $G_{i1}\alpha$ (A1/GFP/Gi), were expressed in CHO cells. The agonist NECA caused a stimulation of [^{35}S]GTPγS binding at both fusion proteins with similar concentration dependence as at the native receptor. However in the presence of pertussis toxin NECA stimulation of [^{35}S]GTPγS binding was only seen at the A1/GFP/Gi fusion protein. The regulation of the adenylyl cyclase and MAP kinase effector systems by both fusion proteins was attenuated following pertussis toxin treatment. These studies demonstrate for the first time the characterisation of a fusion protein between a G-protein coupled receptor, GFP and a G-protein α subunit.

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Key words: G-protein coupled receptor; A_1 adenosine receptor; Green fluorescent protein; Fusion protein

1. Introduction

The human A_1 adenosine receptor couples to members of the $G_{i/o}\alpha$ family of heterotrimeric G-proteins to cause the inhibition of adenylyl cyclase, the stimulation of phoshoinositidase C, the activation of inwardly rectifying K^+ channels and the inhibition of neuronal calcium channels [1–3]. With the exception of $G_z\alpha$ each of these G-proteins can be modified by pertussis toxin catalysed transfer of an ADP-ribose moiety onto a cysteine residue four amino acids from the C-terminus, to result in the attenuation of receptor coupling [4]. To study receptor coupling through a single G-protein we and others have generated mutant G-proteins in which this cysteine residue has been mutated to a serine [4] or glycine [5,6] and have included these mutant G-proteins into G-protein coupled receptor (GPCR)/G-protein fusions. Transient expression of such fusion proteins has demonstrated that the receptor can couple to both endogenous and fused G-protein and that the rank order of agonist efficacy is maintained when studying signalling through the fused G-protein [7–10].

Many reports describe the fusion of the *Aequorea victoria* green fluorescent protein (GFP) to the C-terminus of a GPCR. Such fusion proteins have been used to study receptor trafficking and agonist-mediated desensitisation and internalisation. In such studies an intrinsically fluorescent receptor is created with ligand binding affinity, signalling specificity and phosphorylation and internalisation characteristics, which appear indistinguishable from the wild-type receptor [11–13].

In the present study we have combined the features of GPCR/G-protein and GPCR/GFP fusion proteins by generating a receptor/GFP/G-protein fusion. Fusion proteins consisting of the human A_1 adenosine receptor and the pertussis toxin resistant (Cys351Gly) mutant of rat $G_{i1}\alpha$ (A1/Gi), and between the human A_1 adenosine receptor, GFP, and the Cys351Gly mutant of rat $G_{i1}\alpha$ (A1/GFP/Gi) have been characterised following stable expression in CHO cells. We demonstrate that GFP may be included in a receptor/G-protein fusion with little effect upon the behaviour of the chimeric protein.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium/nutrient mix F12 (50:50) (DMEM/F12), foetal calf serum (FCS), glutamine and neomycin (G418) were from Life Technologies. NECA (5′-*N*-ethylcarboxamidoadenosine) was purchased from Research Biochemical International and adenosine deaminase from Boehringer Mannheim. Forskolin, 3-isobutyl-1-methylxanthine (IBMX) and all other chemicals were purchased from Sigma. Scintillation proximity assay (SPA) beads and [^{35}S]GTPγS (1175 Ci/mmol) were purchased from Amersham. [^{3}H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine) (120 Ci/mmol) was from New England Nuclear.

2.2. Construction and expression of A_1 adenosine receptor fusion proteins

A human codon optimised GFP was amplified by PCR from pEGFP (Clontech) using oligonucleotides 5′-AATCCATGGTGAGCAAGGGCGAGGAG (sense) and 5′-AATCCATGGTGTACAGCTCGTCCATGCCGAGAG (antisense) (*Nco*I restriction sites underlined). During amplification the GFP stop codon was removed. The PCR product was gel purified and subcloned into pCR-script (Stratagene). GFP was released from this vector by digestion with *Nco*I and ligated with similarly digested pCR-script/A1/Gi [10] to generate A1/GFP/Gi. For expression A1/Gi and A1/GFP/Gi were subcloned into the *Eco*RI sites of pCIN [14] and pcDNA3 (Invitrogen).

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Abbreviations: A1/Gi, fusion protein between the human A_1 adenosine receptor and rat $G_{i1}\alpha$; A1/GFP/Gi, fusion protein between the human A_1 adenosine receptor, GFP and rat $G_{i1}\alpha$; CHO, Chinese hamster ovary; GFP, green fluorescent protein; G-protein, guanine nucleotide binding protein; GPCR, G-protein coupled receptor; [^{35}S]GTPγS, guanosine 5′-[γ^{35}S]triphosphate; HEK, human embryonic kidney; MAP kinase, mitogen activated protein kinase; NECA, 5′-*N*-ethylcarboxamidoadenosine

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All cells were maintained in 5% CO_2 at 37°C. HEK 293/T cells, grown to 60–80% confluence in 60 mm culture dishes in DMEM containing 10% FCS and 2 mM L-glutamine, were transfected with pcDNA3 containing either the A_1 receptor, or the A1/Gi or A1/GFP/Gi fusions using LipofectAMINE (Life Technologies) [8]. Following transfection, growth medium was supplemented with adenosine deaminase (2 U/ml). Cells were harvested 48 h after transfection and stored as a cell paste at −80°C. P2 particulate fractions were prepared as described [4].

Chinese hamster ovary (CHO) cells, maintained in DMEM/F12 containing 5% FCS and 2 mM L-glutamine (growth media), were transfected with pCIN containing either A1/Gi or A1/GFP/Gi using the calcium phosphate transfection kit (Invitrogen). Following selection in 1 mg/ml G418 clones were selected according to the level of specific [^{3}H]DPCPX binding. CHO cells stably expressing the human A_1 adenosine receptor were previously generated at Glaxo Wellcome. For studies using pertussis toxin, 50 ng/ml was added to the culture media 18 h prior to harvesting or assay.

2.3. Immunological studies

Antiserum 64 was produced in a New Zealand White rabbit, using a conjugate of a synthetic peptide encoding amino acids 149–158 of the human A_1 adenosine receptor and keyhole-limpet haemocyanin (Calbiochem) as antigen. Membrane samples from transfected cells (10 μg) were resolved by SDS-PAGE using a 4–12% (w/v) acrylamide NuPAGE system (Novex). Following electrophoresis, proteins were subsequently transferred to nitrocellulose, probed with antiserum 64 at 1:1000 dilution and visualised by enhanced chemiluminescence (Supersignal, Pierce).

2.4. Membrane preparations

Cells were harvested in PBS containing 5 mM EDTA and homogenised in 50 mM HEPES, 10 mM $MgCl_2$ and 1 mM EDTA, pH 7.4, containing 1 U/10 ml adenosine deaminase. After pelleting at $48\,000\times g$ membranes were resuspended in buffer containing 1 U/ml adenosine deaminase and stored at −80°C.

2.5. [^{3}H]DPCPX binding

Thawed membrane preparations were pelleted by centrifugation at $100\,000\times g$ for 20 min and resuspended by Dounce homogenisation (20 strokes) on ice in assay buffer (50 mM Tris-HCl, pH 8.26 at 4°C, 10 mM $MgCl_2$ and 1 mM EDTA, 1 U/ml adenosine deaminase) to yield a membrane protein concentration of approximately 0.02 mg/ml. 150 μl of membrane suspension was added to glass tubes containing 50 μl of [^{3}H]DPCPX (0.25–8 nM) and 50 μl of either water or 250 μM R-PIA ([R]-N^6-phenylisopropyladenosine) to define non-specific binding. Duplicate samples were used for each point. Assays were incubated at 37°C for 60 min and terminated by rapid filtration over 0.3% (v/v) polyethylenimine-soaked GF/B filters and washing with ice-cold assay buffer supplemented with 0.01% (w/v) CHAPS using a Brandel cell harvester. Five ml scintillation fluid was added to each filter disc and samples were counted following shaking and overnight incubation at 4°C. Saturation binding data were analysed using a computer-assisted curve-fitting package (Prism, Graphpad).

2.6. [^{35}S]GTPγS binding

Assays were performed exactly as described in [10].

2.7. cAMP assays

Cells were seeded into 96-well plates and maintained for 24 h in growth media. Cells were washed with PBS and incubated with 300 μM IBMX with 1 U/ml adenosine deaminase for 30 min at 37°C prior to being stimulated with forskolin (30 μM) and NECA (0.0003–10 μM) for 15 min. The rest of the assay was performed as in [15].

2.8. MAP kinase activity assay

Cells were grown to subconfluence in 60 mm diameter cell culture dishes. The media were removed, the cells washed twice with PBS, and quiescence media (DMEM/F12, 2 mM glutamine, 1 U/ml adenosine deaminase) added 24 h prior to assay. Cells were stimulated with NECA (10 μM) for 15 min and MAP kinase activity assays were performed as described in [15].

2.9. Confocal laser scanning microscopy

Cells, plated on sterile glass coverslips 2 days before the experiment, were washed with PBS and fixed for 20 min by 4% paraformaldehyde in PBS/5% sucrose, pH 7.2 at room temperature. Fixed cells were washed twice with PBS/10 mM glycine and mounted on microscope slides with 40% glycerol in PBS. Cells were observed using a laser scanning confocal microscope (Zeiss Axiovert 100) using a Zeiss Plan-Apo 63×1.40 NA oil immersion objective and an electronic zoom 3. GFP was excited using a 488 nm argon/krypton laser and detected with 515–540 nm band pass filter. The images were manipulated with Zeiss LSM or Metamorph software.

3. Results

3.1. Expression of A1/Gi and A1/GFP/Gi fusion proteins in CHO cells

The construction of the fusion protein between the human A_1 adenosine receptor and rat Cys351Gly $G_{i1}\alpha$ (A1/Gi) is described in [10]. We now report the construction of a fusion protein between this receptor, GFP, and rat Cys351Gly $G_{i1}\alpha$ (A1/GFP/Gi). To facilitate construction of this fusion the C-terminal amino acid of the receptor has been changed from aspartic acid to alanine and the C-terminal amino acid of GFP from lysine to threonine. Expression of the A_1 receptor, and the A1/Gi and A1/GFP/Gi fusions, as polypeptides of the expected molecular weights of 49, 79 and 115 kDa, was confirmed by immunoblotting membranes of transfected HEK 293/T cells with an antiserum raised to the second extracellular loop of the receptor (Fig. 1).

For characterisation the A1/Gi and A1/GFP/Gi fusions were stably expressed in CHO cells. The level of receptor expression was assessed by specific binding of the antagonist radioligand [^{3}H]DPCPX and cell lines expressing the fusions at similar receptor densities were identified (A1/Gi, 5.13 ± 1.26 pmol/mg protein and A1/GFP/Gi, 4.03 ± 0.59 pmol/mg protein) (Table 1). The K_d for the interaction between [^{3}H]DPCPX and the fusions was comparable to the K_d for the interaction of this ligand with the native receptor (Table 1). No specific binding of [^{3}H]DPCPX was observed in membranes from cells transfected with pCIN alone (data not shown).

The inclusion of GFP within the fusion allows for protein visualisation by confocal microscopy (Fig. 2). The fusion is located on cytoplasmic membranes and at the plasma membrane. Cytoplasmic fluorescence represents protein being syn-

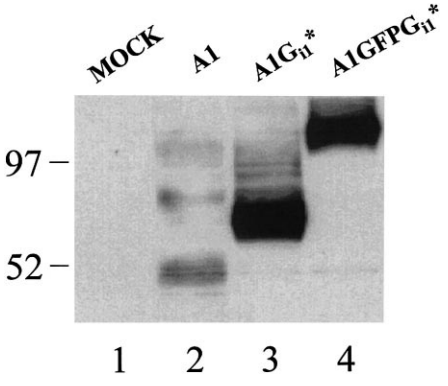

Fig. 1. Expression of A_1 adenosine receptor and the A1/Gi and A1/GFP/Gi fusion proteins. HEK 293/T cells were mock transfected (lane 1), transfected with the human A_1 adenosine receptor (lane 2), the A1/Gi fusion (lane 3) or the A1/GFP/Gi fusion (lane 4). P2 particulate fractions were prepared as described in Section 2 and 10 μg of protein were resolved by SDS-PAGE and immunoblotted with A_1 adenosine receptor-specific antiserum 64. (* denotes Cys351Gly variant of $G_{i1}\alpha$.)

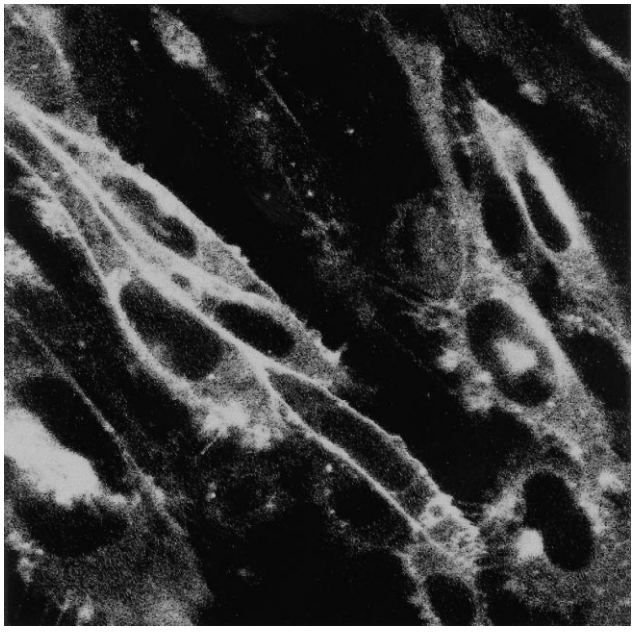

Fig. 2. Cellular distribution of the A1/GFP/Gi fusion protein in CHO cells. CHO cells stably expressing the A1/GFP/Gi fusion construct were imaged in the confocal microscope as described in Section 2.

thesised in the endoplasmic reticulum and being trafficked through the Golgi apparatus.

3.2. Functional activity of the A1/Gi and A1/GFP/Gi fusion proteins in CHO cells

Ligand stimulation of the human A_1 adenosine receptor and the fusions was examined by determining the ability of the agonist NECA to promote the binding of [^{35}S]GTPγS to G_i G-proteins in membranes prepared from the stable cell lines. Agonist activity was studied in naive membranes to examine receptor interaction with endogenous G-protein and in membranes prepared from cells treated with pertussis toxin to examine receptor interaction with the fused G-protein. In the absence of pertussis toxin NECA caused a concentration dependent increase in [^{35}S]GTPγS binding (Fig. 3A). This was concentration dependent with EC_{50} values of 0.73 (0.5–1) nM (A_1 receptor), 2.22 (1.5–3.37) nM (A1/Gi) and 17 (16–19) nM (A1/GFP/Gi) (Fig. 3A). The placement of the agonist curves is consistent with the levels of expression determined by saturation binding analysis (A_1 > A1/Gi > A1/GFP/Gi). Membranes prepared from cells transfected with the A_1 receptor displayed an elevated basal level of [^{35}S]GTPγS binding suggesting constitutive activity in these cells in which the receptor is expressed at 12.96 pmol/mg (Table 1). The addition of

Table 1
Expression levels of the human A_1 adenosine receptor and the A1/Gi and A1/GFP/Gi fusions in the stable CHO cell lines

Cell line	B_{max} (pmol/mg protein)	K_d (nM)
A_1	12.96 ± 2.23	7.32 ± 0.68
A1/Gi	5.13 ± 1.26	2.30 ± 0.28
A1/GFP/Gi	4.03 ± 0.59	4.21 ± 0.87

K_d and B_{max} parameters were obtained from saturation binding studies using the antagonist [^{3}H]DPCPX. Values are presented as mean ± S.D. from three separate experiments.

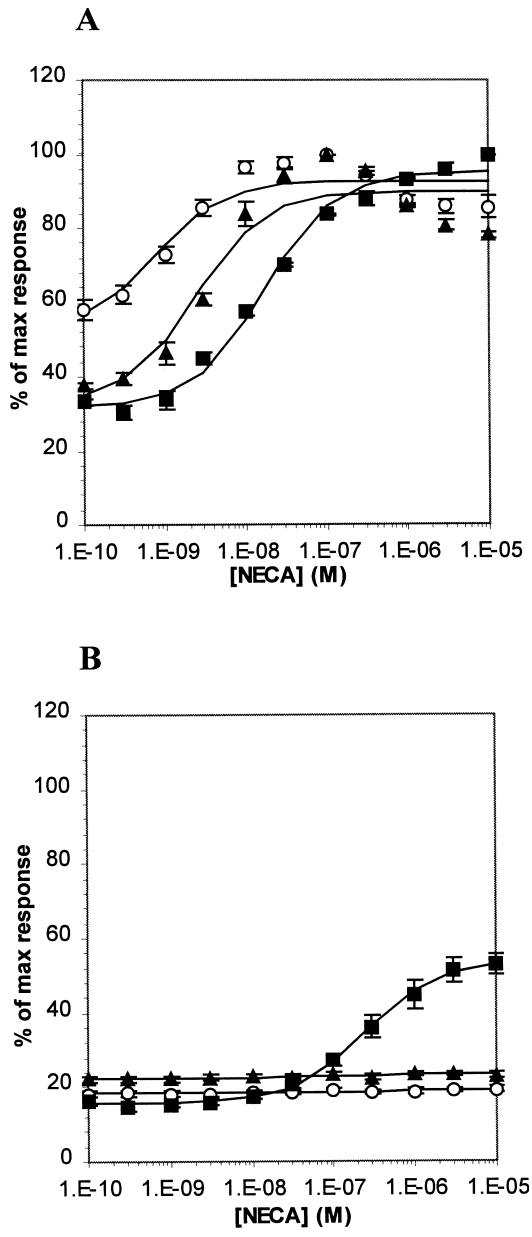

Fig. 3. NECA stimulation of [^{35}S]GTPγS binding to CHO cell membranes. NECA stimulation of [^{35}S]GTPγS binding was assessed on membranes prepared from CHO cells stably transfected with the A_1 adenosine receptor (m), the A1/Gi fusion (s) and the A1/GFP/Gi fusion (n), in the absence (A), and in the presence (B), of pre-treatment for 18 h with 50 ng/ml pertussis toxin. All values are expressed as the mean percent of the maximum NECA response (in the absence of pertussis toxin) of at least three separate experiments performed in duplicate (± S.E.M.).

NECA to membranes prepared from pertussis toxin treated cells expressing either the A_1 receptor or A1/Gi no longer caused the stimulation of [^{35}S]GTPγS binding (Fig. 3B). However, in membranes prepared from similarly treated cells expressing A1/GFP/Gi NECA was able to promote the binding of [^{35}S]GTPγS with an EC_{50} of 257 (193–344) nM.

We next examined the ability of each fusion to regulate adenylyl cyclase. In the absence of pertussis toxin NECA inhibited forskolin stimulated cAMP accumulation in the three cell lines (Fig. 4A). This was concentration dependent with

IC_{50} values of 26 (19–27) nM (A_1 receptor), 68 (38–123) nM (A1/Gi) and 54 (20–144) nM (A1/GFP/Gi). Following pertussis toxin treatment NECA was not able to inhibit forskolin stimulated cAMP indicating that the regulation of adenylyl cyclase is due to receptor, or fusion protein, coupling to endogenous G-proteins (Fig. 4B).

Many $G_i\alpha$ coupled GPCRs have been shown to activate the MAP kinase cascade [10]. This signalling event is thought to be mediated by the G-protein $\beta\gamma$ complex as it can be blocked following overexpression of the $\beta\gamma$ sequestering proteins α-transducin or the C-terminus of the β-adrenergic receptor kinase [17]. In the absence of pertussis toxin, the exposure of CHO cells expressing the human A_1 receptor, or either fusion protein, to 10 μM NECA resulted in a rapid activation of MAP kinase. In each case this effect was abolished following pre-treatment with pertussis toxin (Fig. 5).

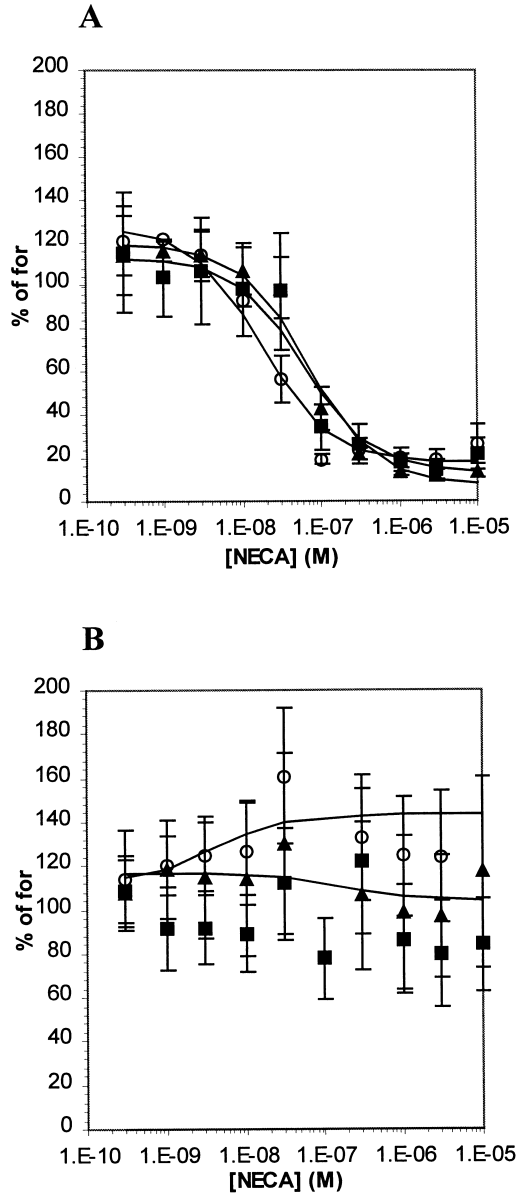

Fig. 4. NECA inhibition of forskolin stimulated cAMP accumulation in CHO cells expressing the fusion proteins. NECA inhibition of forskolin stimulated cAMP accumulation in CHO cells stably expressing the human A_1 adenosine receptor (m), and the A1/Gi (s), and A1/GFP/Gi fusion proteins (n) in the absence (A), and in the presence (B), of pre-treatment for 18 h with 50 ng/ml pertussis toxin. All values are expressed as the mean percentage of the forskolin response of three separate experiments performed in quadruplicate (±S.E.M.).

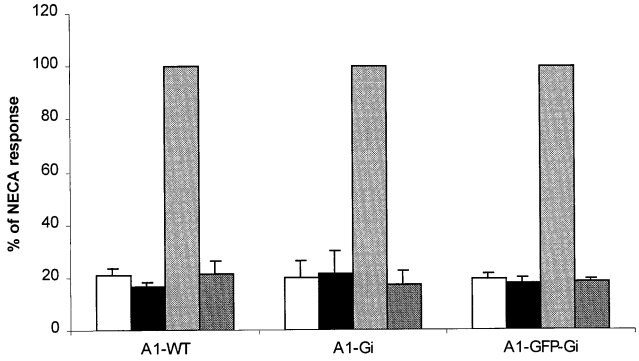

Fig. 5. NECA stimulation of MAP kinase in CHO cells expressing the fusion proteins. NECA activation of MAP kinase in CHO cells stably expressing the human A_1 adenosine receptor, or the A1/Gi or A1/GFP/Gi fusion proteins. Cells were treated with NECA (10 μM) for 15 min in the absence (light grey bars) and presence (dark grey bars) of pre-treatment for 18 h with 50 ng/ml pertussis toxin. Basal activity in the absence (white bar) and presence (black bar) of pertussis toxin is also shown. Bars are the mean percentage of NECA activation in the absence of pertussis toxin for three separate experiments performed in duplicate (±S.E.M.).

4. Discussion

To study the interaction of GPCRs with individual $G\alpha_i$ G-proteins we [4] and others [5,6] have mutated the C-terminal cysteine residue in these G-proteins to render them insensitive to pertussis toxin catalysed ADP-ribosylation. The incorporation of such G-proteins into receptor/G-protein fusion proteins allows the study of receptor interaction with individual G-proteins following pertussis toxin treatment to inactivate the cellular complement of G_i G-proteins. We have used this approach to examine the rank order of efficacy of 40 agonists and partial agonists at fusion proteins between the human A_1 adenosine receptor and pertussis toxin insensitive $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$ and $G_o\alpha$. Using the [^{35}S]GTPγS binding assay we showed that the rank order of efficacy was maintained at each fusion construct although the absolute potencies were 2–3-fold right shifted compared to ligand efficacy at the receptor when coexpressed with the pertussis toxin insensitive G-protein [10].

In a parallel series of studies a number of manuscripts have described the functional expression of GPCR/GFP fusion proteins. The creation of intrinsically fluorescent receptors has facilitated the study of receptor trafficking, desensitisation and internalisation [11–13]. The fusion of GFP to the C-terminus of the receptor appears to have little effect on ligand binding or effector regulation.

The aim of this study was to characterise a fusion protein between the human A_1 adenosine receptor, GFP and rat $G_{i1}\alpha$. This would permit the study of receptor interaction with a specific G-protein while also allowing the direct visualisation of the cellular distribution of the fusion. For functional characterisation the A1/Gi fusion described in [10] and the A1/GFP/Gi fusion described herein were stably expressed in

CHO cells at similar receptor densities (Table 1). Saturation binding analysis indicated that the inclusion of GFP does not effect the ligand binding characteristics of the fusion (Table 1) and confocal microscopy indicated that the protein was found on both internal membranes and at the plasma membrane (Fig. 2).

The ability of the receptor within each fusion to interact with cellular and fused G-protein was examined by determining the ability of the agonist NECA to stimulate high affinity [^{35}S]GTPγS binding in membranes from transfected cells. NECA promoted high affinity [^{35}S]GTPγS binding at both fusion proteins with similar concentration dependence (Fig. 3), demonstrating that in the stable cell lines the receptor in both fusions is capable of interaction with endogenous G-protein. Following pertussis toxin treatment the receptor in the A1/Gi fusion is no longer able to regulate the fused G-protein. This is in contrast to our previous observations in which this fusion, transiently expressed in HEK 293/T cells at 9.6 pmol/mg, remains able to activate the fused G-protein in the presence of pertussis toxin [10]. The G418 resistant pool of cells from which the A1/Gi clone was isolated had a level of expression of 12 pmol/mg. In membranes prepared from this pool NECA was able to promote high affinity [^{35}S]GTPγS binding in the presence of pertussis toxin (data not shown). It appears that to demonstrate functional interaction between the receptor and G-protein in the A1/Gi fusion requires a level of expression greater than 5.13 pmol/mg.

In the presence of pertussis toxin NECA remained able to stimulate high affinity [^{35}S]GTPγS binding at A1/GFP/Gi. At this fusion the EC_{50} for activation of the fused G-protein is 15-fold less potent than for the activation of endogenous G-protein (Fig. 3). This is consistent with previous observations demonstrating that the potency of agonist activation of (Cys-351Gly) $G_{i1}\alpha$ by the porcine α_{2A}-adrenoceptor is 10-fold lower than that for wild-type $G_{i1}\alpha$ [10]. It is intriguing that the receptor in this fusion is able to stimulate the binding of [^{35}S]GTPγS to the fused G-protein in the presence of pertussis toxin (Fig. 3). This may indicate that there is some constraint on receptor/G-protein interaction within these fusions which is relieved by the inclusion of GFP, acting as a spacer molecule, between the receptor and G-protein.

In the absence of pertussis toxin NECA inhibition of forskolin stimulated cAMP accumulation (Fig. 4) and activation of MAP kinase (Fig. 5), at the wild-type receptor and the receptor within both fusions, occurred with very similar concentration dependence. In the presence of pertussis toxin both signalling events were abolished indicating that receptor regulation of the fused G-protein is not sufficient to promote effector regulation. It is unclear why the fusions fail to regulate effector pathways in the presence of pertussis toxin. However, in a similar study a fusion between the α_{2A}-adrenoceptor and Cys351Gly $G_{i1}\alpha$ was unable to regulate adenylyl cyclase and MAP kinase following stable expression in Rat1 fibroblasts [9].

The inability of the fusions to regulate adenylyl cyclase could be explained if the G-protein within the fusion is restrained from interacting with this enzyme. However the absence of receptor regulation of MAP kinase is surprising as GPCR regulation of MAP kinase is thought to be a consequence of the regulation of ras/raf by the G-protein βγ complex [15–17]. While we have not shown that these fusions are able to functionally interact with βγ it is known that an α_{2A}-adrenoceptor/Cys351Gly $G_{i1}\alpha$ fusion protein is able to bind βγ [18]. An explanation may be that $G_i\alpha$ regulation of MAP kinase requires effector regulation by both the Gα and the βγ to subunits.

In conclusion we demonstrate the functional characterisation of the first fusion between a GPCR, GFP and a G-protein α subunit. Inclusion of GFP within the fusion creates an intrinsically fluorescent receptor, which binds ligand with similar affinity to the wild-type receptor and remains capable of functional interaction with endogenously expressed G-protein. Furthermore the inclusion of GFP facilitates functional interaction of the receptor with the fused G-protein. We expect that the inclusion of GFP within receptor/G-protein fusion proteins will expand the utility of such fusions in studies designed to understand the complexities of GPCR signalling, trafficking, desensitisation and internalisation.

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