Measurement of agonist efficacy using an α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein

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Abstract **A fusion protein was constructed between the porcine α_{2A}-adrenoceptor and a pertussis toxin-insensitive ($Cys^{351}Gly$) form of the α subunit of the G protein G_{i1}. Addition of agonist ligands to membranes of COS-7 cells transiently transfected to express this construct, and treated with pertussis toxin prior to cell harvest, resulted in stimulation of both high affinity GTPase activity and enhanced binding of [^{35}S]GTPγS. By considering the fusion protein as an agonist-activated enzyme and measuring V_{max} of GTP hydrolysis a range of agonist ligands displayed varying efficacy in their capacity to activate the receptor-associated G protein with adrenaline = noradrenaline = α-methyl-noradrenaline > UK14304 > BHT933 ≥ xylazine = clonidine. A similar rank order was observed following independent co-expression of the α_{2A}-adrenoceptor and $Cys^{351}Gly$-$G_{i1}\alpha$. These data demonstrate the utility and applicability of using a receptor-G protein fusion protein approach, in which the stoichiometry of receptor and G protein is fixed at 1:1, to measure and further understand the nature of agonist efficacy.**

Key words: G protein; Receptor; Efficacy; Adrenaline

1. Introduction

A molecular understanding of the nature of efficacy [1] of agonists at G protein-coupled receptors (GPCRs) remains a major challenge in GPCR biochemistry and pharmacology [2,3]. Furthermore, as measured estimates of efficacy can be shown to vary with tissue, with levels of expression of a receptor and potentially with the point within a signal transduction cascade at which response is detected [4] then inherent problems exist in providing adequate and unambiguous measurements. One of the earliest, and hence most direct points at which both function and efficacy might be estimated is at the stimulation of the G protein which interacts with a receptor. This may be achieved using assays which measure the exchange of GDP for GTP and/or the subsequent hydrolysis of GTP induced by agonist ligands. This is often constrained, however, by a general lack of information on the absolute levels of expression of the receptor and its cognate G protein and their disposition in relation to one another within cells and at the plasma membrane. To overcome these problems we have generated a fusion protein between the porcine α_{2A}-adrenoceptor [5] and a pertussis toxin-insensitive ($Cys^{351}Gly$) mutant of the α subunit of the G protein $G_{i1}\alpha$ [6]. This defines the stoichiometry of expression of the GPCR and G protein as 1:1 and necessitates their proximity following expression. Following transient expression in pertussis toxin-treated COS-7 cells addition of the α_2-adrenoceptor agonist UK14304 results in stimulation of high affinity GTPase activity, demonstrating the functionality of this construct [7]. We have also demonstrated that creation of fusion proteins between the α_{2A}-adrenoceptor and acylation-deficient forms of $G_{i1}\alpha$ can act to rescue the lack of functional interactions following independent co-expression of these proteins [8]. Herein, we use this construct to evaluate the efficacy of a number of agonists at this GPCR by measuring the ligand-induced rate of GTP turnover by the physically associated G protein. We demonstrate that agonist ligands display a range of efficacy at this construct and that a similar rank order of efficacy is obtained following co-expression of the separated receptor and G protein. We suggest that this strategy may be widely applicable to the designation of efficacy of agonist ligands for specific GPCR-G protein tandems.

2. Materials and methods

2.1. Materials

All materials for tissue culture were supplied by Life Technologies, Inc. (Paisley, Strathclyde, Scotland, UK). [^{3}H]RS-79948-197 (90 Ci/mmol) was purchased from Amersham International. [γ-^{32}P]GTP (30 Ci/mmol) and [^{35}S]GTPγS (1100 Ci/mmol) was obtained from DuPont/NEN. Pertussis toxin (240 μg/ml) was purchased from Speywood. All other chemicals were from Sigma or Fisons plc and were of the highest purity available. Oligonucleotides were purchased from Genosys (Cambridge, UK).

2.2. Construction of $Cys^{351}Gly$-$G_{i1}\alpha$

A pertussis toxin-resistant form of rat $G_{i1}\alpha$ was generated [6] following excision of the wild-type sequence on an *Eco*RI restriction fragment from the vector pGEM2 and insertion into the *Eco*RI site of pBluescript KS$^-$ (pBS) (Stratagene) creating pBS/$G_{i1}\alpha$. To generate pBS/G_{i1}($Cys^{351}Gly$) the 3′ 44 base pairs (bp) of the $G_{i1}\alpha$ open reading frame (ORF) and the 3′ flanking sequence was excised from pBS/$G_{i1}\alpha$ upon digestion with restriction enzymes *Aat*II and *Hin*dIII, and replaced with the synthetic oligonucleotide linker created following annealing of the oligonucleotides 5′-CATCATAAAGAATAACC-TAAAAGACGGTGGTCTCTTCTAAGAATTCA-3′ and 5′-AGCT-TGAATTCTTAGAAGAGACCACCGTCTTTTAGGTTATTCTTT-ATGATGACGT-3′ ($Cys^{351}Gly$ in bold; restriction sites for *Eco*RI, *Hin*dIII and *Aat*II sites underlined) to recreate the 3′ end of $G_{i1}\alpha$. The vector was sequenced following cloning to demonstrate the presence of the cysteine to glycine mutation and the authenticity of the 3′ end of the $G_{i1}\alpha$ open reading frame.

2.3. Construction of the α_{2A}-adrenoceptor-$Cys^{351}Gly$-$G_{i1}\alpha$ fusion construct

The porcine α_{2A}-adrenoceptor [5] was obtained from Dr. L.E. Lim-

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Abbreviations: GPCR, G protein-coupled receptor; G protein, guanine nucleotide binding protein; ORF, open reading frame; DMEM, Dulbecco's modification of Eagle's medium; $Cys^{351}Gly$, codon 351 converted from cysteine to glycine

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bird, Vanderbilt University, TN. The pertussis toxin-resistant $Cys^{351}Gly$ form of rat $G_{i1}\alpha$ was linked to the α_{2A}-adrenoceptor. To do so, the open reading frame (ORF) of the α_{2A}-adrenoceptor cDNA was amplified by PCR using the oligonucleotides: sense 5′-TTGGTACCATGTATCCTTACGACGTTC-3′, antisense 5′-AAGAATTCCATGGCGATCCGTTTCCTGTCCCCACGGC-3′ (restriction sites for *Kpn*I, *Eco*RI and *Nco*I are underlined). The PCR amplified fragment was digested with *Kpn*I and *Eco*RI and ligated to pBluescript through these restrictions sites. Introduction of the *Nco*I site at the 3′ end of the ORF resulted in the C-terminal amino acid of the receptor being altered from Val to Ala and removal of the stop codon. The rat $Cys^{351}Gly$-$G_{i1}\alpha$ cDNA contains two *Nco*I sites; one straddling the ATG start codon and the other 268 bp downstream from this. This 268 bp fragment was removed from $Cys^{351}Gly$-$G_{i1}\alpha$ in pBS by digestion with *Nco*I and the remaining $Cys^{351}Gly$-$G_{i1}\alpha$ pBS cDNA was religated. The shortened cDNA was excised from pBS with *Eco*RI and cloned into the *Eco*RI site of the α_{2A}-adrenoceptor in pBS, adjacent to the 3′ end of the receptor ORF. The 268 bp fragment was then inserted between the *Nco*I sites at the 3′ end of the α_{2A}-adrenoceptor ORF and at the 5′ end of the $Cys^{351}Gly$-$G_{i1}\alpha$ ORF. This resulted in production of an in-frame construct whereby the 3′ end of the α_{2A}-adrenoceptor ORF was exactly adjacent to the 5′ end of the $Cys^{351}Gly$-$G_{i1}\alpha$ ORF. The full fusion construct was then excised from pBS with *Kpn*I and *Eco*RI and ligated into the eukaryotic expression vector pCDNA3 (Invitrogen).

2.4. Cell culture and transfection

COS-7 cells were maintained in DMEM containing 10% (v/v) newborn calf serum (NCS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 60 mm culture dishes and grown to 60–80% confluency (18–24 h) prior to transfection with pCDNA3 containing the relevant cDNA species using lipofectamine reagent (Life Technologies, Inc.). For transfection, 2.5–2.8 μg of DNA was mixed with 10 μl of lipofectamine in 0.2 ml of Opti-MEM (Life Technologies, Inc.) and incubated at room temperature for 30 min prior to the addition of 1.8 ml of Opti-MEM. COS-7 cells were exposed to the DNA/lipofectamine mixture for 5 h. Two ml of 20% (v/v) NCS in DMEM was then added to the cells. Cells were harvested 48 h after transfection. 24 h before harvest they were treated with pertussis toxin (50 ng/ml) to case complete ADP-ribosylation of the α subunits of endogenously expressed G_i-family G proteins [6].

2.5. Preparation of membranes

Plasma membrane-containing P2 particulate fractions were prepared from cell pastes that had been stored at −80°C following harvest. Cell pellets were resuspended in 0.5 ml of 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 (buffer A) and rupture of the cells was achieved with 50 strokes of a hand-held Teflon on-glass homogenizer followed by passage (10 times) through a 25-gauge needle. Cell lysates were centrifuged at 1000×*g* for 10 min in a Beckman TJ-6 centrifuge to pellet the nuclei and unbroken cells and P2 particulate fractions were then recovered by centrifugation of the supernatant at 200 000×*g* for 30 min in a Beckman Optima TLX ultracentrifuge using a Beckman TLA 100.2 rotor. P2 particulate fractions were resuspended in buffer A and stored at −80°C until required. Protein concentrations were determined using the bicinchoninic acid (BCA) procedure using BSA as standard.

2.6. [^{3}H]RS-79948-197 binding studies

Binding assays were initiated by the addition of 5–10 μg of protein to an assay buffer (10 mM Tris-HCl, 50 mM sucrose, 20 mM $MgCl_2$, pH 7.5) containing [^{3}H]RS-79948-197 [9] (0–1 nM). Non-specific binding was determined in the presence of 100 μM idazoxan. Reactions were incubated at 30°C for 45 min, and bound ligand was separated from free by vacuum filtration through GF/C filters. The filters were washed with 3×5 ml of assay buffer, and bound ligand was estimated by liquid scintillation spectrometry.

2.7. High affinity GTPase assays

These were performed essentially as described in [10] with V_{max} estimates being obtained following transformation of enzyme velocity vs. substrate (GTP) concentration experiments as described in [7]. Non-specific GTPase was assessed by parallel assays containing 100 μM GTP.

2.8. Agonist regulation of [^{35}S]GTPγS binding

Binding studies were performed essentially as in [11,12]. Typically, membranes (5 μg) were incubated at 30°C for 30 min in a final volume of 100 μl of reaction mixture comprising 20 mM HEPES-NaOH, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM ascorbate, 10 μM GDP, 5 nM [^{35}S]GTPγS (~350 nCi) in the presence or absence of agonist as described in the text. The non-specific binding component was determined by inclusion of GTPγS to a final concentration of 100 μM. The incubation was terminated by addition of 2.5 ml of ice-cold washing buffer comprising of 20 mM HEPES-NaOH, pH 7.4 and 3 mM $MgCl_2$ and rapid filtration through Whatman GF/C filters followed by three washes (5 ml) with ice-cold wash buffer. Filters were air-dried, dissolved in 5 ml ULTIMA-Gold XR scintillant (Packard), left overnight and counted on a Beckman LS-6500 liquid scintillation analyser. All assays were performed in triplicate.

3. Results

The α_{2A}-adrenoceptor-$Cys^{351}Gly$-$G_{i1}\alpha$ fusion protein was expressed transiently in COS-7 cells. Expression of this polypeptide (between 6–15 pmol/mg membrane protein in individual transfections) was detected by the specific binding of the high affinity ($K_d = 0.41 \pm 0.05$ nM, mean ± S.E.M., $n = 11$) and highly α_2-adrenoceptor selective antagonist [^{3}H]RS-79948-197 (Fig. 1). Immunoblotting with the $G_{i1}\alpha$ specific antiserum I1C also allowed detection of an approx. 100 kDa polypeptide only in membranes of transfected cells (data not shown but see [7,8]). In all experiments, prior to harvest, the cells were treated with pertussis toxin (50 ng/ml, 24 h) to eliminate any potential interactions between the receptor element of the expressed fusion protein and the endogenously expressed G_i-family G proteins [6]. We have shown previously that such pertussis toxin treatment is sufficient to cause complete

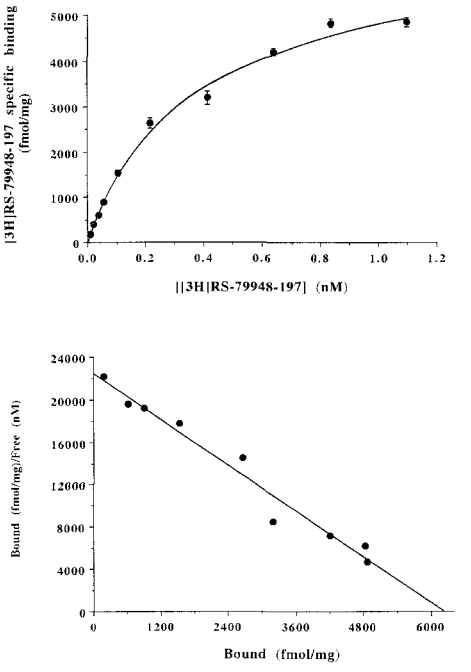

Fig. 1. Expression of an α_{2A}-adrenoceptor-$Cys^{351}Gly$-$G_{i1}\alpha$ fusion protein. P2 particulate membrane fractions of pertussis toxin-treated COS-7 cells transfected with the α_{2A}-adrenoceptor-$Cys^{351}Gly$-$G_{i1}\alpha$ cDNA were subjected to saturation binding studies using [^{3}H]RS-79948-197 (upper panel) as described in Section 2. Specific binding is displayed. This data was then converted to a Scatchard plot (lower panel). Similar studies were performed on all the individual transfections used in these studies (see Section 3 for details).

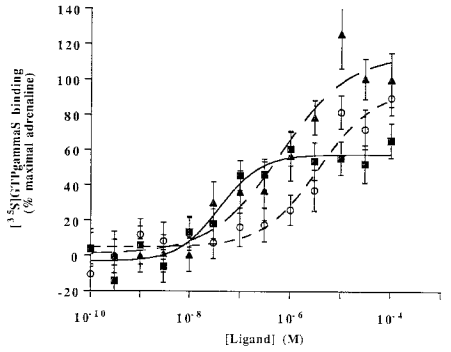

Fig. 2. Agonist-mediated stimulation of [^{35}S]GTPγS binding to α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$. The ability of varying concentrations of adrenaline (filled triangles), noradrenaline (open circles) and UK14304 (filled squares) to stimulate the binding of [^{35}S]GTPγS was measured, as described in Section 2, in membranes of pertussis toxin-treated COS-7 cells transfected to express the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein. Results are presented as means ± error using membranes derived from three separate transfections.

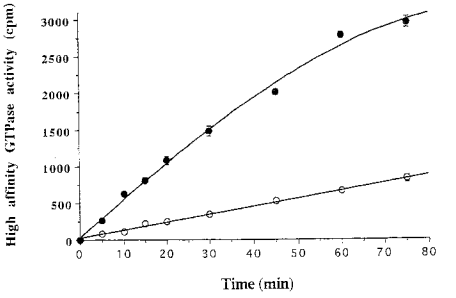

Fig. 3. Time course of basal and agonist-stimulated high affinity GTPase activity in cells expressing α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$. High affinity GTPase activity was measured at various times in membranes of pertussis toxin-treated COS-7 cells transfected to express the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein in the absence of ligand (open symbols) or in the presence of 1×10^{-4} M noradrenaline (filled symbols). Similar results were obtained in three further experiments.

ADP-ribosylation of the entire endogenous cellular population of G_i-like G proteins. Such treatment prevents agonist stimulation of high affinity GTPase activity following expression of the porcine α_{2A}-adrenoceptor in combination with wild-type $G_{i1}\alpha$ but not with Cys^{351}Gly-$G_{i1}\alpha$ [6].

Agonist stimulation of specific binding of [^{35}S]GTPγS to membranes of COS-7 cells could be measured following pertussis toxin treatment of cells transfected to express the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein (Fig. 2) but not in mock transfected cells (data not shown). This was achieved in a concentration-dependent manner (Fig. 2) with EC_{50} values for adrenaline ($7.9\pm1.0\times10^{-7}$ M), noradrenaline ($3.9\pm0.2\times10^{-6}$ M) and UK14304 ($3.7\pm0.2\times10^{-8}$ M) (means ± S.E.M., $n=3$). Although we had previously used UK14304 to demonstrate the functionality of the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein [7,8], the current studies demonstrated UK14304 to function as a partial agonist in comparison to adrenaline with estimated efficacy of some 50–60% of the endogenous ligand. The agonist-induced increase in specific binding of [^{35}S]GTPγS was sufficiently low in absolute terms, however, to limit its potential usefulness in further efficacy estimates. This may reflect that the stoichiometry of the proteins integrated into the fusion protein defines that a maximum of one G protein can be labelled per receptor binding site occupied. This would limit the theoretical labelling potential to one mol [^{35}S]GTPγS per mol of α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein. To overcome this problem we utilised high affinity GTPase activity measurement as an assay which conceptually can function as an accumulation assay over time. In membranes of pertussis toxin-treated, α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein expressing COS-7 cells basal high affinity GTPase activity continued in a linear fashion over at least 75 min (Fig. 3). Addition of adrenaline or noradrenaline (1×10^{-4} M) stimulated this activity. The effect of the agonists was also linear throughout at least a 60 min incubation (Fig. 3) and thus this period was selected for all further studies.

Concentration effect curves for noradrenaline stimulation of high affinity GTPase activity resulted in an EC_{50} of $9.7\pm5.3\times10^{-6}$ M (mean ± S.E.M., $n=3$). Concurrent concentration effect curves performed on the same membrane preparations with UK14304 resulted in a measured EC_{50} of $2.4\pm0.2\times10^{-7}$ M (mean ± S.E.M, $n=3$). However, it was again noticeable that UK14304 acted as only a partial agonist compared to noradrenaline (Fig. 4). A range of α_{2A}-adrenoceptor active ligands were compared to noradrenaline for their capacity to stimulate GTPase activity of the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein tethered G protein. Of those examined, only adrenaline ($EC_{50}=3.2\pm0.5\times10^{-6}$ M) and α-methylnoradrenaline ($EC_{50}=5.5\pm0.6\times10^{-6}$ M) (Fig. 4 and Table 1) also functioned as full agonists with xylazine, BHT933 and clonidine all functioning as partial agonists (Fig. 4 and Table 1). For comparison individual cDNAs encoding the α_{2A}-adrenoceptor and Cys^{351}Gly-$G_{i1}\alpha$ were co-expressed in COS-7 cells and the efficacy of the same series of agonists again assessed following pertussis toxin treatment of the cells (Table 1). All of the ligands shown to be partial agonists at the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein were also partial agonists in comparison to adrenaline for the separated receptor and G protein (Table 1). Although the rank order of efficacy was not different in the two receptor systems examined, the absolute efficacy of all of the partial agonists was

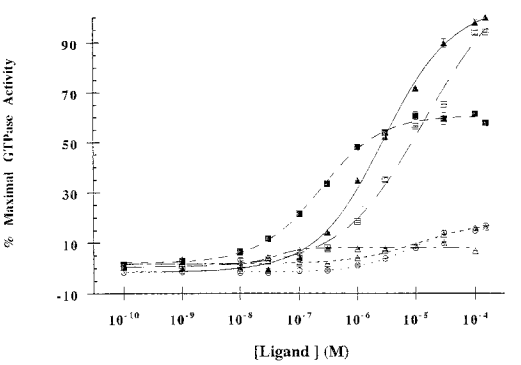

Fig. 4. Efficacy measurement of ligands at the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein. The capacity of varying concentrations of adrenaline (filled triangles), noradrenaline (open squares), UK14304 (filled squares), xylazine (open triangles), BHT933 (open circles) and clonidine to stimulate the high affinity GTPase activity of the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein was assessed following its expression in COS-7 cells and pertussis toxin pretreatment of the cells. Data are presented as the % of the stimulation produced by 1×10^{-4} M adrenaline. Results are means from three independent experiments.

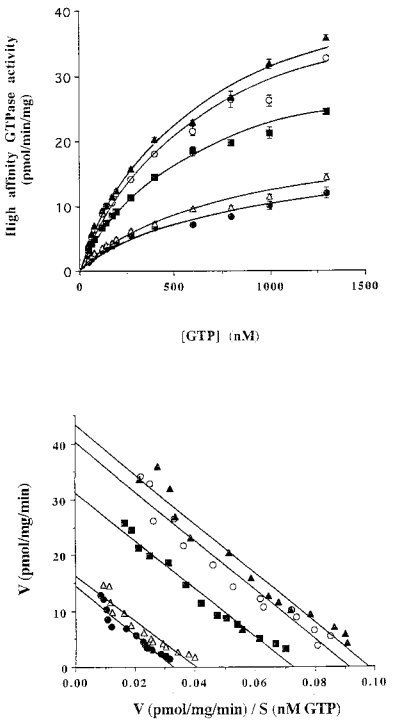

Fig. 5. Partial agonism at the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein is manifest in varying values for the V_{max} of the GTPase activity without alteration in K_m for GTP. High affinity GTPase activity was measured at varying concentrations of GTP (upper panel) in membranes of pertussis toxin-treated COS-7 cells transfected to express the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein in the absence of ligand (filled circles) or in the presence of 1×10^{-4} M adrenaline (filled triangles), α-methylnoradrenaline (open circles), UK14304 (filled squares) or xylazine (open triangles). The data were then transformed as a Eadie-Hofstee plot (lower panel) to allow direct estimation of V_{max}, K_m for GTP and the efficacy of the ligands compared to adrenaline. Similar results were obtained in three further experiments.

greater when examining the separate receptor and G protein compared to the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein (Table 1).

Standard high affinity GTPase activity assays, such as those above, are routinely performed with substrate (GTP) concentrations in the region of 5×10^{-7} M [9]. To assess if the measured differences in efficacy between adrenaline, α-methylnoradrenaline, UK14304 and xylazine truly reflected differences in the enzymic capacity of the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein when the agonist ligand binding site was occupied with these ligands, maximal GTP hydrolysis rates were measured. By treating the α_{2A}-adrenoceptor-Cys^{351}Gly-$G_{i1}\alpha$ fusion protein as an agonist-activated enzyme, assessment of GTP hydrolysis at varying concentrations of GTP (Fig. 5) could be used to assess V_{max} of the construct induced by differing agonists. Such data confirmed that xylazine and UK14304 functioned as partial agonists compared to adrenaline and α-methylnoradrenaline. As such, the partial agonist effects recorded when using a single concentration of GTP as substrate were not a reflection of distinct agonist-induced variations in the K_m of the construct for GTP (Fig. 5) (K_m for basal GTPase activity = $3.2\pm0.2\times10^{-7}$ M and in the presence of UK14304 (1×10^{-5} M) = $3.7\pm0.4\times10^{-7}$ M, means ± S.E.M., $n=9$ in each case) as this was not altered substantially by the identity or efficacy of the agonist.

4. Discussion

Agonist efficacy is a measure of the capacity of a ligand to induce a functional response [1–3]. Although often viewed simply as the 'strength' of the agonist, in molecular terms this can be considered as the capacity of a ligand to maintain or induce a GPCR conformation capable of activating a cognate G protein and thus to subsequently regulate downstream effectors. Assessment of the efficacy of an agonist at a GPCR can thus be measured at a variety of levels within the signal transduction cascade ranging from GPCR-G protein interaction to second messenger production or final physiological response. However, as G protein-coupled systems provide an amplification cascade, assessment of agonist efficacy can vary dependent upon the point of measurement and with alterations in levels of expression of individual protein components of the cascade [4,13]. For example, we have recently demonstrated increases in partial agonist efficacy with increasing levels of expression of the β_2-adrenoceptor in NG108-15 cells, as measured by the ability of the ligands to cause stimulation of adenylyl cyclase activity [4]. This is a reflection that the cellular levels of adenylyl cyclase represent the quantitatively limiting element in the cascade leading from GPCR via $G_s\alpha$ to adenylyl cyclase [13]. Furthermore, in situations in which a GPCR has the capacity to activate multiple G proteins leading to regulation of multiple second messengers [14] then differences in efficacy [15] and even of rank order of potency of agonist ligands have been described for the two

Table 1

Ligand	Efficacy to activate	
	α_{2A}-adrenoceptor–Cys^{351}Gly-$G_{i1}\alpha$ (% of adrenaline)	α_{2A}-adrenoceptor+Cys^{351}Gly-$G_{i1}\alpha$ (% of adrenaline)
Adrenaline	100	100
Noradrenaline	97.0 ± 1.8 (5)	98.7 ± 1.3 (3)
α-Methylnoradrenaline	104.9 ± 4.9 (3)	93.0 ± 3.9 (3)
UK14304	56.3 ± 2.0 (7)	85.3 ± 2.5 (3)
BHT933	19.8 ± 1.7 (5)	44.2 ± 1.8 (3)
Xylazine	16.1 ± 2.7 (7)	28.0 ± 3.1 (3)
Clonidine	14.5 ± 1.2 (2)	26.0 ± 5.3 (3)

The capacity of maximally effective concentrations of ligands with known agonist activity at α_2-adrenoceptors to stimulate high affinity GTPase activity of the α_{2A}-adrenoceptor–Cys^{351}Gly-$G_{i1}\alpha$ fusion protein or of the α_{2A}-adrenoceptor plus Cys^{351}Gly-$G_{i1}\alpha$ was assessed following their transient expression in COS-7 cells.
Efficacy was determined relative to the function of adrenaline (1×10^{-4} M) in parallel assays.
Data represent means ± S.E.M. and the values in parentheses indicate the number of independent experiments performed with each ligand.

functions [16–18]. Such a scenario has been described as receptor channelling [19,20]. Given such observations it is important to try to devise means by which measurements of ligand efficacy should not be hostage to such events because such pharmacology is often considered an indicator of the potential presence of multiple receptor subtypes. Given a large literature on the pharmacology and function of α_2-adrenoceptors (see [21,22] for reviews) we selected the α_{2A}-adrenoceptor for analysis.

We have recently produced a fusion protein between the porcine α_{2A}-adrenoceptor and the α subunit of the G protein G_{i1} by simply linking together the C-terminal tail of the receptor and the N-terminus of the G protein [7]. To allow ease of analysis this was constructed using a modified form of the G protein in which Cys^{351} was modified to Gly [6]. Cys^{351} is the normal acceptor site on the G protein α subunit for ADP-ribose transferred by the ADP-ribosyltransferase activity of pertussis toxin. ADP-ribosylation prevents productive information transfer between an agonist-occupied GPCR and the G protein [23]. As such, any potential interactions between the agonist-occupied fusion protein and the endogenous population of G_i-like G proteins could be prevented, allowing regulation of the attached G protein to be studied in situ following expression of the fusion protein and treatment of the cells with pertussis toxin. Introduction of the $Cys^{351}Gly$ mutation into $G_{i1}\alpha$ reduces the potency of the UK14304-occupied α_{2A}-adrenoceptor to activate the G protein [6]. Using this fusion protein we have shown previously that addition of the α_2-adrenoceptor agonist UK14304 results in an increase in GTPase activity of the attached G protein [7,8].

In the current studies we have extended this analysis to a variety of ligands which display agonist properties at the α_{2A}-adrenoceptor. We demonstrate that variation in agonist efficacy at the α_{2A}-adrenoceptor-$Cys^{351}Gly$-$G_{i1}\alpha$ fusion protein can be assayed at one of the most proximal points of measurement available, i.e. guanine nucleotide exchange and hydrolysis, as variation in GTP turnover rate and hence in V_{max} of the construct (Fig. 5). Initial experiments demonstrated greater hydrolysis of GTP by the α_{2A}-adrenoceptor-$Cys^{351}Gly$-$G_{i1}\alpha$ fusion protein by maximally effective concentrations of noradrenaline compared to UK14304 (Fig. 4), suggesting UK14304 to function as a partial agonist at this construct. Variation in measured efficacy was subsequently observed for a variety of ligands, with the endogenous agonists adrenaline and noradrenaline along with α-methylnoradrenaline all being equally and maximally efficacious (Table 1). By contrast, xylazine, BHT933 and clonidine were only weakly active with efficacy values in the region of 20% of those of the endogenous ligands. Although it is difficult to envisage conceptually, we wished to demonstrate that the measured variation in efficacy between ligands did not represent differences in the affinity of the G protein within the ligand bound fusion protein to bind GTP. Initial experiments were all performed (as are most high affinity GTPase assays) at a single subsaturating concentration of GTP. Measurement of the rate of GTP hydrolysis in response to maximally effective concentrations of the agonists was thus performed at various GTP concentrations. This confirmed that the K_m for GTP of the α_{2A}-adrenoceptor-$Cys^{351}Gly$-$G_{i1}\alpha$ fusion protein was not modified by the identity of the ligand. As such, the agonist-induced V_{max} provided a direct measurement of ligand efficacy (Fig. 5).

The results presented provide the first evidence that a chimaeric GPCR-G protein fusion protein can provide a novel means to measure agonist efficacy at a GPCR. The fixed 1:1 stoichiometry of the GPCR and G protein within the fusion protein further defines an estimate of ligand efficacy which should be independent of levels of receptor and G protein expression, two elements which can vary widely in different tissues. Also, as it should be possible to generate fusion proteins between a receptor and different G protein α subunits, we suggest that fusion proteins such as used herein may provide a means to determine efficacy values for agonist ligands for specific combinations of GRCR and G protein. Furthermore, the capacity to quantitate levels of expression of the fusion protein by saturation [^{3}H]ligand binding studies (Fig. 1) defines the total number of G proteins which can become activated at receptor saturating concentrations of agonist ligand and thus allows direct estimation of GTP turnover number [7] in response to differing agonists.

One concern with the approach employed is that the physical linkage of the G protein to the GPCR might limit or constrain the capacity of agonist ligands to induce GPCR conformations capable of resulting in guanine nucleotide exchange and G protein activation. We thus also measured efficacy of the same series of ligands following the separate co-expression of the α_{2A}-adrenoceptor and $Cys^{351}Gly$-$G_{i1}\alpha$. Although this resulted in increased estimates of efficacy of all of the partial agonists when compared to adrenaline, the rank order of efficacy was unchanged between the fusion protein and the separated receptor and G protein (Table 1). In these experiments, however, we are unable to quantitate the relative levels of GPCR and G protein expression. Furthermore, there is no way to define the number of copies of the G protein activated per receptor.

Interestingly, a variety of experiments have suggested that a 'pre-coupled' state may exist for α_2-adrenoceptors and G proteins [20,24] and the fusion protein construct used herein may inherently represent a 'pre-coupled' state. Equally, little is known about possible constraints placed in GPCR-G protein interactions in more native states by cellular location [25] and potential roles for the cytoskeleton [26,27] for example. Many experiments have indicated the capacity of a range of GPCRs to interact with multiple co-expressed G protein (see [14] for review). Recent evidence has suggested that certain agonists may 'channel' responses due to variations in potency and efficacy to promote or stabilise interactions of GPCRs with different individual G proteins (see [19] for review). It is interesting in this regard that although following expression of the α_{2A}-adrenoceptor in CHO cells Eason et al. [15] were able to demonstrate that all of the ligands they tested (many of which were thus deliberately used in this study) acted as full agonists to promote inhibition of adenylyl cyclase (via G_i), these ligands displayed a range of efficacy for activation of adenylyl cyclase (via G_s). As such, a means to separate and study agonist efficacy at individual GPCR-G protein contacts is clearly required. It will be of considerable interest, in time, to assess the efficacy of a range of ligands at an α_{2A}-adrenoceptor-$G_s\alpha$ fusion construct akin to the fusion protein used herein.

These studies provide for the first time a novel approach to the measurement of ligand efficacy and potentially their efficacy at specific GPCR-G protein tandems by fixing the receptor and G protein stoichiometry in a system and, by their

physical attachment, defining their proximity. We anticipate that this strategy will be widely applicable.

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