An Asp^{79}Asn mutation of the α_{2A}-adrenoceptor interferes equally with agonist activation of individual $G_{i\alpha}$-family G protein subtypes

Richard J. Ward, Graeme Milligan*

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, Davidson Building, University of Glasgow, Glasgow G12 8QQ, UK

Received 2 November 1999

Edited by Philip Randle

Abstract The quantitative effects of an Asp^{79}Asn mutation in the porcine α_{2A}-adrenoceptor on adrenaline-mediated stimulation of the α subunit of individual members of the G_i family of G proteins were assessed by measuring GTP turnover number for fusion proteins between the wild type or mutated receptor and pertussis toxin-resistant forms of each of G_{i1}, G_{i2} and G_{i3}. In each case the receptor mutation limited activation of the G protein to 8–14% of that produced by the wild type receptor. Previous demonstration that in a single cell this mutation selectively interferes with α_{2A}-adrenoceptor regulation of distinct effector end points transduced by G_i family members must therefore reflect differential requirements for amplification or the cellular location of individual, co-expressed, G proteins.

Key words: G protein-coupled receptor; G protein; Adrenaline; GTPase

1. Introduction

The α_{2A}-adrenoceptor is one of the most studied members of the G protein-coupled receptor (GPCR) superfamily which interact preferentially with pertussis toxin-sensitive, G_i-like, heterotrimeric G proteins. This receptor can regulate a wide range of effector end points via interaction with these G proteins including inhibition of adenylyl cyclase [1,2], stimulation of phospholipase D [3], stimulation of ERK mitogen-activated protein activity [4,5], stimulation of K^+ currents [2] and inhibition of Ca^{2+} currents [2]. Furthermore, at least with high receptor expression levels and high receptor occupancy, effector regulation via activation of other G proteins can also be uncovered [6,7].

Interaction of the α_{2A}-adrenoceptor with the full range of pertussis toxin-sensitive G_i family G proteins has been recorded and studied both in liposome reconstitution experiments [8] and following co-transfection of the receptor and appropriate G proteins into mammalian cells [9]. Importantly, both α_{2A}-adrenoceptor-mediated activation of multiple G_i isoforms [1,10] and regulation of multiple effectors by these G proteins [2,3] have been recorded concurrently in individual cells.

The Asp residue at position 79 of the α_{2A}-adrenoceptor, which lies in transmembrane region 2, is the most conserved amino acid in this membrane spanning domain in class I GPCRs and thus in the nomenclature of van Rhee and Jacobson [11] is denoted 2.50. This amino acid is important for allosteric regulation by Na^+ of this [12,13] and other GPCRs. Mutation of this residue to Asn has been shown to result in elimination of agonist-mediated regulation of K^+ currents but not of inhibition of adenylyl cyclase or Ca^{2+} currents when the modified receptor was expressed in AtT20 anterior pituitary cells [2,14]. A possible explanation for these findings was that the mutation selectively interferes with the activation of one specific G_i family G protein. Alternatively, the mutation may reduce the capacity of the receptor to activate all G proteins but the relative expression levels of individual G proteins in cells then determine whether effector regulation can still be recorded [7]. These possibilities have not previously been addressed experimentally.

Herein we use a strategy in which fusion proteins between either the wild type or a Asp^{79}Asn mutant of the porcine α_{2A}-adrenoceptor and pertussis toxin-insensitive forms of each of $G_{i1}\alpha$, $G_{i2}\alpha$ and $G_{i3}\alpha$ allow direct measurement of receptor activation of the G proteins in circumstances in which the molar ratio and proximity of receptor and G protein are identical. We demonstrate that the mutation non-selectively modulates the capacity of the receptor to activate these closely related G proteins with an 85–90% reduction in the capacity of adrenaline to stimulate each G protein.

2. Materials and methods

2.1. Materials

All materials for tissue culture were supplied by Life Technologies, Inc. (Paisley, Strathclyde, UK). [^{3}H]RS-79948-197 (90 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Amersham, Buckinghamshire, UK). γ[^{32}P]GTP (30 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA, USA). Pertussis toxin (240 μg/ml) and all other basic chemicals were purchased from Sigma (Poole, Dorset, UK) or Boehringer-Mannheim (Mannheim, Germany) and were of the highest purity available.

2.2. Construction of the α_{2A}-adrenoceptor–G protein fusion proteins

The porcine α_{2A}-adrenoceptor [15] and the Asp^{79}Asn mutant of this receptor [12] were obtained from Dr L.E. Limbird, Vanderbilt University, Nashville, TN, USA. A Cys^{351}Gly mutant of rat $G_{i1}\alpha$ was linked to the wild type α_{2A}-adrenoceptor as described previously to generate α_{2A}-adrenoceptor–Gly$^{351}G_{i1}\alpha$ [16] and ligated into the *Kpn*I and *Eco*RI sites of the eukaryotic expression vector pcDNA3 (InVitrogen, San Diego, CA, USA). Arg^{351}, Ser^{351}, Val^{351}, Phe^{351}, Leu^{351} and Ile^{351} rat $G_{i1}\alpha$ cDNAs in pcDNA3 [17] were digested with the restriction enzymes *Sac*II and *Eco*RI. The 0.7 kb fragments so produced were recovered and ligated with α_{2A}-adrenoceptor–Gly$^{351}G_{i1}\alpha$ in pCDNA3 from which the equivalent 1.3 kb *Sac*II-*Eco*RI fragment had been removed. This resulted in generation of a series of α_{2A}-adrenoceptor–Xaa$^{351}G_{i1}\alpha$ fusion proteins in pcDNA3. Equivalent strategies were used to generate α_{2A}-adrenoceptor–Ile$^{352}G_{i2}\alpha$, α_{2A}-

*Corresponding author. Fax: (44)-141-330 4620.
E-mail: g.milligan@bio.gla.ac.uk

Abbreviations: GPCR, G protein-coupled receptor

PII: S0014-5793(99)01581-1

adrenoceptor–Ile^{351}G$_{i3}\alpha$ and the Asp^{79}Asn α_{2A}-adrenoceptor–Ile^{351}G$_{i1}\alpha$, –Ile^{352}G$_{i2}\alpha$ and –Ile^{351}G$_{i3}\alpha$ fusion constructs.

2.3. Cell culture and transfection

COS-7 cells were maintained in DMEM containing 10% (v/v) newborn calf serum, 2 mM L-glutamine. Cells were seeded in 100 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.) [16]. For transfection, 5 μg of DNA was mixed with 20 μl of lipofectamine in 1.2 ml of Opti-MEM (Life Technologies, Inc.) and incubated at room temperature for 30 min prior to the addition of 4.8 ml of Opti-MEM. COS-7 cells were exposed to the DNA/lipofectamine mixture for 5 h. 6 ml of 20% (v/v) newborn calf serum in DMEM was then added to the cells. Cells were harvested 48 h after transfection. Cells were treated for the final 24 h prior to cell harvest with pertussis toxin (50 ng/ml).

2.4. Preparation of membranes

Plasma membrane-containing P2 particulate fractions were prepared from cell pastes that had been stored at −80°C following harvest as described previously [18].

2.5. [^{3}H]RS-79948-197 binding studies

Binding assays were initiated by the addition of 5 μg of protein to an assay buffer (75 mM Tris–HCl, 5 mM EDTA, 12.5 mM $MgCl_2$, pH 7.5) containing [^{3}H]RS-79948-197 [9,16,19] (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.

2.6. High affinity GTPase assays

These were performed as described in [16,20,21]. Non-specific GTPase was assessed by parallel assays containing 100 μM GTP. All experiments were performed at least three times on membranes prepared from individual cell transfections.

3. Results

In previous experiments using fusion proteins between the porcine α_{2A}-adrenoceptor and the α subunit of G$_{i1}$ the construct was rendered insensitive to the ADP-ribosyltransferase activity of pertussis toxin by replacement of the acceptor Cys^{351} of the G protein with Gly [16,20]. This was designed to ensure that following pertussis toxin treatment of cells agonist stimulation of high affinity GTPase activity must reflect activation of the receptor-linked G protein. However, following introduction of an Asp^{79}Asn mutation into the α_{2A}-adrenoceptor within this pertussis toxin-resistant fusion protein the capacity of adrenaline to stimulate GTPase activity of the fusion protein was sufficiently poor to make detailed analysis of the effect of the Asp^{79}Asn mutation difficult to quantitate (data not shown).

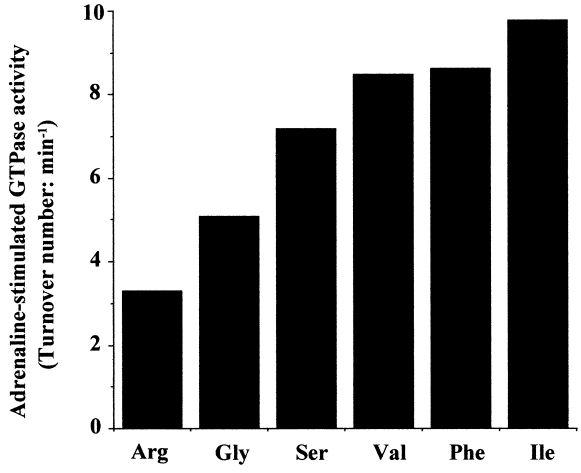

Fig. 1. The identity of residue 351 of G$_{i1}\alpha$ determines adrenaline-stimulated GTPase turnover number of α_{2A}-adrenoceptor–G$_{i1}\alpha$ fusion proteins. α_{2A}-Adrenoceptor–G$_{i1}\alpha$ fusion proteins in which residue 351 of the G protein was either Arg, Gly, Ser, Val, Phe or Ile were expressed transiently in COS-7 cells. Membranes from these transfected cells were used to measure the levels of expression of each construct and the capacity of adrenaline (100 μM) to stimulate high affinity GTPase activity. Extrapolation of the stimulated activity to V_{max} allowed calculation of adrenaline-stimulated GTP turnover number (min^{-1}) for each fusion construct. Results are the means of at least three experiments performed on separate membrane preparations.

In co-transfection experiments utilising the porcine α_{2A}-adrenoceptor and forms of G$_{i1}\alpha$ in which Cys^{351} was replaced by each of the other naturally occurring amino acids Gly^{351}G$_{i1}\alpha$ was relatively poorly activated by agonists at this receptor. By contrast, the presence of a highly hydrophobic amino acid at this position allowed even greater activation than the wild type sequence [17]. We therefore constructed fusion proteins between the wild type porcine α_{2A}-adrenoceptor and a number of residue^{351}-substituted (Arg, Gly, Ser, Val, Phe and Ile) forms of G$_{i1}\alpha$. Each of these fusion constructs was expressed transiently in COS-7 cells which were subsequently treated with pertussis toxin. Membrane fractions were prepared and levels of expression of the constructs recorded in each separate transfection by the specific binding of the high affinity α_{2A}-adrenoceptor antagonist [^{3}H]RS-79948-197. Basal high affinity GTPase activity and its stimulation by

Table 1
Characteristics of the α_{2A}-adrenoceptor–G$_i\alpha$ fusion proteins

Fusion construct	K_d [^{3}H]RS (nM)	K_m GTP (nM) basal	K_m GTP (nM) 100 μM adrenaline	Turnover number (min^{-1})	Asp^{79}Asn turnover number as a percentage of Asp^{79}
α_{2A}G$_{i1}\alpha$C^{351}I	0.55 ± 0.06	340 ± 13	449 ± 17	10.0 ± 0.7	
α_{2A}D^{79}NG$_{i1}\alpha$C^{351}I	0.45 ± 0.03	374 ± 33	306 ± 18	0.8 ± 0.2	8.3 ± 1.5
α_{2A}G$_{i2}\alpha$C^{352}I	0.40 ± 0.10	347 ± 19	291 ± 14	6.5 ± 0.3	
α_{2A}D^{79}NG$_{i2}\alpha$C^{352}I	0.70 ± 0.08	344 ± 16	359 ± 32	0.7 ± 0.3	11.7 ± 4.7
α_{2A}G$_{i3}\alpha$C^{351}I	0.47 ± 0.04	317 ± 17	389 ± 19	5.1 ± 0.5	
α_{2A}D^{79}NG$_{i3}\alpha$C^{351}I	0.50 ± 0.08	351 ± 40	360 ± 20	0.7 ± 0.2	14.0 ± 2.3

Saturation ligand binding studies were employed to measure K_d for [^{3}H]RS-79948-197 at the various fusion proteins and to measure their expression levels. These varied between 1.7 and 14.5 pmol/mg membrane protein in individual transfections. B_{max} values were used in association with adrenaline (100 μM)-stimulated high affinity GTPase activity at V_{max} to calculate GTP turnover values for each construct. Data are presented as means ± S.E.M., $n = 3$ in each case. Adrenaline-stimulated turnover of GTP produced by the Asp^{79}Asn α_{2A}-adrenoceptor as a % of that produced by the wild type receptor was not statistically different ($P > 0.05$) between the three individual subtypes of G$_i$.

adrenaline (100 μM) were then measured at a range of concentrations of GTP [16]. Extrapolation of such data to V_{max} indicated that adrenaline stimulated the maximal GTPase activity of all the individual constructs. However, calculation of adrenaline-stimulated turnover number demonstrated the $Arg^{351}G_{i1}\alpha$-containing fusion protein to be least effectively activated by the agonist and the $Ile^{351}G_{i1}\alpha$-containing fusion protein to be the most effectively activated (Fig. 1).

Based on these observations an $Asp^{79}Asn$ α_{2A}-adrenoceptor–$Ile^{351}G_{i1}\alpha$ fusion protein was constructed and expressed transiently in COS-7 cells. These mutations did not significantly alter the affinity of binding of [^{3}H]RS-79948-197 to this or any of the other fusion proteins used (Table 1). Adrenaline stimulation of the GTPase activity of this construct was then compared directly with that of the wild type α_{2A}-adrenoceptor–$Ile^{351}G_{i1}\alpha$ fusion protein (Fig. 2). Adrenaline was able to cause the $Asp^{79}Asn$ α_{2A}-adrenoceptor to produce only 8.3 ± 1.5% of the GTPase turnover number of the fusion protein-linked $G_{i1}\alpha$ compared to the wild type receptor (Table 1), even though the strategy used ensures that the ratio of receptor to G protein must be maintained at a 1:1 stoichiometry and that the physical proximity of receptor and G protein must be the same in the two constructs. Moreover, the $Asp^{79}Asn$ mutation did not alter the K_m for GTP as substrate (Table 1).

To ascertain whether there were differential effects of the $Asp^{79}Asn$ α_{2A}-adrenoceptor mutation on activation of the other $G_i\alpha$ subtypes, equivalent fusion constructs were generated between both the wild type and $Asp^{79}Asn$ α_{2A}-adrenoceptor and each of $Ile^{352}G_{i2}\alpha$ and $Ile^{351}G_{i3}\alpha$. Following transient expression of wild type and $Asp^{79}Asn$ α_{2A}-adrenoceptor–$Ile^{352}G_{i2}\alpha$, parallel [^{3}H]RS-79948-197 ligand binding and adrenaline (100 μM)-stimulated GTPase measurements (Fig. 3a) indicated that the $Asp^{79}Asn$ substitution equally severely restricted the activation of $G_{i2}\alpha$. This mutation reduced stimulation of the turnover of GTP by $G_{i2}\alpha$ to 11.7 ± 4.7% of that produced by the construct containing the wild type receptor (Table 1). Equivalent experiments with fusion constructs containing $Ile^{351}G_{i3}\alpha$ (Fig. 3b) indicated that

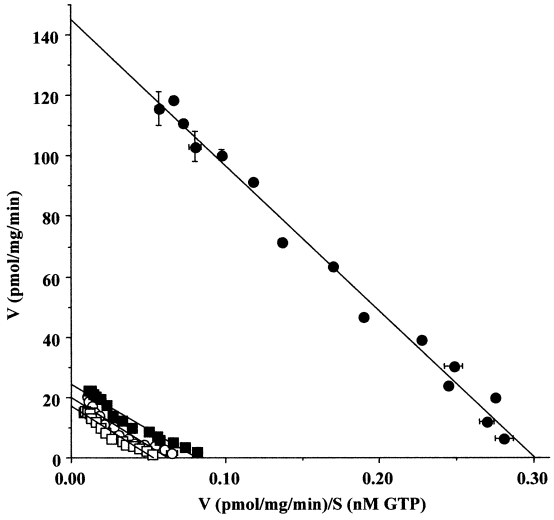

Fig. 2. The effects of an $Asp^{79}Asn$ mutation in the α_{2A}-adrenoceptor on adrenaline-stimulated GTPase activity of α_{2A}-adrenoceptor–$G_{i1}\alpha$ fusion proteins. Wild type α_{2A}-adrenoceptor–$Ile^{351}G_{i1}\alpha$ (circles) and $Asp^{79}Asn$ α_{2A}-adrenoceptor–$Ile^{351}G_{i1}\alpha$ (squares) fusion proteins were expressed in COS-7 cells and membranes prepared. Both basal (open symbols) and adrenaline (100 μM)-stimulated (filled symbols) high affinity GTPase activity was measured at a range of GTP concentrations. Parallel measurement of fusion protein expression levels allowed quantitation of the effect of the $Asp^{79}Asn$ mutation (see Table 1).

the effect of the $Asp^{79}Asn$ substitution of the α_{2A}-adrenoceptor on adrenaline activation of this G protein was as dramatic as that observed with the other G proteins (Table 1).

Previous co-transfection studies using the wild type and $Asp^{79}Asn$ porcine α_{2A}-adrenoceptor and $G_{i2}\alpha$ have indicated that the receptor mutation substantially reduces the EC_{50} for ligand activation of G protein without altering agonist binding affinity [7]. To explore this in detail membranes expressing either wild type or $Asp^{79}Asn$ α_{2A}-adrenoceptor–$Ile^{352}G_{i2}\alpha$ fusion proteins were exposed to varying concentrations of adrenaline (Fig. 4). Although the adrenaline stimulation of

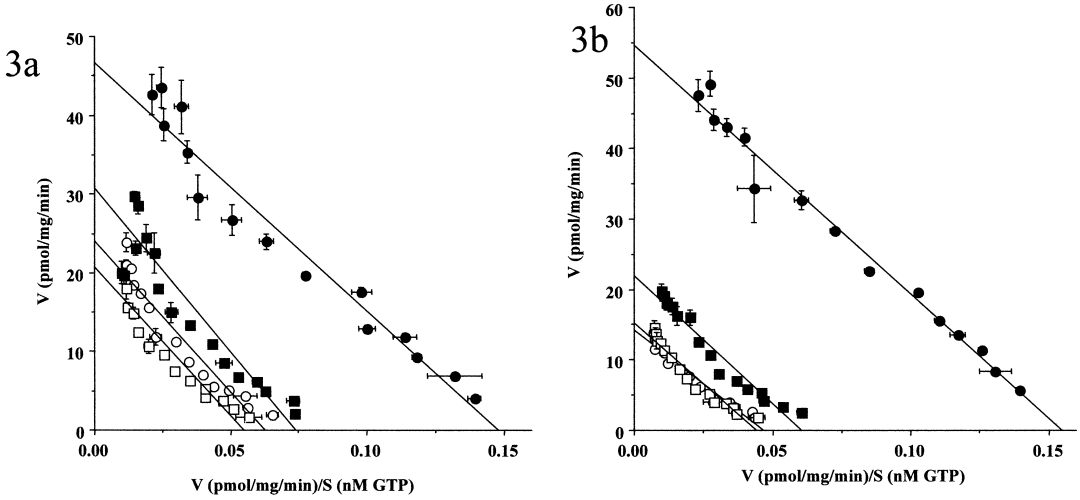

Fig. 3. The effects of an $Asp^{79}Asn$ mutation in the α_{2A}-adrenoceptor on adrenaline-stimulated GTPase activity of α_{2A}-adrenoceptor–$G_{i2}\alpha$ and $G_{i3}\alpha$ fusion proteins. a: α_{2A}-Adrenoceptor–$G_{i2}\alpha$. b: α_{2A}-adrenoceptor–$G_{i3}\alpha$. Wild type α_{2A}-adrenoceptor–$Ile^{351}G_{i2}\alpha$ (a) or –$Ile^{351}G_{i3}\alpha$ (b) (circles) and $Asp^{79}Asn$ α_{2A}-adrenoceptor–$Ile^{351}G_{i2}\alpha$ (a) or –$Ile^{351}G_{i3}\alpha$ (b) (squares) fusion proteins were expressed in COS-7 cells and membranes prepared. Both basal (open symbols) and adrenaline (100 μM)-stimulated (filled symbols) high affinity GTPase activity was measured at a range of GTP concentrations. See Table 1 for quantitative analysis.

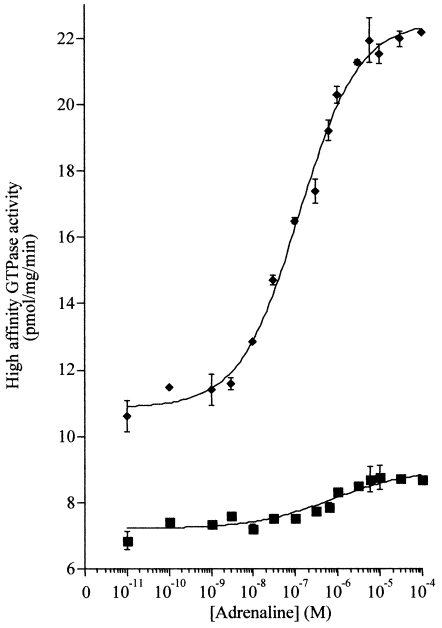

Fig. 4. The effect of an $Asp^{79}Asn$ mutation in the α_{2A}-adrenoceptor on the potency of adrenaline to stimulate $G_{i2}\alpha$. Membranes as in Fig. 3a expressing either the wild type α_{2A}-adrenoceptor–$Ile^{351}G_{i2}\alpha$ (diamonds) or the $Asp^{79}Asn$ α_{2A}-adrenoceptor–$Ile^{351}G_{i2}\alpha$ (squares) fusion proteins were exposed to varying concentrations of adrenaline and high affinity GTPase activity measured at 0.5 μM GTP.

$Asp^{79}Asn$ α_{2A}-adrenoceptor–$Ile^{352}G_{i2}\alpha$ was sufficiently small to make estimates of EC_{50} imprecise, the measured value of 7.6×10^{-7} M was only some 6-fold greater than for stimulation of the wild type α_{2A}-adrenoceptor–$Ile^{352}G_{i2}\alpha$ fusion protein (1.3×10^{-7} M) (Fig. 4).

4. Discussion

Mutation of an Asp residue, predicted to lie within transmembrane helix 2, which is the most highly conserved amino acid in this transmembrane domain, has been performed for a wide range of class 1 GPCRs. In general, mutation of this residue to an Asn results in a decreased efficiency of the GPCR to generate a downstream signal and thus to interact with relevant G proteins [22]. In the case of the α_{2A}-adrenoceptor, although it is capable of interacting with all widely expressed members of the pertussis toxin-sensitive G_i family and of thus regulating a wide range of effector endpoints, data have appeared which could be interpreted as indicating that the $Asp^{79}Asn$ mutation might selectively interfere with contacts between the receptor and different G proteins. For example, following expression in AtT20 pituitary cells, agonist occupancy of the wild type GPCR allowed pertussis toxin-sensitive inhibition of adenylyl cyclase and Ca^{2+} currents and stimulation of K^+ current [2]. However, following expression of an $Asp^{79}Asn$ mutant of this receptor, agonist regulation of the K^+ current was lost but both of the other two endpoints were maintained. Given that earlier antibody uncoupling experiments had suggested key and separate roles for G_{i2} in mediating α_{2A}-adrenoceptor inhibition of adenylyl cyclase in platelet membranes [23] and for G_o in α_2-adrenoceptor control of Ca^{2+} channels in NG108-15 cells [24] then selective uncoupling of G proteins by the $Asp^{79}Asn$ mutation is clearly an attractive hypothesis. An alternative view is that the mutation would non-selectively interfere with coupling to each of the G_i-like G proteins as these are highly similar [7]. In such a situation the apparent selective effect on one measured endpoint might reflect different levels of expression of the individual G proteins and/or the degree of amplification of the initial receptor–G protein interaction required to modulate each endpoint. Neither of these issues has previously been directly addressed.

In recent times we have made substantial use of a fusion protein between the wild type porcine α_{2A}-adrenoceptor and a pertussis toxin-resistant form of $G_{i1}\alpha$ in which the acceptor site for pertussis toxin-catalysed ADP-ribosylation (Cys^{351}) was converted to Gly [16,19,20]. This and other related GPCR–G protein fusion proteins ([25,26], see [27] for review) have a series of advantages for the quantitative analysis of GPCR–G protein interactions and the effects of point mutations in either GPCR or G protein. These include the necessity that the two elements of the fusion protein are present in a 1:1 stoichiometry and that the proximity of the two elements is defined and constrained by their physical linkage. However, in many regards, the key issue is that the fusion proteins function as agonist-activated GTPases and as such can be used for enzyme kinetic analyses. Therefore, for the current study we constructed fusion proteins between either the wild type or $Asp^{79}Asn$ α_{2A}-adrenoceptor and each of $G_{i1}\alpha$, $G_{i2}\alpha$ and $G_{i3}\alpha$.

In initial experiments performed at a single concentration of GTP (0.5 μM) the capacity of a receptor saturating concentration of adrenaline to stimulate the GTPase activity of fusion proteins in which either the wild type or $Asp^{79}Asn$ α_{2A}-adrenoceptor were linked to $Gly^{351}G_{i1}\alpha$ was compared. Although these experiments demonstrated a dramatic decrease in effect via the $Asp^{79}Asn$ α_{2A}-adrenoceptor, the capacity of adrenaline to stimulate this construct was sufficiently poor to make quantitative analysis difficult.

We have recently shown in co-transfection experiments that the identity of the amino acid at residue 351 of $G_{i1}\alpha$ can profoundly affect the extent of receptor-mediated activation [17]. As such, we constructed five further fusion proteins between the wild type α_{2A}-adrenoceptor and forms of $G_{i1}\alpha$ with different amino acids (Arg, Val, Phe and Ile) at residue 351. Adrenaline-stimulated turnover numbers were calculated for each using measurement of ligand-stimulated GTPase activity at V_{max} and of the levels of expression of each construct from saturation ^3H ligand binding studies. A three-fold range of adrenaline-stimulated GTPase turnover numbers was recorded with the $Ile^{351}G_{i1}\alpha$-containing construct producing the highest values. A fusion protein between the $Asp^{79}Asn$ α_{2A}-adrenoceptor and $Ile^{351}G_{i1}\alpha$ allowed sufficient adrenaline stimulation of GTPase activity that accurate estimates of the effect of the receptor mutation on G protein activation could be recorded by calculation of turnover number at V_{max} and comparison with the values for the equivalent fusion protein containing the wild type receptor sequence. Similar $Ile^{351}G_{i3}\alpha$- and $Ile^{352}G_{i2}\alpha$-containing constructs were also much less effectively activated by adrenaline once the $Asp^{79}Asn$ α_{2A}-adrenoceptor was introduced into the fusion proteins.

These results indicate clearly and quantitatively that a $Asp^{79}Asn$ mutation in the α_{2A}-adrenoceptor interferes equivalently with activation of the individual G_i subtypes. Based on the results of Suprenant et al. [2], Limbird and colleagues generated a transgenic mouse in which the wild type α_{2A}-

adrenoceptor gene sequence was replaced with one incorporating the $Asp^{79}Asn$ substitution [28] with the idea of understanding the role of specific receptor–G protein interactions and thus effector systems in producing the wide ranging physiological effects attributed to the α_{2A}-adrenoceptor by classical pharmacology and receptor knockout experiments (see [29] for review). This approach was limited by the poor expression of the $Asp^{79}Asn$ α_{2A}-adrenoceptor compared to the wild type [28] and in essence the transgenic animal behaved very much as anticipated for a true α_{2A}-adrenoceptor knock-out [30]. However, from the results provided herein one must assume that if the expression levels of the mutant had been equivalent to the wild type GPCR, the mouse model could not have provided selective information on the roles and physiological function of activation of G_i subtypes by the α_{2A}-adrenoceptor.

The generation of fusion proteins between a GPCR and closely related G protein α subunits has been integral to this study as they have allowed direct quantitative analysis in circumstances in which the expression ratio of each G protein to the GPCR was constant. Furthermore, analysis of ligand regulation of G protein activation was extrapolated to V_{max} for each construct as estimated concentrations of intracellular guanine nucleotides indicate this to be the likely situation in vivo. This is the first direct quantitative demonstration of the effect of a single point mutation in a GPCR on signal transmission to closely related but individual G proteins. Differential effects of a single GPCR mutation on different effector systems which are mediated by distinct but closely related G proteins thus must result from differential levels of signal amplification required to produce the effect or from differences in G protein expression levels or localisation.

Acknowledgements: This work was supported by the Medical Research Council and the European Union BIOMED 2 programme: 'Inverse agonism: Implications for drug design'.

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