FEBS Letters 355 (1994) 166–170

FEBS 14782

Receptor availability defines the extent of agonist-mediated G-protein down-regulation in neuroblastoma × glioma hybrid cells transfected to express the β2-adrenoceptor

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Received 8 October 1994

Abstract Sustained exposure of neuroblastoma × glioma hybrid, NG108-15, cells transfected to express the human β2-adrenoceptor (clone βN22) to isoprenaline or iloprost (an agonist at the endogenously expressed IP prostanoid receptor) resulted in a substantial and selective down-regulation of the α subunit of the G-protein G_s. Treatment of these cells with the irreversible β-adrenoceptor antagonist bromoacetyl alprenolol menthane diminished both the potency and the maximal ability of isoprenaline but not of iloprost to cause $G_s\alpha$ down-regulation. These results demonstrate that the extent of agonist-mediated $G_s\alpha$ down-regulation is dependent upon the availability of receptor to agonist.

Key words: G-protein; Adrenoceptor; Adenylyl cyclase; Neuroblastoma

1. Introduction

Agonist-mediated selective down-regulation of the G-protein(s) activated upon receptor occupancy has become a well established phenomenon [1]. However, despite this, in many systems little or no detectable regulation is observed. For example, in neuroblastoma × glioma hybrid, NG108-15, cells agonists at each of the endogenously expressed IP prostanoid, adenosine A2 and secretin receptors cause stimulation of adenylyl cyclase but only those at the IP prostanoid receptor cause down-regulation of $G_s\alpha$ [2,3]. This effect is not mimicked by analogues of cAMP [3] and we have hypothesised that G-protein down-regulation may reflect the levels of expression of the individual receptors [4]. As there are technical limitations in the ability to accurately quantitate each of these receptors we have transfected NG108-15 cells to express the human β2-adrenoceptor [5,6] and in the present study regulate agonist access to the receptor population by pretreatment of the cells with varying concentrations of an irreversible β-adrenoceptor antagonist to determine directly the importance of receptor levels in the observation of agonist-mediated down-regulation of $G_s\alpha$.

2. Materials and methods

2.1. Materials

All materials for tissue culture were from Gibco/BRL. [^{3}H]Dihydroalprenolol (56 Ci/mmol) was from Amersham International. Bromoacetyl alprenolol menthane (BAAM) was from RBI (Natick, MA, USA). All other chemicals were from Sigma or BDH and were of the highest purity available.

2.2. Generation of clone βN22

Has previously been described in detail [5–6]. A cDNA encoding the human β2-adrenoceptor was ligated downstream of the β-actin promoter of plasmid pJM16 [7] which harbours a copy of the neomycin resistance gene. 10 μg of this purified DNA was stably transfected into NG108-15 cells using Lipofectin reagent (Gibco/BRL) according to the manufacturers instructions. Clone βN22 was one of those selected and expanded [5–6].

2.3. Cell growth

Clone βN22 cells were grown in tissue culture as previously described [5,6] in the presence of geneticin sulphate (800 μg/ml). Prior to confluency they were either split 1:10 into fresh tissue culture flasks or harvested. Membrane fractions were prepared from cell pastes which had been stored at −80°C following harvest essentially as in [8]. Frozen cell pellets were suspended in 5 ml 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 (buffer A) and rupture of the cells achieved with 25 strokes of a hand-held teflon on glass homogenizer. The resulting homogenate was centrifuged at 500 × g for 10 min in a Beckman L5-50B centrifuge with a Ti 50 rotor to remove unbroken cells and nuclei. The supernatant fraction from this treatment was then centrifuged at 48,000 × g for 10 min and the pellet from this treatment washed and resuspended in 10 ml buffer A. Following a second centrifugation at 48,000 × g for 10 min the membrane pellet was resuspended in buffer A to a final protein concentration between 1–3 mg/ml and stored at −80°C until required.

2.4. Treatment of cells

Cells of clone βN22 were treated in culture with either iloprost (up to 10 μM) (Schering Health Care, Burgess Hill, Sussex, UK) or with isoprenaline (up to 10 μM) (Sigma) including vehicle either without or following pretreatment of the cells with BAAM (up to 10 μM) for 4 h. Preliminary studies indicated that the vehicles and BAAM alone had no effect on levels of $G_s\alpha$ over the time course of the treatments (data not shown).

2.5. Production of antisera and immunoblotting

Antiserum CS was produced by a New Zealand White rabbit following immunization with a glutaraldehyde conjugate of keyhole limpet haemocyanin (Calbiochem) and a synthetic peptide, RMHLRQYELL, which corresponds to the C-terminal decapeptide of all forms of the α subunit of G_s. The specificity of this antiserum for $G_s\alpha$ has previously been demonstrated [9]. Immunoblotting with this antiserum was performed as previously described [10]. Molecular mass determinations were based on pre-stained molecular-mass markers (Bethesda Research Laboratories). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% (w/v) acrylamide) was carried out overnight at 60 V.

2.6. Quantitation of immunoblots

Following SDS-PAGE, proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 2 h in 5% gelatin in phosphate-buffered saline, pH 7.5 (PBS). Primary antisera were added in 1% gelatin in PBS containing 0.2% Nonidet P40 (NP 40) and incubated for

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Abbreviations: BAAM, bromoacetyl alprenolol menthane; DHA, dihydroalprenolol.

SSDI 0014-5793(94)01169-9

at least 2 h. The primary antiserum was then removed and the blot washed extensively with PBS containing 0.2% NP40. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase (Scottish Antibody Production Unit, Wishaw, Scotland) was added (1:200 dilution in 1% gelatin in PBS containing 0.2% NP 40) and incubated with the nitrocellulose for 2 h. The antiserum was then removed and following extensive washing of the blot with PBS containing 0.2% NP40 and finally with PBS alone, the blot was developed using *o*-dianisidine hydrochloride (Sigma) as the substrate for horseradish peroxidase as previously described [10]. The developed immunoblots were scanned with a Biorad model GS-670 Imaging densitometer. Background was subtracted by scanning of equivalent sized areas of nitrocellulose which did not contain immunoreactive protein.

2.7. Binding experiments with [^{3}H]dihydroalprenolol

Were routinely performed with 2.0 nM [^{3}H]dihydroalprenolol ([^{3}H]DHA) at 30°C for 30 min in 20 mM Tris-HCl (pH 7.5), 50 mM sucrose, 20 mM $MgCl_2$ (buffer B) in the absence and presence of 10 μM propranolol to define maximal and non-specific binding respectively. Specific binding, defined as above, represented greater than 90% of the total binding of [^{3}H]DHA. All binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes (5 ml) with ice-cold buffer B.

2.8. Adenylyl cyclase assays

These were performed as described by Milligan et al. [11]. Each assay contained 100 mM Tris-HCl, pH 7.5, 20 mM creatine phosphate, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM cAMP, 1 μM GTP, 10 units creatine phosphokinase and 0.2 mM ATP containing 1 μCi [α-^{32}P]ATP. Separation of radiolabelled cyclic AMP and ATP was achieved using the double column method described by Johnson and Salomon [12].

2.9. Data analysis

All data were analysed using the Kaleidograph (Version 2.1) curve fitting programme driven by an Apple McIntosh computer.

3. Results

Membranes derived from cells of clone βN22, which was generated following transfection of neuroblastoma × glioma hybrid, NG108-15, cells with a plasmid containing a cDNA encoding the human β2-adrenoceptor [5,6], were examined for expression of this receptor by measuring the specific binding of

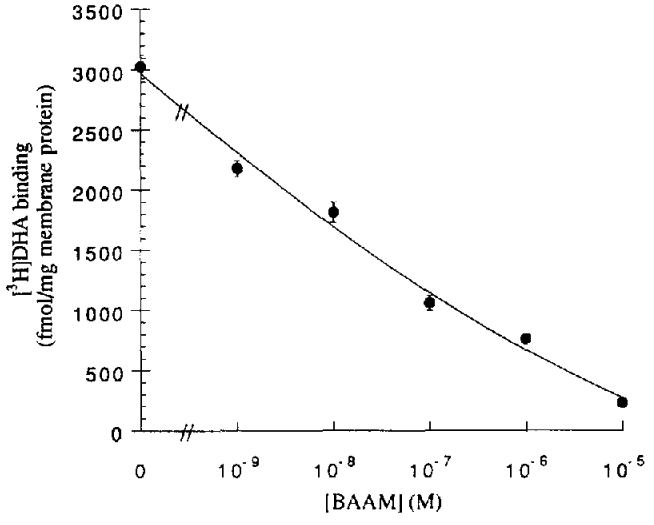


Fig. 1. Treatment of clone βN22 cells with BAAM prevents subsequent binding of [^{3}H]DHA. Clone βN22 cells in tissue culture were untreated or treated with varying concentrations of BAAM for 4 h. Cells were subsequently harvested and membranes prepared. The specific binding of [^{3}H]DHA (2 nM) to these membranes was subsequently assessed as described in section 2.

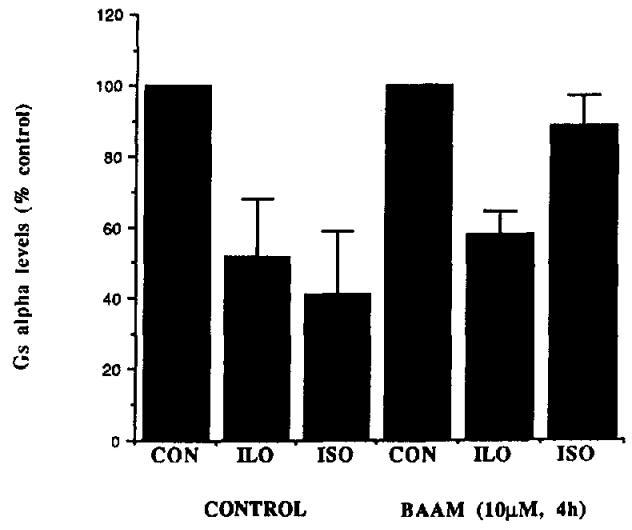


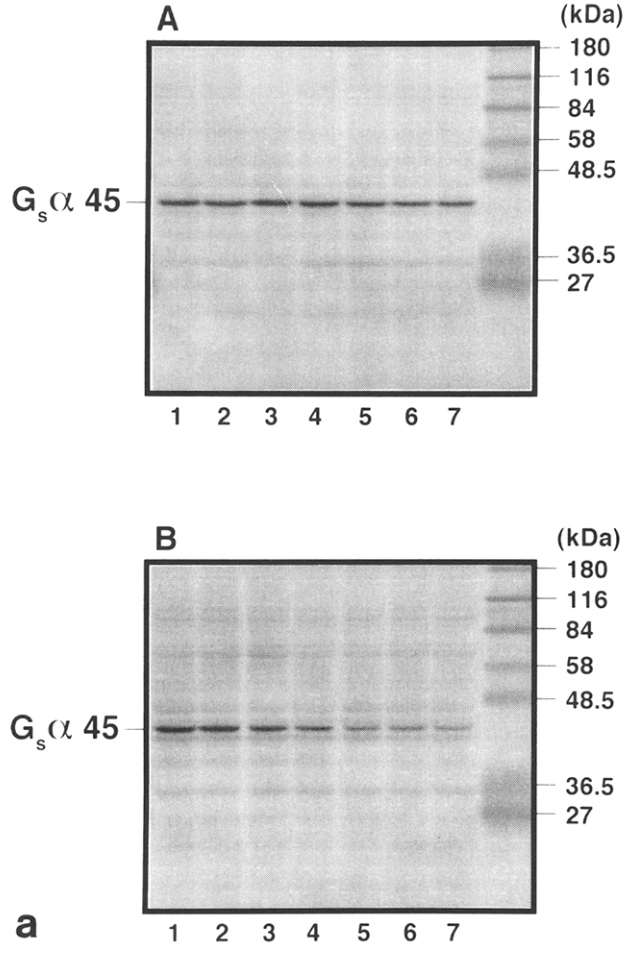
Fig. 2. Treatment of clone βN22 cells with BAAM restricts β2-adrenoceptor but not IP prostanoid receptor agonist-mediated down-regulation of $G_s\alpha$. βN22 cells were untreated (control) or treated with BAAM (10 μM) for 4 h, washed extensively and then either left in the absence of ligand (CON) or challenged with iloprost (10 μM) (ILO) or isoprenaline (10 μM) (ISO) for 8 h. The cells were harvested, membranes generated and immunoblotted for the presence of $G_s\alpha$. Data are presented as means ± S.D. from 3 separate experiments.

the β-adrenoceptor antagonist [^{3}H]DHA (2 nM). Exposure of clone βN22 cells to varying concentrations of the irreversible β-adrenoceptor antagonist BAAM for 4 h prior to cell harvest and membrane preparation resulted in a decrease in the number of detectable specific binding sites for [^{3}H]DHA (Fig. 1). In the absence of BAAM some 3000 fmol/mg membrane protein of β2-adrenoceptor binding sites were available in the passages of the cells used to specifically bind [^{3}H]DHA and this was reduced by approximately 90% by treatment with 10 μM BAAM. Half-maximal reduction in specific [^{3}H]DHA binding was obtained by treatment with some 30 nM BAAM.

Sustained (8 h) exposure of clone βN22 cells to maximally effective concentrations of either the IP prostanoid receptor agonist, iloprost (10 μM) or the β-adrenoceptor agonist isoprenaline (10 μM) resulted in some 50% down-regulation of cellular levels of the α subunit of the stimulatory G-protein of the adenylyl cyclase cascade, G_s (Fig. 2). Such treatments did not alter cellular levels of other G-proteins expressed by these cells (data not shown but see [5]). Following pretreatment of the cells with BAAM (10 μM, 4 h) no alteration in the ability of this concentration of iloprost to down-regulate $G_s\alpha$ was noted. By contrast, a substantial reduction in the effect of 10 μM isoprenaline was observed (Fig. 2). This pattern of reduced effectiveness of isoprenaline to cause down-regulation of $G_s\alpha$ was observed over a range of isoprenaline concentrations (Fig. 3a,b) following treatment with 10 μM BAAM. At all concentrations of isoprenaline tested the degree of down-regulation of $G_s\alpha$ was reduced and there was a substantial shift (some 50-fold) to higher concentrations in the requirement for isoprenaline to cause half-maximal reduction in $G_s\alpha$ levels (Fig. 3b). By contrast, no significant alteration in the dose–effect curve for iloprost-mediated $G_s\alpha$ down-regulation was observed following such treatment with BAAM (data not shown).

Preincubation of cells of clone βN22 with varying concentra-

tions of BAAM (1 nM–10 μM) followed by subsequent exposure to a maximally effective dose of isoprenaline (10 μM) demonstrated that the ability of the agonist to cause $G_s\alpha$ down-regulation was compromised as increasing numbers of the β2-adrenoceptor were eliminated (Fig. 4). Half-maximal reduction in the ability of 10 μM isoprenaline to cause elimination of $G_s\alpha$ was obtained following treatment with 100 nM BAAM (Fig. 4). Even following treatment with 10 μM BAAM the maximal ability of isoprenaline to stimulate adenylyl cyclase activity in



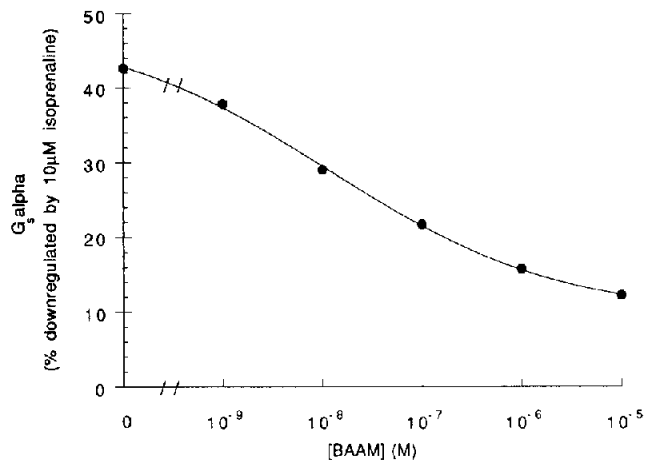


Fig. 4. The effect of BAAM on isoprenaline-induced down-regulation of $G_s\alpha$; dose–effect analysis. Cells of clone βN22 were untreated or treated for 4 h with varying concentrations of BAAM and subsequently with isoprenaline (10 μM, 8 h). Relative levels of $G_s\alpha$ in membranes from these cells were then assessed immunologically. Data is presented as the % of the membrane $G_s\alpha$ which was downregulated by isoprenaline at each concentration of BAAM.

membranes of βN22 cells was only slightly lower than in membranes of untreated cells although the dose–effect curve for isoprenaline was shifted to significantly higher concentrations (Fig. 5). Overall, these results demonstrate that the degree of agonist-mediated down-regulation of $G_s\alpha$ reflects the number of receptors available to agonist.

4. Discussion

Although agonist-induced down-regulation of receptors has been studied for many years and is appreciated to provide a means to regulate cellular sensitivity to agonist ligands [13] the concept that agonist treatment can also regulate the cellular content of G-proteins has taken longer to be accepted and has been more recalcitrant to analysis. In a variety of circumstances sustained exposure of a cell to an agonist ligand does result in a substantial reduction in cellular levels of the G-protein(s) which is activated by the receptor (see [1] for review). However, such effects are not observed for all systems and for all agonists. For example, in the neuroblastoma × glioma hybrid cell line, NG108-15, treatment with agonists at the IP prostanoid receptor but not with agonists at either the adenosine A2 or secretin

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Fig. 3. BAAM treatment of clone βN22 cells reduces the potency of isoprenaline-mediated $G_s\alpha$ down-regulation. Cells of clone βN22 were untreated or treated with BAAM (10 μM, 4 h) and subsequently challenged with varying concentrations of isoprenaline for 8 h. Membranes were prepared and the relative levels of $G_s\alpha$ determined immunologically. (a) *Immunoblots.* (A) Treated with BAAM, (B) not treated with BAAM. Subsequent treatment with isoprenaline: 1, control; 2, 100 pM; 3, 1 nM; 4, 10 nM; 5, 100 nM; 6, 1 μM; 7, 10 μM. (b) *Quantitative analysis.* The data of Fig. 3a was analysed and is displayed as the % of maximal effect of isoprenaline (produced by 10 μM) on membrane levels of $G_s\alpha$. In the example displayed the estimated EC_{50} for isoprenaline-induced down-regulation of $G_s\alpha$ was 1.3 nM in the cells which had not been exposed to BAAM (filled symbols) and 78 nM in the cells which had been pretreated with BAAM (open symbols). Similar results were obtained in two other independent experiments.

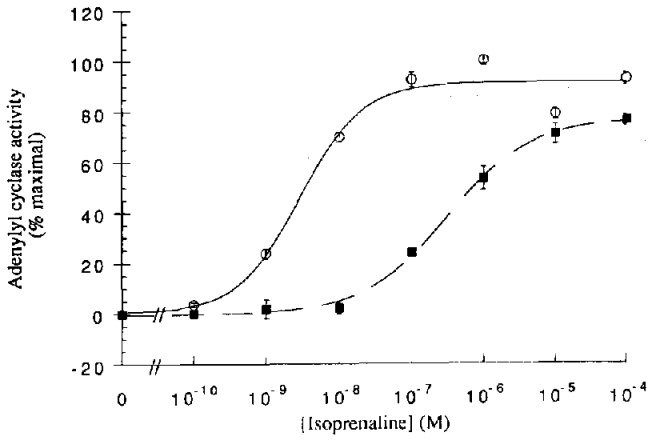

Fig. 5. The effect of BAAM on isoprenaline stimulation of adenylyl cyclase activity. Membranes were prepared from control (open symbols) and BAAM (10 μM, 4 h)-treated (filled symbols) βN22 cells and the ability of varying concentrations of isoprenaline to stimulate adenylyl cyclase activity measured. Results are presented as % of the maximal effect of isoprenaline in membranes of untreated cells.

receptors results in a substantial down-regulation of $G_s\alpha$ even though all of these receptors couple to $G_s\alpha$ and thus cause activation of adenylyl cyclase [2,3]. As addition of analogues of cAMP or agents able to elevate intracellular cAMP in a non-receptor-dependent fashion is unable to mimic the effect of prostanoid agonists [3] we have argued that the differences between the receptor ligands is unlikely to represent the ability of each receptor to activate adenylyl cyclase and may reflect the levels of cellular expression of each receptor [4]. This, however, has been difficult to establish clearly in these cells as only agonist ^{3}H-ligands are available for use in binding studies for the IP prostanoid and secretin receptors and there have been concerns as to whether ligands for the adenosine A2 receptor may label other sites [14] such as the adenosine transporter.

We have recently established clonal cell lines following transfection of NG108-15 cells with a cDNA encoding the human β2-adrenoceptor [5,6] and indeed a clone expressing high levels of the receptor (some 4,000 fmol/mg membrane protein) responded to challenge with isoprenaline by down-regulating levels of $G_s\alpha$ whereas little effect was observed in a lcone expressing much lower levels (300 fmol/mg membrane protein) of the receptor [5]. While such results provide strong supportive evidence for the concept that agonist-mediated G-protein down-regulation is likely to reflect levels of expression of a receptor they suffer from the fact that they had to be performed on different individual clonal isolates. To counteract this concern in the present study we have used a single clone (βN22) which expresses relatively high levels of the β2-adrenoceptor and then limited access of isoprenaline to the receptor by pretreating the cells with varying concentrations of the irreversible β-adrenoceptor antagonist BAAM. We make a number of key observations. (i) BAAM (10 μM, 4 h) treatment of clone βN22 cells results in a substantial reduction in the maximal down-regulation of $G_s\alpha$ which can be achieved by isoprenaline without altering the amount of down-regulation of $G_s\alpha$ produced by occupancy of the IP prostanoid receptor by iloprost (Fig. 2). (ii) Half-maximal down-regulation of $G_s\alpha$ which can be produced by isoprenaline required substantially higher levels of the agonist following treatment with BAAM (10 μM) compared to the control cells (Fig. 3). (iii) Dose–effect curves for iloprost-mediated down-regulation of $G_s\alpha$ were unaffected by treatment with BAAM. (iv) Treatment of clone βN22 cells with a concentration of BAAM sufficient to reduce the amount of [^{3}H]DHA to some 300 fmol/mg membrane protein substantially reduced, but did not eliminate entirely, isoprenaline-induced down-regulation of $G_s\alpha$. Such treatment, however, resulted in only a small reduction in the maximal ability of isoprenaline to stimulate adenylyl cyclase activity. (v) A 50% reduction in the maximal ability of isoprenaline to regulate $G_s\alpha$ levels was produced by treatment of the cells with 100 nM BAAM, a concentration able to block agonist access to some 70% of the receptor population.

These data demonstrate directly that the degree of G-protein down-regulation observed is related to the number of receptors available for the agonist ligand to occupy and not to the ability of this receptor occupancy to result in the activation of adenylyl cyclase. This should be anticipated as previous studies have shown that such effects are restricted to the G-protein activated by the receptor [1,3,15], that the effect is a reflection of enhanced degradation of the G-protein without significant transcriptional of translational control [1,16], that it is not mimicked by treatment with analogues of cAMP [3] and because mutationally activated G-protein α subunits are known to have reduced half-lives compared with the wild type proteins [1,17]. Thus, if greater levels of available receptors are able to activate more copies of the G-protein then greater down-regulation of this polypeptide should be anticipated. However, the observations that reduction of β2-adrenoceptor availability levels in clone βN22 cells to some 300 fmol/mg membrane protein still resulted in a detectable down-regulation of $G_s\alpha$ whereas this was not observed in clone βN17 which expresses this level of receptor endogenously [5] clearly demonstrates that it is unwise to extrapolate results from data obtained in different clonal isolates.

$G_s\alpha$ is expressed at some 1.25×10^6 copies per cell and the IP prostanoid receptor at some 10^5 copies per cell in NG108-15 cells and in the transfected clonal cell lines derived from it [18]. In clone βN22 the β2-adrenoceptor is expressed at some 3–4-fold higher levels than the IP prostanoid receptor [5,6]. We have previously calculated that the IP prostanoid receptor can activate some 65% of the cellular $G_s\alpha$ in NG108-15 cells [19] and therefore if the β2-adrenoceptor is able to activate the G-protein with similar stoichiometry then less than maximal occupancy of this receptor would be expected to result in maximal down-regulation of $G_s\alpha$ as indeed we observe herein.

It should be noted that in this system each 10% down-regulation represents a loss of some 10^5 copies per cell of $G_s\alpha$. The inability to observe detectable G-protein down-regulation in a range of systems may thus be a reflection of a combination of the levels of receptor expression and that of the G-protein. Clearly in cells which express high levels of a G-protein, agonist may be able to cause down-regulation of only a small fraction of the polypeptide and this would be virtually undetectable when measured immunologically. Equally it is unlikely to be observed with agonists at receptors which are expressed at only low levels. This may then provide the explanation for the inability of either secretin or an A2 adenosine receptor agonist to alter $G_s\alpha$ levels detectably in NG108-15 cells [2,20]. The use of irreversible antagonists at a range of receptor systems will allow assessment of whether the conclusions reached in this study are widely applicable.

Acknowledgements: These studies were supported by a MRC project grant to G.M.

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