

# Agonist regulation of cellular $G_s$ $\alpha$ -subunit levels in neuroblastoma $\times$ glioma hybrid NG108-15 cells transfected to express different levels of the human $\beta 2$ adrenoceptor

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Neuroblastoma  $\times$  glioma hybrid NG108-15 cells endogenously express at least three receptors which activate adenylate cyclase via the intermediacy of the stimulatory G-protein,  $G_s$ . Sustained exposure of the cells to agonists at the IP prostanoid receptor results in a substantial decrease in cellular levels of the  $\alpha$ -subunit of  $G_s$  ( $G_s\alpha$ ) [McKenzie and Milligan (1990) *J. Biol. Chem.* **265**, 17084–17093; Adie, Mullaney, McKenzie and Milligan (1992) *Biochem J.* **285**, 529–536]. By contrast, equivalent treatments of the cells with agonists at either the A2 adenosine receptor or the secretin receptor have no measurable effect on cellular amounts of  $G_s\alpha$ . To examine whether this is a feature specific to the IP prostanoid receptor or is related to the level of expression of the individual receptors, NG108-15 cells were transfected with a construct containing a human  $\beta 2$ -adrenoceptor cDNA under the control of the  $\beta$ -actin promoter. Two clones of these cells were examined in detail,  $\beta N22$ , which expressed some 4000 fmol/mg of membrane protein, and clone  $\beta N17$ , which expressed approx. 300 fmol/mg of membrane protein of the

receptor. Exposure of  $\beta N22$  cells to the  $\beta$ -adrenergic agonist isoprenaline resulted maximally in some 55% decrease in membrane-associated levels of  $G_s\alpha$ , without effect on membrane levels of  $G_{12\alpha}$ ,  $G_{13\alpha}$ ,  $G_{16\alpha}$  or  $G_{q\alpha}/G_{11\alpha}$ . Dose–response curves to isoprenaline in  $\beta N22$  cells indicated that half-maximal down-regulation of  $G_s\alpha$  was produced by approx. 1 nM agonist. Equivalent exposure of  $\beta N17$  cells to isoprenaline did not significantly modify levels of any of the G-protein  $\alpha$  subunits, including  $G_s\alpha$ . In  $\beta N22$  cells the IP prostanoid receptor was expressed at similar levels to those in wild-type NG108-15 cells, and treatment with iloprost resulted in a similar down-regulation of cellular  $G_s\alpha$  levels. Iloprost was also effective in causing down-regulation of  $G_s\alpha$  levels in clone  $\beta N17$ . Concurrent addition of both isoprenaline and iloprost to clone  $\beta N22$  resulted in less than additive down-regulation of  $G_s\alpha$ . These results demonstrate that the phenomenon of agonist-induced specific G-protein down-regulation is determined by the levels of expression of the receptor.

## INTRODUCTION

Sustained exposure of cells or tissues to agonists which activate a G-protein-linked receptor frequently results in a time- and concentration-dependent decrease in plasma-membrane, and subsequently cellular, levels of that receptor. This process is called down-regulation, and can contribute to the processes, collectively called desensitization, which limit cellular responsiveness to the maintained presence of extracellular signalling molecules [1,2].

More distal elements of cellular signalling cascades can also provide loci for regulation of either the maximal effectiveness or sensitivity of cellular responses to agonists [3]. Such distal elements, including G-proteins and effector enzymes which control the rate of production of second messengers, have particular attractions as sites of control, as regulation at this level would be likely to modify signal intensity to a range of agents which activate distinct receptors but alter intracellular levels of the same second messenger [4].

We [5,6] and others [7] have recently noted that sustained exposure of neuroblastoma  $\times$  glioma hybrid NG108-15 cells to agonists which activate an IP prostanoid receptor to stimulate adenylate cyclase results in a substantial decrease in cellular levels of the stimulatory G-protein ( $G_s$ ) without altering cellular levels of any other G-protein. These cells express two other defined receptors which stimulate cyclic AMP production. How-

ever, sustained exposure of the cells to agonists at either the A2 adenosine receptor [7,8] or the secretin receptor [8] has no discernible effect on cellular levels of  $G_s$ . To assess whether the prostanoid-mediated effects on  $G_s$   $\alpha$ -subunit ( $G_s\alpha$ ) levels is due to some particular feature of the IP prostanoid receptor, or might be related to levels of expression of receptors which couple to  $G_s$ , in this study we have isolated clones of NG108-15 cells which express either low or high levels of the human  $\beta 2$  adrenoceptor and demonstrate that high-level, but not low-level, expression of this receptor allows agonist-induced down-regulation of  $G_s\alpha$ .

A part of this study has been published in preliminary form [9].

## MATERIALS AND METHODS

### Materials

All materials for tissue culture were from Gibco/BRL. [ $^3$ H]Dihydroalprenolol ([ $^3$ H]DHA; 56 Ci/mmol) was from Amersham International. All other chemicals were from Sigma or BDH and were of the highest purity available.

### Generation and isolation of clones of NG108-15 cells expressing the human $\beta 2$ adrenoceptor

Plasmid pJM16 [10], which harbours a copy of the neomycin-resistance gene, was cut with the restriction enzymes *Bam*HI and

Abbreviations used:  $G_s$ , stimulatory G-protein; DHA, dihydroalprenolol; GTP[S], guanosine 5'-[ $\gamma$ -thio]triphosphate; PGE1, prostaglandin E1;  $pIC_{50}$ ,  $-\log_{10}$  of concn. giving half-maximal inhibition.

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*Xho*I to allow a cDNA encoding the human  $\beta$ 2 adrenoceptor to be ligated downstream of the  $\beta$ -actin promoter of this plasmid. GeneCleaned (Gibco/BRL) human  $\beta$ 2-adrenoceptor cDNA, with 5' *Bam*HI and 3' *Xho*I sites, was ligated into the digested pJM16. Competent *Escherichia coli* were transformed with DNA from the ligation reactions spread on ampicillin (25  $\mu$ g/ml) plates, and ampicillin-resistant colonies were picked. DNA was extracted from bacterial cultures of these colonies with phenol/chloroform and run on an agarose gel (1%, w/v) to demonstrate the presence of the cDNA as insert. The DNA from a colony with the insert present was purified by using CsCl, and then 10  $\mu$ g of this purified DNA was stably transfected into NG108-15 cells by using Lipofectin reagent (Gibco/BRL) according to the manufacturers' instructions. Clones which were resistant to geneticin sulphate (800  $\mu$ g/ml) were selected and expanded. Expression of the  $\beta$ 2 adrenoceptor in membranes from these clones was assessed by the specific binding of the  $\beta$ -adrenoceptor antagonist [ $^3$ H]dihydroalprenolol.

### Cell growth

Wild-type and transfected neuroblastoma  $\times$  glioma hybrid NG108-15 cells were grown in tissue culture as previously described [11], except that the transfected cells were also maintained in the presence of geneticin sulphate (800  $\mu$ g/ml). Before 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  $-80^\circ\text{C}$  after harvest essentially as in [12]. Frozen cell pellets were suspended in 5 ml of 10 mM Tris/HCl/0.1 mM EDTA, pH 7.5 (buffer A), and rupture of the cells was 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 48000  $g$  for 10 min, and the pellet from this treatment was washed and resuspended in 10 ml of buffer A. After a second centrifugation at 48000  $g$  for 10 min, the membrane pellet was resuspended in buffer A to a final protein concentration of 1–3 mg/ml and stored at  $-80^\circ\text{C}$  until required.

### Treatment of cells

NG108-15 cells and the clones derived from these cells were treated in culture with either iloprost (up to 1  $\mu$ M) (Schering Health Care, Burgess Hill, Sussex, U.K.) or with isoprenaline (up to 100  $\mu$ M) (Sigma) including vehicle. Preliminary studies indicated that the vehicles had no effect on levels of  $G_s\alpha$  over the time course of the treatments.

### Production of antisera and immunoblotting

Antiserum CS2 was produced by a New Zealand White rabbit after 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  $G_s\alpha$ . The specificity of this antiserum for  $G_s\alpha$  has previously been demonstrated [13]. Immunoblotting with this antiserum was performed as previously described [11]. Antiserum SG1 was produced in a similar fashion against the C-terminal decapeptide of the  $\alpha$  subunit of rod transducin (KENLKDCGLF) [14]. This antiserum identifies both  $G_i1\alpha$  and  $G_i2\alpha$  as well as transducin  $\alpha$ . However, as we have described previously [11], of these G-proteins only  $G_i2\alpha$  is expressed by NG108-15 cells. Antiserum I3B was generated against the C-

terminal decapeptide of the  $\alpha$  subunit of  $G_{i3}$  (KNNLKECGLY). Antiserum IM1 was generated against amino acids 22–35 (NLKEDGISAAKDV) of forms of  $G_o\alpha$ . The specificities and characterization of these antisera have previously been fully described [15]. Antiserum CQ2 was generated against a synthetic peptide (QLNLKEYNLV) which represents the C-terminal decapeptide which is conserved between  $G_o\alpha$  and  $G_{i1}\alpha$  [16]. As such, this antiserum cannot distinguish between these two polypeptides and has been shown directly to identify both polypeptides equally [17]. Molecular-mass determinations were based on pre-stained molecular-mass markers (Bethesda Research Laboratories) [18]. SDS/PAGE [10% (w/v) acrylamide] was carried out overnight at 60 V.

### Quantification of immunoblots

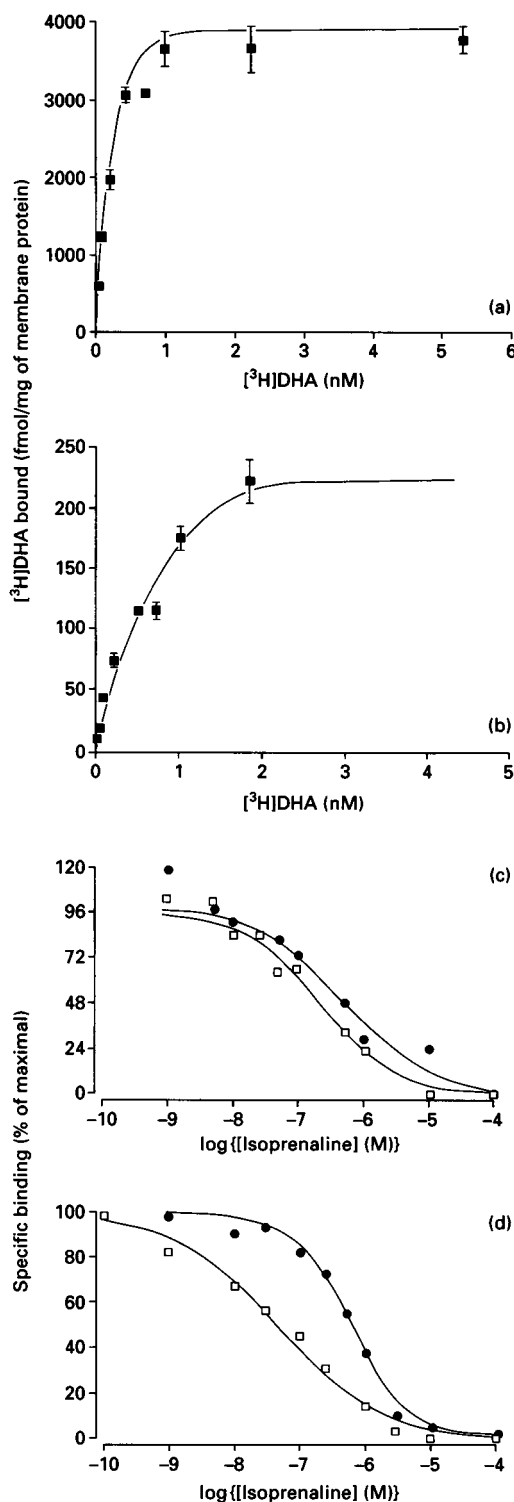
After SDS/PAGE, proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 2 h in 5% gelatin in PBS, pH 7.5. Primary antisera were added in 1% gelatin in PBS containing 0.2% Nonidet P40 and incubated for at least 2 h. The primary antiserum was then removed and the blot washed extensively with PBS containing 0.2% Nonidet P40. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase; Scottish Antibody Production Unit, Wishaw, Scotland, U.K.) was added (1:200 dilution in 1% gelatin in PBS containing 0.2% Nonidet P40) and incubated with the nitrocellulose for 2 h. The antiserum was then removed and, after extensive washing of the blot with PBS containing 0.2% Nonidet P40 and finally with PBS alone, the blot was developed by using *o*-dianisidine hydrochloride (Sigma) as the substrate for horseradish peroxidase as previously described [11]. The developed immunoblots were scanned with a Shimadzu CS-9000 dual-wavelength flying-spot laser densitometer on reflectance mode at 450 nm. Background was subtracted by scanning of equivalent-sized areas of nitrocellulose which did not contain immunoreactive protein. The results were analysed on a Shimadzu FDU-3 central processing unit enabling quantification of the immunoblots. Preliminary experiments were performed to assess the range of linearity of the assay for each antiserum. Amounts of membranes used to assess the effects of isoprenaline and iloprost treatment on levels of the various G-proteins were, in all cases, within the observed linear region.

### Binding experiments with [ $^3$ H]DHA

Displacement experiments were performed routinely with approx. 0.5 nM [ $^3$ H]DHA at  $30^\circ\text{C}$  for 30 min in 20 mM Tris/HCl (pH 7.5)/50 mM sucrose/20 mM  $\text{MgCl}_2$  (buffer B) in the absence or presence of either 100  $\mu$ M isoprenaline or 10  $\mu$ M 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. In experiments designed to assess the maximal binding capacity of membranes for this ligand, concentrations of [ $^3$ H]DHA were varied between 0.1 and 5 nM. All binding experiments were terminated by rapid filtration through Whatman GF/C filters, followed by three washes (each 5 ml) with ice-cold buffer B.

### Binding experiments with [ $^3$ H]prostaglandin E1 (PGE1)

These were performed routinely with 10 nM [ $^3$ H]PGE1 at  $30^\circ\text{C}$  for 30 min in 20 mM Tris/HCl (pH 7.5)/50 mM sucrose/20 mM  $\text{MgCl}_2$  containing 10  $\mu$ M indomethacin (buffer C) in the absence and presence of 10  $\mu$ M PGE1 or 10  $\mu$ M iloprost to define



**Figure 1** (a and b)  $\beta$ 2-adrenoceptor-binding characteristics of clones  $\beta$ N22 (a) and  $\beta$ N17; (c and d) displacement of the specific binding of a single concentration of [ $^3$ H]DHA to membranes of  $\beta$ N22 and  $\beta$ N17 cells by isoprenaline: effect of GTP[S]

The specific binding of different concentrations of [ $^3$ H]DHA was measured in membranes of clones  $\beta$ N22 (a) and  $\beta$ N17 (b). The curves represent the theoretical curves predicted by non-linear least-squares analysis. In the analysis displayed,  $B_{max}$  was estimated by using the LIGAND program [35] to be 3950 fmol/mg of membrane protein in membranes of clone  $\beta$ N22. The estimated  $K_d$  for [ $^3$ H]DHA was 0.22 nM. In membranes of clone  $\beta$ N17,  $B_{max}$  was estimated to be 218 fmol/mg of membrane protein and the  $K_d$  for [ $^3$ H]DHA was 0.42 nM. (c, d) The binding of [ $^3$ H]DHA (0.5 nM) to membranes (5  $\mu$ g) of clone  $\beta$ N22 (c) or  $\beta$ N17 (d) was

maximal and non-specific binding respectively, as described in [6]. Specific binding, defined as above, represented some 80% of the total binding of [ $^3$ H]PGE1. In experiments designed to assess the maximal binding capacity of membranes of NG108-15 cells for this ligand, the specific radioactivity of a single concentration (10 nM) of [ $^3$ H]PGE1 was varied, and measured specific binding was subsequently corrected on this basis. All binding experiments were terminated by rapid filtration through Whatman GF/C filters, followed by three washes (each 5 ml) with ice-cold buffer B.

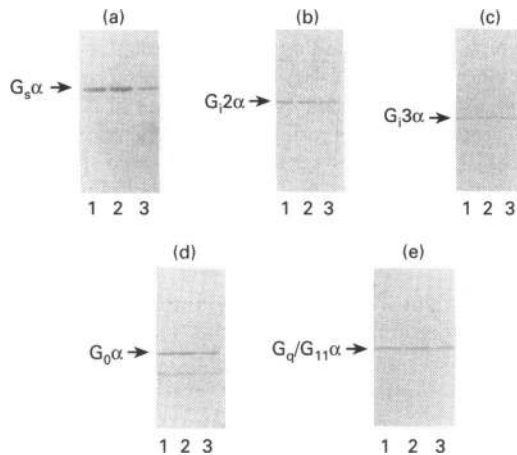
All binding data were analysed by a non-linear least-squares curve-fitting program.

## RESULTS

Neuroblastoma  $\times$  glioma hybrid NG108-15 cells were transfected with a construct of plasmid pJM16 [10] into which a cDNA encoding a human  $\beta$ 2-adrenoceptor cDNA had been ligated to be under the control of the  $\beta$ -actin promoter. Clones from this transfection which were resistant to geneticin sulphate (800  $\mu$ g/ml) were selected and expanded. Clone  $\beta$ N22 demonstrated high levels of expression of the  $\beta$ 2 adrenoceptor ( $B_{max}$  = 3994  $\pm$  700 fmol/mg of membrane protein; mean  $\pm$  S.E.M.,  $n$  = 6), as assessed by the specific binding of [ $^3$ H]DHA, and bound this ligand with high affinity ( $K_d$  = 0.32  $\pm$  0.10 nM) (Figure 1a). Clone  $\beta$ N17 expressed much lower levels of the  $\beta$ 2 adrenoceptor ( $B_{max}$  = 292  $\pm$  62 fmol/mg of membrane protein; mean  $\pm$  S.E.M.,  $n$  = 3), but also bound the [ $^3$ H]ligand with high affinity ( $K_d$  = 0.52  $\pm$  0.20 nM) (Figure 1b). Displacement of the specific binding of [ $^3$ H]DHA from membranes of clone  $\beta$ N22 with the  $\beta$ -adrenoceptor agonist isoprenaline (Figure 1c) resulted in a  $pIC_{50}$  (corrected for receptor occupancy) of 6.80  $\pm$  0.16 (mean  $\pm$  S.E.M.,  $n$  = 4) for this ligand. Although it is often observed that agonist displacement of antagonist binding to G-protein-linked receptors is produced with a pseudo Hill coefficient ( $h$ ) significantly less than 1.0, and that addition of a poorly hydrolysed guanine nucleotide causes a decrease in agonist affinity and a shift in  $h$  to approx. 1.0 (see, e.g., [19]), this was not the case in membranes of  $\beta$ N22 cells (see Figure 1c). The estimated  $h$  value in the absence of guanosine 5'-[ $\gamma$ -thio]triphosphate (GTP[S]) was 0.72  $\pm$  0.05, and in the presence of GTP[S] (100  $\mu$ M) it was 0.78  $\pm$  0.11 (mean  $\pm$  S.E.M.,  $n$  = 4 in each case). GTP[S] also produced only a marginal alteration in the position of the displacement curve for isoprenaline ( $pIC_{50}$  corrected for receptor occupancy = 6.64  $\pm$  0.19; Figure 1c), which was not statistically significant ( $P$  > 0.2). Although receptor theory predicts that guanine nucleotides should decrease the binding affinity of agonists, but not antagonists, and hence produce a rightward shift in the agonist displacement curve [19], such an effect is not observed experimentally in some systems, even for  $\beta$ -adrenoceptors [20,21]. By contrast, in membranes of  $\beta$ N17 cells the measured  $pIC_{50}$  (corrected for receptor occupancy) in the absence of guanine nucleotides (7.37  $\pm$  0.17) was shifted significantly ( $P$  = 0.006) to the right (6.58  $\pm$  0.20) (Figure 1d), and the estimated  $h$  value increased from 0.63  $\pm$  0.10 to 1.00  $\pm$  0.08 on addition of GTP[S] (100  $\mu$ M) to the binding assays.

Immunoblotting membranes from wild-type NG108-15 and clone- $\beta$ N22 cells with an anti-peptide antiserum (CS2) generated

competed for by different concentrations of isoprenaline in the presence (●) or absence (□) of GTP[S] (100  $\mu$ M). Non-specific binding was defined as that in the presence of 100  $\mu$ M isoprenaline. In the experiments displayed,  $pIC_{50}$  (corrected for receptor occupancy) and  $h$  were 7.03 and 0.79 ( $\beta$ N22, no GTP[S]), 6.73 and 0.65 ( $\beta$ N22, + GTP[S]), 7.59 and 0.54 ( $\beta$ N17, no GTP[S]) and 6.52 and 1.04 ( $\beta$ N17, + GTP[S]).

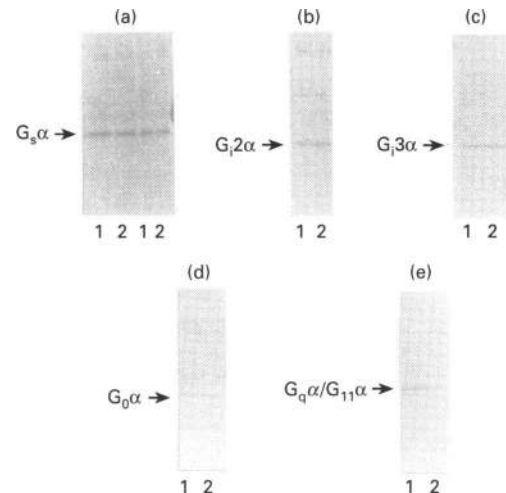


**Figure 2** Levels of  $G_s\alpha$  and other G-proteins in wild-type NG108-15 cells and in clone  $\beta$ N22: effect of isoprenaline treatment

Cells of clone  $\beta$ N22 were treated (2) without or (3) with isoprenaline (0.1 mM) for 16 h. Membranes of these cells and of (1) untreated wild-type NG108-15 cells were resolved by SDS/PAGE (10% acrylamide) and immunoblotted for (a)  $G_s\alpha$  (40  $\mu$ g), (b)  $G_{i2}\alpha$  (50  $\mu$ g), (c)  $G_{i3}\alpha$  (50  $\mu$ g), (d)  $G_o\alpha$  (50  $\mu$ g) and (e)  $G_q\alpha/G_{11}\alpha$  (50  $\mu$ g) with the antisera described in the Materials and methods section. No appreciable differences were noted between wild-type NG108-15 cells and untreated clone- $\beta$ N22 cells (lanes 1 and 2, a–e) in steady-state membrane levels of any of the G-proteins. Isoprenaline treatment of  $\beta$ N22 cells produced a large down-regulation of  $G_s\alpha$  (lanes 2 versus 3, a) but not other G-proteins (lanes 2 versus 3, b–e) (see the text for quantitative analysis).

against a synthetic peptide corresponding to the predicted C-terminal decapeptide of all of the splice variants of  $G_s\alpha$  identified similar steady-state levels of a 45 kDa polypeptide in these two cells lines (Figure 2a, lanes 1 versus 2) which co-migrated with *E. coli*-produced recombinant  $G_s\alpha$  (long form) (results not shown, but see [6]). Treatment of clone  $\beta$ N22 with isoprenaline (0.1 mM, 16 h) resulted in a substantial decrease in membrane-associated  $G_s\alpha$ 45 (45 kDa  $G_s\alpha$  species) (Figure 2a, lane 3 versus 2) to  $45.5 \pm 11\%$  of the control level (mean  $\pm$  S.E.M.,  $n = 13$ ). By contrast, equivalent immunoblots of membranes from untreated isoprenaline-treated  $\beta$ N22 cells with anti-peptide antisera (see the Materials and methods section) able to identify specifically the  $\alpha$ -subunits of  $G_{i2}$ ,  $G_{i3}$ ,  $G_o$  and  $G_q/G_{11}$  in these cells demonstrated that isoprenaline treatment had no effect on steady-state levels of the  $\alpha$ -subunits of any of these other G-proteins:  $G_{i2} = 105 \pm 4$ ,  $G_{i3} = 104 \pm 14$ ,  $G_o = 110 \pm 4$ ,  $G_q/G_{11} = 96 \pm 16\%$  (all values means  $\pm$  S.E.M.,  $n = 3$ ) (Figure 2). Isoprenaline-induced loss of membrane-associated  $G_s\alpha$ 45 in clone- $\beta$ N22 cells was not accompanied by a stable transfer of this polypeptide to the cytoplasm. Immunoblotting equivalent amounts of membrane and cytosol protein from untreated and isoprenaline-treated cells did not result in the appearance of immunodetectable  $G_s\alpha$ 45 in the cytoplasmic fraction (results not shown). Equivalent treatment of clone  $\beta$ N17 with isoprenaline was without effect on membrane-associated levels of  $G_s\alpha$ 45 ( $95 \pm 8\%$ ; mean  $\pm$  S.E.M.,  $n = 5$ ) or the  $\alpha$  subunits of any of the other G-proteins ( $G_{i2} = 102 \pm 9\%$ ,  $G_o = 110 \pm 17\%$ ,  $G_q/G_{11} = 104 \pm 4\%$  (Figure 3).

We have previously noted that wild-type NG108-15 cells express high levels (approx. 1000 fmol/mg of membrane protein) of the IP prostanoid receptor [6]. Both clone  $\beta$ N22 and clone  $\beta$ N17 also expressed high levels of this receptor (Table 1), and in both of these clones maintained exposure to the stable prostanoid agonist iloprost resulted in similar effectiveness of down-regu-



**Figure 3** Levels of  $G_s\alpha$  and other G-proteins in clone  $\beta$ N17: effect of isoprenaline treatment

Cells of clone  $\beta$ N17 were treated (1) without or (2) with isoprenaline (0.1 mM) for 16 h. Membranes of these cells were resolved by SDS/PAGE (10% acrylamide) and immunoblotted for (a)  $G_s\alpha$  (left-hand pair 20  $\mu$ g, right-hand pair 10  $\mu$ g), (b)  $G_{i2}\alpha$  (70  $\mu$ g), (c)  $G_{i3}\alpha$  (50  $\mu$ g), (d)  $G_o\alpha$  (70  $\mu$ g) and (e)  $G_q\alpha/G_{11}\alpha$  (35  $\mu$ g) with the antisera described in the Materials and methods section. No significant alteration in levels of any G-protein  $\alpha$  subunit was recorded (see the text for details).

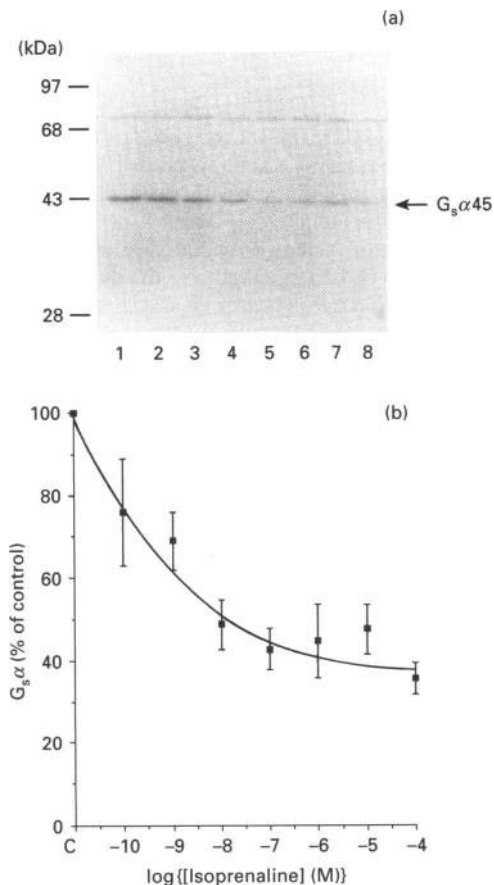
**Table 1** IP-prostanoid-receptor expression and iloprost-induced down-regulation of both the prostanoid receptor and  $G_s$  in  $\beta$ 2-adrenoceptor-expressing NG108-15 cells

Levels of the IP prostanoid receptor in membranes of  $\beta$ N22 and  $\beta$ N17 cells were measured as described in the Materials and methods section after treatment with or without iloprost (1  $\mu$ M) for 16 h (see also [6] for details). Levels of  $G_s\alpha$  were assessed by quantitative immunoblotting with antiserum CS2. Data represent means  $\pm$  S.E.M.:  $n = 4$  for the receptor binding experiments,  $n = 7$  for G-protein down-regulation in  $\beta$ N22 cells, and  $n = 3$  in  $\beta$ N17 cells. Similar values for down-regulation of both the IP prostanoid receptor and  $G_s\alpha$  have previously been obtained for wild type NG108-15 cells [6].

Clone	IP prostanoid receptor in untreated cells (fmol/mg of membrane protein)	Down-regulation by treatment with iloprost (10 $\mu$ M, 16 h) (%)	
		IP prostanoid receptor	$G_s\alpha$
$\beta$ N22	$871 \pm 200$	$49 \pm 6$	$50 \pm 15$
$\beta$ N17	$852 \pm 139$	$53 \pm 4$	$55 \pm 2$

lation of both the IP prostanoid receptor and cellular  $G_s\alpha$ , as previously reported for the wild-type cells (Table 1; see also [6]).

Treatment of clone- $\beta$ N22 cells with different concentrations of isoprenaline for 16 h indicated that a half-maximal decrease in cellular  $G_s\alpha$ 45 levels was obtained with approx. 1 nM agonist (Figure 4). This value is significantly less than that estimated from the binding analyses to be required to occupy half of the  $\beta$ 2 adrenoceptors (see above). Time courses of treatment of  $\beta$ N22 cells with maximally effective concentrations of isoprenaline indicated that half-maximal down-regulation of  $G_s\alpha$ 45 was produced between 2 and 3 h, and that a new steady-state plateau of membrane-associated levels was achieved by 8–10 h



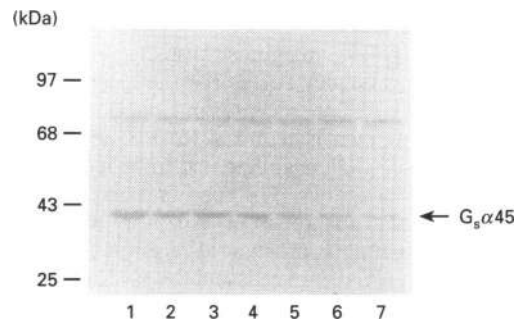
**Figure 4**  $G_s\alpha$  down-regulation in  $\beta$ N22 cells exposed to different concentrations of isoprenaline

(a) Cells of clone  $\beta$ N22 were exposed for 16 h to various concentrations of isoprenaline: 1, control; 2, 0.1 nM; 3, 1 nM; 4, 10 nM; 5, 100 nM; 6, 1  $\mu$ M; 7, 10  $\mu$ M; 8, 0.1 mM. Membranes (30  $\mu$ g) of these cells were then resolved by SDS/PAGE (10% acrylamide) and immunoblotted with antiserum CS2 (1:250 dilution) as primary antiserum. A typical example is displayed. The identity of the polypeptide at 75 kDa labelled in this experiment is unknown, but it is clearly not regulated in amount by isoprenaline treatment of the cells. (b) Quantitative analysis. Immunoblots such as those displayed in (a) were quantified by densitometric analysis. Data are presented as means  $\pm$  S.E.M. ( $n = 3$ ) for experiments derived from individual membrane preparations. Abbreviation: C, control.

(Figure 5). Co-addition of maximally effective concentrations of isoprenaline and iloprost to  $\beta$ N22 cells did not result in an additive down-regulation of  $G_s\alpha$ 45 ( $35 \pm 7\%$  remaining; mean  $\pm$  S.E.M.,  $n = 6$ ) compared with that obtained by either agonist alone (isoprenaline  $46 \pm 11\%$ , mean  $\pm$  S.E.M.,  $n = 13$ ; iloprost  $50 \pm 15\%$ , mean  $\pm$  S.E.M.,  $n = 7$ ).

## DISCUSSION

Neuroblastoma  $\times$  glioma hybrid NG108-15 cells [22] have been widely used to examine G-protein-mediated signal-transduction processes, as they express a wide range of G-proteins and receptor and effector species which interact with them. One of the receptors expressed by these cells is a prostanoid receptor of the IP subtype. We and others have previously noted that sustained exposure of these cells to the prostanoid agonists PGE1 [5] or iloprost [6,7] results in a decrease in cellular levels of  $G_s\alpha$  without alteration in the levels of other G-proteins. NG108-15 cells also



**Figure 5**  $G_s\alpha$  down-regulation in  $\beta$ N22 cells exposed to isoprenaline for different times

Cells of clone  $\beta$ N22 were exposed for various times to isoprenaline (10  $\mu$ M): 1, control; 2, 1 h; 3, 2 h; 4, 4 h; 5, 8 h; 6, 12 h; 7, 16 h. Membranes (30  $\mu$ g) of these cells were then resolved by SDS/PAGE (10% acrylamide) and immunoblotted with antiserum CS2 (1:250 dilution) as primary antiserum. The results displayed are representative of three experiments performed on different membrane preparations.

express endogenously two other identified receptors which interact with  $G_s$  to cause stimulation of adenylate cyclase. However, maintained exposure of the cells to agonists at the A2 adenosine receptor or the secretin receptor does not result in a similar phenomenon of  $G_s\alpha$  down-regulation [7,8]. Furthermore, iloprost treatment of a related cell line NCB20, which also expresses an IP prostanoid receptor, does not result in substantial down-regulation of  $G_s\alpha$  [7]. To address whether the down-regulation of  $G_s\alpha$  in response to prostanoid agonists in NG108-15 cells is related to particular features of the IP prostanoid receptor, or was potentially a reflection of the relative levels of endogenous expression of the various  $G_s$ -linked receptors, in this study we have isolated and examined transfects of NG108-15 cells which express either high or low levels of the human  $\beta$ 2 adrenoceptor.

Incubation of clone- $\beta$ N22 cells with the  $\beta$ -adrenergic agonist isoprenaline resulted in a substantial down-regulation of membrane-associated  $G_s\alpha$ 45, without effect on the levels of the  $\alpha$  subunits of other G-proteins (Figure 2). (A 42 kDa splice variant of  $G_s\alpha$  which co-migrates with *E. coli*-expressed recombinant  $G_s\alpha$  short form can also be detected in membranes of NG108-15 cells (see [5]). However, due to the low levels of expression of this form, it was not routinely detected in the immunoblotting experiments performed in these studies, as to do so requires resolution of large amounts of membrane protein and the consequent saturation of the immunological signal from the 45 kDa  $G_s\alpha$  splice variant, thus preventing the use of quantitative immunoblotting to detect agonist-induced regulation of levels of this polypeptide.) As such, the down-regulation of  $G_s\alpha$ 45 in NG108-15 cells is not inherently restricted to the IP prostanoid receptor and can be mimicked by the expression of another  $G_s\alpha$ -linked receptor. Furthermore, as isoprenaline was unable to produce any significant down-regulation of  $G_s\alpha$  in cells of clone  $\beta$ N17, which expresses only a low number of  $\beta$ 2 adrenoceptors (Figure 3), then the agonist-induced down-regulation is clearly dependent on the level of receptor expression. It has previously been noted that prostanoid-induced down-regulation of  $G_s$  in NG108-15 cells is independent of the generation of cyclic AMP [5], and thus the relative ability of the  $\beta$ 2 adrenoceptors expressed in the two clones to generate cyclic AMP is unlikely to be important for the effect on the  $G_s\alpha$ 45 levels. Moreover, maximal isoprenaline and iloprost stimulation of adenylate cyclase in these two clones was similar (results not shown). In previous

studies, prostanoid-induced down-regulation of  $G_s$  in NG108-15 cells was shown to follow an agonist dose-response curve which mirrored agonist-occupancy curves for the receptor [5]. This is not the same for the  $\beta_2$  adrenoceptor in clone- $\beta N22$  cells, where substantially less agonist is required to produce half-maximal  $G_s\alpha$  down-regulation than is anticipated to be required for half-maximal receptor occupancy. The combination of these observations demonstrates an apparent receptor reserve for  $\beta_2$ -adrenoceptor-, but not IP-prostanoid-receptor-, mediated  $G_s\alpha$  down-regulation in NG108-15 cells and the  $\beta N22$  clone. As noted above, the IP prostanoid receptor is expressed in high levels in NG108-15 cells [5]. Others had initially reported much lower values [23], but a recent publication by Donnelly et al. [24] has produced values similar to our own. Both clone  $\beta N22$  and clone  $\beta N17$  also express high levels of this receptor and, as iloprost-mediated, but not isoprenaline-induced, down-regulation is observed in clone  $\beta N17$ , then clearly the transfection process has not produced an artefact by which receptor-induced regulation of the G-protein is prevented or eliminated. We note that co-addition of isoprenaline and iloprost to cells of clone  $\beta N22$  results in non-additive levels of G-protein down-regulation in comparison with addition of either agonist in isolation. This is likely to be a reflection of the relative levels of expression of the receptors and  $G_s$  (see below). We have previously calculated a stoichiometry of concurrent down-regulation of  $G_s\alpha 45$  and the IP prostanoid receptor in NG108-15 cells of some 8 mol of G-protein:1 mol of receptor at all levels of receptor occupancy [6]. If this represents the ratio of activation of G-protein by receptor, then, as the IP prostanoid receptor is present at approx. 1 pmol/mg of membrane protein and  $G_s\alpha 45$  at some 10 pmol/mg of membrane protein [6], there would appear to be a slight molar excess of  $G_s\alpha$  even when the entire pool of the IP prostanoid receptor is activated by high concentrations of agonist. However, if the  $\beta_2$  adrenoceptor were to show a similar G-protein interaction stoichiometry, it might be anticipated that the entire pool of available  $G_s$  would be activated by agonist occupation of only a fraction of the available  $\beta_2$  adrenoceptors in clone  $\beta N22$ , and as such the dose-response curve for G-protein activation and down-regulation might be anticipated to be to the left of the receptor occupancy curve. Moreover, receptors may not have to activate the entire pool of theoretically available G-protein to produce a maximal response. We have calculated there to be a 70-fold molar excess of  $G_s\alpha$  over the maximal number of  $G_s\alpha$ /adenylate cyclase complexes which can be formed (measured by the high-affinity binding of [ $^3$ H]forskolin in the presence of maximally effective concentrations of either NaF or guanosine 5'-[ $\beta$ -imidotriphosphate] in membranes of NG108-15 cells [25]). Agonist-mediated  $\beta_2$ -adrenoceptor down-regulation has been noted to be impaired in both S49 cyc<sup>-</sup> cells, which do not express  $G_s\alpha$  [26], and in cells expressing mutant  $\beta_2$  adrenoceptors, which couple poorly to  $G_s$  [27]. Such data, in concert with the observation that IP prostanoid receptor and  $G_s$  down-regulation occur concurrently in prostanoid-stimulated NG108-15 cells [6], suggest that it may be the receptor-G-protein complex which is the target for down-regulation.

$G_s$  is not the only G-protein which has been noted to be down-regulated by the maintained presence of an agonist for a relevant receptor. Members of the  $G_i$  family [28,29] and  $G_q$  family [30,31] of G-proteins have been reported to be down-regulated in a similar manner to that reported herein. For the muscarinic M1 acetylcholine receptor when expressed in CHO cells, down-regulation of the G-protein ( $G_q/G_{11}$ ) [30,31] results from agonist-induced enhancement of G-protein turnover, with little or no contribution from transcriptional control [31]. Although formal proof of a similar mechanism is still required for agonist-induced

down-regulation of  $G_s$  in NG108-15 cells, and has been difficult to demonstrate, due to the relatively poor immunoprecipitation properties of the CS antiserum used herein, evidence indicates that neither transcriptional nor translational mechanisms contribute significantly [5]. Furthermore, recent studies by Levis and Bourne [32] have demonstrated enhanced degradation of  $G_s\alpha$  in S49 lymphoma cells concomitant with sustained or constitutive activation of this G-protein.

The significance of receptor-mediated down-regulation of G-proteins is likely to be dependent on the system and the G-protein in question, but can certainly contribute to the generation and maintenance of long-term desensitization [4]. It has been clearly observed that both ethanol [33]- and prostanoid [8]-induced down-regulation in NG108-15 cells results in the heterologous desensitization of receptor stimulation of adenylate cyclase. Furthermore, maintained exposure to certain, but not all, anti-lipolytic agents is able to produce down-regulation of each of  $G_{i1}$ ,  $G_{i2}$  and  $G_{i3}$  in rat white adipocytes either *in vivo* [34] or when maintained in tissue culture [18,28]. Agents which cause down-regulation of the G-proteins produce heterologous desensitization of control of glycerol release, whereas agents which do not down-regulate the G-proteins produce only homologous desensitization [28].

The studies reported in this paper demonstrate that agonist activation of  $\beta_2$  adrenoceptors expressed in NG108-15 cells can cause a down-regulation of cellular levels of  $G_s\alpha$  without altering the steady levels of other G-proteins. This effect is dependent on the level of expression of the receptor, and as such these data are likely to explain the apparent paradox that sustained agonist activation of some, but not all,  $G_s\alpha$ -linked receptors in NG108-15 cells can cause this effect.

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