Biochem. J. (1992) 285, 529–536 (Printed in Great Britain)

Concurrent down-regulation of IP prostanoid receptors and the α-subunit of the stimulatory guanine-nucleotide-binding protein (G_s) during prolonged exposure of neuroblastoma × glioma cells to prostanoid agonists

Quantification and functional implications

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Neuroblastoma × glioma hybrid NG108-15 cells express a high-affinity IP prostanoid receptor. Saturation binding analysis of this receptor, using [^{3}H]prostaglandin E_1 ([^{3}H]PGE_1) as ligand, indicated that it was present at some 1.5 pmol/mg of membrane protein and displayed a dissociation constant for this ligand of 30–40 nM. Prolonged exposure of these cells either to PGE_1 or to iloprost, which is a stable analogue of prostacyclin, caused a 40–70 % decrease in levels of the receptor. The remaining receptors were capable of interacting with the stimulatory G-protein (G_s) of the adenylate cyclase cascade, as saturation analysis of the binding of [^{3}H]PGE_1 indicated that they had a similar affinity for the ^{3}H-labelled ligand, and because the specific binding of [^{3}H]PGE_1 to these receptors was still sensitive to the presence of poorly hydrolysed analogues of GTP. We have recently demonstrated that prolonged exposure of NG108-15 ells to PGE_1 causes a cyclic AMP-independent loss of G_s α-subunit ($G_s\alpha$) from these cells [McKenzie & Milligan (1990) J. Biol. Chem. **265**, 17084–17093]. Steady-state concentration of the larger 45 kDa form of $G_s\alpha$ (which is the predominant form expressed in these cells) was assessed to be 9.6 pmol/mg of membrane protein, and treatment with iloprost decreased levels of this polypeptide to some 3.0 pmol/mg of protein. Time courses of iloprost-mediated down-regulation of the IP prostanoid receptor, loss of $G_s\alpha$ protein as assessed by immunoblotting and loss of $G_s\alpha$ activity as assessed by the reconstitution of NaF stimulation of adenylate cyclase activity to membranes of S49 cyc^- cells by sodium cholate extracts of NG108-15 cells were identical, suggesting that the loss of the IP prostanoid receptor and G-protein occurred in parallel. Each of these effects was half-maximal between 2 and 3 h of exposure to the agonist. Stoichiometry of loss of $G_s\alpha$ and IP prostanoid receptor was unchanged by the percentage receptor occupancy, and quantification indicated the loss of some 7–10 mol of $G_s\alpha$/mol of receptor. This is the first report to demonstrate the temporal concurrence of loss of $G_s\alpha$ and of a receptor which interacts with this G-protein. Chronic activation of the IP prostanoid receptor on these cells results in the development of a heterologous form of desensitization to agents which function to activate adenylate cyclase [Kelly, Keen, Nobbs & MacDermot (1990) Br. J. Pharmacol. **99**, 306–316]. Agonist regulation of $G_s\alpha$ levels in these cells may contribute to this process.

INTRODUCTION

Prolonged exposure of cells or tissues to agonists which activate a G-protein-linked cell-surface receptor is well established to be able to produce down-regulation of the receptor [1]. Such an effect can act to regulate the sensitivity of transmembrane signalling and hence contribute to the development and maintenance of the phenomenon of desensitization [1]. As the presence and activity of each of the receptor, G-protein and effector system are required to produce a cellular response, then regulation in the activities and amounts of either of these components of the transduction cascade which are distal to the receptor could also provide a suitable locus for the production of long-term desensitization.

A family of G-proteins act to transduce information between receptors and effectors [2,3], and receptor control of the stimulation of adenylate cyclase is mediated via $G_s\alpha$, the α-subunit of the stimulatory G-protein, G_s. There is a single gene for $G_s\alpha$, but differential splicing mechanisms can produce up to four distinct forms of the polypeptide [4,5]. However, it is likely that little difference exists in the ability of the individual forms of the G-protein to activate adenylate cyclase [6], although some evidence exists to suggest that the shorter form(s) might have greater functional activity [7]. Experiments to assess the possibility that regulation in the levels or activity of G_s might occur in response to receptor activation have not been widely reported, but we have noted recently that exposure of neuroblastoma × glioma hybrid NG108-15 cells to prostaglandin E_1 (PGE_1), which in these cells produces a large activation of adenylate cyclase, caused a marked decrease in cellular levels of $G_s\alpha$ without substantially altering levels of the α-subunits of G_i2, G_i3 or G_o, or of G-protein β-subunit [8]. This process was not dependent on the generation of cyclic AMP (cAMP) and did not occur by regulation of the expression of the $G_s\alpha$ gene [8].

In this paper we examine the co-ordination of down-regulation of levels of the receptor (which appears to be a IP prostanoid receptor) at which PGE_1 functions and of $G_s\alpha$ after exposure of NG108-15 cells to prostanoid agonists. We also assess the

Abbreviations used: PGE_1, prostaglandin E_1; cAMP, cyclic AMP; PBS, phosphate-buffered saline; NP40, Nonidet P-40; GTP[S], guanosine 5′-[γ-thio]triphosphate.

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stoichiometry of down-regulation of these polypeptides. We demonstrate that time courses and dose–response dependence of the down-regulation of these two polypeptides are identical and that some 7–10 (mean value 8) mol of $G_s\alpha$ are lost from the cell membrane per mol of the IP prostanoid receptor. This ratio is similar to the stoichiometry of activation of $G_s\alpha$ by β-adrenergic receptors, which has been calculated in experiments utilizing reconstitution of the purified components [9]. We further note that the $G_s\alpha$ which remains at the membrane is not inactivated by prostanoid treatment. Such regulation of cellular G-protein levels may contribute to the long-term development of heterologous desensitization to agents which activate adenylate cyclase in these cells.

MATERIALS AND METHODS

Materials

[^{3}H]PGE_1 (53.7 Ci/mmol), [α-^{32}P]ATP and [^{3}H]cAMP were purchased from Amersham International. All materials for tissue culture were from GIBCO/BRL. Iloprost was kindly given by Schering Health Care (Burgess Hill, W. Sussex, U.K.).

Cell growth

Neuroblastoma × glioma hybrid NG108-15 cells were grown in tissue culture as previously described [10]. Before confluency they were either split 1:10 into fresh tissue-culture flasks or harvested. S49 cyc$^-$ cells were grown in Dulbecco's Modified Eagle's Medium containing 10% (v/v) heat-inactivated horse serum and maintained between 5×10^5 and 2×10^6 cells/ml. Membrane fractions were prepared from cell pastes which had been stored at −80 °C after harvest essentially as in [11]. 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/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 °C until required.

Treatment of cells

Treatment of NG108-15 cells with PGE_1 (Sigma) included ethanol (0.1%, v/v) as vehicle. Preliminary studies indicated that this concentration of ethanol had no effect on levels of $G_s\alpha$ over the time course of the treatments [8]. As ethanol has been reported to cause the accumulation of extracellular adenosine [12] and these cells express an adenosine A2 receptor which is linked to the stimulation of adenylate cyclase [13], then preliminary experiments confirmed that co-incubation of the cultures with adenosine deaminase (1 unit/ml) (Sigma) had no effect on levels of $G_s\alpha$ (see [8]). Buffer controls for iloprost treatment of the cells were also shown to have no effect on the cellular levels of $G_s\alpha$ (results not shown).

Production of antisera and immunoblotting

Antiserum CS1 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 [14]. Immunoblotting with this antiserum was performed as previously described [15,16]. Antiserum BN2 was generated against a synthetic peptide corresponding to the *N*-terminal 10 amino acids (MSELDQLRQE) of the β1 subunit of G-proteins. It is able to identify the polypeptides derived from both the β1 and β2 genes. Characterization of this antiserum has previously been defined [17]. Molecular-mass determinations were based on pre-stained molecular-mass markers (Bethesda Research Laboratories). SDS/PAGE in 10% (w/v) acrylamide gels was carried out overnight at 60 V.

Quantification of immunoblots

After SDS/PAGE, proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 3 h in 5% (w/v) gelatin in Dulbecco's phosphate-buffered saline, pH 7.5 (PBS; 0.27 mM-KCl/0.15 mM-KH_2PO_4/137 mM-NaCl/4.0 mM-Na_2HPO_4). Primary antisera were added in 1% gelatin in PBS containing 0.2% Nonidet P40 (NP40) and incubated overnight. 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, U.K.) was added (1:200 dilution in 1% gelatin in PBS containing 0.2% NP40) 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 with *o*-dianisidine hydrochloride (Sigma) as the substrate for horseradish peroxidase as previously described [16]. In a number of cases the developed immunoblots were subsequently treated with a ^{125}I-labelled goat anti-rabbit IgG (Amersham International) (0.1 μCi/ml) in 1% gelatin/PBS/0.2% NP40 for 2 h. After extensive washing with PBS/0.2% NP40 and then with PBS, the blot was air-dried and the coloured bands were excised and radioactivity was assessed in a γ-radiation counter. In such cases, preliminary experiments were performed to assess the range of linearity of the assay for each antiserum (see [8] for details). Such experiments involved immunoblotting and subsequent treatment with ^{125}I-labelled goat anti-rabbit IgG of various amounts of NG108-15 membranes as described above. Amounts of membranes used to assess the effects of PGE_1 treatment on levels of $G_s\alpha$ were in all cases within the observed linear region.

Quantification of the levels of the 45 kDa form of $G_s\alpha$ was achieved in the same manner on immunoblots which contained various concentrations of *Escherichia coli*-expressed $G_s\alpha$ (long form) [18] (this material was kindly given by Dr. Michael Freissmuth, Department of Pharmacology, University of Vienna, Vienna, Austria). On such immunoblots added quantities of $G_s\alpha$ ranged from 0 to 24 ng (0–0.53 pmol). Over such a range of concentrations, ^{125}I-labelled goat anti-rabbit IgG bound to the *o*-dianisidine-stained immunoblots in a manner which was linear with amount of $G_s\alpha$ (see the Results section).

Adenylate cyclase assays

These were performed as described by Milligan *et al.* [19]. Radiolabelled cAMP and ATP were separated by the method of Salomon *et al.* [20].

S49 cyc$^-$ reconstitution assay

Reconstitution assays in S49 cyc$^-$ membranes were performed essentially as described by Milligan & Klee [21]. Sodium cholate (1%, w/v) was used (1 h, 4 °C) to extract protein from membrane preparations of NG108-15 cells. Samples were then centrifuged at 25 lb/in^2 (150000 *g*) for 30 min in an Airfuge (Beckman Instruments) and the supernatant fraction was taken as the soluble extract. A 5 μl portion of extract (containing protein

equivalent to 5 μg of NG108-15 membrane protein) was added to a final volume of 100 μl containing 10 mM-NaF or 10 mM-NaCl, 50 mM-Hepes, pH 8.0, 10 μg of BSA, 10 units of creatine kinase, 6 mM-$MgCl_2$, 0.2 mM-EGTA, 2 mM-2-mercaptoethanol, 1 mM-[^{3}H]cAMP (15000 c.p.m.), 0.2 mM-[α-^{32}P]ATP (2×10^6 c.p.m.) and 10 μg of S49 cyc$^-$ membranes. Samples were incubated at 30 °C for 60 min and [^{32}P]cAMP generated was assayed as above. Differences in the generation of [^{32}P]cAMP in response to NaF and NaCl were taken to represent a measure of the relative activities of $G_s\alpha$.

Binding experiments with [^{3}H]PGE_1

These were performed routinely with 10 nM-[^{3}H]PGE_1 at 25 °C for 30 min in 20 mM-Tris/HCl (pH 7.5)/50 mM-sucrose/20 mM-$MgCl_2$ containing 10 μM-indomethacin (buffer B) in the absence and presence of 10 μM-PGE_1 or 10 μM-iloprost to define maximal and non-specific binding respectively. In preliminary competition displacement experiments, maximal displacement of 10 nM-[^{3}H]PGE_1 was achieved with 1 μM unlabelled PGE_1 or iloprost. No further displacement of the radioligand was achieved with concentrations of PGE_1 or iloprost up to 100 μM. Specific binding, defined as above, represented some 80% of the total binding of [^{3}H]PGE_1. In experiments designed to assess the maximal binding capacity of membranes of NG108-15 cells for this ligand, the specific activity of a single concentration (10 nM) of [^{3}H]PGE_1 was varied and measured specific binding was subsequently corrected on this basis. Data from such experiments were manipulated in accordance with Scatchard [22] to allow assessment of both the total number of binding sites and the affinity of interaction of the ^{3}H-labelled ligand with these sites. In a number of experiments guanosine 5'-[γ-thio]triphosphate (GTP[S]) (100 μM) was added to assess the interaction of the receptor population with the G-protein signalling system. 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 lacking indomethacin.

RESULTS

A receptor on neuroblastoma × glioma hybrid NG108-15 cells for prostanoid agonists has recently been demonstrated to have the pharmacological profile of a receptor for prostacyclin (IP prostanoid receptor) [23]. Activation of this receptor produced stimulation of adenylate cyclase (Fig. 1), and prolonged incubation of the cells with either PGE_1 (Fig. 1) or with iloprost, a stable analogue of prostacyclin, caused a decrease in responsiveness of adenylate cyclase to PGE_1 in membranes produced from these cells.

The IP prostanoid receptor was identified in binding studies using [^{3}H]PGE_1 (Fig. 2). Displacement of the binding of [^{3}H]PGE_1 from this site could be produced with high affinity by both PGE_1 (results not shown) and iloprost (Fig. 2) (IC_{50} corrected for receptor occupancy 14.8 ± 1.0 nM: mean ± S.E.M., $n = 3$). Saturation analysis of this binding site with [^{3}H]PGE_1, under the conditions used for the binding assay, indicated the presence of a single class of some 1500 fmol of receptor/mg of membrane protein. This receptor displayed a dissociation constant for [^{3}H]PGE_1 of some 40 nM (Fig. 3). Overall, measured levels of the IP prostanoid receptor were 1575 ± 83 fmol/mg of membrane protein (mean ± S.E.M., $n = 16$). Treatment of NG108-15 cells with iloprost (1 μM, 8 h) produced a substantial down-regulation of levels of the IP prostanoid receptor (Fig. 3), but the remaining receptors bound [^{3}H]PGE_1 with similar affinity (Fig. 3), suggesting that these receptors were still able to interact with G_s. In a number of individual studies in which NG108-15 cells were

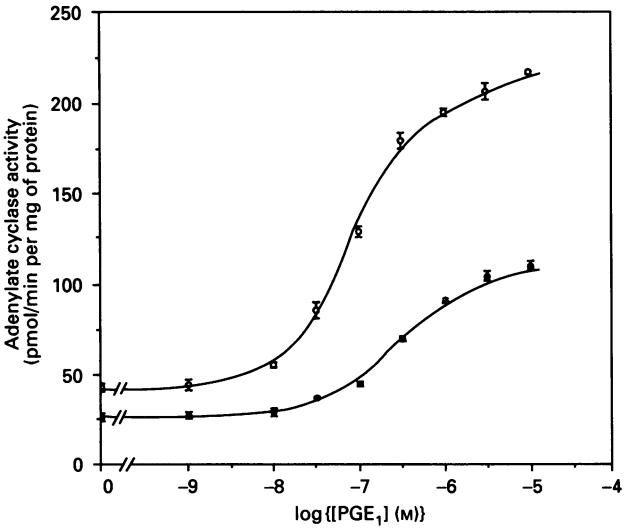

Fig. 1. Ability of PGE_1 to stimulate adenylate cyclase in membranes of untreated and PGE_1-treated NG108-15 cells

NG108-15 cells were treated with (●) or without (○) PGE_1 (10 μM) for 8 h. Membranes were subsequently assayed for PGE_1 regulation of adenylate cyclase activity. Results are presented as means ± S.E.M. for quadruplicate determinations from a single experiment. A second experiment produced similar data.

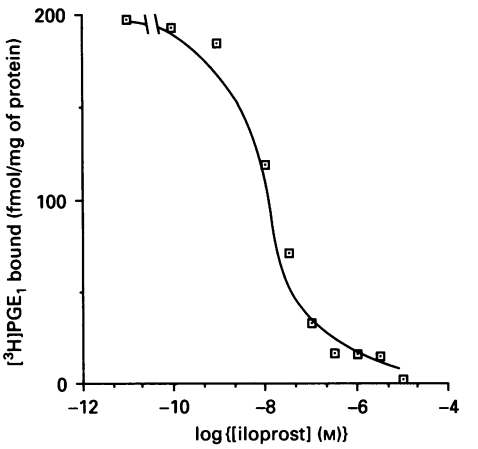

Fig. 2. Specific [^{3}H]PGE_1 binding to membranes of NG108-15 cells

Displacement of the specific binding of [^{3}H]PGE_1 (7.8 nM) from membranes of NG108-15 cells was achieved with various concentrations of iloprost. Based on an estimated K_d for binding of [^{3}H]PGE_1 of 40 nM (see Fig. 3 and the Results section), the density of IP prostanoid receptors in the experiment displayed was 1203 fmol/mg of membrane protein. IC_{50} of iloprost (corrected for receptor occupancy) was 16.7 nM. A series of preliminary competition experiments had defined that non-specific binding of [^{3}H]PGE_1 could be defined adequately by the presence of 10 μM-PGE_1 or 10 μM-iloprost (see the Materials and methods section).

challenged with iloprost, the overall degree of receptor down-regulation was 60.3 ± 3.7% (mean ± S.E.M., $n = 10$). Further confirmation of the interaction of the IP prostanoid receptor with G_s in membranes from both untreated and iloprost-treated NG108-15 cells was obtained when the specific binding of a single sub-saturating concentration of [^{3}H]PGE_1 was measured in the absence and presence of the poorly hydrolysed analogue of GTP, GTP[S] (Table 1). In membranes from both untreated and iloprost-pretreated NG108-15 cells the measured specific binding

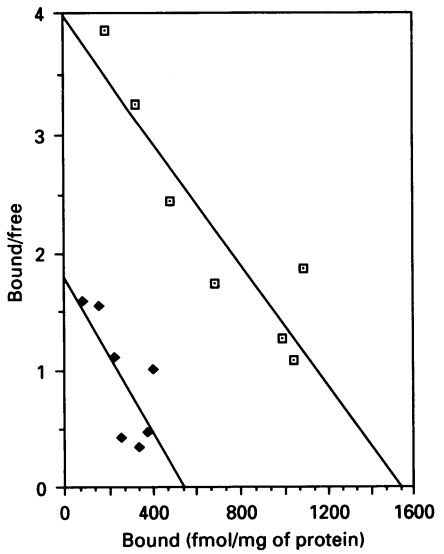

Fig. 3. Saturation analysis of the specific binding of [^{3}H]PGE$_1$ to membranes of untreated and iloprost-treated NG108-15 cells

Analysis of the specific binding of various concentrations of [^{3}H]PGE$_1$ (corrected for specific-radioactivity changes) to membranes of untreated (□) and iloprost-treated (1 μM, 8 h) (◆) NG108-15 cells was performed as described by Scatchard [22]. In the experiment displayed, $B_{max.}$ was 1530 fmol/mg of membrane protein in the membranes from untreated cells and 530 fmol/mg of membrane protein in membranes from iloprost-treated cells. The K_d for binding [^{3}H]PGE$_1$ was 38.5 nM in membranes of untreated cells and 30.4 nM in membranes from iloprost-treated cells. See the Results section for further details.

Table 1. Effect of GTP[S] on the apparent specific binding of [^{3}H]PGE$_1$ to membranes of untreated and iloprost-treated NG108-15 cells

The specific binding of a single concentration (40 nM) of [^{3}H]PGE$_1$, close to the K_d for the ligand, to membranes derived from NG108-15 cells which had been pretreated with either iloprost (1 μM, 8 h) or with vehicle, was measured in the absence and presence of GTP[S] (100 μM). Similar results were produced in two further experiments. As the concentration of the [^{3}H]PGE$_1$ used was close to the K_d for the ligand, the measured number of receptors will be close to 50% of the total population.

	Cells			
	Control		Iloprost-treated	
GTP[S] (100 μM)...	−	+	−	+
Measured [^{3}H]PGE$_1$ specific binding (fmol/mg of protein)	596±88	340±64	284±76	132±40
Decrease by GTP[S] (%)	43.0		53.5	

of [^{3}H]PGE$_1$ was decreased in the presence of GTP[S]. This is a reflection that in the presence of GTP[S] G-protein-coupled receptors adopt a low-affinity conformation for the binding of agonists (see [15] for an example of the use of this phenomenon).

We have previously noted that prolonged treatment of NG108-15 cells with PGE$_1$ causes a decrease in cellular levels of G_s and that this effect was not dependent on the generation of cAMP [8]. Prolonged exposure of the cells to iloprost (1 μM) also produced a marked down-regulation of detectable $G_s\alpha$ whether this was assessed immunologically by immunoblotting membranes of

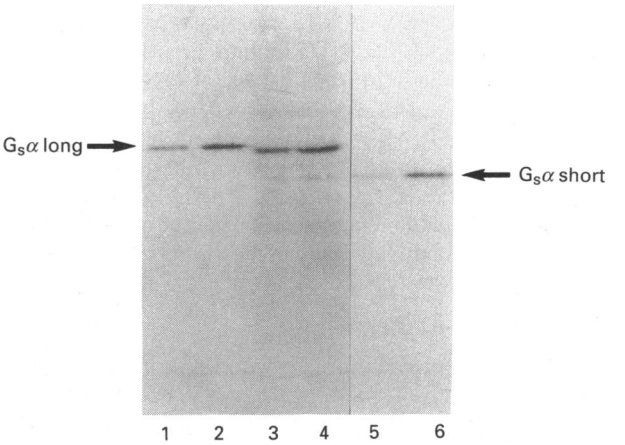

Fig. 4. Quantification of levels of $G_s\alpha$45 in membranes of untreated and iloprost-treated NG108-15 cells

E. coli-expressed $G_s\alpha$ long (45 kDa) and short (42 kDa) forms (see the Materials and methods section) and membranes of untreated and iloprost (1 μM, 8 h)-treated NG108-15 cells were resolved by SDS/PAGE [10% (w/v) acrylamide] and immunoblotted with antiserum CS1 as primary antiserum. Lanes 1 and 2, $G_s\alpha$ long, (1) 6 ng (0.13 pmol) or (2) 18 ng (0.4 pmol); lane 3, membranes (40 μg) from iloprost-treated cells; lane 4, membranes (40 μg) from untreated cells; lanes 5 and 6, $G_s\alpha$ short, (5) 6 ng (0.14 pmol) or (6) 18 ng (0.43 pmol). After colorimetric development, the blot was probed with ^{125}I-labelled goat anti-rabbit IgG, and the coloured bands were excised and counted for γ-radiation. In the experiment displayed, $G_s\alpha$45 in untreated membranes bound 836 c.p.m., whereas that in iloprost-treated membranes bound 494 c.p.m.

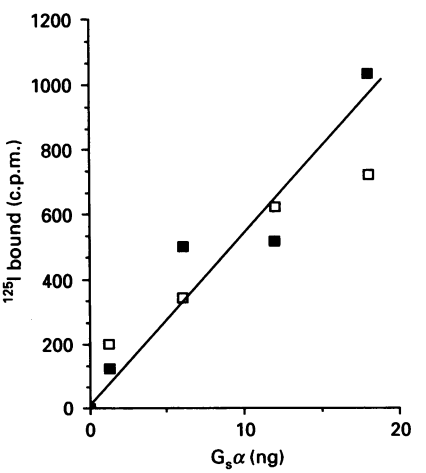

Fig. 5. Quantification, with ^{125}I-labelled goat anti-rabbit IgG, of levels of *E. coli*-expressed $G_s\alpha$ long and $G_s\alpha$ short forms

Various amounts (0–18 ng; 0–0.43 pmol) of *E. coli*-expressed $G_s\alpha$ long (□) and $G_s\alpha$ short (■) forms were resolved by SDS/PAGE and immunoblotted as in Fig. 4. The developed immunoblot was subsequently treated with ^{125}I-labelled goat anti-rabbit IgG, and the coloured bands were excised and counted for γ-radiation. Results are from a single experiment, but similar results over this range of $G_s\alpha$ amounts were observed routinely.

untreated and iloprost-treated NG108-15 cells with an anti-peptide antiserum (CS1) directed against the *C*-terminal decapeptide which is common to all forms of $G_s\alpha$ [14] (Fig. 4) or by the use of cholera-toxin-catalysed [^{32}P]ADP-ribosylation. Quantification of such experiments by cutting out the ^{32}P-

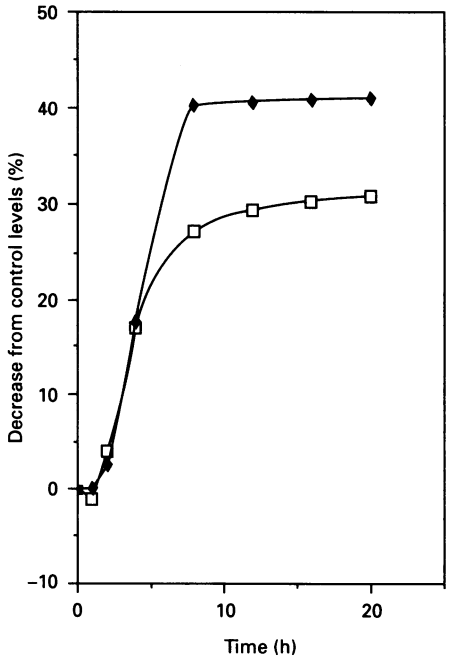

Fig. 6. Concurrent loss of the IP prostanoid receptor and of $G_s\alpha 45$ from NG108-15 cells during treatment with PGE_1

NG108-15 cells in culture were treated with PGE_1 (10 μM) for various times, and levels of the IP prostanoid receptor (◆), measured by the specific binding of [^{3}H]PGE_1, and of $G_s\alpha 45$ (□), measured by the binding of ^{125}I-labelled goat anti-rabbit IgG to immunoblots which utilized antiserum CS1 as primary reagent, were assessed. Results are presented as percentage decreases in levels of the two polypeptides with time. Similar results were observed in experiments derived from three separate time courses.

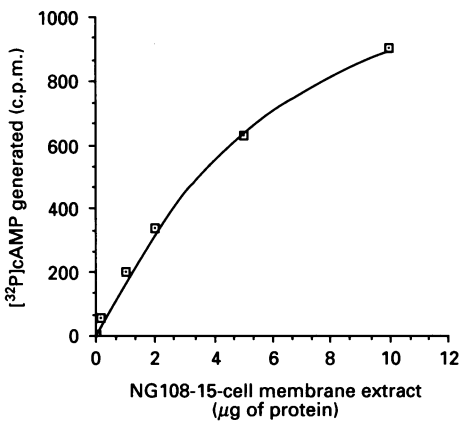

Fig. 7. Reconstitution of NaF-stimulated adenylate cyclase activity to membranes of S49 cyc$^-$ cells: dependence on amount of added exogenous protein

Sodium cholate (1%, w/v) was used to extract protein from membranes of untreated NG108-15 cells. Extracts derived from 0–10 μg of NG108-15 membranes were then added to an S49 cyc$^-$ reconstitution assay (see the Materials and methods section) under conditions in which the sodium cholate concentration in the assay was decreased to 0.05% (w/v). Generation of cAMP in this assay was only observed in the presence of NaF and not when this was replaced with equimolar NaCl. Generation of cAMP was linear with extract derived from between 0.1 and at least 5 μg of NG108-15 membrane protein.

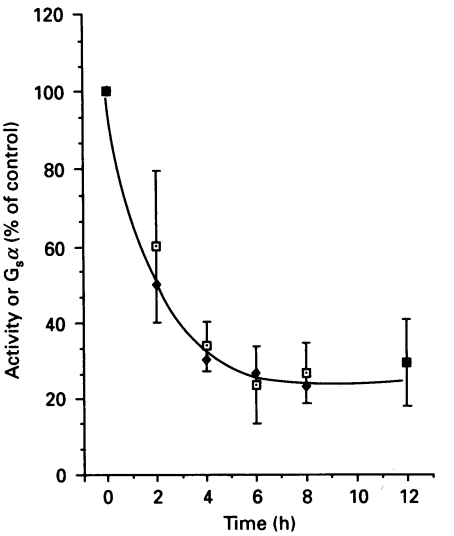

Fig. 8. Time course of the loss of ability of sodium cholate extracts of iloprost-treated NG108-15 cells to reconstitute NaF-dependent adenylate cyclase activity to membranes of S49 cyc$^-$ cells: comparison with the rate of loss of immunologically detected $G_s\alpha$

NG108-15 cells were treated for various times with iloprost (1 μM) and membranes were subsequently prepared. These membranes were immunoblotted for $G_s\alpha 45$ with antiserum CS1 as primary reagent, and the results were subsequently quantified by overlay of the developed immunoblot with ^{125}I-labelled anti-rabbit IgG (□) (see the Materials and methods section). The same membranes were extracted with sodium cholate and the detergent extract from 5 μg of membrane protein was used to reconstitute NaF-dependent adenylate cyclase activity to membranes (10 μg) of S49 cyc$^-$ cells (■). Results for the immunological quantification of $G_s\alpha 45$ are pooled from three individual experiments and represent means ± S.E.M. For the $G_s\alpha$ reconstitution experiments, errors in all cases were within the error bars given for the immunological quantification. In the reconstitution experiments 100% adenylate cyclase activity was equivalent to 42.7 ± 3.1 pmol/min per mg of cyc$^-$ acceptor protein.

radiolabelled bands corresponding to the 45 kDa form of $G_s\alpha$ ($G_s\alpha 45$) followed by liquid-scintillation counting indicated a decrease in labelling in membranes of the iloprost-treated cells by 56% [membranes from control cells 285 ± 13 c.p.m., membranes from iloprost-treated cells 130 ± 2 c.p.m. (means ± S.E.M., n = 5)].

Quantification of the steady-state levels of $G_s\alpha 45$ in membranes of NG108-15 cells was achieved by assessing the binding of a ^{125}I-labelled goat anti-rabbit IgG to colorimetrically developed immunoblots which also contained various amounts of the long form of $G_s\alpha$ which had been expressed in *E. coli* [18] (Fig. 5). This polypeptide migrated as a 45 kDa polypeptide in our gel system, equivalent to the more slowly migrating form of $G_s\alpha$ in NG108-15 cells (Fig. 4). The short form of $G_s\alpha$ which had been expressed in *E. coli* migrated as a 42 kDa polypeptide in our gel system and essentially co-migrated with the short form of $G_s\alpha$ ($G_s\alpha 42$), which is present in membranes of NG108-15 cells, but at considerably lower levels than $G_s\alpha 45$ (Fig. 4). Such quantification indicated that $G_s\alpha 45$ was present at some 9.6 ± 1.5 pmol/mg of membrane protein (mean ± S.E.M., n = 4). Treatment with iloprost (1 μM, 8 h) decreased membrane levels of this polypeptide to 3.0 ± 0.3 pmol/mg of membrane protein (mean ± S.E.M., n = 3), a decline of some 69%.

Time courses of treatment of NG108-15 cells with PGE_1 (10 μM) (Fig. 6) or with iloprost (results not shown) demonstrated that the down-regulation of the IP prostanoid receptor and of

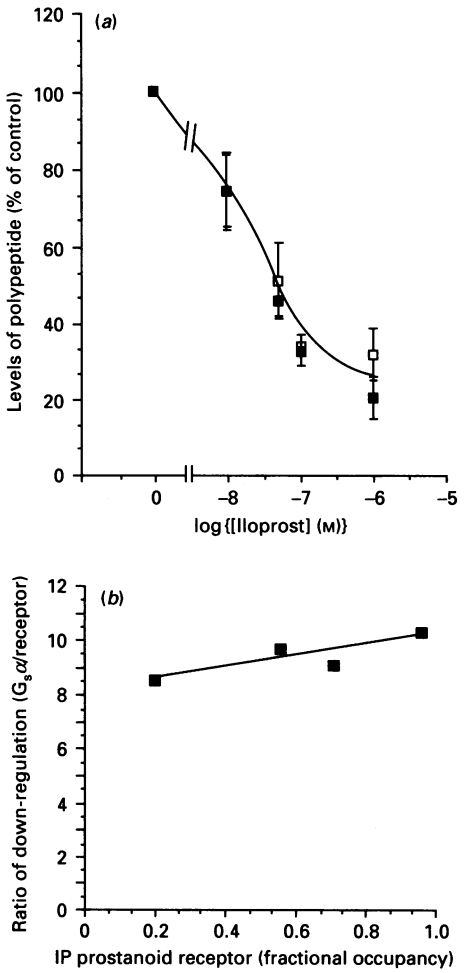

Fig. 9. Degree of receptor occupancy does not modify the stoichiometry of down-regulation of the IP prostanoid receptor and $G_s\alpha 45$

NG108-15 cells were treated with various concentrations of iloprost for 8 h. Membranes were prepared and (*a*) levels of both the IP prostanoid receptor (□) and $G_s\alpha 45$ (■) were quantified. In the experiment displayed, the estimated $B_{max.}$ of the IP prostanoid receptor in membranes from untreated cells was 1.11 pmol/mg of membrane protein and the level of $G_s\alpha 45$ was 9.6 pmol/mg of membrane protein. Based on an estimated K_i of 14.8 nM for iloprost (see Fig. 2), the fractional occupancy of the IP prostanoid receptor during treatment with various concentrations of iloprost was calculated, as were molar decreases of the IP prostanoid receptor and $G_s\alpha 45$. Molar ratios (*b*) of the down-regulation of these two polypeptides remained essentially constant with variation of fractional occupancy of the IP prostanoid receptor. Fractional occupancy of the receptor was varied between 0.20 and 0.96. Ratios of stoichiometry of loss of the two polypeptides ($G_s\alpha$ to IP prostanoid receptor) in the experiment displayed were between 8.5 and 10.1 at the individual points analysed. In two further experiments the ratios of G-protein to receptor loss were between 6.9 and 9.4. Immunoblot assays were used to detect and quantify $G_s\alpha$.

$G_s\alpha 45$ were entirely coincident, with half-maximal effects being observed at 3 h in the case of treatment with PGE_1. In the experiment displayed in Fig. 6, loss of the IP prostanoid receptor reached some 40% of the initial levels, with a loss of $G_s\alpha$ of some 30%. In similar experiments in which iloprost was used as ligand, the percentage loss of both the IP prostanoid receptor and $G_s\alpha$ was greater than that achieved with PGE_1 (see Fig. 9*a*, for example).

Loss of the functional activity of $G_s\alpha$ from membranes of NG108-15 cells during time courses of treatment with iloprost was assessed by the ability of sodium cholate extracts of membranes of these cells to reconstitute adenylate cyclase activity to membranes of S49 cyc^- cells in a fashion which was dependent on the presence of NaF. Preliminary experiments demonstrated that a linear increase in NaF-stimulated generation of cAMP from 10 μg of cyc^- membranes was obtained when the reconstitution assay contained sodium cholate (1%, w/v; 1 h, 4 °C)-extracted protein derived from 0.1–5 μg of NG108-15 cell membranes (Fig. 7). A further increase in cAMP generation was noted when further detergent-extracted protein was added to the reconstitution assay, but this increase was not linearly dependent on protein amount (Fig. 7). Loss of functional $G_s\alpha$ activity in these extracts, as measured by such cyc^- reconstitution assays, paralleled the loss of immunologically detectable $G_s\alpha$ in membranes of the NG108-15 cells (Fig. 8). By contrast, sodium cholate (1%) extraction of protein from NG108-15 cell membranes, as assessed by Coomassie Blue staining of SDS/PAGE gels of the solubilized protein, was not modified by pretreatment of the cells with prostanoid agonists (results not shown).

Treatment of NG108-15 cells with various concentrations of iloprost for 8 h, followed by analysis of the remaining levels of the IP prostanoid receptor and of the 45 kDa form of $G_s\alpha$ (Fig. 9*a*), also indicated that these polypeptides were lost in parallel. The ratio of loss of IP prostanoid receptor to that of $G_s\alpha 45$ was 1:7–10 at all agonist concentrations (Fig. 9*b*), and as such was not modified substantially by the fractional occupancy of the receptor population during agonist treatment.

DISCUSSION

We have noted previously that treatment of neuroblastoma × glioma hybrid NG108-15 cells in tissue culture with PGE_1 causes a marked decrease in cellular levels of $G_s\alpha$ [8]. Although the dose-response curves for PGE_1 stimulation of adenylate cyclase and loss of $G_s\alpha$ were identical, receptor-independent activation of adenylate cyclase did not produce a loss of $G_s\alpha$, eliminating a direct role for cAMP in this process. It was further clear that the loss of $G_s\alpha$ on stimulation of these cells with PGE_1 was specific for this G-protein. No detectable differences in membrane-associated levels of each of G_i2, G_i3, G_o or G-protein β subunit were recorded between untreated and PGE_1-treated cells [8].

It is well established that prolonged exposure of cells to agonists which activate G-protein-linked receptors can cause a decrease in levels of such receptors in the plasma membrane [1]. Therefore in the present study we examined the loss of the prostanoid receptor and $G_s\alpha$ in parallel to assess if these two polypeptides might be co-ordinately down-regulated, and if so to quantify the stoichiometry of this process.

The nature of the prostanoid receptor present on NG108-15 cells has not been unequivocally demonstrated and as, with the exception of a receptor for thromboxane A_2 [24], no receptors for metabolites of arachidonic acid have yet been defined at a molecular level, characterization must remain tentative. However, on the basis of the relative potencies of PGE_1, PGE_2 and iloprost to displace the specific binding of either [^{3}H]PGE_1 or [^{3}H]iloprost, and on the lack of specific binding of [^{3}H]PGE_2 to membranes of these cells, it has been argued that it is a IP prostanoid receptor rather than a receptor for E-series prostaglandins [23]. We noted that NG108-15 cell membranes express high levels (some 1.5 pmol/mg of membrane protein) of this receptor binding site. Levels of $G_s\alpha 45$ were measured to be some 10 pmol/mg of membrane protein (Figs. 4 and 5). The level of the prostanoid receptor which we have noted to be expressed by these cells is markedly greater than recorded previously

[25,26]. We can offer no definitive reason for this, but a number of factors may contribute to the discrepancy. In the previous studies a large number of low-affinity prostanoid binding sites were observed, whereas in our own studies we have not observed this low-affinity high-capacity site. Our own work has used a P2 membrane fraction to analyse prostanoid receptor/G-protein interactions, whereas the studies by MacDermot and co-workers have utilized either a total cell homogenate [26] or a total membrane fraction [25]. Thus it is possible that the low-affinity site and the receptor are not in the same cellular compartment. We believe, however, that the high-affinity prostanoid binding site which we have measured here is a true receptor, for the following reasons. (1) Scatchard analysis of our specific [^{3}H]PGE_1 binding data is consistent with a single population of non-interacting high-affinity sites (Fig. 3). (2) The estimated K_d of this site for PGE_1 (30–50 nM) is the same as the measured EC_{50} for PGE_1 stimulation of adenylate cyclase in these cell membranes [8]. (3) Specific binding of [^{3}H]PGE_1 to this site is decreased by inclusion of poorly hydrolysed analogues of GTP to the binding assay (Table 1), as would be expected for the binding of an agonist to any G-protein-coupled receptor, but not for a non-receptor prostanoid binding site [25]. (4) Binding of [^{3}H]PGE_1 to the population of high-affinity prostanoid binding sites which remain after chronic exposure of the cells to PGE_1 or iloprost is sensitive to guanine nucleotides (Table 1), and hence these receptors can be assumed to remain coupled to the G-protein signalling system of the cell. (5) Prolonged treatment of NG108-15 cells with either PGE_1 or with iloprost caused, as expected, a substantial down-regulation of the prostanoid receptor population (Fig. 3). In previous studies it has been noted that this is true for the high-affinity prostanoid binding site (receptor), but not for the low-affinity (non-receptor?) prostanoid binding site [26].

In time-course experiments in which iloprost was used as agonist, amounts of the receptor stabilized at some 30–40% of the initial levels. As such, this represented a loss of some 1.0 pmol of receptor/mg of membrane protein. In concert, levels of $G_s\alpha45$ were decreased to reach a new steady state of some 3 pmol/mg of membrane protein, a loss of some 7 pmol/mg of membrane protein. The remaining G_s, however, appears to be able to activate adenylate cyclase. This conclusion is based on the observation that the remaining $G_s\alpha$ is able to restore adenylate cyclase activity to membranes of S49 cyc$^-$ cells in a fashion which is completely dependent on the presence of NaF. Further, the maximal reconstitutive ability of sodium cholate extracts of these NG108-15 membranes correlated highly with the proportion of immunologically detectable $G_s\alpha$ remaining after prostanoid treatment of the cells (Fig. 7 and 8).

It has been noted that prostanoid treatment of NG108-15 cells produces heterologous desensitization of receptor-stimulation of adenylate cyclase [26,27]. We noted that iloprost treatment of NG108-15 cells still leaves some 3 pmol of potentially functional $G_s\alpha45$/mg of membrane protein as the new steady state. Gordon and co-workers [13] have reported that each of adenosine-analogue and PGE_1 stimulation of adenylate cyclase, $G_s\alpha$ levels and function and amounts of $G_s\alpha$ mRNA are decreased by similar percentages after treatment of NG108-15 cells with ethanol. Given such data, it is tempting to conclude that $G_s\alpha$ levels provide the limiting factor for the stimulation of adenylate cyclase in these cells. Furthermore, as prolonged incubation of NG108-15 cells with cholera toxin leads to a very marked decrease in cellular steady-state levels of $G_s\alpha$ [28] and a situation in which the expected sustained activation of adenylate cyclase activity owing to cholera-toxin-induced ADP-ribosylation of $G_s\alpha$ is reversed [28], it is then apparent that pharmacological and biochemical manipulation of levels of $G_s\alpha$ in these cells can produce a situation in which there appears to be insufficient $G_s\alpha$ for maximal activation of adenylate cyclase to be achieved. However, given the relatively high levels of apparently unmodified $G_s\alpha$ which remain in membranes of NG108-15 cells after treatment with either ethanol [13] or in these studies with prostanoid agonists, then the receptor-mediated loss of G-protein is unlikely to be sufficient, at least in isolation, to account for the development of heterologous desensitization unless there is distinct cellular compartmentation of this polypeptide. The development of heterologous desensitization in such a situation may well result from cAMP-dependent phosphorylation of receptors which interact with G_s [1].

Incubation of NG108-15 cells with agonists at the A2 adenosine receptor or the secretin receptor does not result in a clearly observed decrease in levels of $G_s\alpha$ [29]. This difference between the effects of activation of the IP prostanoid receptor and the adenosine A2 and secretin receptors may be due to intrinsic differences in the structure and function of these receptors, but may also relate to the levels of expression of these three receptors. We have demonstrated that the IP prostanoid receptor is present in high levels in NG108-15 cells and that prostanoid-mediated down-regulation of the receptor produces a concurrent loss of some 7–10 times as much $G_s\alpha$ (Fig. 9*b*). It is unclear what the levels of the adenosine A2 receptor are on these cells, as attempts to measure them, using [^{3}H]5′-(*N*-ethylcarboxamido)adenosine for example, have been shown to be problematic [30]. However, levels of the secretin receptor are very low [31], being in the region of 15–30 fmol/mg of membrane protein. This is only some 1–2% of the levels of the IP prostanoid receptor. As such, if agonist-induced down-regulation of a receptor were in each case to produce down-regulation of a similar molar ratio of $G_s\alpha$, then this would be impossible to detect with the secretin receptor, owing to the high steady-state levels of $G_s\alpha45$ polypeptide compared with amounts of this receptor. It may be instructive to introduce a molecularly defined receptor which is able to stimulate adenylate cyclase into NG108-15 cells by transfection and then to isolate clones expressing various levels of the receptor to quantify potential co-ordinate down-regulation of the receptor and $G_s\alpha$.

Apparent concurrent down-regulation in rat adipocytes in primary culture of A1 adenosine receptors with a number of pertussis-toxin-sensitive G-proteins has been recorded previously [17,32], although this process is considerably slower than the effects noted herein. Loss of the 'G_i-like' G-proteins in such cells has been noted to be associated with the development of heterologous desensitization of the action of anti-lipolytic hormones [33], and, although alterations of steady-state levels of $G_s\alpha$ have also been recorded [8,13] in NG108-15 cells, this is the first report to demonstrate the temporal concurrence of loss of $G_s\alpha$ and of a receptor which interacts with this G-protein. Although it has been suggested that co-ordinate down-regulation of receptors and G-proteins may occur in a variety of systems [34], it remains for future analysis to ascertain how common a process this is and whether the stoichiometry of this process relates to the stoichiometry of interaction of these polypeptides in the plasma membrane.

These studies were supported by a project grant from the Medical Research Council (U.K.) to G.M. E.J.A. thanks the Science and Engineering Research Council for a studentship.

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Received 17 July 1991/30 January 1992; accepted 7 February 1992