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INHIBITION OF ADP-RIBOSYLTRANSFERASE INCREASES SYNTHESIS OF Gsα IN NEUROBLASTOMA × GLIOMA HYBRID CELLS AND REVERSES ILOPROST-DEPENDENT HETEROLOGOUS LOSS OF FLUORIDE-SENSITIVE ADENYLATE CYCLASE

LOUISE E. DONNELLY,* ROBERT S. BOYD, ROBERT J. WILLIAMS,† EAMONN KELLY* and JOHN MACDERMOT

Department of Clinical Pharmacology, Royal Postgraduate Medical School, Du Cane Road, London W12 0NN; †Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

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Abstract—Exposure of NG108-15 cells to 50 mM nicotinamide [an inhibitor of mono(ADP-ribosyl)transferase] for 18 hr led to an increase in membrane associated Gsα measured either as cholera toxin substrate or by immunoblotting with a specific antiserum. Prolonged exposure of NG108-15 cells to iloprost is followed by homologous loss of iloprost sensitivity, and heterologous loss of fluoride-dependent activation of adenylate cyclase. Nicotinamide reversed the loss of fluoride sensitivity, but failed to restore iloprost-dependent activation of adenylate cyclase. These results with nicotinamide in NG108-15 cells contrasted with those from platelets, which also exhibit heterologous desensitization of fluoride sensitivity following prolonged exposure to iloprost. Treatment of platelets with 50 mM nicotinamide for 18 hr led to an increase of 75.0 ± 19.4% in the amount of membrane associated cholera toxin substrate. However, there was no associated increase in the abundance of Gsα as determined by immunoblotting. Furthermore, in platelets there was no restoration by nicotinamide of the iloprost-dependent loss of fluoride-sensitive adenylate cyclase activity. It follows that heterologous desensitization in platelets is accompanied by inactivation of Gsα, which is retained within the plasma membrane in its inactive state..

The nicotinamide-dependent increase in the abundance of membrane associated cholera toxin substrate and immunoreactive Gsα in NG108-15 cells is associated with an increase of 72.0 ± 20.3% in the levels of mRNA encoding Gsα. The capacity of nicotinamide to increase the abundance of membrane associated Gsα was reversed when the cells were cultured in the presence of 20 μg/mL cycloheximide. These results suggest that the ability of nicotinamide to increase the abundance of Gsα in NG108-15 cells is mediated by *de novo* protein synthesis.

Key words: G-protein; ADP-ribosyltransferase; NG108-15 cells; platelets; desensitization; IP-receptors

Mono(ADP-ribosyl)transferases (E.C. 2.4.2.31) are a family of enzymes which catalyse the hydrolysis of NAD^+ and the subsequent transfer of ADP-ribose on to a suitable amino acid acceptor. Eukaryotic mono(ADP-ribosyl)transferases have been reported in many different cells and tissues including turkey erythrocytes [1], skeletal muscle [2] and thyroid membranes [3]. Although details have emerged regarding the enzymology of purified mono(ADP-ribosyl)transferases [4–7], rather less is known of their intracellular substrates and physiological role. There are reports of mono(ADP-ribosyl)ation of the 78 kDa glucose regulator protein [8], non-muscle actin [7, 9] and EF-2† [10, 11]. It is proposed in each case that the covalent modification alters the biological activity of the acceptor protein. Additional information has emerged from studies exploiting inhibitors of mono(ADP-ribosyl)transferase. Examples include nicotinamide and 3-amino-benzamide [6], which affect tumour necrosis factor-induced cell apoptosis [12]. *Meta*-iodobenzylguanidine, another inhibitor of mono(ADP-ribosyl)transferases [13] modifies calcium homeostasis in rat liver [14].

ADP-ribosyltransferases may alter signal transduction, and bacterial exotoxins from *Vibrio cholerae* and *Bordetella pertussis* possess mono(ADP-ribosyl)-transferase activities that have the capacity to ADP-ribosylate the α subunits of G-proteins and modify adenylate cyclase activity. The α-subunit of the stimulatory G-protein (Gsα) is a substate for cholera toxin, and Giα is a substrate for pertussis toxin. The possibility that G-proteins may be ADP-ribosylated by eukaryotic mono(ADP-ribosyl)transferases was suggested by the finding that a purified arginine-specific mono(ADP-ribosyl)transferase from platelets ADP-ribosylates exogenous Gsα and stimulates adenylate cyclase [15]. A similar activity has also been demonstrated in chicken spleen membranes, where it was shown that a membrane bound enzyme was involved in the ADP-ribosylation of a 45 kDa protein leading to an increase in adenylate cyclase activity [16]. Recently we have demonstrated that

* Corresponding author. Tel. 081-740-3219; FAX 081-749-3439.

† Abbreviations: EF-2, elongation factor-2; PRP, platelet rich plasma; PGE_1, prostaglandin E_1; PGL_2, prostacyclin.

Gsα is an endogenous substrate for mono(ADP-ribosyl)transferase in NG108-15 cells, and that inhibition of the enzyme by nicotinamide was accompanied by an increase in Gsα abundance and activity [17]. The mechanism whereby Gsα levels are regulated by ADP-ribosylation in these cells remains obscure, and has now been further examined in both platelets and NG108-15 cells.

Platelets and NG108-15 cells have similar responses to prostacyclin (or its stable analogue iloprost), which mediates activation of adenylate cyclase. In both cell types, prolonged exposure to PGE_1 or iloprost is followed by desensitization to PGE_1 or iloprost-dependent responses and loss of prostacyclin receptors [18–22]. In addition there is heterologous loss of both adenosine (A_2)-dependent responses and sensitivity to sodium fluoride. The loss of fluoride sensitivity and heterologous desensitization in NG108-15 cells are explained by the finding that prolonged exposure to iloprost or PGE_1 in these cells is followed by elimination of Gsα from the plasma membrane [21–24]. We have now performed experiments to examine the role of endogenous ADP-ribosylation of Gsα in these complex patterns of desensitization. The experiments were performed in both NG108-15 cells and platelets. There is a substantial literature on PGE_1 and iloprost-dependent responses in both NG108-15 cells [17–19, 22, 24] and platelets [21, 23], and differences have emerged in the events that accompany heterologous desensitization in these two cell types. The effects of nicotinamide [an inhibitor of mono(ADP-ribosyl)transferase] have been examined in both NG108-15 cells and platelets, and measurements made of (i) the activity of Gsα, (ii) the abundance of membrane associated Gsα and (iii) the synthesis of new Gsα in NG108-15 cells.

MATERIALS AND METHODS

Materials. [α^{32}P]NAD^+ was obtained from New England Nuclear (Stevenage, U.K.). [α^{32}P]ATP, [^{3}H]cyclic AMP, deoxycytidine 5′[α^{32}P]triphosphate and Hybond-C nitrocellulose were obtained from Amersham International plc (Amersham, U.K.). Anti-rabbit IgG conjugated to horseradish peroxidase was obtained from Dako Ltd (High Wycombe, U.K.). Anti-Gsα and anti-Giα antibodies were obtained from Biomac Ltd (Glasgow, U.K.), and anti-Gβ antibody was obtained from Dupont (Stevenage, U.K.). The following generous gifts were received, Ro20-1724 from Roche Products Ltd (Welwyn, U.K.), iloprost from Schering AG (Berlin, Germany), Gsα common cDNA from Dr R Randall Reed, Johns Hopkins University School of Medicine (Baltimore, MD, U.S.A.) and α-actin cDNA from Dr K Docherty, Department of Medicine, University of Birmingham (U.K.). All other reagents were obtained from Sigma (Poole, U.K.).

Cell culture. NG108-15 neuroblastoma × glioma somatic hybrid cells [25] were cultured in 80 cm^2 flasks in Dulbecco's modified Eagle's minimum essential medium containing 10% (v/v) foetal calf serum, 1 μM aminopterin, 16 μM thymidine and 100 μM hypoxanthine. Cultures were maintained at 37° in a humidified atmosphere of 95% air and 5% CO_2. Membranes were prepared as described previously [22] and stored at −80° until required.

Platelets. Platelets were isolated as described previously [21]. Briefly, blood samples (50–150 mL) were drawn from human volunteers and mixed with 3.15% (w/v) trisodium citrate (9:1, v/v). The blood was centrifuged for 15 min at 500 *g*, and the supernatant PRP was retained. In all experiments HEPES buffer, pH 7.4 was added to the PRP to give a final concentration of 20 mM. Platelet membranes were prepared from PRP as described previously [21] and stored at −80° until required.

Adenylate cyclase assay. Adenylate cyclase activity was measured by a modification [26] of the method of Salomon *et al.* [27]. Briefly, reaction mixtures of 100 μL contained 50 mM Tris–HCl buffer pH 7.4, 5 mM $MgCl_2$, 20 mM creatine phosphate disodium salt, 10 IU creatine kinase, 1 mM cyclic AMP, 0.25 mM Ro20-1724 (a phosphodiesterase inhibitor), 1 mM [α^{32}P]ATP (2 μCi/tube), 4 μM GTP and 20–70 μg membrane protein. The reaction was allowed to proceed at 37° for 20 min, and then terminated by the addition of 800 μL of 6.25% (w/v) trichloroacetic acid. [8-^{3}H]Cyclic AMP (approx. 10,000 cpm in 100 μL of water) was added to each tube, and the mixtures centrifuged at 1500 *g* for 20 min. The [α^{32}P]ATP and [^{32}P]cyclic AMP were then separated using a two-step chromatographic procedure [27]. The losses of [^{32}P]cyclic AMP on the columns were corrected for by measurement of the recovery of [8-^{3}H]cyclic AMP.

Cholera toxin catalysed [^{32}P]ADP-ribosylation of Gsα. NG108-15 cells and platelets were treated for 18 hr in the absence or presence of 10 μM iloprost ± 50 mM nicotinamide, and membranes prepared. Membranes were then subjected to cholera toxin catalysed ADP-ribosylation using [α^{32}P]NAD^+ as reported previously [21]. Reaction mixtures contained 1 mM ATP, 15 mM glycine, 25 mM $MgCl_2$, 10 mM thymidine, 10 μM [^{32}P]NAD (30 Ci/mmol), 10 μg cholera toxin subunit A, 100 μM GTP and 300 mM phosphate buffer pH 7.0. The labelled proteins were resolved by 10% SDS–PAGE and visualized using autoradiography. The relative intensities of the bands were assessed using an LKB-Ultroscan laser densitometer, and quantification performed with the integration facility of Fig.P graphics software (Fig.P. Software Corporation).

Immunoblot analysis. Immunoblots were performed as described previously [17]. Briefly, membrane proteins (50 μg) were resolved by 10% SDS–PAGE and transferred to nitrocellulose membranes. The nitrocellulose was blocked for 2 hr with 3% (w/v) resuspended dried milk protein, and washed three times with PBS containing 0.05% (w/v) BSA and 0.05% (v/v) Tween-20. The nitrocellulose was then incubated for 2 hr with the primary antiserum diluted in PBS containing 0.1% (w/v) BSA, and the blots washed extensively. The blots were then incubated for 1 hr with anti-rabbit IgG, conjugated to horseradish peroxidase (1:1000 dilution) and washed again as above. The immunoreactive bands were visualized using 4-chloro-1-naphthol as described previously [17]. The blots were finally washed, dried and scanned as described above.

Table 1. Effect of 18 hr iloprost ± nicotinamide pretreatment of NG108-15 cells on adenylate cyclase activity

Treatment	Adenylate cyclase activity (pmol cyclic AMP/min/mg protein) Basal	1 μM iloprost	10 mM F
Control	13.9 ± 1.1[1]*	69.2 ± 3.5[3,4]	30.9 ± 2.85[5]
10 μM iloprost	11.0 ± 1.0[1,2]	33.5 ± 4.4[3]	20.6 ± 1.1[5,6]
10 μM iloprost + 50 mM nicotinamide	14.1 ± 0.5[2]	29.6 ± 0.8[4]	33.2 ± 4.3[6]

NG108-15 cells were cultured for 18 hr in the absence or presence of 10 μM iloprost ± 50 mM nicotinamide. Cells were harvested, membranes prepared and adenylate cyclase activity measured as described in Materials and Methods. The enzyme activities relating to measurements made in the presence of 10 mM NaF or 1 μM iloprost are the δv values for the increase in enzyme activity above the basal level. The results are expressed as the means ± SEM, N = 5, except * where N = 15. The shared superscript numbers indicate differences between a pair of mean values ($P < 0.05$). P values were determined by unpaired, two-tailed *t*-tests.

The linearity of band density with protein concentration for both cholera toxin [^{32}P]ADP-ribosylation and immunoblotting were confirmed by resolution of selected concentrations of protein by SDS–PAGE. The results presented in this paper were within the linear range of both techniques.

Northern blot analysis. Total RNA was isolated from NG108-15 cells using a single step procedure [28]. Following denaturation of the RNA (10 μg) with 1 M glyoxal at 50° for 1 hr, RNA was resolved in 1.2% (w/v) agarose gels and transferred to Hybond-C nitrocellulose filters by capillary blotting. Ribosomal RNA (18 and 28S) markers from calf liver were also subjected to gel electrophoresis to determine the size of the hybridized bands. The filters were pre-hybridized at 42° for 2 hr in 50% (v/v) deionized formamide, 0.2% (v/v) polyvinylpyrrolidone, 0.2% (w/v) BSA, 0.2% (v/v) Ficoll, 1 M NaCl, 0.1% (w/v) sodium pyrophosphate, 1% (w/v) SDS, 10% (w/v) dextran sulphate, 0.05 M Tris–HCl, pH 7.5 containing 100 μg/mL denatured salmon sperm. The filters were then hybridized at 42° for 16 hr in the solution above containing ^{32}P-labelled fragments of Gsα common or α-actin. The probes were prepared by oligolabelling of cDNA using hexadeoxynucleotide primers and deoxycytidine 5′[α-^{32}P]triphosphate (sp. act. 3000 Ci/mmol). Following hybridization, the filters were washed with 0.3 M NaCl, 30 mM trisodium citrate, 1% (w/v) SDS at 65° for 1 hr. Blots were then autoradiographed for 48 hr at −80° before development. The intensity of the bands obtained from the autoradiographic plates were quantified by densitometry using a Hoefer GS300 scanning densitometer connected to a pen recorder. From the resulting scan, Gsα-common peaks were cut out and weighed.

Statistical analysis. Data were quantified from both autoradiographs and blots as described earlier, and the effect of any treatment was expressed as a percentage of the control value for each experiment. Data were compared using a Mann–Whitney non-parametric test (or *t*-tests where indicated) and expressed as the mean value ± SEM.

RESULTS

Treatment of NG108-15 cells with 10 μM iloprost for 18 hr resulted in a significant decrease in basal, agonist (iloprost) or fluoride-stimulated adenylate cyclase activity when compared to control values (Table 1). When the cells were pretreated (18 hr) with both 10 μM iloprost and 50 mM nicotinamide, the decrease in iloprost-stimulated adenylate cyclase activity was maintained, but basal enzyme activity and sensitivity to fluoride were restored to control levels. Nicotinamide treatment alone led to a small but significant ($P < 0.05$) increase in iloprost-stimulated adenylate cyclase activity ($\delta V = 69.2 \pm 3.5$ compared with 84.9 ± 4.5, N = 5 pmol cAMP/min/mg protein), with a significant increase ($P < 0.05$) in basal adenylate cyclase activity 13.8 ± 1.1, N = 15 compared with 20.7 ± 2.5, N = 15 pmol cAMP/min/mg protein) and no change in fluoride-sensitive activity.

In order to ascertain whether the reversal of iloprost-dependent loss of fluoride activity by nicotinamide was due to changes in the levels of Gsα, the abundance of Gsα was measured both as cholera toxin substrate and by immunoblotting. We have reported previously [17, 22] that treatment of NG108-15 cells with iloprost leads to a reduction in cholera toxin substrate within cell membranes, and the result is confirmed in Fig. 1. The results in Fig. 1 have been taken from an earlier report [17], and are reproduced here for clarity and comparison with data derived from platelets. Treatment of cells with both iloprost and nicotinamide restored the abundance of cholera toxin substrate back to control levels, and treatment of NG108-15 cells with nicotinamide alone was followed by a significant increase in cholera toxin substrate above the basal level. Immunoblotting of proteins from NG108-15 cell membranes using an antibody raised to the C-terminal decapeptide of Gsα produced qualitatively similar results (Fig. 2). The statistical significance of the results in Fig. 1 have been reported previously [17]. The abundance of immunoreactive Giα or Gβ was unchanged following exposure of NG108-15 cells to iloprost and/or nicotinamide (Fig. 3).

The effect(s) of nicotinamide on the iloprost-dependent loss of both fluoride-stimulated adenylate cyclase activity and membrane associated Gsα was investigated further in platelets. Exposure of platelets

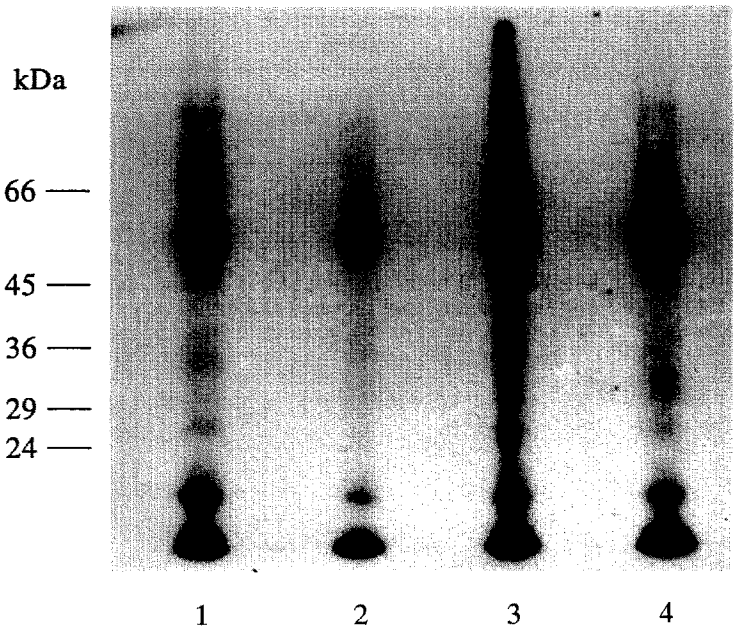

Fig. 1. Cholera toxin [^{32}P]ADP-ribosylation of NG108-15 membranes. NG108-15 cells were cultured in the absence (control, 1), or presence of 10 μM iloprost (2), 50 mM nicotinamide (3) or both 10 μM iloprost and 50 mM nicotinamide together (4) for 18 hr. Cells were harvested and membranes prepared as described in Materials and Methods. The membranes were then subjected to cholera toxin [^{32}P]ADP-ribosylation and the labelled proteins resolved by 10% SDS–PAGE and autoradiography as described in Materials and Methods. This result is a representative of six experiments.

to 10 μM iloprost for 18 hr led to a significant reduction in both agonist and fluoride-stimulated adenylate cyclase activity (Table 2). In contrast to NG108-15 cells, these changes in adenylate cyclase activity were maintained when platelets were treated with iloprost and nicotinamide together (Table 2). Pretreatment of platelets with nicotinamide alone had no effect on basal, fluoride-stimulated or iloprost-stimulated adenylate cyclase activity. The apparent inability of nicotinamide to restore iloprost-dependent loss of fluoride-stimulated adenylate cyclase activity was investigated further by examining the abundance of Gsα in platelets following the various treatments.

Platelets were treated for 18 hr with 10 μM iloprost or carrier alone, and measurements made of the [^{32}P]ADP-ribosylation of the platelet membrane proteins in the presence of cholera toxin. The results revealed a significant ($P < 0.05$) loss of cholera toxin substrate (44.9 ± 7.2%, N = 4) after exposure to iloprost. In contrast, treatment of platelets with nicotinamide resulted in a significant ($P < 0.05$) increase in membrane associated cholera toxin substrate (75.0 ± 19.4%, N = 4). Furthermore, nicotinamide appeared to restore the iloprost-dependent loss of cholera toxin substrate from platelet membranes (Fig. 4). These apparent changes in the abundance of cholera toxin substrate were investigated further by immunoblotting of Gsα. Immunoblots of membrane proteins prepared from platelets that had been treated with iloprost, nicotinamide, or iloprost and nicotinamide together revealed no change in membrane associated Gsα (Fig. 5).

The results in platelets suggested the possibility that the effect of nicotinamide on the activity and abundance of membrane associated Gsα in NG108-15 cells might not be to prevent its elimination, but rather might reflect an increase in *de novo* protein synthesis of Gsα. In order to examine this possibility, NG108-15 cells were treated for 18 hr in the absence or presence of 50 mM nicotinamide, and the abundance of mRNA coding Gsα examined. The abundance of mRNA coding actin was also determined from the samples as a marker of the loading of the gel. Treatment with nicotinamide resulted in a significant ($P < 0.05$) increase in Gsα mRNA (72.0 ± 20.3%, N = 6) (Fig. 6). Finally, experiments were performed to examine the nicotinamide-dependent increase in immunoreactive Gsα in the absence or presence of an inhibitor of protein synthesis. NG108-15 cells were treated for 9 hr in the absence or presence of 50 mM nicotinamide ± 20 μg/mL cycloheximide, and the abundance of membrane associated Gsα measured by immunoblotting. Treatment of NG108-15 cells with nicotinamide for 9 hr resulted in an increase ($P < 0.05$) in Gsα (88.5 ± 44.0%, N = 5), which was reversed by the addition of cycloheximide. Cycloheximide treatment alone had no significant

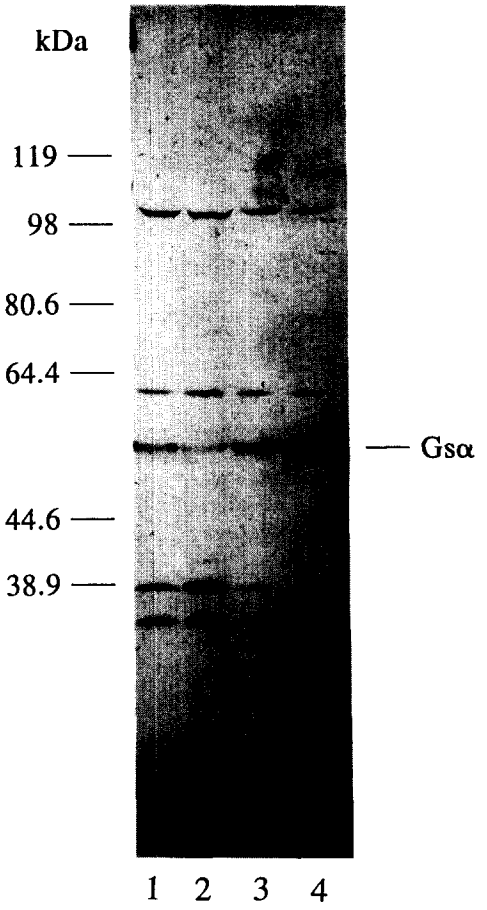

Fig. 2. Gsα immunoblotting of NG108-15 membranes. NG108-15 cells were cultured in the absence (control, 1), or presence of 10 μM iloprost (2), 50 mM nicotinamide (3) or both 10 μM iloprost and 50 mM nicotinamide together (4) for 18 hr. The membrane proteins were resolved by 10% SDS–PAGE and transferred to nitrocellulose and blocked with 3% (w/v) resuspended milk protein for 2 hr. The blots were incubated for 2 hr with anti-Gsα antiserum (1:200 dilution) for 2 hr. The blots were exposed for 1 hr to anti-rabbit IgG (1:1000 dilution) conjugated to horseradish peroxidase and the blots developed as described in Materials and Methods. This result is a representative of six experiments.

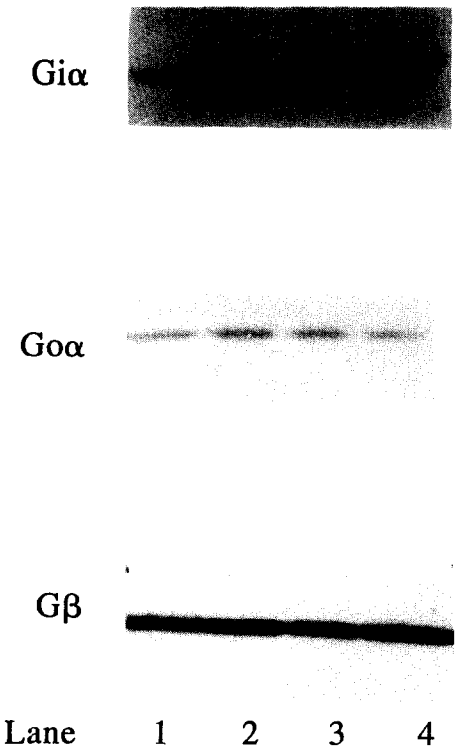

Fig. 3. Giα and Gβ immunoblotting in NG108-15 membranes. NG108-15 cells were cultured in the absence (control, 1), or presence of 10 μM iloprost (2), 50 mM nicotinamide (3) or both 10 μM iloprost and 50 mM nicotinamide together (4) for 18 hr. The membrane proteins were resolved by 10% SDS–PAGE and transferred to nitrocellulose and blocked with 3% (w/v) resuspended milk protein for 2 hr. The blots were incubated for 2 hr with either anti-Giα antiserum (1:200 dilution) or anti-Gβ antiserum (1:1000) for 2 hr. The blots were exposed for 1 hr to anti-rabbit IgG (1:1000 dilution) conjugated to horseradish peroxidase and the blots developed as described in Materials and Methods. This result is a representative of three experiments.

effect on the abundance of membrane associated Gsα. The abundance of Gsα was measured as immunoreactivity within individual lanes loaded with equal amounts of NG108-15 cell membrane protein, and all results were normalized to total cell protein.

We also examined the capacity of cycloheximide to inhibit the increase in fluoride-sensitive adenylate cyclase activity of NG108-15 cells following exposure to nicotinamide. Cells were cultured for 8 hr in the absence or presence of 50 mM nicotinamide, 20 μg/mL cycloheximide or both together. The fluoride-dependent increase in the adenylate cyclase activity was 47.5 ± 6.2, N = 5 pmol cAMP/min/mg protein in control membranes and 72.3 ± 7.1 pmol cAMP/min/mg protein in nicotinamide-treated cells ($P < 0.05$). In cells treated with cycloheximide alone, there was no change in fluoride-sensitive adenylate cyclase activity (39.0 ± 2.4 pmol cAMP/min/mg protein) when compared to control. In those cells treated with both cycloheximide and nicotinamide together, the nicotinamide-dependent increase in fluoride-sensitive adenylate cyclase activity was eliminated (52.3 ± 5.6 pmol cAMP/min/mg protein).

Nicotinamide at a concentration of 50 mM inhibits not only the activity of the mono(ADP-ribosyl)transferase, but also that of the poly(ADP-ribosyl)transferase. The K_i values of nicotinamide

Table 2. Effect of 18 hr iloprost ± nicotinamide pretreatment of platelets on adenylate cyclase activity

Treatment	Adenylate cyclase activity (pmol cyclic AMP/min/mg protein) Basal	1 μM iloprost	10 mM F
Control	98.5 ± 20.6 N = 6	684 ± 77[1,2] N = 6	153 ± 33[3,4] N = 6
10 μM iloprost	82.4 ± 22.9 N = 5	321 ± 43[1] N = 5	40.2 ± 1.6[3] N = 5
10 μM iloprost + 50 mM nicotinamide	105 ± 32 N = 5	328 ± 64[2] N = 5	32.6 ± 7.3[4] N = 4

Platelets were prepared in platelet rich plasma and maintained in the absence or presence of 10 μM iloprost ± 50 mM nicotinamide for 18 hr. Platelets were harvested, membranes prepared and adenylate cyclase activity measured as described in Materials and Methods. The enzyme activities relating to measurements made in the presence of 10 mM NaF or 1 μM iloprost are the δv values for the increase in enzyme activity above the basal level. The results are expressed as the means ± SEM, where the shared superscript numbers indicate differences between a pair of mean values ($P < 0.05$). P values were determined by unpaired, two-tailed *t*-tests.

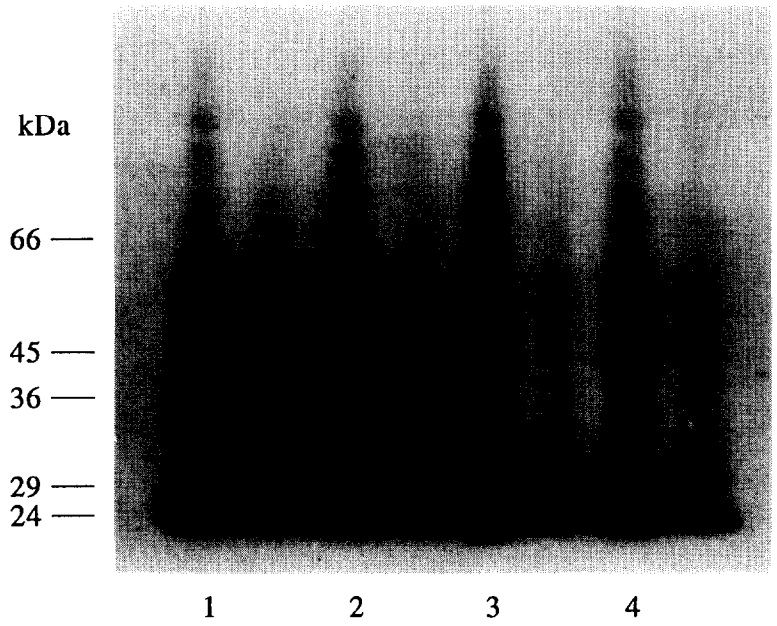

Fig. 4. Cholera toxin [^{32}P]ADP-ribosylation of platelet membranes. Platelets were maintained in the absence (control, 1), or presence of 10 μM iloprost (2), 50 mM nicotinamide (3) or both 10 μM iloprost and 50 mM nicotinamide together (4) for 18 hr. Cells were harvested and membranes prepared as described in Materials and Methods. The membranes were then subjected to cholera toxin [^{32}P]ADP-ribosylation and the labelled proteins resolved by 10% SDS–PAGE and autoradiography as described in Materials and Methods. The additional lanes show the absence of labelling in parallel experiments performed in the absence of cholera toxin. This result is a representative of four experiments.

for the two enzymes are however different. NG108-15 cells were therefore cultured for 18 hr in selected concentrations of nicotinamide, and the abundance of membrane associated Gsα examined by immunoblot. Treatment of cells with 0.5, 1.0, 5.0 or 10 mM nicotinamide had no effect on the abundance of immunoreactive Gsα compared with the control, while exposure of cells to 50 mM nicotinamide

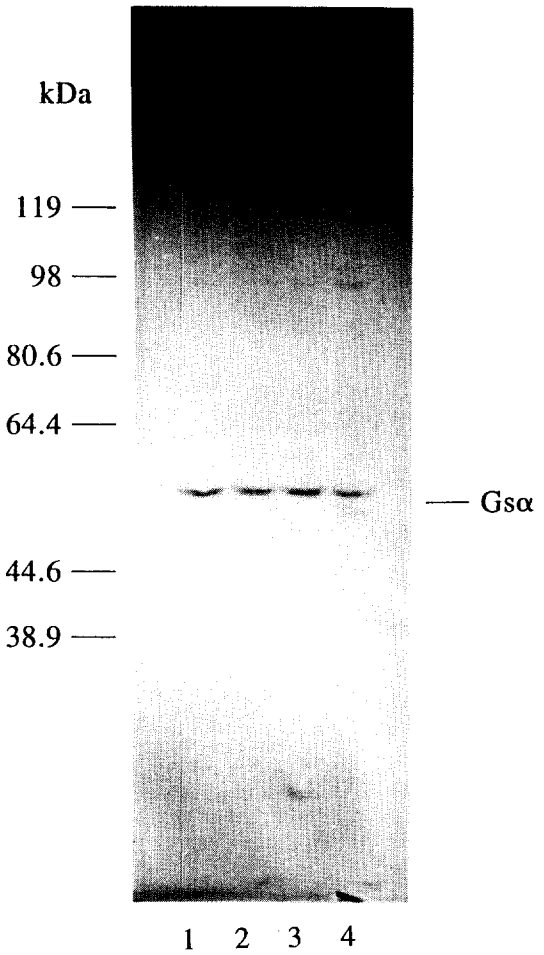

Fig. 5. Gsα immunoblotting of platelet membranes. Platelets were maintained in the absence (control, 1), or presence of 10 μM iloprost (2), 50 mM nicotinamide (3) or both 10 μM iloprost and 50 mM nicotinamide together (4) for 18 hr. The membrane proteins were resolved by 10% SDS–PAGE and transferred to nitrocellulose and blocked with 3% (w/v) resuspended milk protein for 2 hr. The blots were incubated for 2 hr with anti-Gsα antiserum (1:200 dilution) for 2 hr. The blots were exposed for 1 hr to anti-rabbit IgG (1:1000 dilution) conjugated to horseradish peroxidase and the blots developed as described in Materials and Methods. This result is a representative of four experiments.

resulted in a significant ($P < 0.05$) increase (108.2 ± 33.7%, N = 10) above the control.

DISCUSSION

Prostacyclin receptors are eliminated from the cell membrane of NG108-15 cells following prolonged exposure to iloprost or PGE_1. In the same cells there is an accompanying loss of Gsα [17–19, 21, 22, 24, 29]. In the present experiments, the iloprost-dependent loss of membrane associated Gsα in NG108-15 cells was reversed by simultaneous exposure to nicotinamide, an inhibitor of mono(ADP-ribosyl)ation. This was confirmed by measurement of Gsα as cholera toxin substrate and by immunoblotting using a specific Gsα antiserum. The restoration of Gsα following nicotinamide treatment was also accompanied by restored fluoride-stimulated adenylate cyclase activity. However, nicotinamide was unable to restore iloprost-dependent loss of iloprost-stimulated adenylate cyclase activity. The homologous desensitization of iloprost responsiveness reflects loss of PGI_2 receptors [19, 20, 29] rather than reduced abundance of Gsα, and is not affected by nicotinamide [17].

Exposure of platelets to iloprost is also followed by loss of cholera toxin substrate [21, 23]. We have shown that nicotinamide treatment of platelets led to an increase in cholera toxin substrate, confirming the results of other workers [23]. The increase in cholera toxin substrate was similar to that seen in NG108-15 cells. However, in platelets nicotinamide alone had no effect on adenylate cyclase activity which contrasts with results from NG108-15 cells, in which nicotinamide treatment led to an increase in both basal and agonist-stimulated adenylate cyclase activity [17]. Exposure of platelets to iloprost and nicotinamide together appeared to restore the abundance of Gsα (measured as cholera toxin substrate) towards control levels, which confirms a previous report [23]. The increase in cholera toxin substrate in both platelets and NG108-15 cells following exposure to nicotinamide was unaffected by the simultaneous addition of iloprost. The reason for this is at present unclear, but we infer that the predicted loss of cholera toxin substrate due to iloprost has no capacity to modify the availability of cholera toxin substrate following inhibition of the endogenous (eukaryotic) mono(ADP-ribosyl)-transferase.

The iloprost-dependent reduction in cholera toxin substrate seen in platelets was not associated with changes in the abundance of Gsα when measured by immunoblotting, which again confirms the earlier report [23]. This was interpreted at that time as reflecting iloprost-dependent stimulation of ADP-ribosylation of Gsα by an endogenous enzyme, which leads to the subsequent unavailability of Gsα as cholera toxin substrate. Since the abundance of immunoreactive Gsα in platelets is unaltered by exposure to iloprost, there was clearly no associated removal of Gsα from the plasma membrane as seen in NG108-15 cells. The platelet data are of interest in this study because the observed changes in cholera toxin substrate following iloprost exposure cannot be explained solely in terms of Gsα abundance. This implies that Gsα is less available as a substrate for cholera toxin after iloprost exposure (perhaps by ADP-ribosylation during desensitization), although this is not the mechanism of heterologous desensitization. When platelets were treated with iloprost in the presence of nicotinamide, there was no restoration of the iloprost-dependent heterologous loss of fluoride-sensitive adenylate cyclase. Thus, the inactivation of Gsα by iloprost appears to involve a mechanism other than ADP-ribosylation.

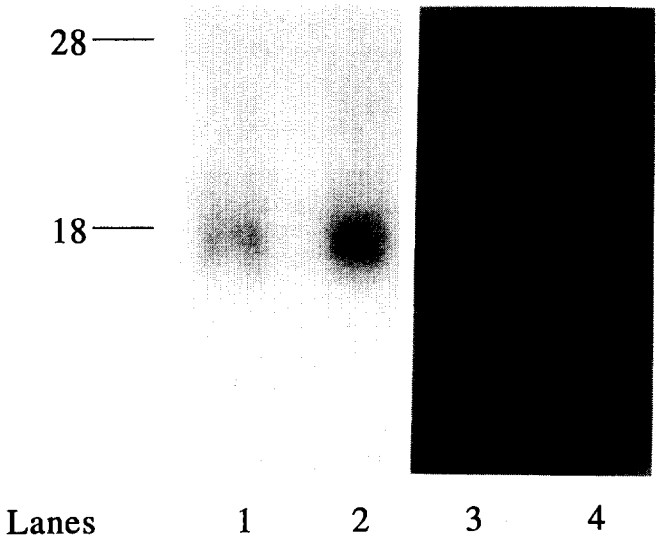

Fig. 6. Northern blot of NG108-15 cells for mRNA encoding Gsα and α-actin. NG108-15 cells were cultured in the absence (1 and 3) or presence (2 and 4) of 50 mM nicotinamide for 18 hr. The cells were harvested and total mRNA extracted as described in Materials and Methods. Total RNA was resolved in a 1.2% (w/v) agarose gel and transferred to nitrocellulose. The filters were pre-hybridized as described in Materials and Methods and finally hybridized with ^{32}P-labelled fragments of Gsα common cDNA (1 and 2) or α-actin cDNA (3 and 4). The blots were washed as described in Materials and Methods and subjected to autoradiography. This figure is a representative of six experiments.

The observation that nicotinamide increased the abundance of Gsα in NG108-15 cell membranes and reversed iloprost-dependent loss of Gsα, suggested initially that ADP-ribosylation of Gsα by a eukaryotic enzyme might target Gsα for removal from the cell membrane. This would be consistent with the finding that persistent cholera toxin ADP-ribosylation of NG108-15 cells is followed by the removal of Gsα from the cell membrane [30, 31]. However, in platelets nicotinamide had no effect on the abundance of immunoreactive Gsα, although nicotinamide treatment increased cholera substrate (presumably by inhibiting endogenous ADP-ribosylation of membrane bound Gsα). Furthermore, treatment of platelets with iloprost had no effect on the abundance of membrane associated immunoreactive Gsα. Thus, it seems improbable that ADP-ribosylation by a eukaryotic enzyme targets Gsα for removal from platelet membranes, but remains a possible mechanism in NG108-15 cells.

The values obtained for Gsα molecular weight (Figs 1, 2, 4 and 5) appear to be somewhat higher than those quoted in certain papers [29, 30] and predicted by the primary sequence, although they are consistent with the finding of a 52 kDa species in other reports [17, 32]. This topic was discussed by Gilman [33] who concluded that the electrophoretic behaviour of the larger form of Gsα is anomalous in SDS polyacrylamide gels, and may well reflect its post-translational modification in intact cells.

An important difference between NG108-15 cells and platelets in the context of these experiments is the inability of platelets to synthesise new protein. We considered the possibility therefore, that the observed differences between NG108-15 cells and platelets were the result of *de novo* protein synthesis in NG108-15 cells. Treatment of NG108-15 cells with nicotinamide was followed by a selective increase in the abundance of both Gsα and the Gsα transcript, with no change in the abundance of Giα and Gβ. However, the observed increase in the Gsα transcript following nicotinamide treatment would not necessarily lead to increased synthesis of Gsα, which prompted experiments which showed that cycloheximide (an inhibitor of protein synthesis) reversed the capacity of nicotinamide to increase membrane associated Gsα, with an accompanying loss of the capacity of nicotinamide to increase the fluoride-sensitive adenylate cyclase.

A eukaryotic mono(ADP-ribosyl)transferase has been shown to ADP-ribosylate EF-2 [11, 34] and inhibit protein synthesis, which suggests the possibility that nicotinamide might reverse this effect. However, this would not readily explain the nicotinamide-dependent increase in Gsα mRNA, nor indeed the selective effect on Gsα expression. The identity of the particular ADP-ribosyltransferase involved in the control of Gsα expression in NG108-15 cells remains obscure, but it is provisionally identified as a mono(ADP-ribosyl)transferase on the basis of the concentrations of nicotinamide required to inhibit the enzyme (>10 mM) and elevate both

Gsα abundance and specific mRNA in these experiments. The IC_{50} of nicotinamide for poly(ADP-ribosyl)transferase is 30 μM [6], from which it follows that at concentrations of nicotinamide of 1–10 mM, the poly(ADP-ribosyl)transferase would be totally inhibited. In contrast, the IC_{50} of nicotinamide for mono(ADP-ribosyl)transferase is 3.4 mM, and thus greater concentrations of nicotinamide are required to inhibit the enzyme. We propose that the increase in abundance of both immunoreactive Gsα and its transcript following exposure of NG108-15 cells to 50 mM nicotinamide may involve inhibition of a mono(ADP-ribosyl)transferase, although clearly other effects of nicotinamide at this concentration might contribute to the effect.

We conclude that mono(ADP-ribosyl)ation of Gsα within the membrane of NG108-15 cells or platelets by a eukaryotic enzyme reduces the capacity of Gsα to serve as a substrate for cholera toxin. Although ADP-ribosylation of Gsα by cholera toxin appears to trigger the elimination of Gsα from the plasma membrane [30, 31], the eukaryotic enzyme appears not to fulfil a similar role in either platelets or NG108-15 cells. The abundance of Gsα within the plasma membrane is tightly regulated, but may be reduced by chronic exposure of NG108-15 cells to iloprost. The elimination of Gsα from the plasma membrane of these cells may be invoked to explain the heterologous loss of fluoride-dependent activation of adenylate cyclase, or indeed activation by other agonists such as adenosine [21, 22]. In NG108-15 cells, inhibition of ADP-ribosylation [most probably a mono(ADP-ribosyl)transferase] during iloprost exposure restores the level of Gsα within the plasma membrane, and reverses the loss of fluoride sensitivity. The mechanism however is unrelated to the elimination pathway(s) for Gsα from the membrane, and involves rather a selective increase in the *de novo* synthesis of Gsα. In platelets, the inactivation of Gsα following exposure to iloprost is not followed by loss of Gsα from the cell surface, and implies that inactive Gsα may be retained within the plasma membrane.

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REFERENCES

1. Moss J and Vaughan M, Isolation of an avian erythrocyte protein possessing ADP-ribosyltransferase activity and capable of activating adenylate cyclase. *Proc Natl Acad Sci USA* **75**: 3621–3624, 1978.
2. Soman G, Mickelson JR, Louis CF and Graves DJ, NAD: Guanidino group specific ADP-ribosyltransferase activity in skeletal muscle. *Biochem Biophys Res Commun* **120**: 973–980, 1984.
3. De Wolf MJS, Vitti P, Ambesi-Impiombato FS and Kohn LD, Thyroid membrane ADP-ribosyltransferase activity. *J Biol Chem* **256**: 12287–12296, 1981.
4. Moss J and Stanley S, Histone-dependent and histone independent forms of an ADP-ribosyltransferase from human and turkey erythrocytes. *Proc Natl Acad Sci USA* **78**: 4809–4812, 1981.
5. Tanuma S, Kawashima K and Endo H, Eukaryotic mono(ADP-ribosyl)transferase that ADP-ribosylates GTP-binding regulatory Gi protein. *J Biol Chem* **263**: 5485–5489, 1988.
6. Rankin PW, Jacobson EL, Benjamin RC, Moss J and Jacobson MK, Quantitative studies of inhibitors of ADP-ribosylation *in vitro* and *in vivo*. *J Biol Chem* **264**: 4312–4317, 1989.
7. Matsuyama S and Tsuyama S, Mono-ADP-ribosylation in brain: purification and characterization of ADP-ribosyltransferases affecting actin from rat brain. *J Neurochem* **57**: 1380–1387, 1991.
8. Leno GH and Ledford BE, ADP-ribosylation of the 78 kDa glucose-regulated protein during nutritional stress. *Eur J Biochem* **186**: 205–211, 1989.
9. Terashima M, Mishima K, Yamada K, Tschuiya M, Wakutani T and Shimoyama M, ADP-ribosylation of actins by arginine-specific ADP-ribosyltransferase purified from chicken heterophils. *Eur J Biochem* **204**: 305–311, 1992.
10. Iglewski WJ, Lee H and Muller P, ADP-ribosyltransferase from beef liver which ADP-ribosylates elongation factor-2. *FEBS Lett* **173**: 113–118, 1984.
11. Lee H and Iglewski WJ, Cellular ADP-ribosyltransferase with the same mechanism of action as diphtheria toxin and *Pseudomonas* toxin A. *Proc Natl Acad Sci USA* **81**: 2703–2707, 1984.
12. Nowicki M, Landon C, Sugawara S and Dennert G, Nicotinamide and 3-aminobenzamide interfere with receptor-mediated transmembrane signalling in murine cytotoxic T cells: independence of golgi reorientation from calcium mobilization and inositol phosphate generation. *Cell Immunol* **132**: 115–126, 1991.
13. Loesberg C, van Rooij H and Smets LA, *Meta*-iodobenzylguanidine (MIBG), a novel high-affinity substrate for cholera toxin that interferes with cellular mono(ADP-ribosylation). *Biochim Biophys Acta* **1037**: 92–99, 1990.
14. Richter C, The prooxidant-induced and spontaneous mitochondrial calcium release: inhibition by *meta*-iodobenzylguanidine (MIBG), a substrate for mono(ADP-ribosylation). *Free Rad Res Commun* **8**: 329–334, 1990.
15. Inageda K, Nishina H and Tanuma S, Mono-ADP-ribosylation of Gs by a eukaryotic arginine-specific ADP-ribosyltransferase stimulates the adenylate cyclase system. *Biochem Biophys Res Commun* **176**: 1014–1019, 1991.
16. Obara S, Yamada K, Yoshimura Y and Shimoyama M, Evidence for the endogenous GTP-dependent ADP-ribosylation of the α-subunit of the stimulatory guanyl-nucleotide-binding protein concomitant with an increase in basal adenylate cyclase activity in chicken spleen cell membrane. *Eur J Biochem* **200**: 75–80, 1991.
17. Donnelly LE, Boyd RS and MacDermot J, Gsα is a substrate for mono(ADP-ribosyl)transferase of NG108-15 cells: ADP-ribosylation regulates Gsα activity and abundance. *Biochem J* **288**: 331–336, 1992.
18. Kenimer JG and Nirenberg M, Desensitization of adenylate cyclase to prostaglandin E_1 or 2-chloroadenosine. *Mol Pharmacol* **20**: 585–591, 1981.
19. Kenimer JG, Desensitization of PGE_1 receptors in neuroblastoma-glioma hybrid cells. *Prostaglandins* **23**: 311–318, 1982.
20. Leigh PJ and MacDermot J, Desensitization of prostacyclin responsiveness in a neuronal hybrid cell line: selective loss of high affinity receptors. *Br J Pharmacol* **85**: 237–247, 1985.
21. Edwards RJ, MacDermot J and Baker AJ, Prostacyclin analogues reduce ADP-ribosylation of the alpha-subunit of the regulatory Gs protein and diminish adenosine (A_2) responsiveness of platelets. *Br J Pharmacol* **90**: 501–510, 1987.
22. Kelly E, Keen M, Nobbs P and MacDermot J,

Segregation of discrete Gs alpha-mediated responses that accompany homologous or heterologous desensitization in two related somatic hybrids. *Br J Pharmacol* **99**: 307–316, 1990.

23. Molina y Vedia L, Nolan RD and Lapetina EG, The effect of iloprost on the ADP-ribosylation of Gsα. *Biochem J* **261**: 841–845, 1989.
24. McKenzie FR and Milligan G, Prostaglandin E_1 mediated, cyclic AMP-independent, down-regulation of Gsα in neuroblastoma × glioma hybrid cells. *J Biol Chem* **265**: 17084–17093, 1990.
25. MacDermot J, Higashida H, Wilson SP, Matzusawa H, Minna J and Nirenberg M, Adenylate cyclase and acetylcholine release regulated by separate serotonin receptors of somatic cell hybrids. *Proc Natl Acad Sci USA* **76**: 1135–1139, 1979.
26. Leigh PJ, Cramp WA and MacDermot J, Identification of the prostacyclin receptor by radiation inactivation. *J Biol Chem* **259**: 12431–12436, 1984.
27. Salomon Y, Londos C and Rodbell M, A highly sensitive adenylate cyclase assay. *Anal Biochem* **58**: 541–548, 1974.
28. Chomczynski P and Sacchi C, Single step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal Biochem* **162**: 156–159, 1987.
29. Adie EJ, Mullaney I, MacKenzie FR and Milligan G, Concurrent down-regulation of IP prostanoid receptors and the alpha-subunit of the stimulatory guanidine-nucleotide-binding protein (Gs) during prolonged exposure of neuroblastoma × glioma cells to prostanoid agonists. Quantification and functional implications. *Biochem J* **285**: 529–536, 1992.
30. MacLeod KG and Milligan G, Biphasic regulation of adenylate cyclase by cholera toxin in neuroblastoma × glioma hybrid cells is due to the activation and subsequent loss of the alpha subunit of the stimulatory GTP binding protein (Gs). *Cell Signal* **2**: 139–151, 1990.
31. Klinz F-J and Costa T, Cholera toxin differentially decreases membrane levels of alpha and beta subunits of G protein in NG108-15 cells. *Eur J Biochem* **188**: 567–576, 1990.
32. Robishaw JD, Russel DW, Harris BA, Smigel MD and Gilman AG, Deduced primary structure of the alpha subunit of the GTP-binding stimulatory protein of adenylate cyclase. *Proc Natl Acad Sci USA* **83**: 1251–1255, 1986.
33. Gilman AG, G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56**: 615–649, 1987.
34. Fendrick JL and Iglewski WJ, Endogenous ADP-ribosylation of elongation factor 2 in polyoma virus-transformed baby hamster kidney cells. *Proc Natl Acad Sci USA* **86**: 554–557, 1989.