G_sα is a substrate for mono(ADP-ribosyl)transferase of NG108-15 cells

ADP-ribosylation regulates G_sα activity and abundance

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NG108-15 neuroblastoma × glioma somatic hybrid cells were permeabilized in the presence of [³²P]NAD⁺ and then cultured for 18 h. Resolution of the cell proteins on polyacrylamide gels revealed [³²P]ADP-ribosylation of five major protein species with molecular mass values of 52 kDa, 44 kDa, 35 kDa, 30 kDa and 25 kDa. A similar pattern of labelling was also seen when NG108-15 cell membranes were incubated with [³²P]NAD⁺ and hydrolysis of the product revealed mono(ADP-ribosyl)ation. Immunoprecipitation of these products with anti-G_sα antiserum revealed a single band identical to cholera toxin substrate. Culture of [³²P]NAD⁺-loaded cells for 18 h in the presence of 50 mM-nicotinamide inhibited the eukaryotic mono(ADP-ribosyl)transferase activity. Inhibition of the eukaryotic enzyme was also accompanied by an increase in the abundance of G_sα, whether measured by Western blotting with anti-G_sα antibody (two separate antisera) or by cholera toxin-dependent [³²P]ADP-ribosylation. There was no accompanying change in the abundance of G_β. The increase in G_sα abundance in nicotinamide-treated NG108-15 cells was accompanied by a 2-fold increase in basal adenylate cyclase activity (measured in the presence of GTP), and by a smaller but significant increase in iloprost-dependent activation of adenylate cyclase. Receptor number or affinity was not affected by nicotinamide, since this treatment did not alter the binding parameters of [³H]iloprost to NG108-15 cell membranes. Short-term exposure of cells to nicotinamide for 1 h revealed no significant difference in either basal or agonist-stimulated adenylate cyclase activity. These results reveal that mono(ADP-ribosyl)ation of G_sα by eukaryotic ADP-ribosyltransferase modifies the abundance and activity of G_sα in NG108-15 cells, and hence may play a role in the hormonal regulation of cell function.

INTRODUCTION

Eukaryotic mono(ADP-ribosyl)transferase activity has been reported in many cells and tissues, including turkey erythrocytes [1], rat liver mitochondria [2] and rabbit muscle sarcolemma [3]. These studies have provided much information on the characteristics and requirements of the various isoforms of the enzyme, and three different isoenzymes have been purified. Transferases A and B have been isolated from the cytosol of turkey erythrocytes, and both have the capacity to transfer the ADP-ribosyl moiety from NAD⁺ on to an arginine acceptor [4,5]. These enzymes differ in their respective *K_m* values for NAD⁺, and also in the effect of chaotropic salts and histones on their activities. In contrast, transferase C is a membrane-bound enzyme which catalyses the transfer of ADP-ribose to a cysteine residue [6]. Studies *in vitro* have shown that transferase A may be inhibited by nicotinamide, a product of NAD⁺ hydrolysis, which is released during ADP-ribosylation [7]. Recently four ADP-ribosyltransferases have been isolated from rat brain which are not inhibited by nicotinamide or thymidine [8]. Despite these detailed studies on the enzymology of purified mono(ADP-ribosyl)transferases, little is known regarding their physiological substrates and functions within the cell.

Many receptors are coupled to enzymes or ion channels by guanine nucleotide-binding regulatory proteins (G-proteins). G-proteins comprise heterotrimeric complexes, having α-, β- and γ-subunits. The α-subunit of each of the G-proteins contains the GTPase catalytic site, and much of our understanding of G-protein function has arisen from studies on the mechanism of the hormonal regulation of adenylate cyclase [9,10].

Many bacterial exotoxins, including those from *Vibrio cholerae*

and *Bordetella pertussis*, possess mono(ADP-ribosyl)transferase activities, and have the capacity to ADP-ribosylate the G-protein α-subunits which regulate adenylate cyclase activity. The α-subunit of the stimulatory G-protein, G_sα, is a substrate for cholera toxin, whereas the α-subunit of the inhibitory G-protein, G_iα, as well as G_oα, are substrates for pertussis toxin. The possibility that G-protein α-subunits might be substrates for endogenous mono(ADP-ribosyl)transferase(s) is suggested by the finding that a cysteine residue in G_iα may be a substrate for purified mono(ADP-ribosyl)transferase C from human platelets [6]. In addition, measurement of ADP-ribosyltransferase activity in intact platelets revealed that a protein with a similar molecular mass to G_sα is a substrate for eukaryotic ADP-ribosyltransferase and that this activity could be stimulated by the prostacyclin analogue iloprost [11]. This observation has been confirmed recently by a report indicating that mono(ADP-ribosyl)transferase A activity isolated from human platelets does indeed ADP-ribosylate exogenous G_sα [12]. The possibility that G_sα may be a substrate for an endogenous ADP-ribosyltransferase has been explored in chicken spleen membranes [13], where it was demonstrated that membranes had the capacity to ADP-ribosylate a 45 kDa protein, resulting in the stimulation of adenylate cyclase activity. These reports suggest that endogenous ADP-ribosyltransferases may play an important role in regulating G-protein activity and thus hormonal regulation of cell function.

In the present study, we have examined (i) the possibility that G_sα contained within NG108-15 cells may be a substrate for the endogenous ADP-ribosyltransferase, and (ii) the role that this covalent modification of G_sα might play in the regulation of the abundance or activity of G_sα in intact cells.

Abbreviations used: PEG, poly(ethylene glycol); PBS, phosphate-buffered saline; PGE₁, prostaglandin E₁.

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MATERIALS AND METHODS

Materials

[α - 32 P]NAD⁺ and anti-G β antibody were obtained from New England Nuclear (U.K.). [α - 32 P]ATP, [3 H]cyclic AMP, [3 H]iloprost and Hybond-C nitrocellulose were obtained from Amersham International. Anti-(rabbit IgG) conjugated to horseradish peroxidase was obtained from Dako Ltd. (U.K.). Anti-G α antibody was obtained from Biomac Ltd. (U.K.). Snake venom phosphodiesterase I was obtained from Worthington Biochemical Corp. (U.S.A.). Polyethyleneimine-treated cellulose t.l.c. plates were obtained from Macherey–Nagel (Düren, Germany). Anti-G α antiserum CT 142 was a gift from Dr. J. C. Ireson, Amersham International, Cardiff, U.K., Ro20-1724 was from Roche Products (U.K.), and iloprost was from Schering AG (Berlin, Germany). GF/C glass filters were obtained from Whatman. All other reagents were obtained from Sigma.

Cell culture

NG108-15 neuroblastoma $\times$ glioma somatic hybrid cells [14] were cultured in 80 cm² flasks in Dulbecco's modified Eagle's minimum essential medium containing 10% (v/v) fetal calf serum, 1 μ M-aminopterin, 16 μ M-thymidine and 100 μ M-hypoxanthine. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Membranes were prepared as described previously [15] and stored at –80 °C until required.

ADP-ribosyltransferase activity of intact NG108-15 cells

ADP-ribosyltransferase activity was measured by a modification of methods reported previously [11]. Cells were harvested in phosphate-buffered saline (PBS) and resuspended in 200 μ l of PBS containing 50% (w/v) poly(ethylene glycol) (PEG)-1500 and 200 μ Ci of [α - 32 P]NAD⁺ (30 Ci/mmol), and maintained at room temperature for 10 min. Thereafter the reaction mixture was diluted by the addition of 10 ml of PBS, and the cells were centrifuged at 500 *g* for 5 min. The cells were washed again a further four times and finally seeded into 35 mm dishes containing medium. The cells were allowed to attach, and then cultured for a further 18 h, harvested in PBS, and the resulting cell pellet solubilized in PAGE sample buffer [0.0625 M-Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) β -mercaptoethanol and 0.01% (w/v) Bromophenol Blue dye]. The proteins were then resolved by electrophoresis in SDS/10%-polyacrylamide gels, and 32 P-labelled proteins were identified by autoradiography [16]. Quantification of the autoradiographs was performed by densitometric analysis using an LKB-Ultrosan laser densitometer and the scans were analysed using the integration facility of Fig.P graphics software (Fig.P. Software Corporation).

ADP-ribosylation activity of NG108-15 cell membranes

Membranes were incubated in 25 mM-Tris/HCl buffer, pH 7.4, containing 0.25 mM-EDTA in the presence of 10 μ M-GTP and 100 μ Ci of [α - 32 P]NAD⁺ for 1 h at 37 °C. The reaction was terminated by the addition of 1 ml of 20% (w/v) trichloroacetic acid. The resulting precipitate was washed twice with ethanol/ether (1:1, v/v) and once with ether alone, prior to solubilization in PAGE sample buffer. The proteins were then resolved by electrophoresis in SDS/10%-polyacrylamide gels, and 32 P-labelled proteins were identified autoradiographically [16].

Toxin-catalysed [32 P]ADP-ribosylation of G α

Membranes were prepared from control cells or cells which had been treated for 18 h with 10 μ M-iloprost and/or 50 mM-

nicotinamide. They were then subjected to cholera toxin-catalysed ADP-ribosylation using [α - 32 P]NAD⁺ as reported previously [16]. The labelled protein was resolved by SDS/10%-PAGE and visualized using autoradiography. The relative intensities of the bands were assessed using an LKB-Ultrosan laser densitometer and quantified as above.

Western blot analysis

Membrane proteins were resolved by SDS/10%-PAGE, and then transferred to nitrocellulose membranes. The nitrocellulose was blocked for 2 h 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 h with the primary antisera diluted in PBS containing 0.1% BSA. The blots were washed three times as above before incubation for 1 h with anti-rabbit IgG conjugated to horseradish peroxidase (1:1000 dilution). The blots were washed again, and the bands were visualized by incubation with 0.5 mg of 4-chloro-1-naphthol/ml in 25 mM-Tris/HCl buffer, pH 7.4, containing 20% (v/v) methanol and 0.05% (v/v) hydrogen peroxide. The blots were finally washed, dried and scanned as described above.

The linearity with protein concentration for both cholera toxin [32 P]ADP-ribosylation and Western blotting 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.

Snake venom phosphodiesterase digestion of ADP-ribosylated samples

The method used here was essentially that reported previously [17]. Cells or membranes were labelled with [α - 32 P]NAD⁺ as described above. The samples were precipitated in 20% (w/v) trichloroacetic acid, and washed twice with ethanol followed by two washes with ether. The resulting precipitate was resuspended in 50 mM-Tris/HCl buffer, pH 9.0, containing 10 mM-MgCl₂ and snake venom phosphodiesterase I (10 units/ml), and incubated at 37 °C overnight. The reaction was terminated by the addition of 20% (w/v) trichloroacetic acid, and the supernatant was extracted with ether. The aqueous phase was then spotted on to polyethyleneimine-treated cellulose t.l.c. plates. The plates were developed in 0.3 M-LiCl. The 32 P-labelled digestion products were revealed autoradiographically and their *R_f* values were compared with those of authentic AMP, NAD⁺ and adenosine 5'-diphosphoribose. The remaining precipitate was solubilized in PAGE sample buffer and any remaining 32 P-labelled proteins were resolved by SDS/PAGE, and identified autoradiographically.

Immunoprecipitation of G α

The immunoprecipitation procedure used here was a modification of published methods [18]. Cells or membranes were labelled with [α - 32 P]NAD⁺ and harvested as described above. The pellet was solubilized by the addition of 150 μ l of 0.1% (w/v) SDS and the mixture was boiled for 5 min. The sample was allowed to cool before the addition of 500 μ l of dilution buffer [1.25% (v/v) Triton X-100, 190 mM-NaCl, 60 mM-Tris/HCl, pH 7.4, 6 mM-EDTA] and 75 μ l of anti-G α antibody. This was maintained at 4 °C for 18 h before centrifugation at 1000 *g* for 2 min. The supernatant was retained and 180 μ l of a 1:1 suspension of Protein A–Sepharose CL-4B in PBS was added, and the mixture was incubated at 37 °C for 2 h. The Sepharose beads were pelleted by a 10 s centrifugation in a microcentrifuge, and the supernatant was discarded. The beads were washed four times with washing buffer [0.1% (v/v) Triton X-100, 0.02% (w/v) SDS, 150 mM-NaCl, 50 mM-Tris/HCl, pH 7.5, 5 mM-

EDTA] and once in the same buffer without detergent. The supernatant was removed carefully, and 80 μ l of PAGE sample buffer was added to the beads and boiled for 3 min. The sample was then subjected to SDS/10%-PAGE and autoradiography.

Adenylate cyclase assay

Adenylate cyclase was measured by a modification [16] of the method reported previously [19]. Briefly, reaction mixtures of 100 μ l contained 50 mM-Tris/HCl buffer, pH 7.4, 5 mM-MgCl₂, 20 mM-phosphocreatine (disodium salt), 10 units of creatine kinase, 1 mM-cyclic AMP, 0.25 mM-Ro20-1724 (a phosphodiesterase inhibitor), 1 mM-[α -³²P]ATP (2 μ Ci/tube), 4 μ M-GTP and 20–70 μ g of membrane protein. The reaction was allowed to proceed at 37 °C for 20 min, and then terminated by the addition of 800 μ l of 6.25 % (w/v) trichloroacetic acid. [8-³H]Cyclic AMP (approx. 10000 c.p.m. in 100 μ l) was added to each tube, and the mixtures were centrifuged at 1500 g for 20 min. The [α -³²P]ATP and [³²P]cyclic AMP were then separated using a two-step chromatography procedure [20]. The losses of [³²P]cyclic AMP on the columns were corrected for by measurement of the recovery of [8-³H]cyclic AMP.

[³H]Iloprost binding

The binding of [³H]iloprost to NG108-15 cell membranes was performed by a modification of a previously reported method [20]. Reaction volumes of 200 μ l contained 50 mM-Tris/HCl buffer, pH 7.4, 10 mM-MgCl₂, 5–100 nM-[³H]iloprost (16.2 Ci/mmol) and 50–120 μ g of membrane protein. Incubations were performed at 25 °C for 45 min and terminated by the addition of 4 ml of ice-cold 50 mM-Tris/HCl buffer, pH 7.4. The membranes were separated by rapid filtration through GF/C glass filters and washed three times with the same buffer. The filters were dried under an infra-red lamp for 1 h and the radioactivity was counted following the addition of 10 ml of scintillant. Specific binding was defined as that displaced by 10 μ M-iloprost at each [³H]iloprost concentration. The data were analysed using the curve-fitting software Graphpad (GraphPAD Software) and fitted to a two-parameter model identifying a single binding site [21].

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 values $\pm$ S.E.M.

RESULTS

Following treatment of the cells with PEG and loading with [α -³²P]-NAD⁺, more than 90 % of the cells adhered to the growth surface within 1 h, and the cells assumed typical neuroblastoma morphology. Under the culture conditions described, five major proteins with molecular masses of 52 kDa, 44 kDa, 35 kDa, 30 kDa and 25 kDa were ADP-ribosylated (Fig. 1). The proteins of molecular masses 30 kDa and 35 kDa were the most heavily ADP-ribosylated. One of the endogenously labelled proteins had a molecular mass (44 kDa) similar to that of cholera toxin substrate, thus indicating that G_{α} may be a substrate for this enzyme activity. When the cells were exposed to 10 μ M-iloprost for 18 h, there was no change in either the intensity

(100.6 $\pm$ 12.6 %, *n* = 7) or the pattern of ADP-ribosylation of the proteins. There was also no effect on ADP-ribosylation if the cells were treated with the cyclic AMP analogue 8-bromo-cyclic AMP (5 mM) (results not shown). In contrast, incubation of cells with 50 mM-nicotinamide, an inhibitor of mono(ADP-ribosylation), resulted in a significant (*P* < 0.05) reduction (54.2 $\pm$ 8.5 %, *n* = 7) in the intensity of labelling of all proteins (Fig. 1), with no selectivity for any one substrate. Iloprost had no effect on the inhibition of ADP-ribosyltransferase activity by nicotinamide (46.2 $\pm$ 10.2 %, *n* = 7) (Fig. 1). The possibility that this apparent inhibition of ADP-ribosyltransferase activity might be the result of the high osmolar concentration of nicotinamide was examined by culturing cells in the presence of 50 mM-choline. However, this did not produce any alteration in the intensity or pattern of the labelled bands when compared with untreated cells (results not shown). Treatment of cells with other known inhibitors of ADP-ribosylation, i.e. benzamide or 5-bromo-2'-deoxyuridine (25 mM or 10 mM), also resulted in a reduction in the intensity of the labelling of all proteins. At lower concentrations (0.1–2 mM), i.e. those reported to inhibit poly(ADP-ribosyl)transferase activity [7], there was no apparent reduction in the labelling of the bands (results not shown).

In order to assess whether iloprost and/or nicotinamide have any effect on the abundance of G_{α} in the cells following such treatment, G_{α} was quantified (as the substrate for cholera toxin ADP-ribosyltransferase) following exposure of cells to either of these effectors. Cholera toxin-catalysed ADP-ribosylation of membranes prepared from cells which had been treated with iloprost revealed a significant (*P* < 0.05) loss (35.6 $\pm$ 8.2 %, *n* = 6) of G_{α} from the membrane when compared with untreated cells (Fig. 2). In contrast, nicotinamide treatment of the cells, followed by cholera toxin ADP-ribosylation of the cell membranes, demonstrated a significant (*P* < 0.05) increase (240.8 $\pm$ 54.2 %, *n* = 6) in membrane associated G_{α} . The capacity of iloprost to decrease the abundance of G_{α} was lowered in the presence of nicotinamide. Although these results suggest differences in abundance of G_{α} in membranes following the various treatments, they might also be obtained if G_{α} remained in the membrane but was no longer available as a substrate for cholera toxin ADP-ribosyltransferase. In order to examine this

Fig. 1. Endogenous ADP-ribosyltransferase activity in NG108-15 cells

NG108-15 cells were permeabilized to [³²P]NAD⁺ by incubation with PEG. The cells were seeded into 35 mm² culture dishes, and allowed to adhere. The cells were cultured for 18 h in the absence (control, C) or presence of 10 μ M-iloprost (I), 50 mM-nicotinamide (N) or both together (NI). Cells were harvested and the cell proteins were resolved by SDS/10%-PAGE. The labelled proteins were detected by autoradiography. This Figure is representative of seven experiments.

Fig. 2. Cholera toxin-catalysed [32 P]ADP-ribosylation of NG108-15 membranes

Cells were harvested, membranes prepared and the membranes subjected to cholera toxin [32 P]ADP-ribosylation, and the labelled proteins were resolved by SDS/10%-PAGE. The corresponding autoradiograph was scanned using an LKB Ultrascan laser densitometer and the scans of untreated cells (C), iloprost-treated cells (I), nicotinamide-treated cells (N) or cells treated with both together (NI) are presented. This Figure is representative of six experiments.

possibility, membranes were further analysed by the technique of Western blotting.

Experiments with an anti- $G_s\alpha$ antibody (Biomac) raised against the C-terminal decapeptide revealed qualitatively similar results to those obtained by cholera toxin ADP-ribosylation. Western blot analysis of iloprost-treated membranes showed a significant loss ($P < 0.05$) ($44.2 \pm 8.3\%$, $n = 6$) of immunoreactive $G_s\alpha$ when compared with control membranes, whereas nicotinamide treatment of the cells resulted in a significant increase ($P < 0.05$) ($221.2 \pm 54.2\%$, $n = 6$) in immunoreactive $G_s\alpha$ (Fig. 3). Again, nicotinamide decreased the effect of iloprost in these cells. The possibilities that ADP-ribosylation might be blocking antibody binding, or that $G_s\alpha$ might have been partially degraded with loss of the C-terminal decapeptide, were addressed by repeating the experiment with an alternative primary antiserum, CT 142, raised against a different region of the $G_s\alpha$ molecule corresponding to amino acid residues 325–339. Similar results were also obtained using this antibody (results not shown). In order to ascertain whether loss of $G_s\alpha$ was accompanied by a decrease in $G\beta$, the abundance of $G\beta$ was determined by Western blotting as above. These results revealed no differences in the levels of $G\beta$ following iloprost or nicotinamide pretreatment of the cells (results not shown).

To confirm that $G_s\alpha$ is a substrate for ADP-ribosyltransferase in NG108-15 cell membranes, membranes were labelled with [α - 32 P]NAD $^{+}$. The membranes were then solubilized and subjected to immunoprecipitation using the Biomac anti- $G_s\alpha$ antibody. Comparisons of immunoprecipitated cholera toxin ADP-ribosylated $G_s\alpha$ and immunoprecipitated $G_s\alpha$ from membranes which had been incubated with [α - 32 P]NAD $^{+}$ were performed (Fig. 4). The autoradiograph shows a band corresponding to the

Fig. 3. Western blotting of NG108-15 membranes with anti- $G_s\alpha$ antiserum

NG108-15 cells were cultured for 18 h in the absence (control, C) or presence of 10 μ M-iloprost (I), 50 mM-nicotinamide (N) or both together (NI). Cells were harvested, and membranes were prepared and subjected to SDS/10%-PAGE. The proteins were transferred to nitrocellulose and blocked with 3% (w/v) resuspended milk protein for 2 h. The blots were incubated with anti- $G_s\alpha$ antiserum (Biomac) at 1:200 dilution, and incubated for a further 2 h. The blots were exposed for 1 h to a second antiserum (1:1000 dilution) of anti-(rabbit IgG) conjugated to horseradish peroxidase. The blots were developed and scanned as described in the Materials and methods section. The alternate left hand lanes show the prestained molecular mass markers at approx. 58 kDa. This Figure is representative of six experiments.

Fig. 4. Immunoprecipitation of [32 P]ADP-ribosylated $G_s\alpha$

NG108-15-cell membranes were prepared and subsequently labelled for 30 min with [32 P]NAD $^{+}$ as described in the Materials and methods section. The membranes were solubilized and anti- $G_s\alpha$ antibody was added. The $G_s\alpha$ /anti- $G_s\alpha$ complexes were immobilized on Protein A-Sepharose, and thereafter the Sepharose beads were washed extensively. The proteins adherent to the Sepharose beads were resolved by SDS/10%-PAGE. Membrane preparations of NG108-15 cells were also [32 P]ADP-ribosylated with cholera toxin, and subjected to the same immunoprecipitation procedure. The proteins were resolved by SDS/10%-PAGE, and the labelled bands were identified by autoradiography. Lane 1 contains total labelled membrane proteins, lane 2 contains immunoprecipitated $G_s\alpha$ from labelled membranes and lane 3 contains immunoprecipitated $G_s\alpha$ from cholera toxin-labelled membranes. This Figure is representative of five experiments.

cholera toxin substrate, $G_s\alpha$. A similar band was also demonstrated which showed that $G_s\alpha$ is a substrate for an endogenous ADP-ribosyltransferase in these cell membranes. Experiments performed with non-immune rabbit serum failed to immunoprecipitate $G_s\alpha$ after labelling with either cholera toxin or eukaryotic mono(ADP-ribosyl)transferase. Analysis of the

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

NG108-15 cells were cultured in the absence or presence of 50 mM-nicotinamide for 18 h. Cells were harvested, membranes prepared and adenylate cyclase activity measured as described in the Materials and methods section. Enzyme activities presented for measurements in the presence of NaF or iloprost are the δv values given by the increase in enzyme activity above the basal level. The results are expressed as the means $\pm$ S.E.M. of n values given in parentheses; ** $P < 0.01$, * $P < 0.05$ for differences from control, determined by unpaired two-tailed t tests.

Additions	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)	
	Control	Nicotinamide
Basal	11.6 $\pm$ 1.2 (9)	21.2 $\pm$ 2.7 (12)**
10 mM-F ⁻	48.7 $\pm$ 3.8 (5)	54.2 $\pm$ 4.9 (5)
1 μ M-Iloprost	65.7 $\pm$ 5.4 (7)	83.1 $\pm$ 5.9 (8)*

Table 2. Effect of 1 h of nicotinamide pretreatment of NG108-15 cells on adenylate cyclase activity

NG108-15 cells were cultured in the absence or presence of 50 mM-nicotinamide for 1 h. Cells were harvested, membranes prepared and adenylate cyclase activity measured as described in the Materials and methods section. Enzyme activities presented for measurements in the presence of NaF or iloprost are the δv values given by the increase in enzyme activity above the basal level. Results are expressed as the means $\pm$ S.E.M., $n = 4$.

Additions	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)	
	Control	Nicotinamide
Basal	9.9 $\pm$ 0.5	9.61 $\pm$ 0.65
1 μ M-Iloprost	91.5 $\pm$ 4.5	82.2 $\pm$ 8.6

Table 3. Binding parameters for the association of [³H]iloprost and NG108-15 cell membranes following 18 h of pretreatment with iloprost or nicotinamide

NG108-15 cells were cultured in the absence or presence of 10 μ M-iloprost or 50 mM-nicotinamide and cell membranes were prepared. The specific binding of [³H]iloprost was measured at concentrations between 5 and 100 nM. The data were analysed as described in the Materials and methods section. The binding parameters presented are the means $\pm$ S.E.M. ($n = 3$) for each parameter, * $P < 0.05$ for differences from control, determined by a two-tailed unpaired t test.

Pretreatment	[³ H]Iloprost binding	
	B_{max} (fmol/mg of protein)	K_D (nM)
Control	1437 $\pm$ 164	4.99 $\pm$ 1.19
10 μ M-Iloprost	734 $\pm$ 149*	16.28 $\pm$ 5.65
50 mM-Nicotinamide	1592 $\pm$ 115	3.93 $\pm$ 0.33

ADP-ribosylated products from both whole cells or membranes with snake venom phosphodiesterase revealed 5'-AMP as the major digestion product (> 90%) (results not shown), which

indicates that the proteins were mono(ADP-ribosyl)ated. A more specific analysis was also performed using immunoprecipitated $G_s\alpha$, which indicated once again that 5'-AMP was the most abundant product.

The effect of ADP-ribosylation by the endogenous enzyme on the function of $G_s\alpha$ was examined by measurement of adenylate cyclase activity in membranes from control cells or from cells following 18 h of nicotinamide pretreatment (Table 1). Nicotinamide treatment led to a significant increase in the basal and iloprost-stimulated adenylate cyclase activities (measured in the presence of GTP). The short-term effect of nicotinamide pretreatment of cells on adenylate cyclase activity was also examined (Table 2). These results indicated that treatment of cells for 1 h with 50 mM-nicotinamide did not result in any significant differences in either basal or agonist-stimulated adenylate cyclase activity.

The possibility that the increase in agonist-stimulated adenylate cyclase activity following treatment with nicotinamide was due to increased receptor numbers was investigated by measuring [³H]iloprost binding (Table 3). Pretreatment of cells with 50 mM-nicotinamide had no effect on either the K_D or the B_{max} of [³H]iloprost binding when compared with control cells. Pretreatment of cells with 10 μ M-iloprost did result in a significant decrease in B_{max} , but not in K_D .

DISCUSSION

These results reveal covalent modification of $G_s\alpha$ by endogenous enzyme(s) in intact NG108-15 cells and in cell membranes. Immunoprecipitation has shown that $G_s\alpha$ is mono(ADP-ribosyl)ated, and that NG108-15 cells have the capacity to ADP-ribosylate several proteins including $G_s\alpha$. These results are consistent with those seen in electroporated platelets where several proteins were ADP-ribosylated, including one with a similar molecular mass to $G_s\alpha$ [11]. However, in the previous report [11] the results showed that platelet ADP-ribosyltransferase was stimulated in the presence of iloprost, which contrasts with our results showing an apparent lack of effect of iloprost on endogenous ADP-ribosyltransferase activity in NG108-15 cells. These contradictory results may be due to differences in cell type. However, the responsiveness of both platelets and NG108-15 cells are similar in many respects, including the capacity of iloprost to activate adenylate cyclase and mediate the loss of prostacyclin receptors [15,16]. Prolonged exposure of platelets to iloprost leads to a decrease in cholera toxin substrate in the membrane, which was interpreted as reflecting a loss of $G_s\alpha$ from the membrane [16]. In the present report we show a loss of $G_s\alpha$ from the membranes of NG108-15 cells following iloprost treatment, using both cholera toxin ADP-ribosylation and Western blotting with two different primary antisera. These results confirm those reported for NG108-15 cells following prostaglandin E_1 (PGE₁) treatment [22]. The same group [22] showed that the loss of $G_s\alpha$ from the membrane was not due to inhibition of transcription of the $G_s\alpha$ gene or to decreased protein synthesis, but to translocation of $G_s\alpha$ from the membrane to the cytosol.

The physiological role of endogenous mono(ADP-ribosyl)-transferases in eukaryotic cells is unclear. Mono(ADP-ribosyl)-transferase activities from rat brain have been shown to ADP-ribosylate exogenous actin and prevent polymerization of G-actin in a manner similar to *Clostridium botulinum* C2 toxin [8]. The possibility that the removal of $G_s\alpha$ from the plasma membrane might be mediated through the ADP-ribosylation of the protein by a eukaryotic enzyme is suggested by a similar effect mediated by cholera toxin [23–26]. The persistent activation of $G_s\alpha$ by cholera toxin is followed by elimination of $G_s\alpha$ from

the membrane, and incubation of NG108-15 cells with cholera toxin for 24 h leads to a reduction in $G_s\alpha$ abundance [26]. However, there is some uncertainty arising from previous reports regarding the effect of cholera toxin on G-protein subunits other than $G_s\alpha$. The decrease in membrane-bound $G_s\alpha$ following cholera toxin treatment of NG108-15 cells was not accompanied by loss of $G\beta$ or any of the pertussis toxin-sensitive G-proteins, $G_{i\alpha_2}$, $G_{i\alpha_3}$ or $G_o\alpha$ [25]. However, others [26] have reported simultaneous loss of $G_o\alpha$ and $G\beta$ in NG108-15 cells. We have shown that inhibition of endogenous ADP-ribosylation leads to increased $G_s\alpha$ in the cell membrane. It follows that ADP-ribosylation of membrane-bound $G_s\alpha$ by eukaryotic mono(ADP-ribosyl)transferase might be one mechanism by which $G_s\alpha$ is targeted for removal from the membrane. Iloprost treatment of NG108-15 cells leads to loss of $G_s\alpha$, but could not be shown to activate endogenous ADP-ribosyltransferase activity under the present conditions, although it has been reported to do so in platelets [11]. Thus the mechanism by which iloprost lowers the abundance of membrane-associated $G_s\alpha$ is most probably not mediated through endogenous ADP-ribosylation.

We have shown in NG108-15 cells that prolonged inhibition of ADP-ribosyltransferase by nicotinamide leads to an increase in $G_s\alpha$, which in turn significantly increased both basal and iloprost-stimulated adenylate cyclase activity. This effect was not due to any alterations in the binding of [3H]iloprost to cells which had been treated with nicotinamide, though iloprost did lead to a significant decrease in B_{max} , as reported previously with PGE_1 [20]. A recent report [12] has indicated that short-term ADP-ribosylation of $G_s\alpha$ may stimulate adenylate cyclase activity in human platelet membranes. A similar effect has also been reported in chicken spleen membranes [13]. However, short-term inhibition of ADP-ribosyltransferase in NG108-15 cells with nicotinamide for 1 h did not have any effect on adenylate cyclase activity.

The results reported here provide evidence for covalent modification of $G_s\alpha$ within an intact living cell as a consequence of endogenous enzyme activity. $G_s\alpha$ is a physiological acceptor for ADP-ribosyl groups derived from NAD^+ , which are transferred as a consequence of eukaryotic mono(ADP-ribosyl)transferase activity. This covalent modification of $G_s\alpha$ within cells affects the functional response of $G_s\alpha$, and hence may have a role in the hormonal regulation of cellular function.

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