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Cholera toxin treatment produces down-regulation of the α-subunit of the stimulatory guanine-nucleotide-binding protein (G_s)

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The effect of activation of the α-subunit(s) of the stimulatory guanine-nucleotide-binding protein, G_s, on levels of this polypeptide(s) associated with the plasma membrane of L6 skeletal myoblasts was ascertained. Incubation of these cells with cholera toxin led to a time- and concentration-dependent 'down-regulation' of both 44 and 42 kDa forms of $G_s\alpha$ as assessed by immunoblotting with an anti-peptide antiserum (CS1) able to identify the extreme *C*-terminus of G_s. The effect of cholera toxin was specific for G_s; levels of $G_i\alpha$ in membranes of cholera toxin-treated cells were not different from untreated cells. Down-regulation of G_s was absolutely dependent upon prior ADP-ribosylation, and hence activation of G_s and was not mimicked by other agents which elevate intracellular levels of cyclic AMP. Pretreatment with pertussis toxin, which catalyses ADP-ribosylation of G_i but not of G_s, did not down-regulate either G_i or G_s, demonstrating that covalent modification by ADP-ribosylation is alone not a signal for removal of G-proteins from the plasma membrane.

INTRODUCTION

A family of guanine-nucleotide-binding proteins (G-proteins) function to transduce information from agonist-activated cell surface receptors to effector systems which include both enzymes which generate intracellular second messengers and ion channels [1,2]. The first of these G-proteins to be identified and isolated was that responsible for controlling receptor-mediated stimulation of adenylate cyclase. This G-protein (G_s), in common with other G-proteins, is a heterotrimer consisting of distinct α-, β- and γ-subunits, but within a single tissue it appears that at least two distinct forms of the α-subunit of this G-protein can be generated. These two polypeptides appear to result from differential splicing of pre-mRNA transcribed from a single gene [3,4]. The identification of $G_s\alpha$ is frequently achieved by utilizing the known ability of cholera toxin to catalyse a [^{32}P]NAD^+-dependent [^{32}P]ADP-ribosylation of the α-subunit(s) of G_s [5]. Estimates of the molecular mass of the forms of $G_s\alpha$ following such chemical modification have ranged between 42 and 52 kDa.

Given that the role of G_s is to allow information transfer between two proteins which are intrinsic to the plasma membrane of eukaryotic cells, then the physical location of G_s should also be at the plasma membrane. However, Rodbell [6] has proposed that the α-subunit of G_s might be released from the plasma membrane following activation, perhaps to catalyse distinct functions at other sites within the cell. Little evidence has accrued to support this concept, but we have recently noted, by the use of site-directed anti-peptide antisera, that the α-subunit(s) of G_s can indeed be released from membrane preparations following chronic activation of the G-protein with poorly hydrolysed analogues of GTP [7]. In further work, using similar protocols, we have also observed the release of the α-subunits of two other G-proteins, G_i and G_o [8,9]. In this study we have addressed whether such a release of G_s might occur in whole cells. An appropriate means to persistently activate G_s *in situ* is to treat the cells or tissue with holomeric cholera toxin. We note that treatment of L6 skeletal myoblasts in tissue culture with cholera toxin produces a reduction in levels of G_s in membranes derived from these cells. This is dependent upon both length of exposure and the concentration of cholera toxin used. This down-regulation occurs with a slower time course than cholera-toxin-catalysed ADP-ribosylation of G_s, demonstrating that activation of the G-protein must precede down-regulation. Further, the effect of cholera toxin is specific for G_s as no alteration in the levels of membrane-associated G_i were observed in parallel experiments.

MATERIALS AND METHODS

Cell culture

L6 skeletal myoblasts were cultured in Dulbecco's modified Eagle's medium (Gibco, Paisley, Scotland, U.K.) containing 10% (v/v) heat-inactivated foetal calf serum (Gibco) as previously described [10]. In a number of cases the cells were treated with either cholera toxin (Sigma) (up to 1000 ng/ml) or pertusis toxin (Porton Products, Porton Down, Wiltshire, U.K.) (100 ng/ml)

Abbreviations used: G-protein, guanine-nucleotide-binding protein; G_s and G_i, the G-proteins associated with stimulation and inhibition, respectively, of adenylate cyclase; PAGE, polyacrylamide-gel electrophoresis; GDP[S], guanosine 5'-[γ-thio]diphosphate; GTP[S], guanosine 5'-[γ-thio]triphosphate; Gpp[NH]p, guanosine 5'-[βγ-imido]triphosphate; App[NH]p, adenosine 5'-[βγ-imido]triphosphate; KLH, keyhole-limpet haemocyanin.

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for up to 24 h. Cells were harvested from tissue culture flasks and stored as cell pastes at −80 °C before disruption in 10 mM-Tris/HCl, pH 7.5/0.1 mM-EDTA (buffer A) using a glass-on-Teflon homogenizer. The homogenate was then centrifuged at 1000 *g* for 10 min to remove cellular debris, and the supernatant from this was recentrifuged for a further 10 min at 40000 *g* to generate a membrane pellet [11]. This pellet was resuspended in buffer A and stored at −80 °C until use. Protein was assessed by the method of Lowry *et al.* [12] using bovine serum albumin as standard.

Immunological analysis

The individual forms of the α-subunit of G_s were identified in membranes of L6 cells by Western blotting using antiserum CS1. This antiserum was produced in a New Zealand White rabbit by using a synthetic peptide (RMHLRQYELL), which corresponds to the *C*-terminal decapeptide of all forms of $G_s\alpha$, conjugated to keyhole-limpet haemocyanin (KLH) as antigen. We have previously described similar protocols for the generation of a series of other anti-G-protein anti-peptide antisera [13–15]. A second anti-$G_s\alpha$ antiserum, CT142, was a gift from Dr. J. Ireson, Amersham International, Cardiff, Wales. This antiserum was generated against the synthetic peptide TPEPGEDPRVTRAKY, which corresponds to amino acids 325–339 of $G_s\alpha$. Antiserum SG1 was produced in a similar fashion against a KLH-conjugate of the peptide KENLKDCGLF, which corresponds to the *C*-terminal decapeptide of rod transducin. We have previously used a similar conjugate to generate antisera AS6 and AS7 [13]. Antiserum SG1 shows a similar profile of immunoreactivity to antisera AS6 and AS7 and as such identifies forms of G_i as well as transducin. As transducin is limited in distribution to photoreceptor-containing tissues, this antiserum can be used to identify forms of G_i in all other tissues. Immunoblotting with these antisera was performed as previously described [13–17].

Analysis of the guanine-nucleotide-mediated release of the α-subunits of G_s from membranes of L6 cells was performed as previously described [8,9]. Briefly, membranes were incubated in a buffer (20 mM-Tris/HCl, pH 7.5/20 mM-$MgCl_2$/1 mM-dithiothreitol/100 μM-EDTA) in the presence of various analogues of guanine nucleotides (100 μM) (Boehringer Mannheim) for 60 min at 37 °C. At the termination of incubation, soybean trypsin inhibitor (25 μg) was added as a soluble protein carrier and the samples separated into supernatant and membrane-associated fractions by centrifugation at 172.5 kPa (150000 *g*) for 2 min in an Airfuge (Beckman Instruments). Protein in the supernatant fraction was collected by deoxycholate/trichloracetic acid precipitation (final concentrations. 0.0125% and 6%, w/v) [18] before addition of sample buffer and resolution using SDS/polyacryamide-gel electrophoresis (SDS/PAGE) [10% (w/v) acrylamide]. The residual membrane pellet was solubilized with sample buffer and added directly to the gels.

ADP-ribosylation

This was performed with thiol-preactivated cholera toxin (2.5 μg/assay) and [^{32}P]NAD^+ (New England Nuclear), in the presence of added exogenous GTP, as previously described [19], on membranes derived from L6 cells as described above.

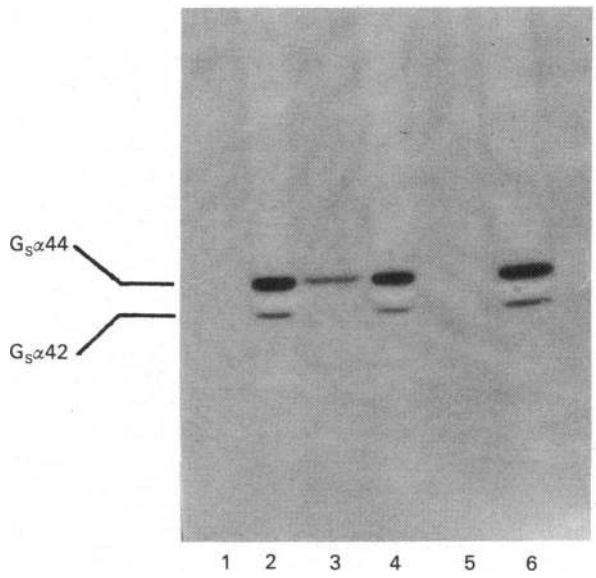

Fig. 1. Gpp[NH]p promotes the release of $G_s\alpha$ from membranes of L6 cells

Membranes of L6 skeletal myoblast cells (100 μg) were analysed for guanine-nucleotide-mediated release of the α-subunits of G_s as described in the Materials and methods section and in [7]. The membranes were incubated with App[NH]p (lanes 1 and 2), Gpp[NH]p (3 and 4) or GDP[S] (5 and 6) (all 100 μM) for 30 min at 37 °C. Lanes 1, 3 and 5 represent the released material, lanes 2, 4 and 6 the residual membrane-associated material. The immunoblot displayed was produced using antiserum CS1 (1:200 dilution) as primary reagent. Whilst the signal from the 42 kDa form of $G_s\alpha$ in lane 3 is too faint to see in this reproduction, a similar proportion of this polypeptide was released from the membrane in response to Gpp[NH]p as the 44 kDa form.

RESULTS

Antiserum CS1 was produced against a synthetic peptide corresponding to the *C*-terminal decapeptide of the α-subunit(s) of G_s. Immunoblotting of membranes derived from L6 skeletal myoblasts with this antiserum demonstrated the expression of two forms of $G_s\alpha$ with molecular masses of 44 and 42 kDa. The 44 kDa form was present in higher amounts than the 42 kDa form. Incubation of membranes of L6 cells with the poorly hydrolysed analogue of GTP, Gpp[NH]p, led to release of the α-subunits of G_s from the membrane (Fig. 1). This release process was specific for the analogue of GTP, as neither the GDP analogue, GDP[S], nor the ATP analogue, App[NH]p, was able to mimic this effect (Fig. 1). In an attempt to assess whether activation of G_s within intact L6 cells would lead to a similar removal of $G_s\alpha$ from the membrane, we treated L6 cells in tissue culture with or without cholera toxin (100 ng/ml) for 24 h. Immunoblotting of membranes derived from the cholera toxin-pretreated cells with antiserum CS1 displayed distinctly lower immunoreactivity corresponding to the 44 kDa form of $G_s\alpha$ in comparison with membranes from the untreated cells (Fig. 2). Lower amounts of immunoreactive $G_s\alpha42$ were also noted in the cholera toxin-pretreated membranes, although the degree of decrease was not as marked as with the 44 kDa form

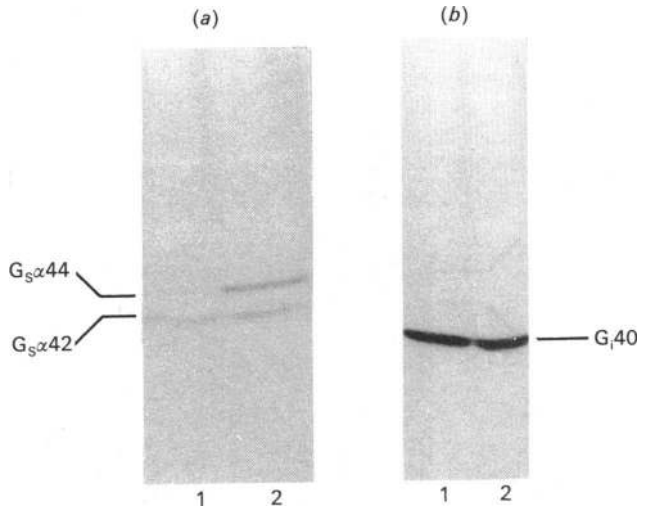

Fig. 2. Immunological estimation of amounts of $G_s\alpha$ and $G_i\alpha$ in membranes of L6 cells: the effect of cholera toxin

L6 cells were treated with either cholera toxin (100 ng/ml) (lane 1) or vehicle (lane 2) for 24 h prior to harvest and membrane preparation. Either 200 μg of the membranes was immunoblotted with antiserum CS1 ($G_s\alpha$) (*a*), or 100 μg was immunoblotted with antiserum SG1 ($G_i\alpha$) (*b*).

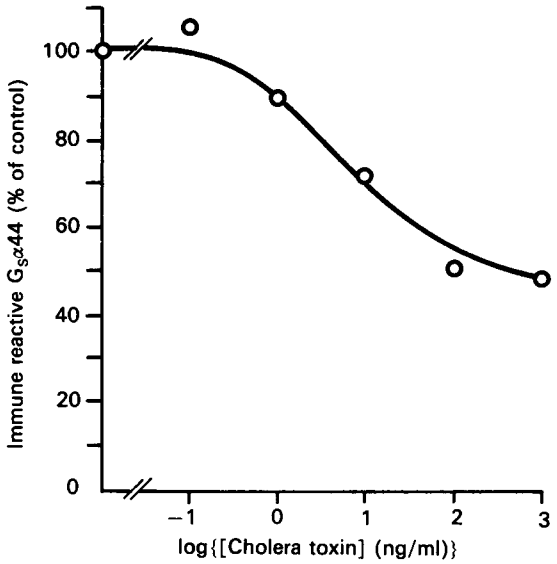

Fig. 3. Down-regulation of $G_s\alpha$ by treatment with cholera toxin: concentration dependence

L6 cells were treated with a variety of concentrations of cholera toxin for 24 h. Immunoblotting of SDS/PAGE-resolved membranes (200 μg) derived from the treatments was performed with antiserum (1:200 dilution) as primary reagent. Quantification of the immunoblot was performed as described in [26].

(Fig. 2). Both the 44 and 42 kDa forms of $G_s\alpha$ migrated with slightly reduced mobility through SDS/PAGE following pretreatment of the cells with cholera toxin (Fig. 2; see also Fig. 6). We have previously reported that ADP-ribosylated forms of both G_i and transducin migrate with a reduced mobility in comparison with the unmodified forms [13], presumably due to the extra charge provided by the covalent modification. Levels of $G_i\alpha$, as assessed by immunoblotting with antiserum SG1, were similar in the membranes from the untreated and cholera toxin-pretreated cells (Fig. 2).

Pretreatment of L6 cells in culture with varying concentrations of cholera toxin for 24 h before harvest and membrane preparation indicated that half-maximal reduction in levels of $G_s\alpha$ occurred with some 5 ng of cholera toxin/ml (Fig. 3). In the experiments displayed in Fig. 3, some 50% of $G_s\alpha 44$ was lost from the membrane within this time period. In a number of other experiments this reduction was up to 80%. A second anti-peptide antiserum which identifies $G_s\alpha$ (CT142) produced similar results to CS1 when used in Western blots of membranes of untreated and cholera toxin-treated cells (results not shown).

One question related to the possibility that ADP-ribosylated $G_s\alpha$ displayed lower immunoreactivity to antiserum CS1 than the unmodified form. In an effort to assess this point we incubated membranes of untreated L6 cells *in vitro* with combinations of thiol-preactivated cholera toxin and high concentrations of NAD^+, and then immunoblotted the samples with antiserum CS1. In each case, the signal from both 44 and 42 kDa forms of $G_s\alpha$ was not reduced in comparison with membranes incubated in the absence of both preactivated cholera toxin and NAD (Fig. 4). Toxin-catalysed ADP-ribosylation *in vitro* is however an inefficient process and thus it was possible that only a small proportion of the available $G_s\alpha$ was modified by this procedure. As such,

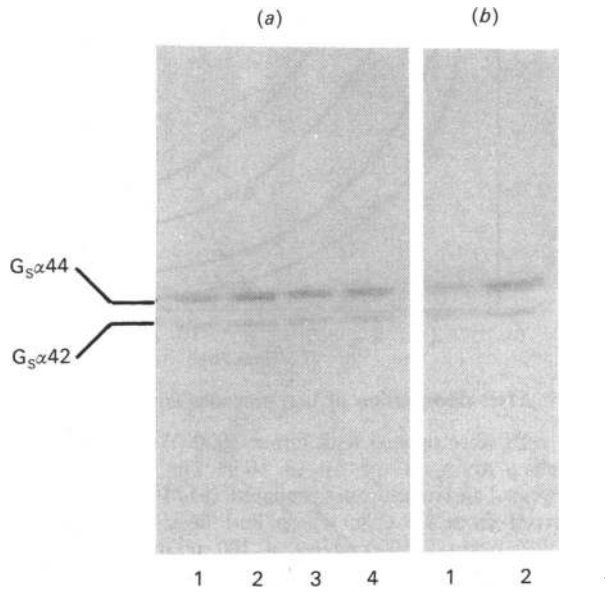

Fig. 4. Antiserum CS1 identifies equally ADP-ribosylated and non-modified forms of $G_s\alpha$

(*a*) Membranes of L6 cells (200 μg) were incubated *in vitro* in the absence of both cholera toxin and NAD (lane 1), with NAD (5 mM) (lane 2), with cholera toxin (2.5 μg) and NAD (5 mM) (lane 3) or with cholera toxin (2.5 μg) but without NAD (lane 4). In each case all other reagents normally used in cholera toxin-catalysed ADP-ribosylations [17,18] were present. The samples were incubated for 2 h at 37 °C and then processed for SDS/PAGE. (*b*) As an internal control, membranes (200 μg) derived from either cholera toxin-treated (100 μg/ml, 24 h) (lane 1) or untreated (lane 2) L6 cells were resolved and immunoblotted with antiserum CS1 on the same gel.

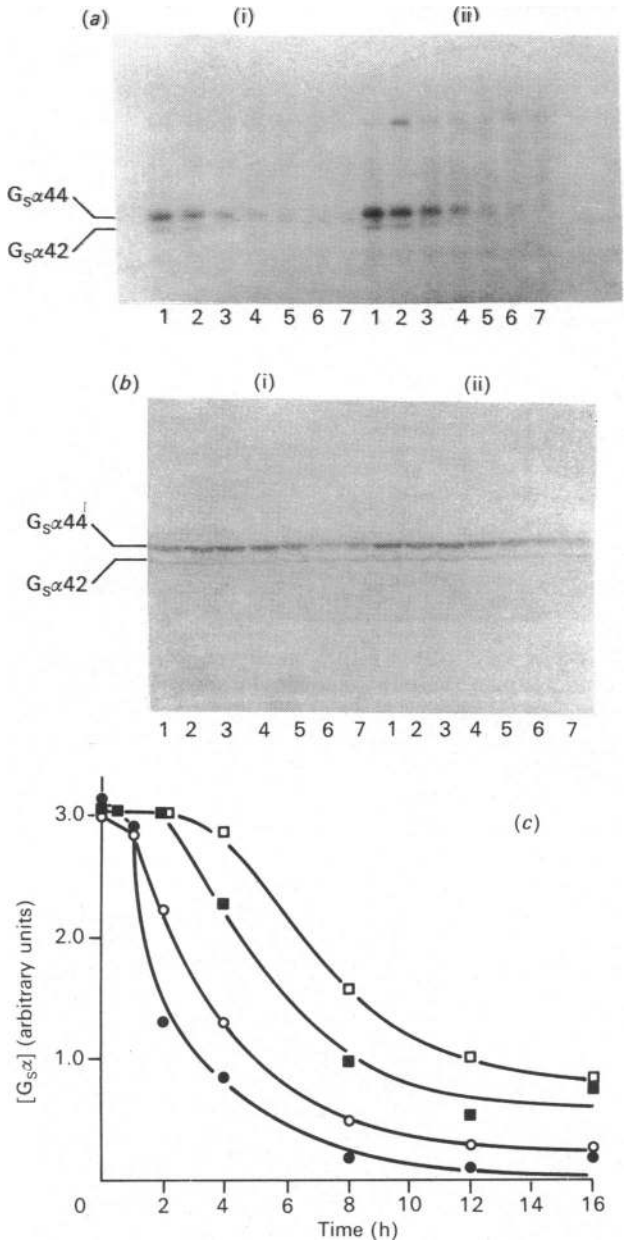

Fig. 5. ADP-ribosylation of $G_s\alpha$ precedes down-regulation

L6 cells were treated with either 1000 (i) or 100 (ii) ng of cholera toxin/ml for up to 16 h. The cells were then harvested and membranes prepared. (*a*) Membranes (20 μg) derived from L6 cells which had been pretreated with cholera toxin (i, 1000 ng/ml; ii, 100 ng/ml) for 0 (lane 1), 1 (2), 2 (3), 4 (4), 8 (5), 12 (6) or 16 (7) h were ADP-ribosylated *in vitro* with [^{32}P]NAD$^+$ and fresh preactivated cholera toxin for 2 h at 37 °C. At termination of incubation the samples were collected by sodium deoxycholate/trichloroacetic acid precipitation [18], resolved by SDS/PAGE [10% (v/v) acrylamide] and autoradiographed. (*b*) Levels of $G_s\alpha$ in membranes derived from L6 cells following treatment with cholera toxin: time-dependence of down-regulation. Membranes (200 μg) derived from the treatments detailed in Fig. 5(*a*) were resolved on SDS/PAGE and immunoblotted using a 1:200 dilution of antiserum CS1 as primary reagent. (*c*) Comparison of time courses of cholera toxin-catalysed ADP-ribosylation and down-regulation of $G_s\alpha44$. The data relating to $G_s\alpha44$ from Figs. 5(*a*) and 5(*b*) were quantified by densitometric analysis of either the autoradiogram displayed in Fig. 5(*a*) and of a film positive produced from the immunoblot displayed in Fig. 5(*b*) as described previously [26]. Levels of $G_s\alpha$ in membranes available for cholera toxin-catalysed ADP-ribosylation *in vitro* following pretreatment of the cells with 1000 ng (●) or 100 ng (○) of cholera toxin/ml, and levels of immunoreactive $G_s\alpha$ after pretreatment with 1000 (■) or 100 (□) ng of cholera toxin/ml are shown.

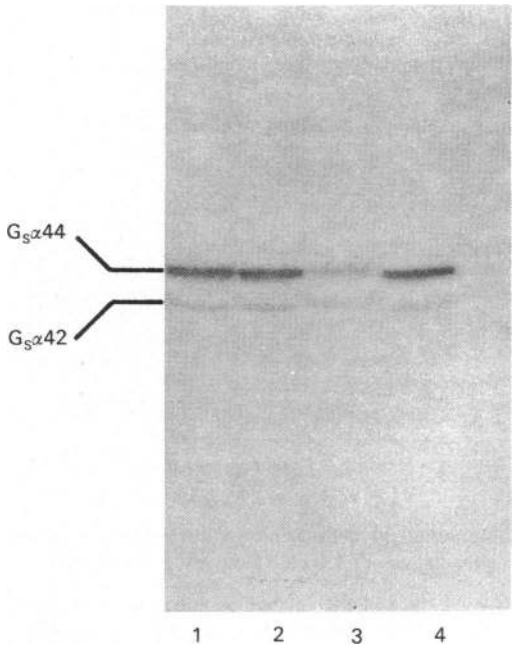

Fig. 6. Pertussis toxin treatment does not down-regulate membrane associated levels of $G_s\alpha$

L6 cells were treated with the pertussis toxin vehicle (lane 1), pertussis toxin (100 ng/ml) (lane 2), cholera toxin (100 ng/ml) (lane 3) or the cholera toxin vehicle (lane 4) for 24 h. Membranes (200 μg) derived from these cells were immunoblotted with antiserum CS1.

a more definitive means to ascertain that ADP-ribosylated $G_s\alpha$ was identified by antiserum CS1 was required (see below and Fig. 5).

To assess whether ADP-ribosylation by cholera toxin, and hence activation of $G_s\alpha$, had to occur before the down-regulation process, we treated L6 cells for varying times with either 100 or 1000 ng of cholera toxin/ml. Membranes prepared from these cells were either challenged *in vitro* with fresh, thiol-activated cholera toxin and [^{32}P]NAD$^+$ (Fig. 5*a*) or were immunoblotted with antiserum CS1 following separation on SDS/PAGE (Fig. 5*b*). Autoradiography of the membranes following [^{32}P]NAD$^+$-dependent ADP-ribosylation indicated that in the cells pretreated with 1000 ng of cholera toxin/ml, ADP-ribosylation *in vivo* using unlabelled NAD$^+$ as the substrate had proceeded more quickly than when 100 ng of cholera toxin/ml had been used and that >80% of the available $G_s\alpha$, of both the 44 and 42 kDa forms, had been modified within 2 h. In contrast, a reduction in immunoreactivity to antiserum CS1 was not noted at that time. A 50% reduction in immunoreactivity only occurred following between 4 and 8 h treatment with the toxin. As such, the time course of down-regulation indicated that this was subsequent to that of ADP-

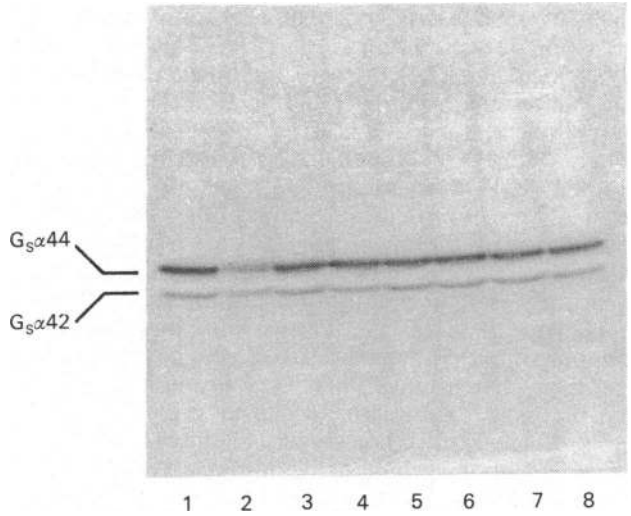

Fig. 7. Down-regulation of $G_s\alpha$ by cholera toxin only requires activation of the G-protein and is not dependent upon cyclic AMP generation

L6 cells were incubated for 24 h with cholera toxin (100 ng/ml) (lane 2), cholera toxin A subunit (100 ng/ml) (lane 3), cholera toxin B subunit (100 ng/ml) (lane 4), dibutyryl cyclic AMP (10 mM) (lane 5), forskolin (0.1 mM), isoprenaline (10 μM) (lane 7) or without treatment (lanes 1 and 8). Membranes (200 μg) prepared from these cells were immunoblotted using antiserum CS1 (1:200 dilution) as primary reagent.

ribosylation and activation (Fig. 5*c*) and that ADP-ribosylated $G_s\alpha$ was adequately identified by antiserum CS1.

In contrast with the results obtained with cholera toxin, pretreatment of L6 cells with pertussis toxin (100 ng/ml) did not affect levels of either the 44 or 42 kDa forms of $G_s\alpha$ (Fig. 6), nor did it affect levels of $G_i\alpha$ (results not shown) even though the toxin catalysed incorporation of ADP-ribose into the entire pool of G_i (results not shown). It was possible that the down-regulation of the forms of $G_s\alpha$ might be related to the increased levels of intracellular cyclic AMP produced following activation of G_s by cholera toxin. To assess this we treated L6 cells for 24 h with either holomeric cholera toxin, with the separated A or B subunits of the toxin, with the β-adrenergic agonist isoprenaline, with the diterpene forskolin or with dibutyryl cyclic AMP. Immunoblotting of membranes derived from cells following the various treatments with antiserum CS1 indicated that levels of both the 44 and 42 kDa forms of $G_s\alpha$ were reduced only in cells treated with the holomeric toxin (Fig. 7). None of these treatments reduced levels of membrane-associated G_i (results not shown). As such, the down-regulation process was independent of cyclic AMP levels in the cell and was absolutely dependent upon the ability of the holomeric toxin to allow transport of the A subunit into the cell.

DISCUSSION

The ability of cholera toxin to catalyse a [^{32}P]NAD$^+$-dependent [^{32}P]ADP-ribosylation on an arginine residue in the α-subunit(s) of G_s provided the first convenient means of detection of this G-protein [1,2]. This covalent modification inhibits the GTPase activity which would normally function to deactivate the activated α-subunit [1,2]. As such, following ADP-ribosylation, G_s becomes persistently activated. Whilst incorporation of radioactivity from [^{32}P]NAD$^+$ thus allows easy identification of the presence of $G_s\alpha$, there are limitations to the use of this assay as a means of quantification of levels of this protein. These stem both from the requirement for a proteinaceous cofactor, ADP-ribosylation factor [20], for the reaction and from the alterations in kinetics of the reaction dependent upon the state of the G-protein, i.e. whether in the holomeric state or as the free α-subunit [19]. Further, it is impossible to detect G_s with assays of this type if the protein has previously been ADP-ribosylated. Thus, the inability of activated cholera toxin, in the presence of [^{32}P]NAD$^+$, to incorporate radioactivity into the polypeptides of $G_s\alpha$ after cholera toxin treatment of a tissue or cell is taken as evidence that all the G_s has previously been modified. In this paper we demonstrate that the true situation is more complex and that sustained activation of G_s by treatment of a cell with cholera toxin does not simply maintain an activated population of $G_s\alpha$ at the plasma membrane. Rather, this activation process can lead to the removal from the membrane and hence down-regulation of a substantial proportion of this polypeptide.

In these experiments we have used an anti-peptide antiserum, CS1, which is able to identify the extreme *C*-terminus of the different forms of $G_s\alpha$. Up to four individual forms of $G_s\alpha$ can potentially be generated by the differential splicing of nuclear RNA derived from an apparently single gene [4]. However, in all cases, the *C*-terminal region is identical and as such, antiserum CS1 would be anticipated to identify all of these forms. In the L6 skeletal myoblast cell line we have been able to identify both large (44 kDa) and small (42 kDa) forms of $G_s\alpha$, both by cholera toxin-catalysed ADP-ribosylation and by immunoblotting with antiserum CS1 (Fig. 5). In each experimental protocol the 44 kDa form predominated in quantity over the 42 kDa form. Treatment of the L6 cells in tissue culture produced a reduction in the levels of immunoreactive forms of $G_s\alpha$ in membranes derived from these cells. This event was dependent both upon the period of treatment with the toxin (Fig. 5) and also upon the concentration employed (Fig. 3). Whilst this appeared to represent a down-regulation of levels of $G_s\alpha$, the observations might have been very simply explained as an artefact of the assay if we were able to demonstrate that ADP-ribosylated $G_s\alpha$ was less well identified by the antiserum than the unmodified form. On a purely subjective basis it appeared unlikely that this would provide a satisfactory explanation since the site of ADP-ribosylation on $G_s\alpha$ is an arginine residue (position 213 in the primary sequence), whilst the antiserum was generated against a synthetic peptide corresponding to the *C*-terminal decapeptide (amino acids 385–394). Thus it appeared unlikely that addition of ADP-ribose at a position which is relatively remote from the antigenic site (particularly following SDS/PAGE and electroblotting) would interfere with antibody–antigen recognition. Moreover, we have previously characterized anti-peptide antisera which identify the *C*-terminal decapeptide of forms of G_i which are able to interact at least as effectively with G_i following ADP-ribosylation catalysed by pertussis toxin [13]. This is despite the fact that the cysteine residue which is the acceptor amino acid for the ADP-

ribose in $G_i\alpha$ is within the region identified by the antibody.

To test this assumption more rigorously we ADP-ribosylated membranes of untreated L6 cells with preactivated cholera toxin and a high concentration of NAD^+ and then immunoblotted these membranes with antiserum CS1 (Fig. 4). An equivalent reactivity was observed with both forms of $G_s\alpha$ in the untreated and the ADP-ribosylated samples *in vitro*. A more definitive confirmation of these conclusions was obtained in the course of experiments designed to assess whether activation of G_s and the down-regulation process were sequential (Fig. 5). In time courses of treatment of L6 cells with 1000 ng of cholera toxin/ml, more than 80% of the forms of $G_s\alpha$ became ADP-ribosylated within 2 h. In contrast, no diminution of the immunoreactivity of either form of the G-protein was noted in the membranes at this time point (Fig. 5).

Rodbell [6] has proposed that activation of G-proteins might result in the release of the relatively hydrophilic α-subunits from their site of membrane attachment and their translocation into the cytoplasm, where they might function in ways distinct from their roles at the plasma membrane. Whilst little evidence has been obtained experimentally to support such a notion, recent reports have noted the presence of G-proteins in both microsomal and, to a lesser extent, cytosolic locations [21].

We have, however, been unable to demonstrate an accumulation of immunoreactive $G_s\alpha$ in the cytoplasm of cells during time courses of cholera toxin treatment (results not shown), despite the loss of immunoreactivity from total membrane preparations. This may be a reflection of rapid degradation of $G_s\alpha$ following activation-induced release and might argue against a specific role for a G-protein subsequent to release. A second possibility is that reduction in immunoreactivity in the membranes following cholera toxin treatment of cells is due either to a proteolytic cleavage of $G_s\alpha$ *in situ* such that the *C*-terminal antigenic epitope is removed or that, subsequent to ADP-ribosylation, a covalent modification takes place close to the *C*-terminus, such that antiserum CS1 is now unable to identify $G_s\alpha$. That a second anti-peptide anti-$G_s\alpha$ antiserum (CT142), which identifies a region some 70 amino acids from the *C*-terminus, gave similar results to antiserum CS1, implies that proteolytic cleavage *in situ* would have to remove a considerable length of the polypeptide for this idea to be consistent with the observations noted herein. Moreover, these results are suggestive that the concept that a further covalent modification, subsequent to ADP-ribosylation, acts to prevent interaction of antigen with both antisera is unlikely. As demonstrated in Fig. 1, guanine-nucleotide-activated $G_s\alpha$ is released from the membrane of these cells in a form which is immunologically indistinguishable from the membrane-associated form.

Whilst cholera toxin and guanine nucleotide-induced activation of G_s represent distinct mechanistic processes they are believed to be functionally identical. On this basis we would also argue that proteolytic processing of $G_s\alpha$ *in situ* is unlikely, although we cannot prove that it is impossible.

All G-proteins which have currently been identified have an invariant arginine residue in an equivalent position to that which is the site of cholera toxin-catalysed ADP-ribosylation in G_s. Whilst it is possible to use cholera toxin to catalyse ADP-ribosylation of forms of G_i *in vitro* under strictly defined conditions [22,23], such a reaction does not occur *in vivo* [23]. As such it would be anticipated that cholera toxin treatment of cells would not lead to an activation of G_i. In all of the experiments with cholera toxin we did not observe any down-regulation of G_i. Equally, pertussis toxin does not catalyse ADP-ribosylation of G_s, and no alterations in levels of this G-protein were noted following treatment of L6 cells with this agent. Pertussis toxin does of course catalyse ADP-ribosylation of $G_i\alpha$, but the functional consequences of the modification are different from those of cholera toxin on G_s. Pertussis toxin prevents the coupling of relevant receptors to G_i and as such prevents agonist-mediated activation of G_i. It is thus not surprising that pertussis toxin treatment did not reduce membrane levels of G_i (results not shown) if down-regulation is dependent upon chronic activation of a G-protein.

For Rodbell's theory of G-proteins as programmable messengers [6] to have relevance, it would be necessary to demonstrate that hormone activation of a G-protein would lead to the release of an activated α-subunit. We attempted to address this point by incubating L6 cells with the β-adrenergic agonist isoprenaline. Isolation of membranes following this treatment demonstrated no reduction in the membrane complement of $G_s\alpha$ (Fig. 7). This lack of effect of the agonist may in this case, however, reflect a rapid desensitization of the agonist at the level of the receptor. Further studies will be required to address this point.

It is unclear why the down-regulation should be markedly slower than activation of the G-protein (Fig. 5). In the case of both G_i and G_o, the α-subunits of these proteins have *N*-terminally attached myristic acid [24], which may be as important as the β-subunit in the attachment of the α-subunit to the membrane. However, in the case of G_s no such fatty acylation has been noted, although other, as yet unidentified, linkages may exist and indeed $G_s\alpha$ is known to be resistant to Edman degradation, suggesting that it has a blocked *N*-terminus. Thus it might reasonably have been anticipated that the dissociation of α- from β-subunit might be sufficient to allow release of the α-subunit of G_s.

Cholera toxin-induced down-regulation of $G_s\alpha$ is not restricted to L6 skeletal myoblast cells. We have noted a similar pattern of reduction in immunologically detectable $G_s\alpha$ in membranes following cholera toxin treatment in each of rat glioma C6, neuroblastoma × glioma hybrid, NG105-15, and in monocytic U937 cells (results not shown). Further, we have noted that chronic exposure to cholera toxin leads to a reduction in both basal and forskolin-amplified adenylate cyclase activity in NG108-15 cells. This correlates highly with the reduction in membranes of immunodetectable $G_s\alpha$ (K. G. MacLeod & G. Milligan, unpublished work).

A recent report has indicated that as well as acting to stimulate adenylate cyclase, G_s is able to activate dihydropyridine-sensitive Ca^{2+} channels [25]. Down-regulation of G_s might be anticipated to modulate maximal responsiveness of both of these effector systems. Further experimentation will help to define the consequences of this process.

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