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GTP analogues promote release of the α subunit of the guanine nucleotide binding protein, G_i2, from membranes of rat glioma C6 BU1 cells

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The major pertussis-toxin-sensitive guanine nucleotide-binding protein of rat glioma C6 BU1 cells corresponded immunologically to G_i2. Antibodies which recognize the α subunit of this protein indicated that it has an apparent molecular mass of 40 kDa and a pI of 5.7. Incubation of membranes of these cells with guanosine 5'-[βγ-imido]triphosphate, or other analogues of GTP, caused release of this polypeptide from the membrane in a time-dependent manner. Analogues of GDP or of ATP did not mimic this effect. The GTP analogues similarly caused release of the α subunit of G_i2 from membranes of C6 cells in which this G-protein had been inactivated by pretreatment with pertussis toxin. The β subunit was not released from the membrane under any of these conditions, indicating that the release process was a specific response to the dissociation of the G-protein after binding of the GTP analogue. Similar nucleotide profiles for release of the α subunits of forms of G_i were noted for membranes of both the neuroblastoma × glioma hybrid cell line NG108-15 and of human platelets. These data provide evidence that: (1) pertussis-toxin-sensitive G-proteins, in native membranes, do indeed dissociate into α and βγ subunits upon activation; (2) the α subunit of 'G_i-like' proteins need not always remain in intimate association with the plasma membrane; and (3) the α subunit of G_i2 can still dissociate from the β/γ subunits after pertussis-toxin-catalysed ADP-ribosylation.

INTRODUCTION

The existence of a considerable number of pertussis-toxin-sensitive signal-transducing guanine nucleotide-binding proteins (G-proteins) has recently been demonstrated by using either protein purification protocols [1,2] or analysis of cDNA clones [3–5]. Each of the individual G-proteins consists of α, β and γ subunits. The α subunits are unique to each G-protein, but they are highly homologous, and this has made it difficult to identify particular species unambiguously. In an attempt to address this problem, we have generated polyclonal antisera against purified G-proteins [6,7] and more recently against peptide sequences predicted from cDNA clones to be present in specific G-proteins [8–10].

G-proteins are located on the cytoplasmic face of the plasma membrane and link receptors to second-messenger-generation systems. Although it has been demonstrated that treatment of G-proteins with poorly hydrolysed analogues of GTP, in detergent extracts of membranes, can cause dissociation of the G-proteins into α+β/γ subunits, similar effects have not been reported for native membranes. It has been proposed, however, that G-protein α subunits might be released from the plasma membrane after stimulation to act as soluble 'programmable messengers' to interact with intracellular effector systems [11]. For this hypothesis to have credence, dissociation of the subunits of membrane-bound G-proteins must occur upon binding of GTP, as the β subunit is a hydrophobic polypeptide which is not likely to be found in the cytoplasm. We have recently addressed this question, in an indirect manner, in membranes of both human neutrophils and bovine brain [12], and were unable to find any evidence of guanine nucleotide-mediated release of pertussis-toxin-sensitive G-proteins over a short time span. In the present paper, however, we demonstrate that GTP analogues, but not analogues of either GDP or ATP, do cause a slow release of the membrane complement of the α, but not the β, subunit of G_i2 from rat glioma C6 BU1 cells in a time-dependent fashion. Similar results were obtained with membranes of cells of the neuroblastoma × glioma hybrid cell line NG108-15 and with human platelets.

On the basis of the slow time course of release, we do not believe that this process is related to short-term responses to receptor activation which are mediated via G-proteins. It does, however, allow demonstration that: (1) binding of a GTP analogue promotes dissociation of the α and β/γ subunits of 'G_i-like' proteins within the membrane; (2) the α subunit of ADP-ribosylated forms of 'G_i-like' proteins can dissociate from β/γ subunits upon binding of a GTP analogue; and (3) a longer-term

Abbreviations used: G-protein, guanine nucleotide-binding protein; GDP[S], guanosine 5'-[thio]diphosphate; p[NH]ppG, guanosine 5'-[βγ-imido]triphosphate; GTP[S], guanosine 5'-[thio]triphosphate; p[CH_2]ppG; guanosine 5'-[βγ-methylene]triphosphate.

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event, perhaps involving demyristoylation of the α subunit, allows the release of the polypeptide from the plasma membrane under conditions in which the α and β/γ subunits are no longer in intimate association.

MATERIALS AND METHODS

Materials

Production of the anti-peptide antisera used in this study has previously been described in detail [8–10,13]. Antisera AS6 and AS7 were raised in individual rabbits against the *C*-terminal decapeptide (KENLKDCGLF) of the α subunit of transducin. As well as transducin, these antisera recognise the *C*-terminal sequences of the α subunits of both G_i1 and G_i2, which have identical *C*-terminal decapeptides [3,14] differing in but a single position from that of transducin [14]. As transducin is limited in distribution to photoreceptor-containing tissues, these antisera can be used as specific probes for the 'G_i-like' subfamily of pertussis-toxin-sensitive G-proteins. These two antisera have identical specificities, but AS7 has a higher titre than AS6. Antiserum LE2 was raised against amino acids 160–169 (LERIAQSDYI) of G_i2 α as cloned from C6 glioma cells [5]. This sequence has three differences from the equivalent sequence of G_i1 α, and the antiserum does not recognize G_i1 α [8,15]. None of these antisera cross-react with the α subunit of G_o. The β subunit, which appears to be common to a number of G-proteins, was detected with antiserum RV6 [6]. This antiserum was raised against a mixture of holomeric pertusis-toxin-sensitive G-proteins purified from bovine brain [1], but contains antibodies against the β subunit only [6]. Sources of all materials for one- and two-dimensional electrophoresis have previously been described [15,16]. All nucleotide analogues were purchased from Boehringer–Mannheim. Pertussis toxin was purchased from Porton Products (Porton Down, Wilts., U.K.).

Methods

Rat glioma C6 BU1 cells were grown in tissue culture in Dulbecco's Modified Eagle's Medium supplemented with 5% (v/v) foetal-calf serum (Imperial Laboratories), and membranes were prepared from them as previously described [17]. These membranes were stored at −80 °C before use. In some cases the cells were incubated with pertussis toxin (25 ng/ml) for 16 h before harvest. NG108-15 cells were grown as previously described [18], and membranes were prepared as for the C6 cells. Membranes were incubated in a buffer (20 mM-Tris/HCl, pH 7.5, 20 mM-$MgCl_2$, 1 mM-dithiothreitol, 100 μM-EDTA) for various times at 37 °C in the presence of various nucleotide analogues (100 μM, except where otherwise stated). At the end of the incubation soya-bean trypsin inhibitor (25 μg) was added, and the samples were separated into supernatant and particulate fractions by centrifugation at 20 lb/in^2 (126000 *g*) for 2 min in a TL100 (30°) rotor in an Airfuge (Beckman Instruments). Protein in the supernatant fraction was collected by deoxycholate/trichloroacetic acid (final concns. 0.0125% and 6%, w/v) precipitation before addition of sample buffer and addition to SDS/polyacrylamide (10%) gels. The residual membrane pellet was solubilized with sample buffer and added directly to the gels.

Two-dimensional electrophoresis was performed by modifying the procedure of O'Farrell [19] as described by Backlund *et al.* [15]. Immunoblotting of the gels was as previously described [6,9] with 1:200 dilutions of each of the primary antisera. The immunoblots were quantified by densitometric scanning, with a Bio-Rad gel scanner linked to an Olivetti M24 personal computer, of film positives produced from the blots [20].

RESULTS

Immunoblotting of SDS/polyacrylamide gels of membranes of C6 BU1 cells with either antiserum AS6 or antiserum LE2 identified apparently a single polypeptide of 40 kDa (Fig. 1). These reactivities co-migrated, as immunoblotting with a mixture of the two antisera equally stained only a single band of 40 kDa in these membranes (results not shown). As antisera AS6 and AS7 do not discriminate between the α subunits of G_i1 and G_i2, we then applied the greater resolution of two-dimensional electrophoresis to test if more than one 'G_i-like' protein was present in these membranes. Antiserum AS7 identified a single spot in the region of 40 kDa having a pI close to 5.7 (Fig. 2). Antiserum LE2 also recognized only a single spot of this apparent M_r and with the same pI (results not shown). As a range of pertussis-toxin-sensitive G-proteins can be resolved under these conditions [15], these data indicate the expression of detectable amounts of only a single G_i-like protein in these cells, and that this protein represents G_i2.

Incubation of C6 BU1-cell membranes for 20 min in the presence of GDP[S] (100 μM), followed by their separation into particulate and supernatant fractions and immunoblotting with antiserum AS7, demonstrated that all of the α subunit of G_i2 remained tightly associated with the membrane (Fig. 3). However, when the experiment was performed with p[NH]ppG (100 μM) as

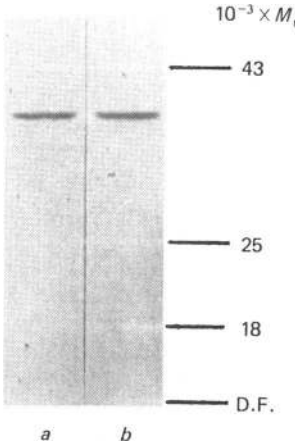

Fig. 1. Immunochemical identification of the 40 kDa G-protein of glioma C6 BU1 cells as the α subunit of G_i2

Membranes of rat glioma C6 BU1 cells (50 μg) were separated on SDS/polyacrylamide gels (10% acrylamide) at 50V. The proteins were transferred to nitrocellulose, which was blocked with 3% gelatin and immunoblotted with 1:200 dilutions of antisera LE2 (*a*) or AS6 (*b*) as described [10]. Both antisera stained a polypeptide of 40 kDa which, on the basis of the specificity of these antisera (see under 'Materials' and [8]), represents the α subunit of G_i2. Abbreviation: D.F., dye front.

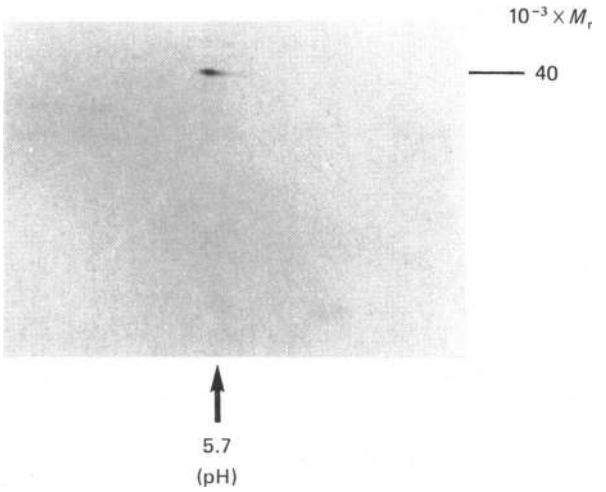

Fig. 2. Characterization of the α subunit of G_i2 by two-dimensional electrophoresis

Membranes of C6 BU1 cells (150 μg) were subjected to two-dimensional electrophoresis as described in [15]. The proteins were then transferred to nitrocellulose and immunoblotted as described in Fig. 1 by using a 1:200 dilution of antiserum AS7 as first antibody. A single spot of 40 kDa, with pI 5.7, was identified by the antiserum.

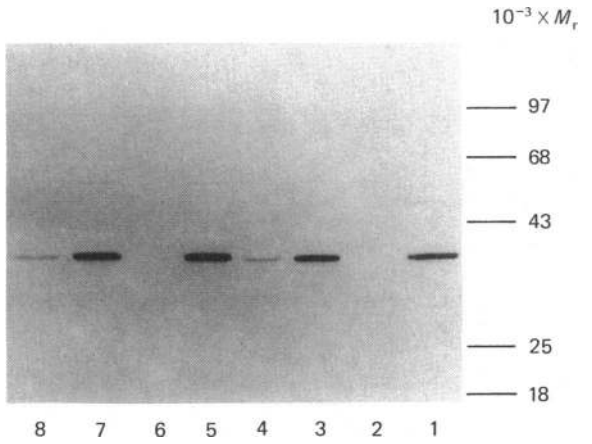

Fig. 3. Release of the α subunit of G_i2 from membranes of C6 BU1 cells by p[NH]ppG

Membranes of untreated (1–4) or pertussis-toxin-pretreated (5–8) C6 BU1 cells (100 μg) were incubated for 20 min as described under 'Methods' with either p[NH]ppG (100 μM) (3, 4, 7, 8) or GDP[S] (100 μM) (1, 2, 5, 6) and then separated into particulate (1, 3, 5, 7) or supernatant fractions (2, 4, 6, 8). All samples were resolved by SDS/polyacrylamide-gel electrophoresis and immunoblotted with a 1:200 dilution of antiserum AS7 as described in Fig. 1.

the nucleotide, a proportion of the α subunit of G_i2 was released from the membrane and was found in the supernatant fraction (Fig. 3). Two other GTP analogues, GTP[S] and p[CH_2]ppG, also caused release of a proportion of the α subunit of G_i2 from the membranes with an order of potency GTP[S] ≥ p[NH]ppG > p[CH_2]ppG (results not shown). In a series of experiments the proportion of the G-protein that was released from

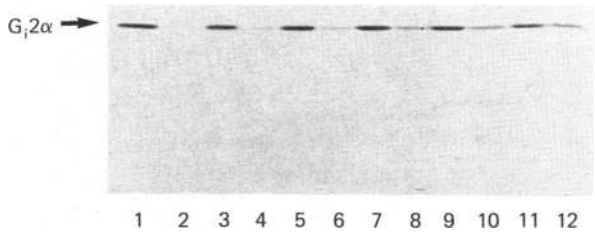

Fig. 4. Time course of the release of the α subunit of G_i2 from membranes of C6 BU1 cells

Membranes of C6 BU1 cells were treated with p[NH]ppG (100 μM) as described under 'Methods' for up to 2 h. Samples were then separated into particulate (1, 3, 5, 7, 9, 11) and supernatant (2, 4, 6, 8, 10, 12) fractions, resolved by SDS/polyacrylamide-gel electrophoresis and immunoblotted with a 1:200 dilution of antiserum AS7. Times of incubation: 1, 2, 0 min; 3, 4, 20 min; 5, 6, 40 min; 7, 8, 60 min; 9, 10, 90 min; 11, 12, 120 min.

the membrane within 20 min varied from 5 to 15% as assessed by densiometric scanning of film positives produced from the immunoblots [19]. In every experiment, however, no detectable $G_i2\alpha$ subunit was released in the absence of the GTP analogue. Equally, replacement of p[NH]ppG with the equivalent ATP analogue p[NH]ppA produced no release of immunoreactive G-protein (results not shown). GTP was able to produce very little release of the α subunit of G_i2 in these experiments, although an increase in release was noted when the experiments were performed with GTP in the presence of both phosphocreatine and creatine kinase to act as a regenerating system (results not shown). The limited effects of GTP, in comparison with those of the poorly hydrolysed analogues of GTP, are likely to be due both to the hydrolysis of GTP by the GTPase activity of the membrane preparation and also because the maintenance of GDP at the nucleotide-binding site after hydrolysis of GTP will produce a deactivated state of the G-protein.

Guanine-nucleotide-mediated release of the α subunit of the pertussis-toxin-sensitive G-protein was observed from membranes of C6 BU1 cells which had been pretreated with pertussis toxin (25 ng/ml) for 16 h (Fig. 3), conditions which have previously been shown to produce complete ADP-ribosylation and hence functional inactivation of this G-protein [16,17]. These results imply that it is not the ability of the ADP-ribosylated α subunit to dissociate from β/γ subunits which compromises the functional role of relevant pertussis-toxin-treated G-proteins. Time courses in which incubation of the membranes with p[NH]ppG were performed for up to 2 h showed that release of the α subunit of G_i2 continued throughout this period (Fig. 4). Even at these longer time points no release was observed in the presence of GDP[S] or p[NH]ppA (results not shown), indicating that this was not simply a reflection of destruction of the membrane structure. In no case, in the presence of either GTP or GDP analogues, was the β subunit detected in the supernatant fractions when immunoblots of membrane and supernatant fractions were analysed with antiserum RV6 (Fig. 5). In all experiments the β subunit remained tightly associated with the membrane.

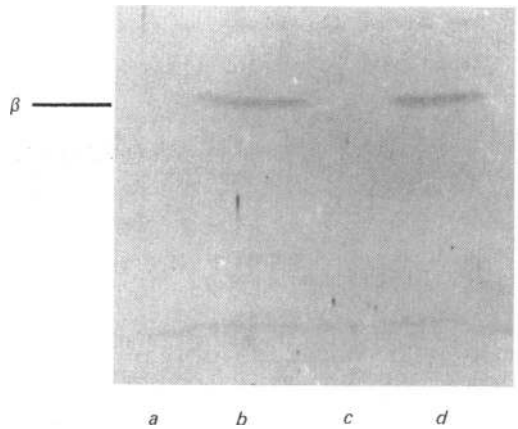

Fig. 5. Anchorage of the β subunit in C6 BU1 membranes after incubation with p[NH]ppG

C6 BU1 membranes (100 μg) were treated with either GDP[S] (100 μM) (lanes *a*, *b*) or p[NH]ppG (100 μM) (lanes *c*, *d*) for 20 min as described under 'Methods'. The samples were then separated into supernatant (*a*, *c*) and particulate (*b*, *d*) fractions and immunoblotted with a 1:100 dilution of antiserum RV6 after separation by SDS/polyacrylamide-gel electrophoresis and transfer to nitrocellulose. In each case, all of the β subunit detected was associated with the membrane fraction.

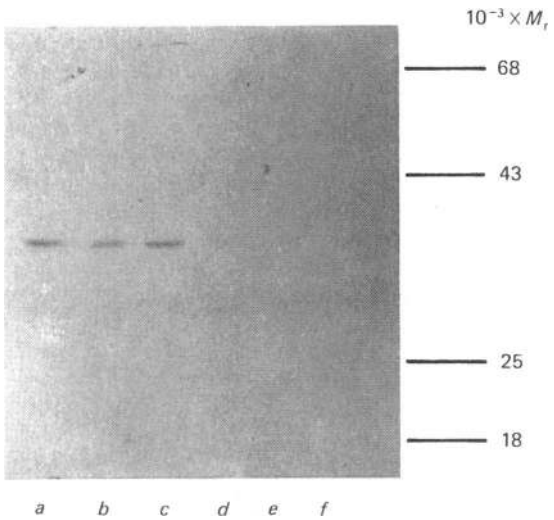

Fig. 6. Concentration-dependence of p[NH]ppG-mediated release of G_i2 from membranes of C6 BU1 cells

Membranes of C6 BU1 cells (100 μg) were incubated with GDP[S] (10 μM) and different concentrations of p[NH]ppG: (*a*) 100 μM, (*b*) 10 μM, (*c*) 1 μM, (*d*) 100 nM, (*e*) 10 nM, (*f*) 0. The samples were then treated as in Fig. 3. Only samples corresponding to the supernatant fractions are displayed.

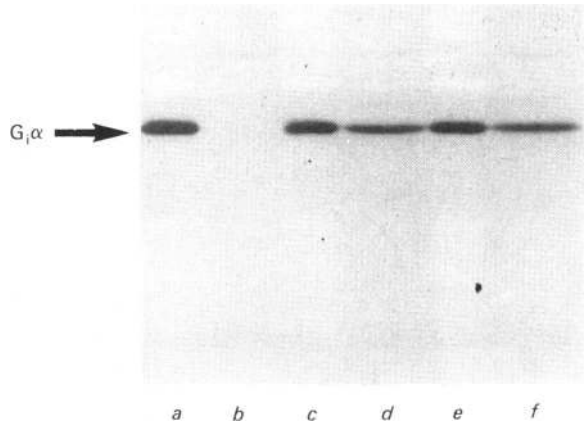

Fig. 7. Nucleotide specificity of the release of the α subunit of 'G_i' from membranes of neuroblastoma × glioma hybrid cells

Membranes of NG108-15 cells (100 μg) were incubated for 60 min with GDP[S] (*a*, *b*), GTP[S], (*c*, *d*) or p[NH]ppG (*e*, *f*). Each nucleotide was present at 100 μM. At the termination of incubation, samples were treated as in Fig. 3 and immunoblotted with an antiserum against the *C*-terminal decapeptide of 'G_i-like' proteins. Lanes *a*, *c* and *e* represent the membrane fractions and lanes *b*, *d* and *f* the supernatant fractions.

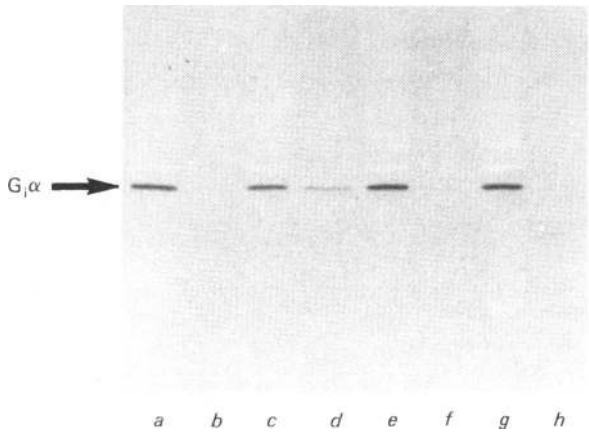

Fig. 8. Nucleotide specificity of the release of the α subunit of 'G_i' from membranes of human platelets

Membranes of human platelets (100 μg) were incubated for 60 min with p[NH]ppA (*a*, *b*), p[NH]ppG (*c*, *d*), GDP[S] (*e*, *f*) or with water (*g*, *h*). The concentration of each nucleotide was 100 μM. Samples were treated as described in Fig. 7. Lanes *a*, *c*, *e*, *g* represent the residual pellets. Lanes *b*, *d*, *f*, *h* are the equivalent supernatant fractions.

In the presence of 10 μM-GDP[S], increasing concentrations of p[NH]ppG released the α subunit of G_i2 from membranes of C6 BU1 cells when assays were carried out for 20 min. Release could be detected with 100 nM-p[NH]ppG and was maximal in the presence of 10 μM nucleotide (Fig. 6).

Release of forms of G_i from membranes was not restricted to the C6 BU1 cells. Release of the α subunit was noted with exactly the same profile of nucleotide specificity in membranes derived from cells of the neuroblastoma × glioma hybrid cell line NG108-15, where both p[NH]ppG and GTP[S] but not GDP[S] promoted release of some 50% of the 'G_i-like' immunoreactivity from the membrane in a 60 min incubation (Fig. 7). Furthermore, a similar release, promoted by p[NH]ppG of the α subunit of a form of G_i was noted

from membranes of human platelets, where some 15% of the total membrane complement was released during a 60 min incubation (Fig. 8).

DISCUSSION

A range of guanine nucleotide-binding proteins have been identified [1–5]. These proteins transduce information from the interaction of agonists with the binding site of receptors on the extracellular face of the plasma membrane. They do so by modulating effector systems at the cytoplasmic aspect of the plasma membrane which control the concentrations of intracellular second messengers.

Studies on the hydrodynamic characteristics of G-proteins have indicated that, at least in solution in the presence of detergents, activation and the binding of poorly hydrolysed analogues of GTP leads to dissociation of the heterotrimeric structure of the G-protein into separate $\alpha + \beta/\gamma$ subunits [21]. It has not previously been possible to ascertain whether such a physical separation of the individual subunits occurs in the native membrane, although it is widely assumed that this must indeed take place. As the α subunits of G-proteins appear to be relatively hydrophilic entities, it has been proposed that the more hydrophobic β/γ subunits might serve to anchor the α subunits to the membrane [22], and it has been further suggested that the α subunits might be released into the cytoplasm upon activation of the G-protein, to act as 'programmable messengers' [11]. Relatively little evidence has been presented in support of such a proposal, although it has been noted that a proportion of the α subunit of G_s can be released from rat liver plasma membranes after treatment with cholera toxin [23]. For such a hypothesis to be tenable, it is necessary that dissociation of the holomeric G-protein can occur within the milieu of the plasma membrane. We have previously noted that there is no significant guanine-nucleotide-mediated release of pertussis-toxin-sensitive G-proteins from either bovine brain membranes (G_i1 and G_o) or from human neutrophils (G_i2) [12] over short time periods. However, in the present experiments we demonstrate that in membranes of C6 BU1 cells, in neuroblastoma × glioma hybrid NG108-15 cells and in membranes of human platelets a proportion of the cellular complement of the α, but not the β, subunit of a pertussis-toxin-sensitive G-protein is released when the membranes are incubated with analogues of GTP, but not GDP or ATP. In each of the tissues studied here, the release process is relatively slow, but it does continue throughout the time periods studied.

In these studies we have detected the presence of the G-protein subunits by using specific polyclonal [6] and anti-peptide antisera [8–10]. This has obviated the previous requirement to tag covalently the G-protein α subunits by using either pertussis- or cholera-toxin-catalysed ADP-ribosylation [23]. Using this immunological approach, we have also been able to follow the distribution of the β subunit (Fig. 5), which would not have been possible by utilizing bacterial toxin-catalysed ADP-ribosylation, as only the α-subunits of G-proteins are substrates for these toxins.

It appears that the attachment of the α subunit of G-proteins to the plasma membranes of cells is crucially dependent on the integrity of the *N*-terminal region of the polypeptide. Tryptic cleavage of an approx. 2 kDa fragment from the *N*-terminus releases the α subunit from the plasma membrane (see, e.g., [12]). This may reflect either that the *N*-terminus is directly attached to the plasma membrane, perhaps via an *N*-terminal myristoylation [24], or that the *N*-terminus is of direct importance in the interaction of the α and β subunits [25]. The fact that the released form of the α subunit in these experiments migrated identically with the remaining membrane-bound form indicates that release was not due to the proteolytic removal of a peptide from the *N*-terminus of the α-subunit polypeptide [12]. It equally could not represent removal of a *C*-terminal peptide, as we have used antisera which recognize the *C*-terminus of the protein in the immunoblotting experiments. One possibility is that release is dependent on the turnover of myristic acid, which appears to be associated with the *N*-terminus of at least some G-proteins [24]. A potentially related phenomenon has been noted in relation to the interaction of $p21^{n\text{-}ras}$ with membranes, which appears to be dependent on the integrity of attached palmitic acid [26]. We wish to address this question, but, as the antisera used here are poor in immunoprecipitation assays, this is not a trivial problem.

ADP-ribosylation of pertussis-toxin-sensitive G-proteins prevents receptor-mediated modulation of second-messenger-effector systems. It is often assumed that, as pertussis-toxin-catalysed ADP-ribosylation is decreased in the presence of poorly hydrolysed analogues of GTP, then pertussis toxin has a preference for the holomeric G-proteins as substrate. A further conclusion often drawn from this observation is that the α-subunit with attached ADP-ribose is unable to dissociate from the β/γ subunits. Here, using the release assay, we demonstrate that this assumption is incorrect and that the covalently modified G-protein α subunit can indeed dissociate upon binding of a GTP analogue (Fig. 3). It is much more likely, based on the observations that the receptor-recognition site of G-proteins appears to be located at the extreme *C*-terminus of the α subunit [18,27] and that the site of pertussis-toxin-catalysed ADP-ribosylation is also at the extreme *C*-terminus of this polypeptide, that the lack of agonist modulation of second-messenger generation after treatment with pertussis toxin is because the receptor and G-protein cannot interact in a functional manner to promote the dissociation of the G-protein.

The molecular identity of the 'G_i-like' protein of C6 BU1 cells has not until now been unequivocally demonstrated. cDNA clones corresponding to a 'G_i-like' protein and to G_o have previously been identified from another clone of the C6 cell line [5]. In the present study we have employed *C*-terminal anti-peptide antisera (AS6, AS7) which recognize all 'G_i-like' proteins, and an anti-peptide antiserum (LE2) raised against amino acids 160–169 of the 'G_i-like' protein cloned from C6 cells (now called G_i2), to study the guanine-nucleotide-mediated release of G_i2 from membranes of these cells. All of the anti-peptide antisera used in this study, which recognize the α subunits of either all 'G_i-like' proteins (AS6, AS7) or only G_i2 (LE2), recognized only a single polypeptide in both one- and two-dimensional gels. As the different 'G_i-like' G-proteins can be resolved by two-dimensional electrophoresis [15], this argues strongly that only a single 'G_i-like' protein is expressed in detectable quantities in this cell line. Thus antiserum AS7, which has the highest titre of the antisera currently available to us, was utilized in most of the experiments,

and in this cell line can be used to detect G_i2. The particular clone of C6 cells used in this study expresses very low amounts of G_0 [28], and thus it has not been possible to study the effect of guanine nucleotides on the distribution of this G-protein in this cell line. We have, however, recently performed studies similar to those described herein to demonstrate release of the α subunit of G_0 from membranes of NG108-15 cells [29]. The release of $G_0\alpha$ was also entirely specific for analogues of GTP, and release was detectable within 5 min.

These experiments can offer no information on a prospective physiological role (if any) of the released α subunit of G_i2, but demonstrate that such a process can indeed occur. The time courses of the experiments presented here would, however, suggest that the release of the G-protein from the plasma membrane is not related to short-term second-messenger-mediated functions.

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