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GTP ANALOGUES CAUSE RELEASE OF THE ALPHA SUBUNIT OF THE GTP BINDING PROTEIN, G_O, FROM THE PLASMA MEMBRANE OF NG108-15 CELLS

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Incubation of membranes of neuroblastoma x glioma hybrid, NG108-15 cells with GDPβS followed by immunoblotting of resolved membrane and supernatant fractions with specific anti-peptide antisera showed essentially all of the α subunit of G_o to be associated with the membrane. Similar experiments with poorly hydrolyzed analogues of GTP caused release of a significant fraction (some 50% within 60 minutes) of $G_o\alpha$ into the supernatant. This was not mimicked by analogues of ATP. Antisera directed against peptides corresponding to the extreme N and C-termini of $G_o\alpha$ demonstrated that the released polypeptide was not proteolytically clipped. These experiments show that the α subunit of G_o need not be invariably bound to the plasma membrane and that guanine nucleotide

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Abbreviations

G-protein; guanine nucleotide binding protein. G_o; a G-protein of unknown function which is particularly abundant in brain. Gpp[NH]p; guanyl-5'-yl imidodiphosphate. GTPγS; guanosine-5'-O-(3-thiotriphosphate). GMP-PCP; guanylyl(β,γ-methylene)-diphosphonate. GDPβS ; guanosine-5'-O-(2-thiophosphate). SDS-PAGE; sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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activation can release the alpha subunit of G_o from its site of membrane attachment.

A considerable number of heterotrimeric signal transducing G-proteins have recently been identified [1,2]. In each case they appear to be associated with the cytoplasmic aspect of the plasma membrane and function to link cell-surface receptors to second messenger-generation systems. Despite suggestions to the contrary [3], it is generally assumed that the individual subunits of the G-proteins remain in intimate contact with the membrane throughout their cycle of activation and deactivation which is promoted by the binding of GTP and terminated by its subsequent hydrolysis. Here we use a range of anti-peptide antisera directed against different epitopes of the α subunit of G_o to demonstrate that a proportion of the membrane complement of this polypeptide can be released from the membrane, in a time dependent manner, upon activation of this G-protein.

Materials and Methods.

Neuroblastoma x glioma hybrid, NG108-15 cells were grown in tissue culture in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) foetal bovine serum essentially as previously described [4]. The cells were differentiated by changing the medium to one containing 2% foetal bovine serum and 1mM dibutyryl cAMP six days before cell harvest. Membranes were prepared [4] and stored at -70^0 until use. In release studies, membranes were incubated in a buffer (20 mM Tris/HCl, pH 7.5, 20mM $MgCl_2$, 1mM Dithiothreitol, 100uM EDTA) for varying times at 37^o in the presence of various nucleotide analogues (all purchased from Boehringer-Mannheim) (100uM unless otherwise indicated). At termination of incubation, soybean trypsin inhibitor (25ug) was added as a soluble carrier protein and the samples separated into supernatant and particulate fractions by centrifugation at 20 p.s.i. (approx.126,000 x g) for 2 minutes using a TL 100 (30^0) rotor in an airfuge (Beckman Instruments). Protein in the supernatant fraction was collected by deoxycholate/trichloroethanoic acid precipitation prior to addition of sample buffer and addition to SDS-PAGE (10% (w/v) acrylamide). The residual membrane pellet was solubilized with sample buffer and added directly to the gels. Immunoblotting of the resolved proteins was performed as previously described [5,6]. The antisera used in these studies were raised in New Zealand white rabbits following immunization with synthetic peptides

corresponding to parts of the sequence of $G_o\alpha$ which had been coupled to keyhole limpet haemocyanin. These were antiserum IM1, corresponding to amino acids 22-35 of $G_o\alpha$, antiserum ON1, corresponding to amino acids 1-16 and antiserum OC1, corresponding to amino acids 345-354. Each antiserum was used in these experiments at a 1:200 dilution. In some cases quantitation of the immunoblots was achieved by densitometric scanning of film positives produced from the blots using a Bio-Rad Gel Scanner linked to an Olivetti M24 personal computer [6].

Results

Immunoblotting of resolved membrane and supernatant fractions was performed, following incubation of membranes of NG108-15 cells for 60 minutes with a range of nucleotide analogues, using an antipeptide antiserum (OC1) generated against the C-terminal decapeptide of the α subunit of G_o. In the presence of GDPβS (100uM), essentially all of the α subunit of G_o remained in intimate association with the membrane (Fig. 1 lanes I,J). In contrast however, incubation of the membranes with Gpp[NH]p

Figure 1.
Nucleotide specificity in the release of the α subunit of G_o from membranes of neuroblastoma x glioma hybrid cells.
Membranes (75ug) of NG108-15 cells were incubated with various nucleotide analogues (100uM) (lanes A,B = App[NH]p, lanes C,D = Gpp[NH]p, lanes E,F = GTP in the presence of creatine phosphate (25mM) and creatine phosphokinase (15 units/assay), lanes G,H = GTP, lanes I,J = GDPβS, lanes K,L = water) for 60 minutes as described in Methods. The samples were separated into membrane and supernatant fractions, resolved on SDS-PAGE and immunoblotted with antiserum OC1 (C-terminal decapeptide of $G_o\alpha$) as first antibody. Lanes A,C,E,G,I and K are the membrane fractions, lanes B,D,F,H,J and L represent the supernatant fractions.

(100uM) led to the release of some 50% of the α subunit of G_o from the membrane into the supernatant (Fig 1 lanes C,D). The equivalent analogue of ATP, App[NH]p, did not mimic the effect of the GTP analogue (Fig.1 lanes A,B) but other analogues of GTP, including GTPγS and GMP-PCP did (data not shown). These results demonstrate that this process was specific both for guanine nucleotides and for the triphosphate form. In contrast however, GTP did not produce substantial release, presumably due to hydrolysis of the triphosphate to GDP during the assay (Fig. 1, lanes G,H). However, release by GTP could be potentiated to some extent by the inclusion of a regeneration system (Fig. 1 lanes E,F).

The mobility of the released α subunit of G_o in SDS-PAGE appeared to be identical to that of the remaining membrane-associated form. However, to confirm that release of the α subunit of G_o from the membranes in response to Gpp[NH]p was not due to a proteolytic clip which removed the N-terminal region, equivalent experiments to those shown in Fig. 1 were performed by immunoblotting the resolved membrane and supernatant fractions with either the anti C-terminal region antiserum or with a second antipeptide antiserum (ON1) raised against the N-terminal 16 amino acids of the alpha subunit of G_o. This epitope is within the region which is selectively removed by trypsin treatment of GTP-liganded G-protein α subunits [17]. Using this antiserum, equivalent results were obtained with the different nucleotide analogues (Fig. 2 and data not shown). Similarly, a third antiserum (IM1) which we have previously characterized [17] and which is directed against a peptide within the internal sequence of $G_o\alpha$, produced the same pattern (data not shown).

Time courses of the release of the α subunit in response to Gpp[NH]p demonstrated measurable release of $G_o\alpha$ from the membranes within 5 minutes and that this process continued in an essentially linear manner up to at least 60 minutes. However, even following a 60 minute incubation

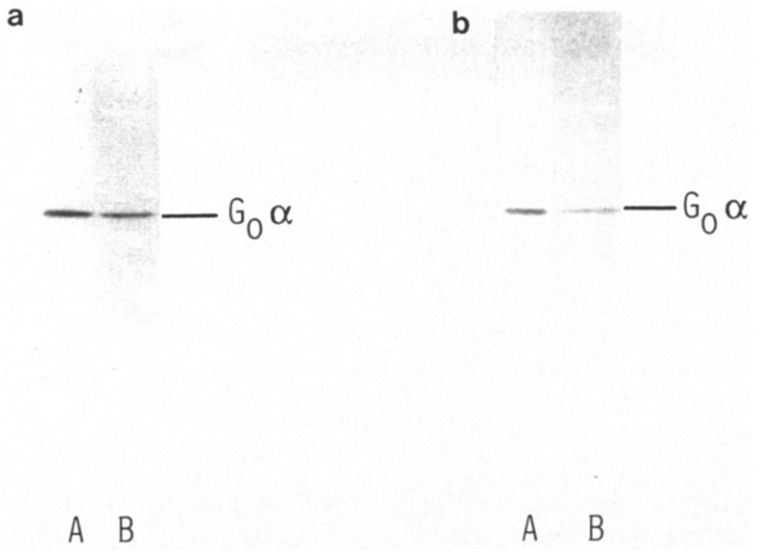

Figure 2.
Both membrane bound and soluble forms of $G_0\alpha$ are identified by antisera directed against either the N or C-termini.
Membranes (100ug) of NG108-15 cells were incubated with Gpp[NH]p (100uM) for 60 minutes and then separated into membrane associated (lane A) and supernatant (lane B) fractions, resolved on SDS-PAGE and immunoblotted with either ON1 (Fig. 2a) or OC1 (Fig. 2b) as first antibody.

with Gpp[NH]p, some 50% of the $G_0\alpha$ remained in association with the membrane fraction (Fig. 3).

In the presence of GDPβS (100uM), increasing concentrations of Gpp[NH]p caused release of increasing amounts of $G_0\alpha$. Under these conditions,

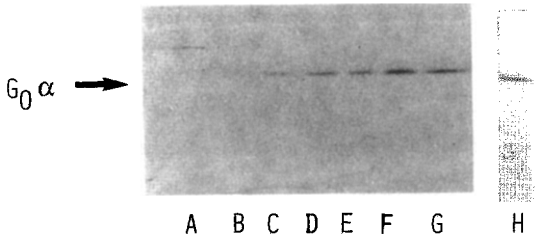

Figure 3.
Time course of Gpp[NH]p mediated release of $G_0\alpha$ from membranes of NG108-15 cells.
Membranes (75ug) of NG108-15 cells were incubated with Gpp[NH]p (100uM) for varying times (0-60 minutes) and then separated into membrane and supernatant fractions which were resolved on SDS-PAGE. The gel was then immunoblotted with antiserum OC1. Lane A, supernatant after incubation for 60 minutes with GDPβS (100uM), lanes B-G, supernatants after incubation with Gpp[NH]p (100uM) for 0, 5, 10, 20, 40, 60 minutes, lane H, immunoreactivity remaining associated with the membrane pellet following 60 minutes incubation with Gpp[NH]p (100uM).

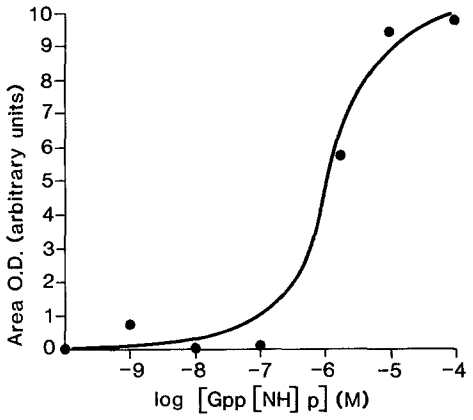

Figure 4
Concentration dependence of Gpp[NHp] mediated release of $G_o\alpha$ from membranes of NG 108-15 cells.
Membranes (75ug) of NG108-15 cells were incubated with GDPβS (100uM) and varying concentrations of Gpp[NH]p (0-100uM) for 60 minutes. The supernatants were collected and resolved as in Fig. 1 and immunoblotted with antiserum OC1. A film positive of the blot was produced. This was scanned on a densitometer as described in Methods to construct the figure in which the areas under the densitometer peak is plotted against Gpp[NH]p concentration.

release was half maximal at 1uM and maximal in the presence of 100uM Gpp[NH]p (Fig. 4).

Discussion

It has recently been demonstrated that a considerable number of highly homologous pertussis toxin-sensitive G-proteins can be expressed [2,7]. As these all have very similar apparent Mr on SDS-PAGE, then it is essentially impossible to unambiguously identify the individual forms by analysis of pertussis toxin-catalysed ADP-ribosylation of membrane preparations followed by SDS-PAGE and autoradiography. To counter this problem a number of workers, including ourselves, have generated antipeptide antisera which are capable of recognizing individual forms of the pertussis toxin-sensitive G-proteins [8-10]. In this report we use three such antisera directed against different regions of the α subunit of G_o. G_o is a widely distributed protein [11] which is particularly abundant in neural tissue [12], and whilst it has been suggested to couple δ opioid

receptors to the modulation of Ca^{2+} channel function [13], its role remains a matter of debate. The neuroblastoma x glioma hybrid cell line, NG108-15, was selected for these studies as it has previously been shown to express G_o [14].

The suggestion that the α subunit of G-proteins might be released from the plasma membrane of cells upon activation was originally proposed by Rodbell [3] to account for the observation that transfer of G_s appeared to occur by the simple mixing of membranes from erythrocytes and from cyc^- S49 lymphoma cells. Furthermore, in the case of G_s, it has been noted that treatment with cholera toxin causes release of a proportion of $G_s\alpha$ from the membrane [15]. One technical difficulty with attempts to assess release of pertussis toxin-sensitive G-proteins from the membrane is that the separated α subunits are not substrates for pertussis toxin-catalysed ADP-ribosylation in the absence of β/γ subunits [16]. Thus, it is only now, with the availability of selective antisera, that this question can be adequately addressed.

Here we demonstrate that the α subunit of G_o can indeed be released from membranes of NG108-15 cells. This process appears to be a specific response to activation of the G-protein as it is promoted by analogues of GTP but not by analogues of either GDP or of ATP. Furthermore, the released α subunit migrated with identical mobility as the membrane-bound form in SDS-PAGE and anti-peptide antisera directed against both the N and C-termini of the polypeptide detected the soluble form. These observations argue strongly that there is no requirement for a proteolytic cleavage of the G-protein coincident with the release process, although tryptic hydrolysis of GTP-liganded forms of the α subunits of G-proteins can indeed occur, to produce a clipped form which is some 2kDa smaller [17].

The nature of the interaction of the α subunits of the pertussis toxin-sensitive G-proteins with the plasma membrane remains an unresolved issue. Tryptic cleavage of a 2kDa N-terminal peptide causes the release of the α subunit of both G_o and of G_i [17], but it is unclear whether this is a reflection of the subsequent inability of the remaining polypeptide to interact with β/γ subunits or whether it is due to the removal of N-terminally-linked myristic acid [18].

It is not possible at this juncture to suggest whether there is a potential physiological function for the released α subunit or even to define the mechanism by which binding of the GTP analogues causes release from the membrane. However, the specificity of the release process noted here suggests that the function of G-proteins may not be limited to interactions at the level of the plasma membrane.

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