Hormonal regulation of G_i2 α -subunit phosphorylation in intact hepatocytes

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Hepatocytes contain the G_i2 and G_i3 forms of the 'G_i-family' of guanine-nucleotide-binding proteins (G-proteins), but not G_i1. The anti-peptide antisera AS7 and I3B were shown to immunoprecipitate G_i2 and G_i3 selectively, and the antiserum CS1 immunoprecipitated the stimulatory G-protein G_s. Treatment of intact, ³²P-labelled hepatocytes with one of glucagon, TH-glucagon ([1-*N*- α -trinitrophenylhistidine, 12-homoarginine]glucagon), Arg-vasopressin, angiotensin-II, the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate) and 8-bromo-cyclic AMP elicited a time- and dose-dependent increase in the labelling of the α -subunit of immunoprecipitated G_i2 which paralleled the loss of ability of low concentrations of the non-hydrolysable GTP analogue guanosine 5'-[β -imido]triphosphate (p[NH]ppG) to inhibit forskolin-stimulated adenylate cyclase activity ('G_i'-function). The immunoprecipitation of phosphorylated G_i2- α -subunit by the antiserum AS7 was blocked in a dose-dependent fashion by the inclusion of the C-terminal decapeptide of transducin, but not that of G_z (a 'G_i-like' G-protein which lacks the C-terminal cysteine group which is ADP-ribosylated by pertussis toxin in other members of the G_i family), in the immunoprecipitation assay. No labelling of the α -subunits of either G_i3 or G_s was observed. α -G_i2 was labelled in the basal state and this did not change over 15 min in the absence of ligand addition. In contrast to the monophasic dose-effect curves seen with vasopressin, angiotensin and TPA, the dose-effect curve for the glucagon-mediated increase in the labelling of α -G_i2 was markedly biphasic where the loss of G_i function paralleled the high-affinity component of the labelling of α -G_i2 caused by glucagon. TPA, TH-glucagon, angiotensin-II and vasopressin achieved similar maximal increases in the labelling of α -G_i2, which was approximately half that found after treatment of hepatocytes with either high glucagon concentrations (1 μ M) or 8-bromo-cyclic AMP. Analysis of the phosphoamino acid content of immunoprecipitated α -G_i2 showed the presence of phosphoserine only. Incubation of hepatocyte membranes with [γ -³²P]ATP and purified protein kinase C, but not protein kinase A, led to the incorporation of label into immunoprecipitated α -G_i2. This labelling was abolished if membranes were obtained from cells which had received prior treatment with ligands shown to cause the phosphorylation of α -G_i2 in intact cells. We suggest that there are two possible sites for the phosphorylation of α -G_i2; one for C-kinase and the other for an unidentified kinase whose action is triggered by A-kinase activation.

INTRODUCTION

Guanine-nucleotide-binding proteins (G-proteins) are involved in the coupling of numerous hormone and neurotransmitter receptors to their specific effector systems [1]. The receptor-mediated stimulation and inhibition of adenylate cyclase are mediated by two distinct G-proteins, termed G_s and G_i, respectively. These G-proteins are heterotrimers consisting of α , β and γ subunits. The $\beta\gamma$ complexes appear to be the same for G_s and G_i, whereas the α -subunits are unique and can be distinguished by the ability of specific bacterial toxins to catalyse their NAD⁺-dependent ADP-ribosylation. Thus cholera toxin catalyses the ADP-ribosylation and activation of G_s whereas pertussis toxin ADP-ribosylates and inactivates G_i [1].

Molecular cloning studies, and subsequent biochemical and immunological analyses, have shown that a family of 'G_i-like' proteins exists [1–3]. This comprises three different, but very similar, pertussis-toxin-sensitive G-proteins termed G_i1, G_i2 and G_i3 [3]. In addition to mediating inhibition of adenylate cyclase, there is evidence that members of this family are involved in the

regulation of a variety of signalling systems, including those which involve phosphoinositidase C, phospholipase A₂ and K⁺ channels [4–7]. However, it is not clearly understood which of these G_i-like proteins regulate which specific effector systems.

The phosphorylation of the α -subunit of a solubilized purified G_i preparation by purified protein kinase C (C-kinase) was first demonstrated by Jakobs and co-workers [8]. More recently, both the purified human insulin receptor tyrosyl kinase [9,10] and cyclic AMP-dependent protein kinase (A-kinase) [11] have been shown to phosphorylate the α -subunit of purified solubilized G_i preparations. In contrast with these experiments done with purified solubilized preparations we have, in a preliminary study, provided evidence that the α -subunit of G_i can be phosphorylated in intact hepatocytes treated with either 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or glucagon, but not with insulin [12].

The phosphorylation of G_i was suggested, by Jakobs and co-workers [13], to lead to inactivation of G_i, as α_2 -adrenergic inhibition of platelet adenylate cyclase was blocked by treatment with TPA, a phorbol ester which activates protein kinase C.

Abbreviations used: G-protein, guanine-nucleotide-binding protein; G_i, inhibitory G-protein controlling adenylate cyclase activity; G_s, stimulatory G-protein controlling adenylate cyclase activity; G_z, a 'G_i-like' G-protein which lacks the C-terminal cysteine group which is ADP-ribosylated by pertussis toxin in other members of the G_i-family; EC₅₀, the concentration of ligand at which half maximal effects were observed; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TH-glucagon, [1-*N*- α -trinitrophenylhistidine, 12-homoarginine]glucagon; p[NH]ppG, guanosine 5'-[β -imido]-triphosphate.

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However, C-kinase has been shown to cause the phosphorylation and inactivation of a number of receptors, including various adrenoceptors [14]. It is thus unclear as to whether TPA was exerting its inhibitory effect on α_2 -adrenoceptor function by receptor phosphorylation or by a presumed action on G_i . However, we have shown [12,15] that the ability of low concentrations of the non-hydrolysable GTP analogue guanosine 5'-[β -imido]triphosphate (p[NH]ppG) to inhibit forskolin-stimulated adenylate cyclase activity, an index of G_i function which is independent of receptor activation, was abolished in membranes obtained from hepatocytes which had been pretreated with either TPA or glucagon. These results indicate that G-protein phosphorylation may be a general mechanism of 'cross-talk' between different signalling systems, perhaps providing a basis for heterologous desensitization and/or synergism.

In this study we have determined which form of G_i is phosphorylated in intact hepatocytes, and have evaluated the time- and dose-dependence of action of ligands functioning through C- and A-kinase systems in causing alterations in G_i functioning and phosphorylation. We also show that such ligands do not elicit the phosphorylation of α - G_s .

MATERIALS AND METHODS

[32 P]P_i was obtained from Amersham International, [32 P]NAD was from New England Nuclear and pertussis toxin was from PHLS Centre for Applied Microbiology, Porton Down, Wilts., U.K. TPA was from Cambridge Bioscience, Cambridge, U.K. TH-glucagon ([1-*N*- α -trinitrophenylhistidine,12-homo-arginine]glucagon) was prepared as described before [16]. Hormones and Protein A-agarose were from Sigma. Synthetic peptides for the production of anti-peptide antisera and for the immunocompetition experiments were purchased from BioMac, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K. All other biochemicals were from Boehringer, and other chemicals were of AR grade from BDH. Purified protein kinase C (α , β and γ isoforms) was prepared as described before [17] and the catalytic subunit of protein kinase A was purified to apparent homogeneity from bovine heart [18].

Hepatocyte preparation and harvesting

Hepatocytes were prepared according to [19] using livers from 225–250 g fed male Sprague–Dawley rats as previously described [20]. For phosphorylation studies, cells (10^6 cells/ml) were pre-incubated for 50 min at 37 °C in Krebs–Henseleit (50 μ M-potassium phosphate/0.2–0.5 mCi of [32 P]P_i) buffer supplemented with 2.5% BSA, 2.5 mM-CaCl₂ and 15 mM-glucose. Cells were gassed with O₂/CO₂ (19:1) for 30 s every 15 min. The ATP content of the isolated hepatocytes was determined by the luciferase method using a neutralized HClO₄ extract [20].

Ligands were added in less than 1% of the total incubation volume and, after an appropriate time, the reactions were stopped by the addition of 4 vol. of ice-cold Krebs–Henseleit buffer. The cells were harvested by centrifugation (1000 g, 3 min) and washed once in cold buffer prior to immunoprecipitation of G_i . For adenylate cyclase assays, cells were incubated in normal Krebs–Henseleit and the incubations were quenched by the addition of an equal volume of ice-cold 1 mM-KHCO₃, pH 7.2, and kept on ice until processed further as described below in order to provide a membrane fraction for the assays.

Preparation of hepatocyte membranes

A washed membrane fraction was obtained from the hepatocytes as described previously [21], and membranes were used within 2 h of preparation.

Assay of adenylate cyclase

Adenylate cyclase was assayed as described previously [20,22]. Incubations contained 1.5 mM-ATP, 5 mM-MgSO₄, 10 mM-theophylline, 1 mM-EDTA, 7.5 mg of phosphocreatine/ml, 1 mg of creatine kinase/ml and 25 mM-triethanolamine/KOH, pH 7.4. The cyclic AMP produced was determined in a binding assay using the regulatory subunit of A-kinase [20,23]. Assays were linear under all conditions, and initial rates were analysed.

Assays for G_i function

G_i function was assessed by determining the ability of low concentrations of p[NH]ppG (1 nM) to inhibit adenylate cyclase activity in the presence of the diterpene forskolin (100 μ M) as previously described [15,24,25].

Labelling of α - G_i and α - G_s

Pertussis-toxin- and cholera-toxin-catalysed ADP-ribosylation of isolated hepatocyte membranes was performed as described previously using [32 P]NAD⁺ and thiol-preactivated toxins [24–27]. Reactions (30 °C, 90 min) were terminated by the addition of ice-cold 10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA, and labelled membranes were collected by centrifugation (14000 g, 10 min, 4 °C).

Immunoprecipitation of G_i and G_s

Cells (from phosphorylation incubations) or membranes (from toxin-labelling incubations) were extracted by the addition of 1 ml of a buffer containing 1% Triton X-100, 0.1% SDS, 10 mM-EDTA, 100 mM-NaH₂PO₄, 10 mM-NaF, 100 μ M-Na₃VO₄, 10 mM- β -glycerophosphate, 1 mM-phosphoserine, 1 mM-phosphothreonine, 2 mM-phenylmethanesulphonyl fluoride 10 μ g of leupeptin/ml, 10 μ g of aprotinin/ml and 50 mM-Hepes, pH 7.2. After 1 h at 4 °C, non-solubilized material was removed by centrifugation (14000 g, 10 min, 4 °C).

Labelled α - G_i 2 was immunoprecipitated using AS7 antiserum and α - G_s 3 was immunoprecipitated using I3B antiserum. α - G_s was immunoprecipitated using CS1 antiserum. The production, characterization and use of these antisera have been described previously [25,28]. AS7 was raised in rabbits against the C-terminal decapeptide of the α -subunit of transducin and recognizes both α - G_i 1 and α - G_i 2, in addition to transducin. However, since hepatocytes express neither transducin nor α - G_i 1 (S. Griffiths & M. D. Houslay, unpublished work), AS7 can be used as a specific reagent for the immunoprecipitation of α - G_i 2 in rat hepatocytes. The I3B antiserum was raised in rabbits against the C-terminal decapeptide of α - G_i 3, and recognizes α - G_i 3 with slight cross-reactivity with α - G_o [28,29]. Hepatocytes, however, do not express G_o ([28]; M.D. Houslay & G. Milligan, unpublished work) and thus we have used I3B to specifically immunoprecipitate G_i 3. The antiserum CS1 was raised in rabbits against the C-terminal decapeptide of α - G_s and recognizes both the 45 kDa and 52 kDa forms of α - G_s [28].

A 10 μ l portion of antiserum was added to 1 ml of cell extract and samples were incubated for 12 h at 4 °C. After this period, 50 μ l of protein A-agarose (25 μ l of packed gel) was added and the incubation was continued for a further 2 h. The immune complex was collected as Protein A-agarose pellets by centrifugation (14000 g, 2 min, 4 °C) and the pellets were washed three times in a buffer containing 1% Triton X-100, 0.1% SDS, 100 mM-NaCl, 100 mM-NaF, 50 mM-NaH₂PO₄ and 50 mM-Hepes, pH 7.2, by repeated centrifugation as above.

In experiments where synthetic peptides were tested for their ability to displace α - G_i 2 from the anti-peptide antibodies in AS7, then peptide addition (10 μ l) was made to the cell extract immediately before the addition of the antiserum AS7. The peptides used represented the C-terminal decapeptide of trans-

ducin (KENLKDCGLF) and the C-terminal peptide of the G-protein G_z (a 'G_i-like' G-protein which lacks the C-terminal cysteine group which is ADP-ribosylated by pertussis toxin in other members of the G_i-family) (QNNLKYGILC). The bold typeface indicates the four amino acids in the decapeptide of G_z which are distinct from those of transducin. The C-terminal decapeptide domain of α -G_i2 only differs from that of transducin by a single amino acid (KNNLKDCGLF). The transducin peptide was dissolved and added to the assay in water, whereas the G_z peptide was added in 1% glacial acetic acid; this did not affect the final pH of the incubation.

Phosphorylation of α -G_i2 in hepatocyte membranes using purified kinases

In order to examine the phosphorylation of α -G_i2 by purified A-kinase, hepatocyte membranes (100 μ g of protein) were incubated (30 min, 30 °C) with the catalytic unit of A-kinase (1 unit/ml), 10 mM-MgCl₂, 100 μ g of aprotinin/ml, 100 μ g of leupeptin/ml, 10 μ M-ATP, 5×10^6 c.p.m. of [γ -³²P]ATP and 50 mM-Tris/HCl, pH 7.4. In some instances, the heat-stable inhibitor of A-kinase (Walsh inhibitor, 20 units/ml) was included in the incubation. Incubations with purified C-kinase contained 15 units of C-kinase/ml, 10 mM-MgCl₂, 100 μ g of aprotinin/ml, 100 μ g of leupeptin/ml, 10 μ M-ATP, 5×10^6 c.p.m. of [γ -³²P]ATP, 0.5 mg of phosphatidylserine/ml, 1.5 mM-CaCl₂, 10 ng of TPA/ml and 50 mM-Hepes, pH 7. In some instances the phosphatidylserine, CaCl₂ and TPA were replaced by 1 mM-EGTA.

SDS/PAGE, autoradiography and phosphoamino acid analysis

Protein A-agarose pellets were resuspended in Laemmli sample buffer [30] and placed in a boiling water bath for 3 min. Samples were then centrifuged (14000 g, 2 min) and the supernatants taken for SDS/PAGE. This was performed at 300 V and 60 mA for 2 h using 10% acrylamide gels. After electrophoresis, gels were fixed in 10% trichloroacetic acid for 1 h prior to drying and were then subjected to autoradiography. Gels were scanned and analysed quantitatively with a Bio-Rad Video densitometer connected to an Olivetti M21 computer driven by the Bio-Rad-1D analysis software package. Labelled bands of interest were excised, and radioactivity was determined by Cherenkov counting. Phosphoamino acids were analysed as described [31], using samples eluted from the excised gel chips.

RESULTS

Immunoprecipitation of G_i2, G_i3 and G_s from rat hepatocyte membranes

Hepatocytes express both α -G_i2 and α -G_i3 but not α -G_i1, as determined both by specific immunoblotting and by Northern blot analysis of mRNA fractions using specific oligonucleotide probes [32]; S. Griffiths & M. D. Houslay, unpublished work). We have previously shown that the antiserum AS7, directed against the C-terminal decapeptide of transducin, can specifically immunoprecipitate a labelled 40 kDa protein from Triton X-100 detergent-extracts of hepatocyte membranes which had previously been subjected to pertussis-toxin-catalysed [³²P]NAD⁺-dependent ADP-ribosylation [12]. This is presumably α -G_i2. As shown in Fig. 1, the antiserum I3B immunoprecipitated a [³²P]ADP-labelled protein of similar molecular size (41 kDa), namely α -G_i3.

Of the total radioactivity (100%) found migrating on SDS/PAGE gels in the 40–41 kDa region due to pertussis-toxin-catalysed ADP-ribosylation with [³²P]NAD⁺, approx. 40% could be removed by prior immunoprecipitation of the extracts with the antiserum AS7 and around 10% by prior immunoprecipitation with the antiserum I3B. This indicates that our

Fig. 1. Immunoprecipitation of G-proteins using antisera AS7 I3B and CS1

Membranes were ADP-ribosylated using pertussis toxin and [³²P]NAD (tracks 1 and 2) or cholera toxin (tracks 3 and 4), solubilized and subjected to immunoprecipitation, SDS/PAGE and autoradiography as described in Materials and methods section. Track 1, α -G_i2 immunoprecipitated using AS7; track 2, α -G_i3 precipitated using I3B; track 3, α -G_s immunoprecipitated using CS1. No antiserum was present in track 4. The results shown are from a single experiment representative of three.

solubilization, and subsequent immunoprecipitation, protocols afforded a recovery of approx. 50% of the total [³²P]ADP-ribosylated species, namely G_i2 and G_i3.

Also shown in Fig. 1 is the immunoprecipitation, using antiserum CS1, of both the 42 kDa and the 45 kDa forms of G_s, which had been radiolabelled by treatment of intact membranes with [³²P]NAD⁺ and thiol-preactivated cholera toxin prior to solubilization and subsequent immunoprecipitation. As with the two forms of G_i, approx. 50% of total α -G_s was solubilized and subsequently immunoprecipitated using the antiserum CS1.

Agonist-induced phosphorylation of α -G_i2 in intact hepatocytes

Having established the ability of these antisera to immunoprecipitate particular G-proteins specifically, we then used the antisera to examine whether the α -subunits of G_i2, G_i3 and G_s could become phosphorylated in intact hepatocytes. This was done by incubating hepatocytes with [³²P]P_i and, in some instances, challenging the intact hepatocytes with specific ligands.

Using the antiserum AS7, which here immunoprecipitates the α -subunit of G_i2 only, we found that label was incorporated into this G-protein α -subunit under basal conditions (Fig. 2a). The degree of incorporation of [³²P]P_i was, however, markedly increased by incubation for short times with glucagon (10 nM), TH-glucagon (10 nM), Arg-vasopressin (3 nM), angiotensin-II (3 nM), TPA (10 ng/ml) or 8-bromo-cyclic AMP (100 μ M) (Fig. 2a; Table 1). In contrast with this, we observed no evidence to suggest any labelling, and hence phosphorylation, of the α -subunit of either G_i3 (Fig. 2b) or G_s (Fig. 2c) under basal conditions when antisera specific for the α -subunits of these two G-proteins were used in the immunoprecipitation experiments. Neither did we observe any (< 10%) altered labelling of either α -G_s or α -G_i3 after treatment with any of the above listed ligands.

It is possible that if such proteins did become phosphorylated then this covalent modification might interfere with subsequent

Fig. 2. Immunoprecipitation of G-proteins from ^{32}P -labelled hepatocytes

Hepatocytes were labelled with ^{32}P and then challenged with: 1, no agonist; 2, TPA (10 ng/ml), 15 min; 3, 100 μM -8-bromo-cyclic AMP, 15 min; 4, 3 nM-vasopressin, 5 min; 5, 3 nM-angiotensin II, 5 min; 6, 10 nM-glucagon, 5 min; 7, 10 nM-TH-glucagon, 5 min. After harvesting hepatocytes, they were subjected to detergent extraction, immunoprecipitation with antiserum, SDS/PAGE and autoradiography performed as described in the Materials and methods section. The autoradiographs shown are from typical experiments which were performed at least three times with similar results. (a) Antiserum AS7, which immunoprecipitates $\alpha\text{-G}_{i2}$, (b) antiserum I3B, which immunoprecipitates $\alpha\text{-G}_{i3}$, and (c) antiserum CS1, which immunoprecipitates $\alpha\text{-G}_s$.

recognition by the specific antisera, and this would result in a decrease in the amount immunoprecipitated. In order to address this point, hepatocytes were incubated either alone, for 5 or 15 min at 37 °C, or with one of TPA (10 ng/ml, 15 min), 8-bromo-cyclic AMP (100 μM , 15 min) and glucagon (10 nM, 5 min) before washed membrane fractions were prepared for labelling with ^{32}P and either (thiol-preactivated) cholera or pertussis toxin. These preincubations had no discernible effect (< 10%) on the ability either of cholera toxin to label the 42 kDa and 45 kDa forms of $\alpha\text{-G}_s$, or of pertussis toxin to catalyse the

incorporation of label into the 40/41 kDa species. Using these membranes we were able to show that pretreatment of intact hepatocytes with these various ligands had no effect (< 10% change) on the amount of ^{32}P ADP-ribosylated material, corresponding to the α -subunits of G_{i2} , G_{i3} and G_s , which could be solubilized and then immunoprecipitated by the various specific antisera used in this study.

We assessed the specificity of AS7 in immunoprecipitating phosphorylated $\alpha\text{-G}_{i2}$ by adding synthetic peptides to the immunoprecipitation assay. The peptides used were the C-terminal decapeptide of transducin and the C-terminal decapeptide of G_z . Hepatocytes were labelled with ^{32}P , as above, and $\alpha\text{-G}_{i2}$, which had been phosphorylated under basal conditions, was immunoprecipitated. As can be seen (Fig. 3), increasing concentrations of the C-terminal decapeptide of transducin blocked the immunoprecipitation of $\alpha\text{-G}_{i2}$, whereas the G_z decapeptide did not.

As well as determining the phosphorylation of $\alpha\text{-G}_{i2}$ we also evaluated the inhibitory function of G_{i1} , as assessed using the well-established ability of low concentrations of the non-hydrolysable GTP analogue p[NH]ppG to inhibit the catalytic activity of adenylate cyclase (G_i function) which has been amplified by the diterpene forskolin in isolated membrane fractions [15,24,25,27]. Thus the presence of 1 nM-p[NH]ppG inhibits the

Table 1. Ligand-induced changes in the phosphorylation of $\alpha\text{-G}_{i2}$ and in G_i function

Intact hepatocytes were challenged with the indicated ligands and both the labelling of $\alpha\text{-G}_{i2}$ and G_i function were assessed as described in the Materials and methods section. EC_{50} values were obtained from dose-effect curves as shown in Fig. 4, and s.d. values are for three separate experiments done on different cell preparations. Maximal labelling refers to the incorporation of ^{32}P into $\alpha\text{-G}_{i2}$ from hepatocytes challenged with 3 nM-vasopressin (5 min), 3 nM-angiotensin (5 min), 10 ng of TPA/ml (5 min), 1 μM -glucagon (5 min), 10 nM-TH-glucagon (5 min) or 100 μM -8-bromo-cyclic AMP (15 min) in three to six separate experiments on different cell preparations. The degree of maximal labelling given here is directly comparable for the various ligands, as detailed in the legend to Fig. 5. n.a., not applicable. * EC_{50} values for the two apparent components of the action of glucagon in increasing the labelling of $\alpha\text{-G}_{i2}$.

Ligand	Maximal labelling (% increase)	EC_{50} for ^{32}P labelling	EC_{50} for loss of G_i function
Vasopressin	138 ± 25	0.7 ± 0.3 nM	1.1 ± 0.4 nM
Angiotensin	135 ± 25	0.5 ± 0.35 nM	0.2 ± 0.16 nM
TPA	103 ± 21	0.4 ± 0.2 nM	0.7 ± 0.3 nM
Glucagon	219 ± 20	36 ± 12 pM*	25 ± 15 pM
		20 ± 8 nM*	
TH-glucagon	88 ± 10	0.36 ± 0.3 nM	0.75 ± 0.25 nM
8-Bromo-cyclic AMP	297 ± 54	4.0 ± 2.2 μM	1.8 ± 1.2 μM
TPA + 8-bromo-cyclic AMP	282 ± 22	n.a.	n.a.

Fig. 3. Displacement of AS7-immunoprecipitated $\alpha\text{-G}_{i2}$ using a C-terminal decapeptide of transducin

Hepatocytes were labelled with ^{32}P under basal conditions and then subjected to detergent extraction and subsequent immunoprecipitation with the antiserum AS7. Immunoprecipitation was done in the absence of any added peptide (track 5) and in the presence of either the C-terminal decapeptide of transducin at 0.01 $\mu\text{g}/\text{ml}$ (track 10), 0.1 $\mu\text{g}/\text{ml}$ (track 9), 1 $\mu\text{g}/\text{ml}$ (track 8), 10 $\mu\text{g}/\text{ml}$ (track 7) or 100 $\mu\text{g}/\text{ml}$ (track 6), or the C-terminal decapeptide of G_z at 0.1 $\mu\text{g}/\text{ml}$ (track 4), 1 $\mu\text{g}/\text{ml}$ (track 3), 10 $\mu\text{g}/\text{ml}$ (track 2) or 100 $\mu\text{g}/\text{ml}$ (track 1). The structure of the peptides used is given in the Materials and methods section. The data are from a typical experiment.

Fig. 4. Time course of α -G_i2 phosphorylation and inactivation in response to ligand challenge of intact hepatocytes

Labelled hepatocytes were challenged with (a) TPA (10 ng/ml), (b) 3 nM-vasopressin, (c) 1 μ M-glucagon or (d) 8-bromo-cyclic AMP. Activity of adenylate cyclase shows the effect of 10 nM-p[NH]ppG on forskolin-stimulated adenylate cyclase activity (G_i function) (○) expressed as the percentage of the control activity assessed in the absence of p[NH]ppG (100 %). Labelling (●) is expressed as a percentage of that observed in the basal state (set at zero), i.e. in the absence of any hormonal challenge. Samples were subjected to immunoprecipitation with AS7, PAGE and autoradiography. Results are for a typical experiment performed three times. In this experiment cells from the same animal were labelled and analysed similarly. Thus the degree of labelling elicited by the various ligands is directly comparable.

activity of membrane adenylate cyclase activity, which has been stimulated using 100 μ M-forskolin, by some 30–40 %.

The increased α -G_i2 phosphorylation induced by TPA (10 ng/ml) and vasopressin (10 nM) was maximal after 5 min of incubation (Figs. 4a and 4b), whereas the 8-bromo-cyclic-AMP-induced phosphorylation attained a plateau after some 15 min of incubation (Fig. 4d). In each instance, the increased labelling of α -G_i2 paralleled the fall in G_i function. Addition of 8-bromo-cyclic AMP (100 μ M) and TPA (10 ng/ml) together to intact hepatocytes showed no additive increase in the labelling of α -G_i2 (Table 1). Phosphorylation of α -G_i2 induced by 1 μ M-glucagon attained maximal levels after around 5 min of incubation. However, loss of G_i function appeared to precede the time at which maximal labelling was achieved (Fig. 4c).

Dose-effect curves for the ligand-induced phosphorylation of α -G_i2 and the loss of G_i function were also examined (Fig. 5). In the case of TPA, vasopressin, TH-glucagon and 8-bromo-cyclic AMP, these curves paralleled each other (Figs. 5a, 5b and 5d-f)

and yielded similar EC₅₀ values (concn. of ligand causing half-maximal effects) (Table 1). In contrast, the dose-effect curve for the glucagon-mediated ligand-induced phosphorylation of α -G_i2 was unusual in that it appeared to be biphasic (Fig. 5c). Thus incubation with low concentrations of glucagon, in the 0.01–10 nM range, resulted in an increase in labelling of some 2-fold, with an EC₅₀ of 36 pM (Fig. 5c), whereas further increases in glucagon concentrations, up to 1 μ M, resulted in a maximal 3-fold increase in the labelling of α -G_i2. The EC₅₀ for this second phase was around 20 nM (Fig. 5c). Loss of G_i function in membranes from glucagon-challenged cells appeared to be associated with the high-affinity component of the action of glucagon on the labelling of α -G_i2 (Fig. 5c), yielding an EC₅₀ value of around 25 pM (Table 1).

Interestingly, the maximal increases in phosphorylation induced by TPA, angiotensin-II, TH-glucagon and vasopressin, as well as by low concentrations (1 nM) of glucagon, were approximately half the levels achieved by challenge of hepatocytes with

Fig. 5. Dose-effect curves for the agonist-induced phosphorylation of α -G₂ in intact hepatocytes

Hepatocytes were incubated with the indicated concentrations of (a) TPA (15 min), (b) vasopressin (5 min), (c) glucagon (5 min), (d) TH-glucagon (5 min), (e) angiotensin-II (5 min) and (f) 8-bromo-cyclic AMP (15 min). 'Zero' indicates no ligand present. Results are shown for labelling of α -G₂ (●), expressed as a percentage change relative to that occurring in the basal state (set at zero). This method of data expression was chosen so as to eliminate inter-experimental alterations in the magnitude of the detected basal phosphorylation signal. Thus the degree of labelling caused by the various agents is directly comparable in the terms expressed here. The effect of 10 nM-p[NH]ppG on forskolin-stimulated adenylate cyclase activity (G_i -function) (○) is expressed as the percentage of the adenylate cyclase activity assessed in the absence of p[NH]ppG (100 %); this was 56–64 pmol/min per mg of protein. Samples were processed and results quantified as described in the legend to Fig. 3 with means $\pm$ S.E.M. from three separate experiments.

either 8-bromo-cyclic AMP or micromolar concentrations of glucagon (Table 1).

The phosphoamino acid analysis of ^{32}P -labelled α -G₂, which had been solubilized and immunoprecipitated from hepatocytes challenged with either TPA or 8-bromo-cyclic AMP, is shown in Fig. 6. In both cases the major phosphoamino acid was phosphoserine and there was no detectable phosphothreonine or phosphotyrosine. Similar results, i.e. the presence of ^{32}P -

labelled phosphoserine, were found in α -G₂ immunoprecipitated from untreated cells and those treated with the other ligands used in this study (results not shown).

Phosphorylation of α -G₂ in isolated hepatocyte membranes using purified protein kinases

Hepatocyte membranes (10 μg in a total volume of 100 μl) were incubated for 30 min at 30 $^{\circ}\text{C}$ with either purified C-kinase

Fig. 6. Phosphoamino acid analysis of α -G_i2

Phosphoamino acids were analysed by one-dimensional t.l.c. as described in the Materials and methods section. Samples were from hepatocytes treated with TPA (10 ng/ml, 15 min) (track 1) or 8-bromo-cyclic AMP (100 μ M, 15 min) (track 2). The slowly migrating spots represent phosphopeptides [31]. The data are typical from one experiment done three times.

Fig. 7. Phosphorylation of α -G_i2 in hepatocyte membranes treated with purified C-kinase

Hepatocyte membranes were incubated with (a) purified C-kinase as described in the Materials and methods section. Extraction, immunoprecipitation with AS7, PAGE and autoradiography were performed as described in the Materials and methods section. (a) Track 1 gives the result when 10 mM-EGTA was added to the assay and in track 2 shows incubations done in the presence of TPA (10 ng/ml), 1.5 mM-CaCl₂ and phosphatidylserine (0.5 mg/ml). (b) Membrane fractions were obtained from hepatocytes which either had not been exposed to the exogenous stimulatory ligands (control; track 1) or had been exposed to one of TPA (10 ng/ml for 15 min; track 2), 8-bromo-cyclic AMP (100 μ M for 15 min; track 3), vasopressin (10 nM for 5 min; track 4) and glucagon (10 nM for 5 min; track 5) prior to incubation with purified protein kinase C together with [³²P]ATP and TPA (10 ng/ml), 1.5 mM-CaCl₂ and phosphatidylserine (0.5 mg/ml). The data are typical from one experiment done three times.

Table 2. Action of purified protein kinase C on the labelling of α -G_i2 in isolated hepatocyte membranes

Membrane fractions were obtained from hepatocytes which either had not been exposed to the exogenous stimulatory ligands (None) or had been exposed to one of a variety of indicated agents. The isolated membrane fractions were then incubated with purified protein kinase C together with [³²P]ATP, TPA (10 ng/ml), 1.5 mM-CaCl₂ and phosphatidylserine (0.5 mg/ml). The membranes were then extracted and α -G_i2 was immunoprecipitated with AS7. The immunoprecipitated samples were then subjected to SDS/PAGE with subsequent autoradiography as described in the Materials and methods section. Data are means $\pm$ S.D. for three separate experiments on different cell preparations. Labelling is expressed as a percentage decrease compared with the control (set as none, i.e. no decrease).

Hepatocyte pretreatment	Inhibition of C-kinase action on α -G _i 2 phosphorylation (%)
None	(None)
TPA (10 ng/ml, 15 min)	79 $\pm$ 14
8-Bromo-cyclic AMP (100 μ M, 15 min)	78 $\pm$ 15
Glucagon (1 μ M, 5 min)	75 $\pm$ 17
Vasopressin (3 nM, 5 min)	99 $\pm$ 2

(15 units/ml) or the catalytic subunit of A-kinase (1 unit/ml) in the presence of [³²P]ATP as described in the Materials and methods section. After incubation, α -G_i2 was solubilized and immunoprecipitated using the antiserum AS7. Treatment with C-kinase, but not A-kinase (results not shown), resulted in the incorporation of ³²P into immunoprecipitated α -G_i2 (Fig. 7). The A-kinase preparation was apparently homogeneous and acted on histones in a manner which was fully inhibited (> 90%) by inclusion in the incubation of the heat-stable inhibitor of A-kinase (Walsh inhibitor [33]).

Phosphorylation of α -G_i2 by C-kinase was completely abolished if 10 mM-EGTA was included in the incubation medium instead of TPA, CaCl₂ and phosphatidylserine (Fig. 7a).

When hepatocytes were treated with the various ligands employed in this study prior to membrane preparation, we failed to observe any C-kinase-mediated phosphorylation of α -G_i2 in membranes obtained from such pretreated cells (Fig. 7b, Table 2).

DISCUSSION

Adenylate cyclase is controlled by hormone receptors exerting either stimulatory or inhibitory actions [1]. These actions are mediated by two distinct G-proteins, namely G_s, which transduces stimulatory effects and G_i, which transduces inhibitory effects. G_i was originally identified as having an α -subunit of approx. 40 kDa which could be ADP-ribosylated through the action of pertussis toxin, an effect which led to the functional inactivation of G_i [1]. Although it is now appreciated that a family of G_i-like proteins exists [1–3], termed G_i1, G_i2 and G_i3, the question of whether all or only particular species of these G-proteins can mediate the functional inhibition of adenylate cyclase activity is still unresolved.

Inhibition of adenylate cyclase can be seen in hepatocyte membranes, where only G_i2 and G_i3 occur, indicating that either one or both of these species can mediate this effect upon adenylate cyclase activity. The data presented here show that it is the α -subunit of G_i2, and not that of G_i3, which becomes phosphorylated and inactivated in response to the challenge of intact hepatocytes with a variety of ligands capable of activating either

or both A-kinase and C-kinase. It is possible that our inability to demonstrate phosphorylation of hepatic α -G₃ could be due to the relatively low abundance of this protein. However, the existence of a potential site for the C-kinase-dependent phosphorylation of α -G₂ at Ser-306, which is not found in α -G₁, α -G₃ or α -G_s, might offer support to our contention that it is α -G₂ which is specifically phosphorylated by C-kinase action in intact hepatocytes.

Vasopressin and TPA, agents which are known to stimulate protein kinase C (but not protein kinase A) in hepatocytes [34–36] here elicit both the rapid phosphorylation of α -G₂ and the loss of G_i function, as assessed by the ability of low concentrations of the non-hydrolysable GTP analogue p[NH]ppG to inhibit the catalytic activity of adenylate cyclase. As both time courses and dose-effect curves for these effects were very similar, we suggest that phosphorylation of α -G₂ leads to ablated G_i function.

Treatment of hepatocytes with the membrane-permeable analogue of cyclic AMP, 8-bromo-cyclic AMP, also elicited phosphorylation of α -G₂ and loss of G_i function (Figs. 4 and 5). Although this compound will activate A-kinase, there is good evidence that treatment of hepatocytes with 8-bromo-cyclic AMP [37] and a related analogue [38] can persistently elevate intracellular Ca²⁺ concentrations to levels comparable with those found using angiotensin, vasopressin and glucagon. Furthermore, treatment of intact hepatocytes with 8-*p*-chlorophenylthio-cyclic AMP has also been demonstrated [35] to lead to the stimulation of inositol phospholipid metabolism in hepatocytes. Whether these cyclic AMP analogues elicit a direct effect on inositol phospholipid metabolism which leads to a Ca²⁺ flux, or whether they act initially on a Ca²⁺ channel leading to a rise in the intracellular concentration of Ca²⁺, which can then stimulate inositol phospholipid metabolism, remains to be determined. Thus it is likely that 8-bromo-cyclic AMP will cause the activation of protein kinase C either by elevating the intracellular concentration of diacylglycerol or by a marked elevation in the intracellular [Ca²⁺] which, of itself, is able to activate this enzyme [39]. Thus 8-bromo-cyclic AMP may exert actions by stimulating both protein kinases A and C and possibly also the Ca²⁺/calmodulin-dependent protein kinase. Certainly, this compound appears to activate C-kinase, or a kinase which phosphorylates α -G₂ at or near to the site of phosphorylation by C-kinase, as we were unable to see any additive effect with TPA (Table 1) in intact hepatocytes, and purified C-kinase became unable to phosphorylate α -G₂ in membranes from hepatocytes which had been pretreated with 8-bromo-cyclic AMP.

Challenge of hepatocytes with 8-bromo-cyclic AMP appeared to elicit the incorporation of approximately twice the amount of label into α -G₂ compared with that found using agents such as TPA and vasopressin, which have been established to stimulate C-kinase and not A-kinase [34,36,39]. One explanation for this is that α -G₂ could serve as a substrate for both C- and A-kinase. G_i preparations containing a mixture of forms have been shown to serve as substrates for pure C-kinase *in vitro* [8] and our studies, using membrane-bound G-proteins and pure C-kinase, would support this (Fig. 2). However, we failed to observe any ability of purified A-kinase to phosphorylate membrane-bound α -G₂. This contrasts with one study using a purified G_i preparation [11], yet is consistent with a more recent study [40]. We, however, believe it unlikely that A-kinase can act to phosphorylate α -G₂ directly, as inspection of the primary sequence [3] shows no 'classical' site for serine phosphorylation. Nevertheless, we consider that there are likely to be at least two sites of phosphorylation of α -G₂. This is because (i) treatment of hepatocytes with either 8-bromo-cyclic AMP or glucagon caused the incorporation of almost twice as much label into α -G₂ compared with treatments employing any of the classical ligands

which activate C-kinase and not A-kinase, (ii) the effects of 8-bromo-cyclic AMP and TPA on the labelling of α -G₂ in intact hepatocytes were not additive (Table 1), and (iii) the dose-effect curve for the glucagon-mediated labelling of α -G₂ (Fig. 5c) was clearly biphasic in nature, whereas that for TH-glucagon, which does not increase intracellular cyclic AMP concentrations and appears to function by activating C-kinase [41], was clearly monophasic. In this regard, loss of G_i function appeared to follow the high-affinity component of the action of glucagon in the labelling of α -G₂, which is consistent with previous studies attributing to this component the cyclic-AMP-independent actions of glucagon suggested to lead to C-kinase activation [15,41]. However, elevation of glucagon concentrations above nanomolar levels caused activation of adenylate cyclase and enhanced accumulation of cyclic AMP with further increases in the incorporation of label into α -G₂. Levels attained were comparable with those found when hepatocytes were challenged with maximally effective doses of 8-bromo-cyclic AMP (Table 1). Appropriately low concentrations of glucagon thus appeared to segregate the presumed C-kinase-mediated actions of this hormone in causing the phosphorylation and inactivation of α -G₂ from its ability to activate adenylate cyclase and increase intracellular cyclic AMP concentrations. This was not the case for 8-bromo-cyclic AMP, which may be because the effects which underlie its ability to increase intracellular Ca²⁺ and perhaps activate C-kinase are dependent upon A-kinase activation. It is possible then that A-kinase does not phosphorylate α -G₂ directly, but elicits the phosphorylation of this G-protein either through another kinase or by involving another protein. In each case it must be presumed that these are not present in the washed membrane preparations used in our studies.

Crouch & Lapetina [42] showed that the antiserum AS7 could immunoprecipitate a phosphorylated 40 kDa protein from hepatocytes which had been challenged with thrombin, an agent capable of stimulating inositol phospholipid metabolism. Our studies are consistent with this, although platelets contain G₁, which, like G₂, can be immunoprecipitated by AS7. However, recently Manning and co-workers [43] failed to detect phosphorylation of α -G₂ in human platelets but found, rather, that the α -subunit of G_s became phosphorylated. This G-protein is highly related to the G_i family but has the striking distinction of not being a substrate for ADP-ribosylation by pertussis toxin because it lacks the C-terminal cysteine residue that is modified by this toxin [44]. In whole liver, some investigators have found [45] mRNA coding for α -G_s and others have not [44]. However, we have been unable to detect α -G_s in hepatocyte membranes by immunoblotting procedures using an anti-peptide antiserum specific for this G-protein (M. D. Houslay & G. Milligan, unpublished work). The antibody AS7 used in this study was raised against the C-terminal decapeptide of transducin [28] and does not cross-react with α -G_s. It can clearly be seen (Fig. 3) that phosphorylated α -G₂ was displaced from immunoprecipitates using the C-terminal decapeptide of transducin, which differs from that of α -G₁ by a single residue, but was not displaced by the C-terminal decapeptide of G_s, which differs by four amino acids. Indeed, such specificity is consistent with the finding that AS7 does not detect α -G₃ [28], which differs by three residues from transducin in the C-terminal decapeptide region. Thus we feel that it is highly improbable that the phosphorylated species that we have immunoprecipitated in these studies using the antiserum AS7 is anything other than α -G₂.

The physiological role of this hormone-mediated modification of α -G₂ remains to be determined. As GTP concentrations in hepatocytes are around 600 μ M [46], these might be expected to elicit a tonic inhibitory effect upon adenylate cyclase activity which may be removed upon challenge with hormones that

activate C-kinase. Whether the phosphorylation of α -G_i2 actually uncouples receptor-mediated inhibition of adenylate cyclase remains to be seen. We have recently shown in adipocytes [47] that diabetes elicits the loss of p[NH]ppG-mediated inhibition of adenylate cyclase activity but not that mediated by A₁ adenosine receptors or those for nicotinate.

Our studies thus indicate that α -G_i2 can mediate inhibitory actions upon adenylate cyclase in hepatocyte membranes and that the functioning of this G-protein can provide a point for 'cross-talk' between two distinct signalling pathways, namely that controlling intracellular cyclic AMP concentrations and those leading to the activation of C-kinase.

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REFERENCES

- Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649
- Milligan, G. (1988) *Biochem. J.* **255**, 1–13
- Jones, D. T. & Reed, R. R. (1987) *J. Biol. Chem.* **262**, 14241–14249
- Cockcroft, S. (1987) *Trends Biochem. Sci.* **12**, 75–78
- Jelsema, C. L. & Axelrod, J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3623–3627
- Yatani, A., Codina, J., Brown, A. M. & Birnbaumer, L. (1987) *Science* **235**, 207–211
- Yatani, A., Mattera, R., Codina, J., Gref, R., Okabe, K., Padrell, E., Iyengar, R., Brown, A. M. & Birnbaumer, L. (1988) *Nature (London)* **336**, 680–682
- Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jakobs, K. H. (1985) *Eur. J. Biochem.* **151**, 431–437
- O'Brien, R. M., Houslay, M. D., Milligan, G. & Siddle, K. (1987) *FEBS Lett.* **212**, 281–288
- Krupinski, J., Rajaram, R., Lakonuhok, M., Benovic, J. L. & Cerione, R. A. (1988) *J. Biol. Chem.* **263**, 12333–12341
- Watanabe, Y., Imaizumi, T., Misaki, N., Iwakura, K. & Yoshida, H. (1988) *FEBS Lett.* **236**, 372–374
- Pyne, N. J., Murphy, G. J., Milligan, G. & Houslay, M. D. (1989) *FEBS Lett.* **243**, 77–82
- Jakobs, K. H., Bauer, S. & Watanabe, Y. (1985) *Eur. J. Biochem.* **151**, 425–430
- Houslay, M. D. (1989) *Curr. Opin. Cell Biol.* **1**, 669–674
- Murphy, G. J., Gawler, D., Milligan, G., Wakelam, M. J. O., Pyne, N. J. & Houslay, M. D. (1989) *Biochem. J.* **259**, 191–197
- Bregman, M. D., Trivedi, D. & Hruby, V. (1980) *J. Biol. Chem.* **255**, 11725–11733
- Parker, P. J., Stabel, S. & Waterfield, M. (1984) *EMBO J.* **3**, 953–959
- Reimann, E. M. & Beham, R. A. (1983) *Methods Enzymol.* **99**, 51–93
- Smith, S. A., Elliott, K. R. F. & Pogson, C. I. (1978) *Biochem. J.* **176**, 817–825
- Heyworth, C. M. & Houslay, M. D. (1983) *Biochem. J.* **214**, 93–98
- Houslay, M. D. & Elliott, K. R. F. (1979) *FEBS Lett.* **104**, 359–363
- Houslay, M. D., Metcalfe, J. C., Warren, G. B., Hesketh, T. R. & Smith, G. A. (1976) *Biochim. Biophys. Acta* **436**, 489–494
- Whetton, A. D., Needham, L., Dodd, N. J. F., Heyworth, C. M. & Houslay, M. D. (1983) *Biochem. Pharmacol.* **32**, 1601–1608
- Heyworth, C. M., Hanski, E. & Houslay, M. D. (1984) *Biochem. J.* **222**, 189–194
- Gawler, D., Milligan, G., Spiegel, A. M., Unson, C. G. & Houslay, M. D. (1987) *Nature (London)* **327**, 229–232
- Johnson, G. L. & Bourne, H. F. (1977) *Biochem. Biophys. Res. Commun.* **78**, 792–798
- Heyworth, C. M., Whetton, A. D., Wong, S., Martin, B. R. & Houslay, M. D. (1985) *Biochem. J.* **228**, 593–603
- Mitchell, F. M., Griffiths, S. L., Saggerson, E. D., Houslay, M. D., Knowler, J. T. & Milligan, G. (1989) *Biochem. J.* **262**, 403–408
- Price, S. R., Tsai, S. C., Adamik, R., Angus, C. W., Serrenti, I. M., Tsuchia, M., Moss, J. & Vaughan, M. (1989) *Biochemistry* **28**, 3803–3807
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Cooper, J. A., Sefton, B. M. & Hunter, T. (1983) *Methods Enzymol.* **99**, 387–405
- Milligan, G. & Unson, C. G. (1989) *Biochem. J.* **260**, 837–841
- Walsh, D. A., Asby, C. D., Gonzales, C., Calkins, D., Fischer, E. H. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1977–1985
- Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) *Biochem. J.* **212**, 733–747
- Bockino, S. B., Blackmore, P. F. & Exton, J. H. (1985) *J. Biol. Chem.* **260**, 14201–14207
- Garrison, J. C., Johnsen, D. E. & Campanile, C. P. (1984) *J. Biol. Chem.* **259**, 3283–3294
- Staddon, J. M. & Hansford, R. G. (1989) *Eur. J. Biochem.* **179**, 47–52
- Blackmore, P. F. & Exton, J. H. (1986) *J. Biol. Chem.* **261**, 11056–11063
- Nishizuka, Y. (1983) *Trends Biochem. Sci.* **8**, 13–16
- Premont, R. T. & Iyengar, R. (1989) *Endocrinology (Baltimore)* **125**, 1151–1160
- Wakelam, M. J. O., Murphy, G. J., Hruby, V. J. & Houslay, M. D. (1986) *Nature (London)* **323**, 68–71
- Crouch, M. F. & Lapetina, E. G. (1988) *J. Biol. Chem.* **263**, 3363–3371
- Carlson, K. E., Brass, L. F. & Manning, D. R. (1989) *J. Biol. Chem.* **264**, 13298–13305
- Fong, H. K. W., Yoshimoto, K. K., Eversole-Cire, P. & Simon, M. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3066–3070
- Matsoka, M., Itoh, H., Kozasa, T. & Kaziro, Y. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5384–5388
- Heyworth, C. M., Wallace, A. V. & Houslay, M. D. (1983) *Biochem. J.* **214**, 99–110
- Strassheim, D., Milligan, G. & Houslay, M. D. (1990) *Biochem. J.* **266**, 521–526

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