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# Treatment of intact hepatocytes with either the phorbol ester TPA or glucagon elicits the phosphorylation and functional inactivation of the inhibitory guanine nucleotide regulatory protein $G_i$

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The antiserum AS7 can specifically immunoprecipitate α-$G_i$ from membrane extracts as well as from a mixture of purified α-$G_i$ and α-$G_o$ as ascertained using [$^{32}$P]ADP-ribosylated G-proteins. Using this antiserum to immunoprecipitate α-$G_i$ from hepatocytes labelled with $^{32}$P it was evident that α-$G_i$ was phosphorylated under basal (resting) conditions. Challenge of hepatocytes with the tumour promoting phorbol ester TPA, however, elicited a marked enhancement of the phosphorylation state of α-$G_i$. This was accompanied by the loss of inhibitory effect of $G_i$ on adenylate cyclase, as judged by the inability of low concentrations of p[NH]ppG to inhibit forskolin-stimulated adenylate cyclase activity. Such actions were mimicked by treatment of hepatocytes with either glucagon or TH-glucagon, an analogue of glucagon which is incapable of activating adenylate cyclase and elevating intracellular cyclic AMP concentrations. Pre-treatment of hepatocytes with either glucagon, TPA or insulin did not affect the ability of pertussis toxin to cause the $NAD^+$-dependent, [$^{32}$P]ADP-ribosylation of α-$G_i$ in membrane fractions isolated from such pre-treated hepatocytes. We suggest that protein kinase C can elicit the phosphorylation and functional inactivation of α-$G_i$ in intact hepatocytes. As pertussis toxin only causes the ADP-ribosylation of the holomeric form of $G_i$, it may be that phosphorylation leaves α-$G_i$ in its holomeric state.

Adenylate cyclase; Guanine nucleotide regulatory protein; Phosphorylation; Phorbol ester; Protein kinase; Desensitization; Glucagon; (Hepatocyte)

## 1. INTRODUCTION

The functioning of many receptors is mediated through the action of distinct guanine nucleotide regulatory proteins (G-proteins), which serve to couple receptors to their appropriate signal generation system [1–3]. In the case of adenylate cyclase two distinct G-proteins are involved. These are $G_s$, which interacts with receptors which serve to stimulate adenylate cyclase, and $G_i$, which interacts with those receptors which inhibit the functioning of this enzyme. Both of these G-proteins have been isolated and characterised as heterotrimers ($\alpha,\beta,\gamma$) possessing apparently identical $\beta,\gamma$ subunits but with distinct $\alpha$-subunits. The $\alpha$-subunits of these two G-proteins can be identified separately using cholera and pertussis toxins, which cause the $NAD^+$-dependent ADP-ribosylation of $\alpha$-subunits of $G_s$ and $G_i$, respectively [1–3]. Such toxins, however, can also interact with other G-proteins. One example is the G-protein $G_o$, which is found in a number of cell types. This species is related to $G_i$ in that it provides a substrate for ADP-ribosylation by pertussis toxin but, however, does not inhibit adenylate cyclase [1–3].

Activation of $G_s$ leads to the dissociation of its $\alpha$-subunit ($\alpha$-$G_s$) which interacts with and stimulates adenylate cyclase. Inhibition of adenylate cyclase, by $G_i$, is elicited both through the interaction of $\alpha$-$G_i$ with adenylate cyclase and

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*Abbreviations:* p[NH]ppG, guanylyl 5′-imidodiphosphate; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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by the release of $\beta,\gamma$ subunits from $G_i$, which inhibits the dissociation of $G_s$ by mass action [1–3].

Incubation of solubilized, purified $G_i$ with purified protein kinase C has been shown [4] to lead to the phosphorylation of $\alpha$-$G_i$. Similarly, we [5] and others [6] have shown that incubation of pure preparations of $G_i$ and $G_o$ with a purified human insulin receptor preparation leads to the phosphorylation of $\alpha$-$G_i$. It has been suggested that phosphorylation of $G_i$ by protein kinase C may lead to its inactivation as incubation of platelets with tumour promoting phorbol esters, which activate protein kinase C, blocked the ability of $\alpha_2$-adrenoceptors to inhibit adenylate cyclase activity [7].

Here we show that, under resting conditions, $\alpha$-$G_i$ in hepatocytes is in a phosphorylated state. Whilst $G_i$ is functional in membranes prepared from these cells, the prior challenge of intact hepatocytes with either TPA, glucagon or TH-glucagon, an analogue of glucagon which does not stimulate adenylate cyclase activity, led to an increase in the phosphorylation state of $\alpha$-$G_i$ and to its functional inactivation.

## 2. MATERIALS AND METHODS

Inorganic [$^{32}$P]phosphate was obtained from Amersham International, Amersham, England. [$^{32}$P]$NAD^+$ was obtained from New England Nuclear, England. Collagenase type-II was from Worthington Biochemical Co., England. TPA was from Cambridge Bioscience, England. Hormones were from Sigma, England. All other biochemicals were from Boehringer Ltd, England. TH-glucagon was a kind gift from Drs V. Hruby and D. Trivedi, Dept of Chemistry, Univ. of Arizona, USA. Islet activating protein/pertussis toxin was from PHLS Centre for Applied Microbiology, Porton Down, England. Immunoprecipitin (protein A) was obtained from BRL, England. All other chemicals were of AR grade from BDH Ltd, England.

### 2.1. *Hepatocyte preparation, incubation and harvesting*

Hepatocytes were prepared according to [8] using 225–250 g fed male Sprague-Dawley rats, as described before by us [9]. Cells (3–5 mg dry wt/ml) were preincubated at 37°C for at least 50 min in Krebs Henseleit (50 $\mu$M potassium phosphate, 1 mCi $^{32}P_i$) buffer also containing 2.5% BSA, 2.5 mM $CaCl_2$ and 10 mM glucose, and gassed with $O_2/CO_2$ (19.1) for 30 s every 10 min. The ATP content of the isolated hepatocytes was determined by the luciferase method on a neutralised $HClO_4$ extract [9].

Ligands were added to the incubation vessel in a volume which was less than 1% of the total incubation volume. After an appropriate time the reactions were stopped by addition of an equal volume of ice-cold buffer A, composed of 0.25 M sucrose, 10 mM Tris-HCl, final pH 7.4; 1 mM EDTA, 1 mM EGTA, 10 mM $\beta$-glycerophosphate, 50 mM sodium fluoride, 0.1 mM PMSF and 2 mM benzamidine. The cells were harvested by centrifugation at 1000 rpm for 3 min and washed twice in buffer A. After this procedure cells were taken for immunoprecipitation of $\alpha$-$G_i$.

When cells were isolated for the purpose of adenylate cyclase assays, the hepatocytes were pre-incubated in normal Krebs Henseleit. Incubation of the hepatocytes was then quenched by adding an aliquot to an equal volume of ice-cold 1 mM $KHCO_3$, pH 7.2 which was kept on ice until processed further as below.

### 2.2. *Preparation of liver and hepatocyte membranes*

A washed membrane fraction was obtained from the hepatocytes as detailed previously described by us [10]. In all cases membranes were used within 2 h of their preparation. In some experiments, membranes were prepared from whole liver homogenates [11].

### 2.3. *Assay of adenylate cyclase*

Adenylate cyclase was assayed as described previously by us [9,11]. Assays contained (final concentrations) 1.5 mM ATP, 5 mM $MgSO_4$, 10 mM theophylline, 1 mM EDTA, 7.5 mg phosphocreatine/ml, 1 mg creatine kinase/ml and 25 mM triethanolamine/KOH, pH 7.4. The cyclic AMP produced was assessed in a binding assay using cyclic AMP binding subunit of A-kinase [9,12]. Assays were linear under all conditions and initial rates were analysed.

### 2.4. *Labelling and assay of $G_i$*

Pertussis toxin-catalysed ADP-ribosylation both of purified $G_i/G_o$ and of isolated adipocyte and hepatocyte membranes was performed as before [13] using [$^{32}$P]$NAD^+$ and thiol preactivated pertussis toxin. In some instances reactions were terminated by addition of ice-cold 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and taken for immunoprecipitation. In others reactions were terminated by addition of Laemmli [14] sample buffer as described before [13], with subsequent electrophoresis and autoradiography.

A mixture of purified soluble $G_i/G_o$ was prepared from bovine brain as described before by one of us [15].

Detection of functional $G_i$ was assessed as before [13,16–18] by demonstrating the ability of low concentrations of p[NH]ppG (1 nM) to inhibit basal adenylate cyclase that had been previously amplified by the diterpene forskolin (100 $\mu$M).

### 2.5. *Immunoprecipitation of $G_i$*

Phosphorylated $\alpha$-$G_i$ was immunoprecipitated using a 1:50 dilution of AS7 antiserum [19], an antibody produced against the last 10 amino acids of the C-terminal end of rod transducin. This sequence differs in but a single amino acid from the equivalent C-terminal region of $\alpha$-$G_i$ found in hepatocytes. This antiserum has been used successfully, by us, to identify $\alpha$-$G_i$ in both purified and membrane preparations from these cells by immunoblotting [18].

In order to immunoprecipitate $\alpha$-$G_i$ from hepatocytes a cell extract was first made. This involved taking 250 $\mu$l of cells (50 mg protein) and adding them to 135 $\mu$l of PBS (10 mM $KH_2PO_4/K_2HPO_4$; pH 7.4, 0.9% NaCl) and 100 $\mu$l of buffer B containing (final concentrations) 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 1 mM EDTA,

0.15 M NaCl. To this mixture, 12.5 μl of gelatin (2%, w/v) was added and finally 2.5 μl of antiserum AS7 (1:200 dilution) added and left at 4°C for 12 h. When purified $G_i/G_o$ was used for immunoprecipitation then 170 ng of a mixture of purified $G_i/G_o$ was present in 135 μl PBS and 100 μl buffer B to which 2.5 μl antiserum was added as detailed above. The mixture was then centrifuged at 4°C and 14000 × $g_{av}$ for 10 min to remove any insoluble material. The supernatant obtained subsequently was removed, and 25 μl (w/v, 10%) protein A added to it and this mixture left at 4°C for 1.5 h. Samples were diluted 1:1 PBS prior to protein A being collected by centrifugation at 14000 × $g_{av}$ for 2 min at 4°C and washed once in PBS, then once in buffer B and finally once in PBS.

2.6. *SDS-PAGE and autoradiography*

The material immunoprecipitated using immunoprecipitin (protein A) was resuspended in Laemmli sample buffer [14] and placed in a boiling water bath for 3 min. After this time the samples were centrifuged for 2 min at 14000 × $g_{av}$. The supernatants were taken for SDS-PAGE. This was performed for 2 h at 300 V and 60 mA using gels of 10% (v/v) acrylamide. After electrophoresis, the gels were fixed in 10% TCA for 1 h prior to drying and then subjected to autoradiography. The labelled bands of interest were excised from the gel and subjected to Cherenkov counting.

## 3. RESULTS AND DISCUSSION

We [13,18] and others [1,3,17] have shown previously, using a variety of methods, that hepatocyte plasma membranes contain $\alpha$-$G_i$. This can be detected functionally by employing low concentrations of p[NH]ppG to inhibit basal adenylate cyclase activity which has been amplified using the diterpene forskolin. This takes advantage of the fact that $G_i$ has a higher affinity for p[NH]ppG than $G_s$, thus allowing $G_i$ to be selectively activated [1–3,16]. $\alpha$-$G_i$ has also been detected in hepatocyte membranes using pertussis toxin-catalysed ADP-ribosylation [13,20]. Similarly, the presence of $\alpha$-$G_i$ has been determined in hepatocytes using a specific antiserum (AS7) raised against the C-terminal decapeptide of the related G-protein, transducin [18].

The antiserum AS7 thus offers a potential means to detect whether $\alpha$-$G_i$ is indeed phosphorylated in situ in the intact hepatocyte. In order to determine as to whether AS7 could be used to immunoprecipitate $\alpha$-$G_i$ specifically, we first attempted to show whether AS7 could selectively immunoprecipitate $\alpha$-$G_i$ from a mixture of purified $G_i$ and its related G-protein $G_o$. These proteins, which had been purified from bovine brain, were then labelled covalently by pertussis toxin-catalysed [$^{32}$P]NAD-dependent ADP-ribosylation. Fig.1 shows that the antiserum AS7 immunoprecipitated specifically the 41 kDa subunit that is $\alpha$-$G_i$. Similarly, the antipeptide antiserum OC1, which interacts specifically with $\alpha$-$G_o$ in Western blotting analyses [21], specifically immunoprecipitated the 39 kDa $\alpha$-subunit of $G_o$ (fig.1a). If non-immune/pre-immune antisera were employed in such experiments then we failed to observe any label in the immunoprecipitate. Such an observation, coupled with the ability of AS7 and OC1 to immunoprecipitate $\alpha$-$G_i$ and $\alpha$-$G_o$ selectively, indicates that these two antisera can be used for specific immunoprecipitations. Immunoblotting with the antiserum OC1, however, showed that hepatocyte membranes did not appear to contain detectable concentrations of $\alpha$-$G_o$ (not shown).

In order that the ability of AS7 to immunoprecipitate $\alpha$-$G_i$ could be determined using solubilized membrane preparations, rather than purified components, we used pertussis toxin to label isolated membranes by [$^{32}$P]ADP-ribosylation. Using AS7 we were able to show that this antiserum immunoprecipitated a [$^{32}$P]ADP-ribosylated 41 kDa protein from these membranes (fig.1b). Again, normal rabbit serum/pre-immune serum failed to elicit any immunoprecipitation.

When AS7 was used to immunoprecipitate material from solubilized extracts of $^{32}$P-labelled hepatocytes then we observed a single labelled band which presumably reflected $\alpha$-$G_i$ (fig.2). This suggests that $\alpha$-$G_i$ is phosphorylated under 'normal', basal conditions in intact hepatocytes. If, however, hepatocytes were preincubated with the tumour-promoting phorbol ester TPA (10 ng/ml, 15 min) then a marked increase in the degree of phosphorylation was observed (fig.2; table 1). This increase in phosphorylation was accompanied by a small decrease in the apparent mobility of $\alpha$-$G_i$ on SDS-PAGE (fig.2). This could result from the presence of the negatively charged phosphate group, making $\alpha$-$G_i$ migrate differently, perhaps by effecting a change in the conformation of $\alpha$-$G_i$ or allowing it to bind a different amount of SDS. A small decrease in mobility of $\alpha$-$G_i$, upon SDS-PAGE, has also been noted by one of us [22] following pertussis toxin-catalysed ADP-ribosylation of $\alpha$-$G_i$. This small shift in mobility which accompanied the increased labelling of $\alpha$-$G_i$

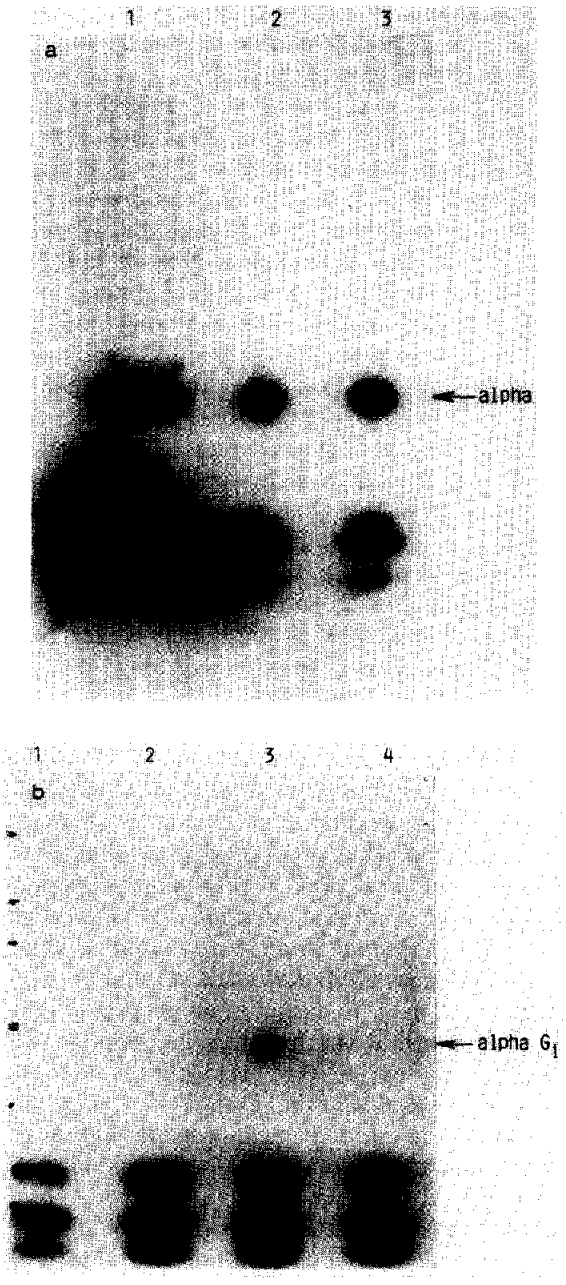


Fig.1. The antiserum AS7 specifically immunoprecipitates $\alpha$-$G_i$. (a) A purified, homogeneous preparation of $G_i$ and $G_o$, from bovine brain, was ADP-ribosylated using pertussis toxin and [$^{32}$P]$NAD^+$. This mixture was then incubated with either the antiserum AS7, which is specific for $\alpha$-$G_i$, or with the antiserum OC1, which is specific for $\alpha$-$G_o$. Immunoprecipitation and subsequent analysis by SDS-PAGE and autoradiography was then performed as detailed in section 2. Track '1' shows the ADP-ribosylated mixture of $G_i$ and $G_o$ before immunoprecipitation, track '2' shows material immunoprecipitated by OC1 and track '3' material immunoprecipitated by AS7. $\alpha$-$G_i$ migrated with an apparent molecular mass of 41 kDa and $\alpha$-$G_o$ with that of 39 kDa. (b) Membranes were either ADP-ribosylated using pertussis toxin and [$^{32}$P]$NAD^+$ as in (track 2 and track 3) or were not treated with pertussis toxin (track 1 and track 4). Membranes were then solubilized and subjected to immunoprecipitation as described above using either pre-immune serum (track 1 and track 2) or AS7 (track 3 and track 4). Track '3' shows that ADP-ribosylated $\alpha$-$G_i$ was immunoprecipitated and migrated as a single band with an apparent molecular mass of 41 kDa. This shows a typical experiment of one performed three times.

might indicate that the TPA elicited phosphorylation occurred on a site distinct from that which is phosphorylated under basal conditions.

Presumably the effect of TPA is mediated here by its known ability to activate protein kinase C, which it does by mimicking the conformation of diacyl glycerol [23]. Such a contention would certainly be consistent with previous observations [4] which have shown that pure preparations of $G_i$ can be phosphorylated by protein kinase C.

We also observed that if hepatocytes were treated with glucagon (10 nM, 5 min), then this too caused an increase in the phosphorylation state of $\alpha$-$G_i$ (fig.2). Such an action could be mimicked using TH-glucagon (10 nM, 5 min), an analogue of glucagon which is incapable of both activating adenylate cyclase and of increasing the intracellular concentration of cyclic AMP in hepatocytes [24,25]. However, both glucagon and TH-glucagon have been shown capable of achieving small stimulations of inositol phospholipid metabolism [25] and glucagon has been found to increase the intracellular concentration of diacylglycerol in intact hepatocytes [26]. Such observations would imply that both glucagon and TH-glucagon might trigger the phosphorylation of $\alpha$-$G_i$ by producing diacylglycerol which subsequently activates protein kinase C.

The phosphorylation of $\alpha$-$G_i$ which was elicited by these agents did not, however, affect the ability of pertussis toxin to catalyse the $NAD^+$-dependent, [$^{32}$P]ADP-ribosylation of $\alpha$-$G_i$ in membrane fractions isolated from such pre-treated hepatocytes. As is shown in fig.3, the labelling of the 41 kDa $\alpha$-subunit of $G_i$ was unaffected by pretreatment with all of these various agents. This indicates that phosphorylation does not prevent ADP-ribosylation from occurring. Indeed, as per-

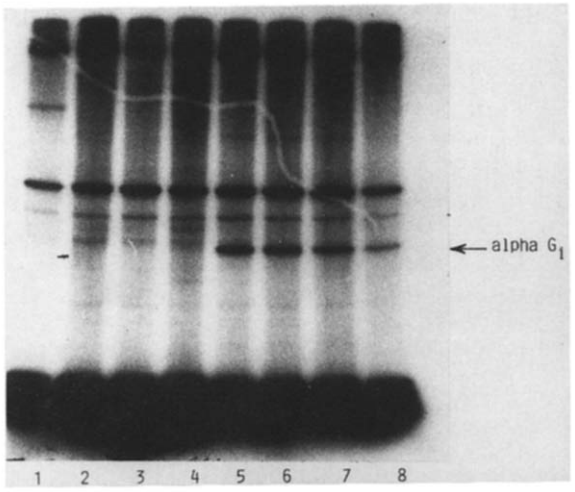


Fig.2. The phosphorylation of $\alpha$-$G_i$ in intact hepatocytes. Hepatocytes were labelled to equilibrium with $^{32}P$. They were then challenged with one of TPA (10 ng/ml, 15 min), glucagon (10 nM, 5 min) or TH-glucagon (10 nM, 5 min). After this a solubilized membrane extract was made for immunoprecipitation analysis as described in section 2. This figure shows an autoradiograph of immunoprecipitates prepared using the antiserum AS7 (tracks 5–8), which immunoprecipitates $\alpha$-$G_i$, and an experiment using a control serum preparation (tracks 1–4). Tracks '1,8' are with no challenge of the hepatocytes with ligands; tracks '2,7' after challenge with TPA; tracks '3,6' after challenge with TH-glucagon and tracks '4,5' after challenge with glucagon. This shows a typical experiment of one performed at least three times using different cell preparations. It is clear that some phosphorylated proteins were precipitated non-specifically in this experiment, due to interaction with the immunoprecipitin, as shown in tracks 1–4. This shows a typical experiment of one performed three times.

tussis toxin causes the ADP-ribosylation of $G_i$ only when this G-protein is in its holomeric state [27] then the unchanged level of pertussis toxin-catalysed ADP-ribosylation of $\alpha$-$G_i$ indicates that phosphorylation does not modify the interaction between $\alpha$-$G_i$ and the $\beta$-$\gamma$ complex which would lead to the dissociation of $G_i$. Furthermore, if dissociation had occurred then one might have expected $\beta$-subunits to have been released by pretreatment with the various ligands, which would lower the basal activity of adenylate cyclase activity; which is not the case [9,24].

The ability of TPA, glucagon and TH-glucagon to phosphorylate $\alpha$-$G_i$ may offer an explanation for our recent observations [28] that $G_i$ function in hepatocytes is lost in membranes from cells which had been pre-treated with these ligands. Here we

Table 1

Increase in phosphorylation of $\alpha$-$G_i$ by treatment of hepatocytes with either TPA, glucagon or TH-glucagon

| Ligand treatment | % increase in labelling of $\alpha$-$G_i$ |
|---|---|
| TPA | 66.0 ± 4.2 |
| TH-glucagon | 77.5 ± 17.6 |
| Glucagon | 108.2 ± 10.5 |

Hepatocytes were labelled to equilibrium with $^{32}P$ and then challenged with either TPA (10 ng/ml, 15 min), glucagon (10 nM, 5 min) or TH-glucagon (10 nM, 5 min). $\alpha$-$G_i$ was immunoprecipitated using the antiserum AS7 and analysed by SDS-PAGE and autoradiography as described in section 2. The %-age increase in labelling of $\alpha$-$G_i$ is shown relative to its labelling under basal (resting) conditions. Errors are SE for $n$ = 3 separate experiments using different cell preparations. The degree of labelling was ascertained by Cherenkov counting of the radioactive bands which had been excised from the gels. Analysis of the 60 kDa phosphoprotein which was precipitated in experiments using both AS7 and non-specific antiserum showed that pre-incubation with the various ligands did not alter its level of phosphorylation (< 10%)

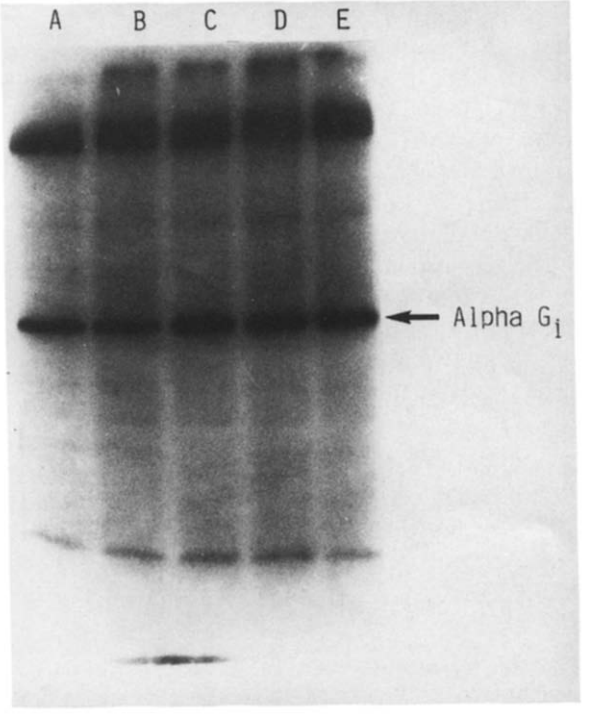


Fig.3. Pertussis toxin catalysed ADP-ribosylation of $\alpha$-$G_i$ in intact hepatocytes. Pertussis toxin catalysed ADP-ribosylation of $\alpha$-$G_i$ was performed using isolated hepatocyte membranes and [$^{32}P$]$NAD^+$, as described in section 2. Hepatocytes had been pre-treated beforehand for 5 min at 30°C in the absence of any hormone (track A); insulin (10 nM) (track B); glucagon (10 nM) (track C); TPA (10 ng/ml for 15 min) (track D); no hormone (track E).

Table 2

Inactivation of functional $G_i$ by treatment of hepatocytes with ligands which stimulate the phosphorylation of $\alpha$-$G_i$

| Treatment of hepatocytes | %-age inhibition of adenylate cyclase activity |
|---|---|
| None | 45 ± 5 |
| TPA | −3 ± 3 |
| Glucagon | −10 ± 7 |
| TH-glucagon | −5 ± 6 |

Hepatocytes were either used untreated (control) or were treated with one of TPA (10 ng/ml, 15 min), glucagon (10 nM, 5 min) and TH-glucagon (10 nM, 5 min) prior to the preparation of a washed membrane fraction. This was used to assess functional $G_i$ activity by monitoring the ability of low concentrations of p[NH]ppG to inhibit basal adenylate cyclase that had been amplified using the diterpene forskolin. Data shows the %-age inhibition elicited by 1 nM p[NH]ppG upon adenylate cyclase activity stimulated by 100 $\mu$M forskolin. Errors are SD for $n = 3$ experiments done on separate cell preparations. The specific activity of forskolin-stimulated adenylate cyclase activity was 62.3 ± 4.5 pmol cAMP produced/min per mg membrane protein

have assessed functional $G_i$ by taking advantage of the fact [1–3] that $G_i$ has a much higher affinity for p[NH]ppG than has $G_s$. Thus, low concentrations of p[NH]ppG can be used to inhibit basal adenylate cyclase that has been amplified using the diterpene forskolin. Under the conditions used to elicit the phosphorylation of $\alpha$-$G_i$ then the ability of this G-protein to inhibit adenylate cyclase activity is shown to be abolished (table 2). It would thus seem that the phosphorylation of $\alpha$-$G_i$, elicited by agents known to activate protein kinase C in intact cells, leads to the functional inactivation of $\alpha$-$G_i$.

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