Okadaic acid identifies a phosphorylation/dephosphorylation cycle controlling the inhibitory guanine-nucleotide-binding regulatory protein G_i2

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Recently, the α -subunit of the inhibitory guanine-nucleotide-binding protein G_i2 (α -G_i2) has been shown to be a substrate for phosphorylation both by protein kinase C and also by other unidentified kinase(s) which are activated as a result of elevated cyclic AMP levels in intact rat hepatocytes [Bushfield, Murphy, Lavan, Parker, Hruby, Milligan & Houslay (1990) *Biochem. J.* 268, 449–457]. Here we show that the incorporation of [³²P]P_i into α -G_i2 was enhanced 3-fold by incubation of intact hepatocytes with the tumour promoter and protein phosphatase (1 and 2A) inhibitor, okadaic acid. This action was both time- and concentration-dependent and was accompanied by a loss of guanine-nucleotide-induced inhibition of adenylate cyclase. The increased labelling of α -G_i2 induced by okadaic acid was partially additive with that elicited by 8-bromo cyclic AMP, but not with that elicited by the protein kinase C activator phorbol 12-myristate 13-acetate. We suggest that, in the absence of hormones, the activity of α -G_i2 is under the control of a dynamic phosphorylation/dephosphorylation system involving protein kinase C and protein phosphatases 1 and/or 2A. This highlights the regulation of kinases and phosphatases as both providing potentially important mechanisms for causing 'cross-talk' between different signalling systems, in this instance controlling cellular responsiveness through regulation of α -G_i2 phosphorylation.

INTRODUCTION

The receptor-dependent stimulation and inhibition of adenylate cyclase is mediated by two distinct guanine-nucleotide-binding proteins (G-proteins) termed G_s and G_i respectively. These G-proteins are heterotrimers consisting of $\beta\gamma$ complexes, which appear to be the same for G_s and G_i, together with unique α -subunits. Molecular cloning studies, and subsequent biochemical and immunological analyses, have shown that a family of G_i-like proteins exists [1–4] which comprises three highly related pertussis-toxin-sensitive G-proteins termed G_i1, G_i2 and G_i3 [3], together with the pertussis-toxin-insensitive G_z [5,6]. 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. These include the regulation of phosphoinositidase C, phospholipase A₂ and K⁺ channels [3,4,7,8]. Although it is not known which of these G_i-like proteins regulate each of these specific effector systems in intact cells, recent evidence indicates that at least G_i2 may act as an inhibitory G-protein controlling adenylate cyclase activity [9–11].

G-proteins can serve as potential key points for the initiation of 'cross-talk' between various different signalling pathways. As such, modulation of their functioning can result in the phenomena of desensitization and/or potentiation of specific signalling systems. Such cross-talk represents an important mechanism for the control of cellular responsiveness and may be mediated by hormone-induced G-protein phosphorylation. Using intact hepatocytes we have examined the hormonal regulation of the phosphorylation state of the G-proteins which control the activity of adenylate cyclase [11–13]. Treatment of intact hepatocytes

with a range of ligands capable of activating protein kinase C [including phorbol 12-myristate 13-acetate (PMA), vasopressin and angiotensin II] and/or cyclic AMP-dependent protein kinase (glucagon and 8-bromo cyclic AMP) induced the selective serine-specific phosphorylation of the α -subunit of G_i2 (α -G_i2), but did not affect either α -G_i3 or α -G_s [11]. We have proposed that there are two sites of phosphorylation on α -G_i2, one acted upon by protein kinase C and phosphorylation at the other site being mediated by an as yet unidentified kinase whose activation, we suggest, is triggered in response to increased cyclic AMP levels. Interestingly, treatment of hepatocytes with either glucagon or 8-bromo cyclic AMP appeared to trigger phosphorylation at both sites on α -G_i2, presumably because both pathways were stimulated (see ref. [11] for discussion).

Recently, a novel tool has become available for the study of protein phosphorylation in intact cells. This is okadaic acid, a polyether fatty acid which forms a major toxic component associated with diarrhoeic shellfish poisoning [14,15]. As with phorbol esters, okadaic acid acts as a tumour promoter, presumably by increasing the phosphorylation of key regulatory proteins. However, unlike phorbol esters, okadaic acid exerts its effect by inhibiting protein phosphatases 1 and 2A [16,17] rather than by activating protein kinase C. In the present study we have used okadaic acid in order to demonstrate that inhibition of protein phosphatase activity under basal conditions, i.e. in the absence of hormonal challenge, suffices to increase the phosphorylation of α -G_i2 at what we suggest is primarily the protein kinase C site on this G-protein. This, we propose, exposes a dynamic phosphorylation/dephosphorylation cycle which controls the activity of this key G-protein.

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; PMA, phorbol 12-myristate 13-acetate; p[NH]ppG, guanosine 5'-[β -imido]triphosphate.

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EXPERIMENTAL

Materials

[32 P]P_i was obtained from Amersham International. PMA was from Cambridge Bioscience, Cambridge, U.K. Hormones and Protein A-agarose were from Sigma. All other biochemicals were from Boehringer, and other chemicals were of AR grade from BDH. Antiserum AS7 prepared as previously described [18,19] was a gift from Dr. Graeme Milligan, Glasgow University.

Hepatocyte preparation and harvesting and membrane preparation

Hepatocytes were prepared as previously described [20] using 220–250 g fed male Sprague–Dawley rats. 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 % (w/v) BSA, 2.5 mM-CaCl₂ and 10 mM-glucose. Cells were gassed with O₂/CO₂ (19:1) for 30 s every 10 min. Ligands were added in less than 1 % of 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, 2 min) and washed once in cold buffer before immunoprecipitation of specific G-proteins. For adenylate cyclase assays, cells were incubated in normal Krebs–Henseleit buffer and the incubations were quenched by the addition of an equal volume of ice-cold 1 mM-KHCO₃, pH 7.2, and were kept on ice until processed further. A washed membrane fraction was obtained from the hepatocytes as described previously [21,22], and membranes were used within 2 h of preparation.

Assay of adenylate cyclase and G_i function

Adenylate cyclase was assayed as described previously [19]. Incubations contained 1.5 mM-ATP, 5 mM-MgSO₄, 10 mM-theophylline, 1 mM-EDTA, phosphocreatine (7.5 mg/ml), creatine kinase (1 mg/ml) and 25 mM-triethanolamine/HCl, pH 7.4. The cyclic AMP produced was determined in a binding assay using the regulatory subunit of cyclic AMP-dependent protein kinase [19]. Assays were linear under all conditions and initial rates were analysed. G_i function was assessed by determining the ability of low concentrations of guanosine 5'-[β -imidotriphosphate (p[NH]ppG; 10 nM) to inhibit adenylate cyclase activity in the presence of the diterpine forskolin (100 μ M) as previously described [11,22–24].

Immunoprecipitation of G_i

This was performed as described before [11,13]. Briefly, cells (10^6 /ml) 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, leupeptin (10 μ g/ml), aprotinin (10 μ g/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_i2 was immunoprecipitated using AS7 antiserum. The production and characterization of this antiserum has been described previously [18,19]. AS7 was raised in rabbits against the C-terminal decapeptide of the α -subunit of transducin and recognizes both α -G_i1 and α -G_i2 in addition to transducin, but does not recognize either α -G_i3 or α -G_s. However, since in hepatocytes neither transducin nor α -G_i1 can be detected by immunoblotting or by analysis of mRNA [25,26], AS7 can be used as a specific tool to immunoprecipitate α -G_i2.

Antiserum (10 μ l) 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. 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.

We have previously demonstrated the ability of this antiserum to immunoprecipitate α -G_i2 selectively from rat hepatocytes. Precipitation was critically dependent upon the presence of the specific antiserum in the incubation. It was not due to non-specific binding of phosphoproteins to immunoglobulins or to Protein A-agarose and could be competed for by the C-terminal decapeptide from α -transducin but not the C-terminal decapeptide from α -G_s [11,13].

SDS/PAGE and autoradiography

Protein A-agarose pellets were resuspended in Laemmli sample buffer [27] and placed in a boiling water bath for 3 min. Samples were then centrifuged (14000 g, 2 min) and the supernatants were taken for SDS/PAGE. This was performed at 300 V and 60 mA for 2 h using 10 % (w/v) acrylamide gels. After electrophoresis, gels were fixed in 10 % (w/v) trichloroacetic acid for 1 h before 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 Čerenkov counting.

Calculation of stoichiometry of phosphorylation

In order to determine the stoichiometry of labelling of α -G_i2 in intact hepatocytes, we measured the amount of α -G_i2 present in the incubation, the specific radioactivity of [γ - 32 P]ATP in the cells and the amount of radioactivity incorporated into immunoprecipitated α -G_i2. Cells were incubated for various times with [32 P]P_i and samples were then divided into two portions. One half was taken for immunoprecipitation of α -G_i2, and the other half was taken for the measurement of ATP content by the luciferase method, as described previously [20]. The specific radioactivity of the [γ - 32 P]ATP was determined as described [28]. The amount of radioactivity associated with α -G_i2 was measured by Čerenkov counting of the excised band from a dried gel after autoradiography. In separate unlabelled samples, the amount of α -G_i2 protein present was determined by quantitative immunoblotting using purified brain G_i as standard [18] and by analysis of the recovery of α -G_i2 in the immunoprecipitation procedure as previously described [11].

RESULTS AND DISCUSSION

Okadaic acid treatment caused a rapid time-dependent (Fig. 1) and concentration-dependent (Fig. 2) increase in the phosphorylation of α -G_i2 in [32 P]P_i-labelled intact rat hepatocytes. The elevation in α -G_i2 phosphorylation reached a peak after 5–10 min of incubation with 100 nM-okadaic acid (Fig. 1). The EC₅₀ value (concentration producing a half-maximal response) was $\sim$ 10 nM (Fig. 2). These concentrations are similar to, or somewhat lower than, those previously shown to produce increases in the phosphorylation of other hepatocyte proteins and to activate gluconeogenesis and glucose output from hepatocytes (around 10 nM–1 μ M) [17].

Phosphorylation of α -G_i2 in intact cells appears to have functional significance, being coupled to loss of the ability of guanine nucleotides to inhibit adenylate cyclase in hepatocyte membranes prepared from these cells. In this instance, the non-hydrolysable GTP analogue p[NH]ppG was used at low concen-

Fig. 1. Time course of okadaic acid-induced phosphorylation of α - G_i2

A typical autoradiograph from a representative experiment is shown in (a) and the effects of okadaic acid treatment on the stoichiometry of labelling of α - G_i2 are shown in (b). Hepatocytes were labelled with [32 P] P_i and then incubated for the indicated times with either vehicle (track 0) or 100 nM-okadaic acid. Hepatocytes were harvested and then subjected to detergent extraction, immunoprecipitation with antiserum AS7, SDS/PAGE and autoradiography, as described in the Experimental section. Results are expressed as means $\pm$ range ($n = 2$ experiments performed in duplicate using hepatocytes from different rats).

Fig. 2. Concentration-effect curve for okadaic acid-induced phosphorylation of α - G_i2

A typical autoradiograph from a representative experiment is shown in (a) and the effects of okadaic acid treatment on the stoichiometry of labelling of α - G_i2 are shown in (b). Samples were incubated for 20 min with either vehicle (track 0) or the indicated concentrations of okadaic acid. Results in (b) are expressed as means $\pm$ ranges ($n = 2$ determinations). The methods used are described in the Experimental section.

trations to inhibit selectively adenylate cyclase, as reported by various investigators [22–24]. Here we see that pre-treatment of hepatocytes with okadaic acid led to a complete loss of this inhibitory effect of p[NH]ppG (Table 1). In intact cells adenylate cyclase may be under the tonic inhibitory control of G_i2 due to the high (around 600 μ M) concentrations of GTP present [29]. Hence, the alteration of the balance between the specific kinase and phosphatase activities that control the level of phosphorylation of α - G_i2 might be expected to modulate hepatocyte responsiveness to stimulatory or inhibitory hormones [11,18,22].

Based on the results of experiments performed previously, using mixtures of ligands able to stimulate cyclic AMP-dependent protein kinase and protein kinase C in hepatocytes, we have suggested that two sites of phosphorylation may exist on α - G_i2 [11]. These are a site for protein kinase C-mediated phosphoryl-

ation and a second site which is acted upon by an as yet unidentified protein kinase whose ability to phosphorylate α - G_i2 is elicited by increased cyclic AMP levels. In order to try and assess which of these two possible phosphorylation sites might be affected by okadaic acid treatment, we examined the effects of okadaic acid under the basal conditions and in combination with various agents previously shown to induce the phosphorylation of α - G_i2 . As shown in Table 2, the ligands employed in this study can be divided into two groups based on the maximal degree of phosphorylation of α - G_i2 induced. Under resting (basal) conditions the stoichiometry of α - G_i2 phosphorylation in [32 P] P_i -loaded hepatocytes was 0.39 ± 0.06 mol of [32 P] P_i /mol of α - G_i2 (mean $\pm$ S.E.M., $n = 5$ separate cell preparations). As reported before [11], in the absence of okadaic acid, treatment of hepato-

Table 1. Effects of pre-treatment of hepatocytes with okadaic acid on the inhibition of adenylate cyclase by p[NH]ppG in membranes

Intact hepatocytes were incubated (37 °C) with the vehicle dimethyl sulphoxide (0.1% of incubation volume, 20 min), okadaic acid (100 nM, 20 min), PMA (10 ng/ml, 15 min), or 8-bromo cyclic AMP (300 μ M, 15 min). Incubations were terminated by the addition of an equal volume of ice-cold 1 mM-KHCO₃, pH 7.2, a washed membrane fraction was prepared from the hepatocytes and G_i function was assessed by determining the ability of low concentrations of p[NH]ppG (10 nM) to inhibit adenylate cyclase activity in the presence of the diterpine forskolin (100 μ M), as described in the Experimental section. Data are means $\pm$ S.E.M. for two experiments each performed in triplicate. Values in parentheses indicate percentage inhibition of adenylate cyclase activity by p[NH]ppG.

Treatment	Adenylate cyclase activity (pmol/min per mg of protein)	
	-p[NH]ppG	+p[NH]ppG
Vehicle	21.9 $\pm$ 0.5	16.0 $\pm$ 0.8 (27)
Okadaic acid	19.9 $\pm$ 1.4	20.9 $\pm$ 0.6 (-5)
PMA	21.5 $\pm$ 1.8	23.2 $\pm$ 0.9 (-8)
8-Bromo cyclic AMP	24.3 $\pm$ 1.5	24.8 $\pm$ 0.7 (-2)

Table 2. Maximal ligand-induced increases in phosphorylation of α -G_i2

The increases in the stoichiometry of phosphorylation of α -G_i2 resulting from treatment of intact rat hepatocytes with PMA (10 ng/ml, 15 min), vasopressin (10 nM, 5 min), glucagon (1 μ M, 5 min) or 8-bromo cyclic AMP (300 μ M, 15 min) in the presence or absence of okadaic acid (1 μ M) were determined as described in the legend to Fig. 1 and in the Experimental section. Where indicated okadaic acid was present throughout the total incubation period (65 min). Data shown are means $\pm$ S.E.M. for three to seven separate determinations.

Treatment	Stoichiometry of labelling of α -G _i 2 (mol/mol)	
	Control	+ Okadaic acid
Basal	0.39 $\pm$ 0.06	1.20 $\pm$ 0.14
PMA	0.62 $\pm$ 0.09	1.10 $\pm$ 0.11
Vasopressin	0.64 $\pm$ 0.07	1.15 $\pm$ 0.06
Glucagon	0.88 $\pm$ 0.10	1.67 $\pm$ 0.11
8-Bromo cyclic AMP	0.92 $\pm$ 0.10	1.85 $\pm$ 0.10

cytes with exclusive protein kinase C activators such as vasopressin and PMA increased the labelling of α -G_i2 to around 0.62 $\pm$ 0.09 and 0.64 $\pm$ 0.07 mol/mol respectively. In contrast with this, treatment of hepatocytes with agents capable of activating both cyclic AMP-dependent protein kinase and (presumably) protein kinase C [11], such as glucagon at high concentrations (1 μ M) and 8-bromo cyclic AMP (300 μ M), increased the labelling of α -G_i2 to 0.88 $\pm$ 0.10 and 0.92 $\pm$ 0.10 mol/mol respectively (Table 2). However, okadaic acid added together with either PMA or vasopressin was no more effective than treatment of hepatocytes with okadaic acid alone when a maximal labelling stoichiometry of 1.2 $\pm$ 0.14 mol/mol (Table 2) was achieved. In marked contrast with this, an additive effect on the phosphorylation of α -G_i2 was seen when okadaic acid-treated hepatocytes were exposed to either 8-bromo cyclic AMP or high glucagon concentrations, giving a stoichiometry of labelling of 1.85 $\pm$ 0.10 and 1.67 $\pm$ 0.11 mol of P_i/mol of α -G_i2 respectively.

We would like to suggest that the lack of additivity between protein kinase C activators and okadaic acid is because under basal conditions, i.e. in the absence of any hormonal challenge, labelling of α -G_i2 is primarily due to the action of protein kinase C. Thus, in the presence of okadaic acid, inhibition of phosphatase activity allows this site to become fully labelled. This suggests that, under resting conditions, the functioning of α -G_i2 is regulated by a dynamic phosphorylation/dephosphorylation system involving protein phosphatases 1 and/or 2A and (primarily) protein kinase C. Certainly, the finding that cyclic AMP levels in hepatocytes under resting conditions are very low [20,30] would be consistent with phosphorylation of this site not being important under basal conditions. The relatively high basal labelling, and the rapid nature of the enhancement of α -G_i2 labelling by okadaic acid, indicate that there may be a high steady-state level of both phosphorylation and dephosphorylation occurring at the proposed protein kinase C site on α -G_i2. The rapid turnover of phosphate on this protein may thus be akin to so-called 'futile cycles' which are seen at key points in metabolic pathways and which may contribute rapid responsiveness to such control points. This may indicate that the control of phosphorylation of α -G_i2 is of some importance in the regulation of cellular functioning.

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