Insulin inhibits the phosphorylation of α -G_i-2 in intact hepatocytes

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Challenge of intact hepatocytes with insulin reduced the level of phosphorylated α -G_i-2 found under basal (resting) conditions. At maximally effective concentrations of insulin the steady-state labelling of α -G_i-2 was reduced by ~ 21 %. Insulin achieved this in a time- and dose-dependent fashion, exhibiting an IC₅₀ value of 109 ± 22 pM. The increased labelling of α -G_i-2 seen after challenge of cells with phorbol 12-myristate 13-acetate was also attenuated by insulin. Treatment of hepatocytes with the protein

phosphatase inhibitor okadaic acid increased the labelling of α -G_i-2 in a fashion which was insensitive to the action of insulin. It is suggested that insulin may reduce the level of phosphorylation of α -G_i-2 by stimulating intracellular protein phosphatase activity and that this action may offer a molecular explanation for the ability of insulin to inhibit adenylate cyclase activity in hepatocytes by increasing the level of non-phosphorylated α -G_i-2.

INTRODUCTION

Members of a family of G-proteins serve to transduce the activity of many cell-surface receptors by allowing them to couple to specific effector, signal generating, systems (see e.g. [1]). The receptor-mediated control of adenylate cyclase is effected by two distinct heterotrimeric G-proteins termed, generically, G_s (stimulatory) and G_i (inhibitory). The 'G_i' family is formed from three highly related, pertussis toxin-sensitive, G-proteins termed G_i-1, G_i-2 and G_i-3 [see 1]. The specificity of coupling of these G-proteins is not yet fully resolved; however, it is generally accepted that at least G_i-2 can mediate inhibitory actions upon adenylate cyclase [2–7].

We [8–10] and others [11–13] have shown that protein kinase C (PKC) activation can lead to the phosphorylation of the α -subunit of the G_i-2 isoform in cells such as hepatocytes, U-937 cells, NG108 cells and platelets, although it does not appear to be characteristic of all cells, implying that this phenomenon may be cell-specific [see 14–16]. In intact hepatocytes, treatment with a range of hormones capable of activating PKC, as well as phorbol 12-myristate 13-acetate (PMA), resulted in the serine-specific phosphorylation of α -G_i-2, but did not affect the labelling of either α -G_i-3 or α -G_s [6,17]. It would seem that G_i-2 may be controlled, in hepatocytes, by a phosphorylation/dephosphorylation cycle [9], as exposure of hepatocytes to the phosphoprotein phosphatase (1 and 2A) inhibitor, okadaic acid, also increased the phosphorylation of α -G_i-2 [9]. α -G_i-2 is, however, subject to multi-site phosphorylation, as ligands capable of activating protein kinase A, such as glucagon and 8-bromo-cyclic AMP, elicit phosphorylation at a separate serine site from that modified by PKC [6,17].

Insulin has been shown to be able to lower intracellular levels of cyclic AMP (cAMP) in hepatocytes [18]. It appears to be able to do this both through eliciting the activation of a type-III cAMP phosphodiesterase and by causing the inhibition of adenylate cyclase activity [18,19]. The mechanism through which

insulin exerts its effect upon adenylate cyclase activity is unknown. However, it has been suggested to occur at the level of control by G-proteins in that its expression is absolutely dependent upon the presence of GTP [19]. α -G_i-2 has been shown to mediate the tonic inhibition of adenylate cyclase that can be achieved by the action of GTP [6]. Such an action is abolished upon the phosphorylation of this G-protein α -subunit [6]. Here we provide evidence which indicates that insulin can reduce the level of phosphorylated α -G_i-2 which, by increasing the pool size of α -G_i-2 able to inhibit adenylate cyclase, may provide an explanation as to how insulin can exhibit a small inhibitory effect on hepatocyte adenylate cyclase activity.

MATERIALS AND METHODS

Materials

Inorganic [³²P]phosphate was obtained from Amersham International, U.K. PMA was from Cambridge Bioscience, U.K. Insulin and protein A-agarose were from Sigma, U.K. Okadaic acid was from Mona Bioproducts, Hawaii, U.S.A. All other biochemicals were from Boehringer Ltd., U.K. and all other chemicals were of AR grade from BDH Ltd., U.K.

Hepatocyte preparation and labelling conditions

Hepatocytes were prepared as previously described [19,20] using 220–250 g fed male Sprague–Dawley rats. Cells (10⁶–10⁷ per ml) were pre-incubated for 65 min at 37 °C in Krebs–Henseleit buffer (see [17]) supplemented with 2.5 % (w/v) BSA, 2.5 mM CaCl₂ and 10 mM glucose and containing 50 μ M potassium phosphate and 1 mCi ³²P_i (see [6,21]). Cells were gassed with O₂/CO₂ (19:1) for 30 s every 10 min. 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 ten volumes of ice-cold Krebs–Henseleit buffer (see [17]). The cells were harvested by centrifugation (100 g for 2 min).

Abbreviations used: G_i, inhibitory G-protein controlling adenylate cyclase activity; G_s, stimulatory G-protein controlling adenylate cyclase activity; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; cAMP, cyclic AMP.

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Immunoprecipitation of α -G_i-2

This was performed as described previously [6,17]. Briefly, a pellet of cells (10^6 /ml) was extracted by the addition of 1 ml of a buffer containing 1% (v/v) Triton-X 100, 10 mM EDTA, 100 mM NaH_2PO_4 , 100 μM Na_3VO_4 , 2 mM phenylmethanesulphonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 nM okadaic acid 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 1867 antisera [17]. Antiserum 1867 was raised [17] in rabbits against the C-terminal decapeptide of the α -subunit of G_i-2 (conjugated to keyhole limpet haemocyanin) and, because of absolute identity over this sequence [see 21], would be expected to recognize α -G_i-1 as well as α -G_i-2. However, since α -G_i-1 cannot be detected in hepatocytes by immunoblotting with specific antisera or by analysis of mRNA [6,22], this antiserum can be used as a specific tool to immunoprecipitate α -G_i-2 in hepatocytes [17]. Antiserum (50 μl) was added to 1 ml of this cell extract and samples were incubated for 12 h at 4 °C. After this period, 50 ml of protein A-agarose (25 μl of packed gel in PBS) was added and the incubation continued for a further 2 h at 4 °C. Immune complexes were collected as protein A-agarose pellets by centrifugation (4 °C, 100 g, 1 min) and the pellets were washed twice in a buffer containing 1% (v/v) Triton-X 100, 0.1% (w/v) SDS, 100 mM NaCl, 50 mM NaH_2PO_4 and 50 mM Hepes, pH 7.2, and once with the above buffer but lacking SDS. Immunoprecipitation of α -G_i-2, detected by subsequent immunoblotting or the presence of a ~40 kDa phosphorylated species, only occurred in the presence of a specific antiserum in the incubation mixture. It was not apparent if immunoglobulins from pre-immune sera or sera raised against a decapeptide to α -G_s were used, nor if protein A-agarose alone was employed. Furthermore, it could be competed out with the C-terminal decapeptide from either α -transducin or α -G_i-2 but not the C-terminal decapeptide from α -G_s, a heteromeric G-protein, [see 17]. Tryptic phosphopeptide analyses were done exactly as described in [17] using the high temperature/prolonged hydrolysis procedure which resolves three ^{32}P -labelled peptides (C1, C2 and C3) from α -G_i-2 immunoprecipitated from cells under basal conditions and after stimulation of endogenous PKC activity using PMA. These reflect a single site of phosphorylation with C3 being the smallest peptide [17].

Gel electrophoresis and autoradiography

Before SDS/PAGE, protein A-agarose pellets were resuspended in Laemmli sample buffer [23] 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 60 mA for 2 h using 10% acrylamide gels. After electrophoresis, gels were dried and subjected to autoradiography. Gels were scanned and analysed quantitatively using a Shimadzu C5-9000 dual wavelength flying spot scanning densitometer. Labelled bands of interest were excised and radioactivity was determined by Cerenkov counting.

RESULTS AND DISCUSSION

A proportion of G_i-2 is phosphorylated on its α -subunit in hepatocytes under resting (basal) conditions [6,17] and this can be identified in hepatocytes which have been incubated with $^{32}\text{P}\text{P}_i$. Such labelling occurs at a single serine site, thought to be Ser¹⁴⁴, whose phosphorylation is mediated by the action of PKC [17]. Increased labelling at this site can be achieved not only

Table 1 Insulin-mediated actions upon the phosphorylation of α -G_i-2 in hepatocytes

Hepatocytes were labelled with $^{32}\text{P}\text{P}_i$ as described in the Materials and methods section. Equal amounts of protein (see Materials and methods section) were then treated for the indicated times with ligands before harvesting and extraction. Immunoprecipitated α -G_i-2 was subjected to SDS/PAGE with subsequent autoradiography used to locate labelled α -G_i-2 on the gels. Bands containing labelled α -G_i-2 were excised and subjected to Cerenkov counting. Data are shown here as percentage changes in the labelling of α -G_i-2 determined relative to that noted under basal conditions (absence of any added ligand): i.e. a 100% increase would be a doubling of the amount of label incorporated and -50% would be a reduction in labelling by one half. *n* is the number of separate experiments done and errors are given as S.D. Significance of inhibition (given in parentheses) assesses the significance of the inhibitory effect of insulin as assessed using *t*-test analysis; ns, not significant.

Ligand	Added alone	Plus insulin	<i>n</i> (Significance of inhibition)
None (basal)	—	-21 ± 3	18 (0.001)
Okadaic acid	112 ± 16	111 ± 15	4 (ns)
PMA	23 ± 4	12 ± 3	10 (0.01)
PMA + okadaic acid	88 ± 9	97 ± 15	4 (ns)

Figure 1 Time-dependent reduction in the labelling of α -G_i-2, elicited by insulin

Hepatocytes were labelled with $^{32}\text{P}\text{P}_i$ as described in the Materials and methods section. Equal amounts of protein (see Materials and methods section) were then treated for the indicated times with insulin (100 nM) before harvesting and extraction. Immunoprecipitated α -G_i-2 was subjected to SDS/PAGE with subsequent autoradiography used to locate labelled α -G_i-2 on the gels, as shown in some detail in [6,8–10,17,24,29]. Bands containing labelled α -G_i-2 were excised and subjected to Cerenkov counting. Data are shown here as the percentage change in the labelling of α -G_i-2 elicited by insulin for an experiment done five times with errors given as S.D.

through the activation of PKC by, for example, PMA ([17]; Table 1), but also by inhibition of protein phosphatase activity elicited by okadaic acid ([17]; Table 1). This shows that, under resting conditions in hepatocytes, α -G_i-2 is at the centre of an active phosphorylation/dephosphorylation cycle which determines the magnitude of the phosphorylated α -G_i-2 pool [15,24]. Such a system allows the ratio of non-phosphorylated/phosphorylated α -G_i-2 to be determined through processes which affect the activity of either appropriate PKC isoforms or phosphatases [15]. This system can be expected to influence the activity of adenylate cyclase, as only the non-phosphorylated form of α -G_i-2 can effect a small inhibitory effect on this enzyme,

Figure 2 Dose-dependent reduction in the labelling of α -G_i-2 elicited by insulin

Hepatocytes were labelled with [32 P] P_i , as described in the Materials and methods section. Equal amounts of protein (see Materials and methods section) were then treated for 15 min with the indicated concentrations of insulin before harvesting and extraction. Immunoprecipitated α -G_i-2 was subjected to SDS/PAGE with subsequent autoradiography used to locate labelled α -G_i-2 on the gels, as shown in some detail in [6,8–10,17,24,29]. Bands containing labelled α -G_i-2 were excised and subjected to Cerenkov counting. Data are shown here as the percentage change in the labelling of α -G_i-2 elicited by insulin for an experiment done five times with errors given as S.D.

a situation which can be expected to occur tonically in intact hepatocytes due to the high intracellular levels of GTP that are present [see 15].

Here we show that treatment of hepatocytes with the hormone insulin reduced the labelling of the α -G_i-2 pool in hepatocytes (Figures 1 and 2). Insulin achieved this in a time-dependent fashion, with a new steady-state level of labelling occurring around 5 min after challenge of the cells with maximally effective doses of insulin (Figure 1). This level of labelling was some 20–25% lower than that seen in untreated cells (Table 1) and was achieved in a dose-dependent fashion (Figure 2) with an IC_{50} value of 109 ± 22 pM insulin ($n = 4$ separate experiments; errors are S.D.). This value is consistent with an action mediated through high-affinity insulin receptors (see [25,26]) and is comparable with values reported for the effects of insulin on hepatocyte processes which include the activation of cAMP phosphodiesterase activity and the inhibition of adenylate cyclase (see [18,19,27]).

In hepatocytes, under resting conditions, we have been able to immunoprecipitate a phosphorylated fraction of α -G_i-2 [6,9,17]. Extensive digestion of this immunoprecipitated species with trypsin then allowed us to identify three labelled phosphopeptides, called C1, C2 and C3 [17]. These reflect partial digestion products containing a single labelled residue which has been deduced to be Ser¹⁴⁴ [17]. We see here (Figure 3) that an identical mobility pattern was obtained for labelled tryptic phosphopeptides from hepatocytes challenged with insulin with that found in the absence of added insulin. However, analysis of the degree of labelling of this family of peptides showed a reduction of 19–26% (range; $n = 3$ separate experiments) compared with that seen for this peptide family isolated from cells which had not been treated with insulin. This shows that insulin solely elicits a reduction in the labelling at the C-site

Figure 3 Labelled phosphopeptide maps of hepatocyte α -G_i-2 done in the presence of insulin

Hepatocytes were pre-labelled with [32 P] P_i and α -G_i-2 was specifically immunoprecipitated before subsequent treatment with trypsin to generate peptides, which were then resolved by 2-D chromatography, as described in some detail in [17]. This shows the labelling pattern for extracts made from cells treated with insulin (100 nM) for 5 min. The labelled spots identified migrated in an identical fashion with those found using hepatocytes which had not been challenged with insulin (results not shown; see [17]). As described in [17], these are a doublet of C1 and C2, which migrate very close together, and the species C3. In no instances were additional radiolabelled spots identified and all of the labelling in these spots was shown to be due to phosphoserine and not phosphotyrosine (results not shown; determination as in [17]). The relative labelling of the three spots was unchanged by insulin action. This is consistent with the contention [17] that these species reflect partial degradation products containing a single modified serine residue, Ser¹⁴⁴ [17] with C3 as the smallest peptide and with C2 being larger than C1. SF denotes the solvent front. The 2,4-dinitrophenyl (DNP)-lysine marker is indicated. These data are typical of an experiment done three times.

(C-peptide group) and does not cause any associated phosphorylation of α -G_i-2 at another site. It was important to identify this as studies *in vitro* have shown that purified α -G_i-2 can serve as a substrate, albeit a poor one, for tyrosyl phosphorylation by the purified insulin receptor [28]. However, such tyrosyl phosphorylation *in vitro* only appeared to occur when the G-protein was in its holomeric, GDP-bound state, was also of low stoichiometry and required high concentrations of G-protein [28]. Our studies here indicate that such tyrosyl phosphorylation is unlikely to be of importance *in situ* in the intact cell, in agreement with earlier studies which have failed to identify the tyrosyl phosphorylation of α -G_i-2 in intact hepatocytes [8,10,11].

In order to determine whether insulin could exert its inhibitory action on α -G_i-2 phosphorylation in hepatocytes under chronic stimulation of PKC, we challenged the cells with PMA. This is known to activate PKC and has been shown to increase the steady-state labelling of α -G_i-2 [6,9,17]. Despite this, insulin was seen to attenuate the degree of labelling of α -G_i-2 (Table 1). In marked contrast, however, when the protein phosphatase (1 and 2A) inhibitor okadaic acid [29] was added to the cell incubations, insulin failed to decrease the labelling of α -G_i-2 in either the absence or presence of PMA (Table 1).

Potentially, insulin could attenuate the labelling of α -G_i-2 in a number of ways. These might include the inhibition of PKC, the activation of protein phosphatase activity or by interacting with α -G_i-2 in some fashion such that it attenuates the ability of this G-protein to undergo phosphorylation. However, that insulin failed in its ability to attenuate the phosphorylation of α -G_i-2 in the presence of the protein phosphatase inhibitor okadaic acid, might indicate that insulin reduced the level of phosphorylation of α -G_i-2 through the stimulation of protein phosphatase activity. In this regard, there are a number of reported instances where

insulin has been shown to decrease the phosphorylation of proteins in, for example, both hepatocytes and adipocytes through processes shown to involve protein phosphatase activation [29,30].

Insulin has been shown to exert an inhibitory effect upon adenylate cyclase activity in hepatocytes. However, the effect is small and is certainly not a feature that is seen in the majority of cells which express insulin receptors (see [16,19,26,31]). Insulin appears, however, to exert an inhibitory effect which is of a similar order to that seen for the GTP-triggered inhibitory action of G_i and which is both similarly GTP-dependent and blocked by the action of pertussis toxin [19,32]. The insulin receptor, though, has a structure which bears no resemblance to G-protein linked species (see [25,26]) and is thus unlikely to be able to couple directly to G_i . However, by lowering the amount of phosphorylated α - G_i -2 in hepatocytes, insulin might be expected to increase the pool of non-phosphorylated α - G_i -2 able to exert a tonic GTP-mediated inhibitory effect on adenylate cyclase. This could account for the small inhibitory effect that insulin exerts in intact hepatocytes due to the high endogenous GTP levels which occur there (see [31]). Indeed, the level of phosphorylation of α - G_i -2 in hepatocytes is $\sim 40\%$ of the total [24]. Thus, as insulin appears to cause a reduction of this phosphorylated pool by $\sim 25\%$, the level of the active, non-phosphorylated α - G_i -2 can be expected to rise by $\sim 20\%$. This level of change is very similar to the degree of inhibition of adenylate cyclase that can be determined in hepatocytes [32,33]. Such a mechanism also offers an explanation for the observation that insulin inhibition of adenylate cyclase is not a ubiquitous event, as it would depend not only upon the occurrence of insulin receptors but also on the presence of a pool of phosphorylated G_i whose subsequent dephosphorylation was sufficient to elicit a functional inhibition, a feature which would depend upon the size of the G_i pool and the isoforms of adenylate cyclase present. Thus the ability of insulin to inhibit adenylate cyclase may in itself be a highly cell specific effect.

This work was supported by an SERC-CASE (SKB) award to N.J.M., by a grant from the MRC to M.D.H. and by an equipment grant from the Wellcome Trust to M.D.H.

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