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INSULIN AFFECTS THE ABILITY OF G_i TO BE ADP-RIBOSYLATED BUT DOES NOT ELICIT ITS PHOSPHORYLATION IN INTACT HEPATOCYTES

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SUMMARY Insulin inhibited the ability of activated pertussis toxin to catalyse the ADP-ribosylation of alpha-G_i in isolated plasma membranes in either the absence of added guanine nucleotides or in the presence of GTP. In contrast, when the non-hydrolysable GTP analogue guanylyl-5'-imido-diphosphate (p[NH]ppG) was added to ribosylation mixtures, to inhibit the action of pertussis-toxin in catalysing the ADP-ribosylation of alpha-G_i, then the addition of insulin attenuated the action of p[NH]ppG causing an increase in alpha G_i ribosylation. Pre treatment of intact hepatocytes with insulin had no effect on the subsequent ability of thiol-preactivated pertussis toxin to cause the ADP-ribosylation of alpha G_i using isolated membranes from such cells. The ability of p[NH]ppG to inhibit forskolin-stimulated adenylate cyclase activity was attenuated in the presence of insulin. Insulin did not cause the phosphorylation of alpha-G_i in either intact hepatocytes or in isolated membranes.

INTRODUCTION Insulin exerts a variety of actions upon target cells, although the precise molecular mechanism through which it achieves such effects has yet to be firmly established. The insulin receptor, however, does express a potent tyrosyl kinase activity and although putative substrates have been identified the physiological significance of these events has still to be determined [1-3].

Many receptors are coupled to distinct guanine nucleotide regulatory proteins (G proteins) which serve to transduce actions upon specific signal generation systems, such as adenylate cyclase [4,5]. We have provided evidence indicating that the insulin receptor might interact with the G-protein system and, in particular, that there might be a specific G-protein which transduced certain of insulin's actions [2,3,6]. Since then it has been shown by us [7] and others [8,9] that the purified insulin receptor can cause the phosphorylation of various purified G-proteins such as G_i, G_o, transducin and $p21^{ras}$ when they are in their GDP-bound form. More recently

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we [10] have shown that the alpha subunit of G_i can be phosphorylated in intact cells upon activation of protein kinase C.

In this study we show that whilst insulin does not elicit the phosphorylation of G_i in intact hepatocytes, it does attenuate the ability of p[NH]ppG to inhibit the pertussis toxin-catalysed ADP ribosylation of G_i in isolated hepatocyte membranes.

MATERIALS AND METHODS Forskolin was from Calbiochem, Cambridge, U.K. Purified homogenous pertussis toxin was a kind gift from Professor John Freer, Department of Microbiology, University of Glasgow, U.K. Bovine insulin was from Eli Lilly & Co., Indianapolis, IN. USA. All other biochemicals were from Boehringer (UK), Lewes, East Sussex, U.K. All general chemicals were of A.R. grade from BDH Chemicals, Poole, Dorset, U.K. Radiochemicals were from Amersham International, Amersham, Bucks, U.K.

Male Sprague Dawley rats (200-270g) were used. Hepatocytes were prepared and incubated as described in detail previously by us [11] as was the preparation of a washed membrane fraction [12,13]. Adenylate cyclase activity was assessed from linear timecourses as described in detail previously by us [11]. ADP-ribosylation of hepatocyte membranes using thiol pre activated pertussis toxin was done as described in detail before by us [14]using 10 min incubation at 30°C. Immunoprecipitation of the alpha subunit of G_i from either hepatocytes labelled with ^{32}P or membranes with ^{32}P-gamma-ATP was done as described before by us [10]. This employed the antiserum AS7 produced against the C-terminal decapeptide of rod transducin. This sequence differs in but a single amino acid from the equivalent C-terminal region of alpha G_i 2, the dominant G_i species in rat hepatocytes. It will also recognise alpha G_i 1, which is not expressed in rat hepatocytes. Also, isolated [11] hepatocyte membranes (10ug) were incubated under similar conditions to the ribosylation assay with final concentrations of 20uM-ATP, 10uCi [^{32}P]-gamma ATP, 5mM $MgCl_2$ and 20mM-Tris HCl final pH7.9 in a final volume of 300ul for 5 and 10 min at 30°C. The reaction was terminated by the addition of the immunoprecipitation cocktail with subsequently resolution by SDS-PAGE autoradiography [10].

RESULTS AND DISCUSSION There is a growing body of evidence which suggests that the insulin receptor can interact with the inhibitory guanine nucleotide regulatory protein G_i. The first indications came from studies which showed that the ability of insulin to stimulate a high affinity cyclic AMP phosphodiesterase in both hepatocytes [15] and adipocytes [16] could be blocked by pre-treatment of the cells with pertussis toxin. Thereafter, itwas demonstrated [7,9] that pure G_i could be phosphorylated by the pure human insulin receptor tyrosyl kinase.

We have previously shown [17] that insulin can attenuate the ability of cholera toxin to cause the ADP-ribosylation of a circa 25kDa in hepatocyte membranes. Using a similar approach we show here that insulin can attenuate the ability of pertussis toxin to cause the ADP-ribosylation of the alpha-subunit of G_i in hepatocyte membranes (Fig.1a). Such an inhibition of ADP-ribosylation was seen when either no exogenous guanine nucleotide was added to the ribosylation assay or when GTP (10^{-4}M) was present (Fig.1a).

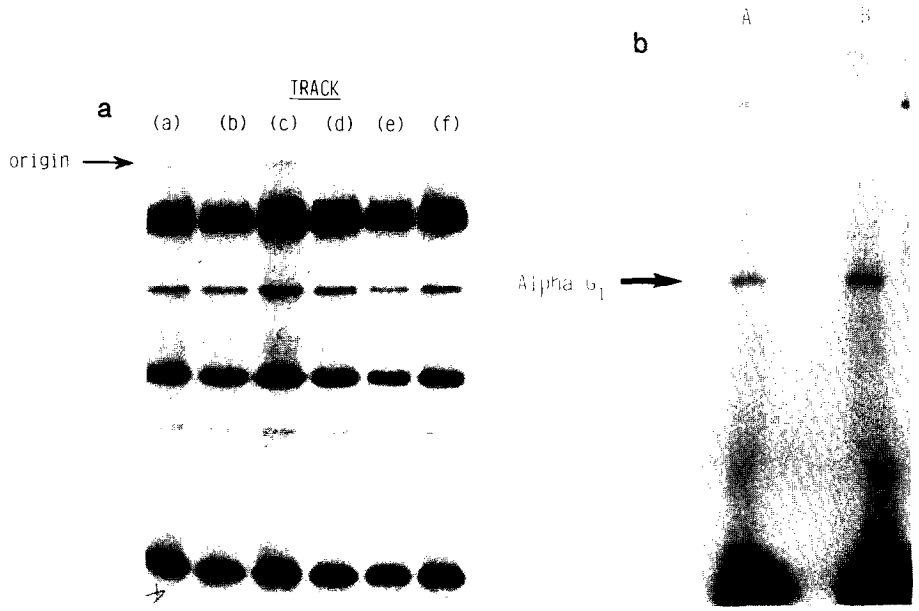

Figure 1. ADP-ribosylation of alpha-G_i by pertussis toxin
(a) Isolated hepatocyte membranes were treated as described before [14] with thiol pre-activated pertussis toxin and [^{32}P]-NAD$^+$. This was done for 10 min at 30°C over which time there was a linear incorporation of label into the 40kDa alpha subunit of G_i (labelled band in the centre of each track). Gel tracks each contained 50ug of membrane protein. Incubations were done in either the absence (tracks a,c and e) or presence (tracks b,d and f) of 10^{-9}M insulin. In tracks 'a,b' no addition of guanine nucleotides were made; in tracks 'c,d' GTP (10^{-4}M) was added and in tracks 'e,f' p[NH]ppG (10^{-9}M) was added to the ribosylation assay. Data shows a typical experiment. (b) Intact hepatocytes either were (track A) or were not (track B) pre-treated for 10 min with insulin (10^{-9}M) prior to making a washed membrane fraction. Pertussis toxin catalysed ADP-ribosylation was then performed as above in the absence of added guanine nucleotides. Data shows a typical experiment.

During our study we noted that Rothenberg and Kahn [18] also observed that insulin could reduce the pertussis toxin-catalysed ADP-ribosylation of G_i when GTP was present. In our hands, the insulin-catalysed reduction in labelling was 35+/-6% in the absence of GTP and 32+/-7% in the presence of GTP (n=6; errors are S.D.)

It has been shown by others [19] that when G_i is activated by non-hydrolysable guanine nucleotide analogues, such as p[NH]ppG, it dissociates and the free guanine nucleotide bound alpha-subunit provides a poor substrate for ADP-ribosylation by pertussis toxin. Indeed, we see here that treatment of hepatocyte membranes with the non-hydrolysable GTP-analogue p[NH]ppG markedly inhibits (60-70%, range) the ADP-ribosylation of G_i (Fig.1a) as might be expected. Rothenberg and Kahn [18] suggested that the inhibition of ribosylation of G_i ensued because insulin promoted G_i dissociation. However, we show here that if insulin was added together with p[NH]ppG then the labelling of alpha-G_i was actually greater (21%+/-4%; n=6, errors are S.D.) than that observed when pertussis toxin-catalysed

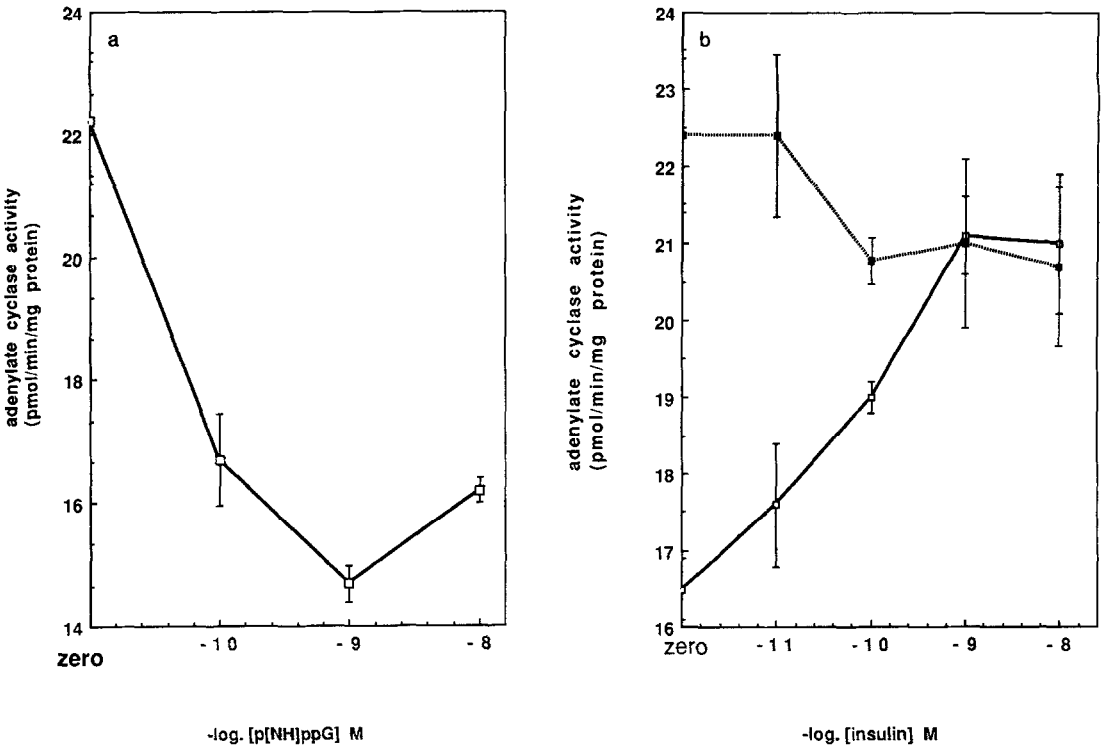

Figure 2. p[NH]ppG-mediated inhibition of adenylate cyclase activity
(a) Adenylate cyclase activity was determined from linear time courses at 30°C in the presence of the diterpene, forskolin (0.1mM). This yielded an activity of 22.2pmol/min/mg protein. Assays were then done in the presence of increasing concentrations of p[NH]ppG in order to activate the inhibitory G-protein G_i, selectively [21,22]. (b) Adenylate cyclase was assayed with forskolin (0.1mM) and increasing concentrations of insulin in either the absence (■) or presence (□) of 10^{-8}M p[NH]ppG.

labelling was done with p[NH]ppG alone. Thus insulin cannot be simply eliciting the dissociation of G_i. Indeed, if insulin did promote G_i dissociation, then insulin might be expected to inhibit adenylate cyclase activity by a G_i-mediated process. However, we have demonstrated that whilst insulin does inhibit adenylate cyclase activity in both intact hepatocytes and in isolated membranes [20] it does so by a process which does not appear to involve G_i [21].

That insulin appears to modify the ability of p[NH]ppG to attenuate ADP-ribosylation of G_i suggests that actions of this hormone might alter the conformation of G_i. The inhibitory effect of G_i on adenylate cyclase can be assessed [22] in membranes by following the ability of low concentrations of p[NH]ppG to inhibit the activity of adenylate cyclase which has been amplified by the diterpene forskolin (Fig.2a). We note here, that this inhibitory effect of p[NH]ppG could be attenuated by insulin in a dose dependent fashion with a K_a 7.9+/-1.5x10^{-11}M for p[NH]ppG (Fig.2b).

It may be that, under these conditions, the G_i mediated inhibition of forskolin-stimulated adenylate cyclase activity reflects predominantly the

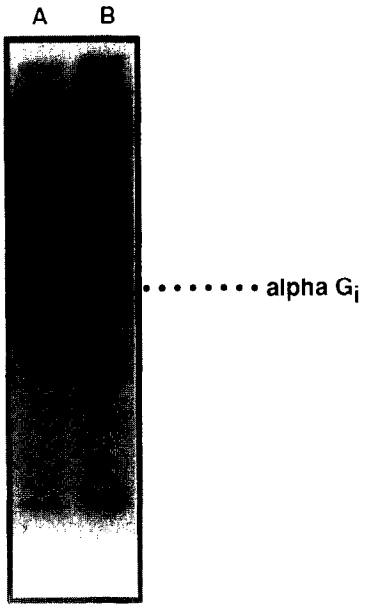

Figure 3. Immunoprecipitation of alpha-G_i from [^{32}P]-labelled hepatocytes
Isolated hepatocytes were labelled with [^{32}P]-P_i and then either not challenged (track A) or challenged (track B) with insulin (10^{-8}M) for 10 min at 30°C. Hepatocytes were harvested, alpha G_i-specifically immunoprecipitated and analysed by SDS-PAGE as described in detail previously by us [10].

action of the alpha subunit of G_i on the catalytic unit of adenylate cyclase because G_s-stimulation, whose blockade can be effected by beta-gamma complexes, is only apparent at much higher concentrations of p[NH]ppG [21,22]. Thus our experiments might imply that insulin leads to an altered conformation of G_i which reduces its inhibitory potency. This appears to be mediated via high affinity insulin receptors.

We attempted to define whether the actions of insulin were mediated by it causing the phosphorylation of G_i. However, using either isolated membranes (data not shown) or intact hepatocytes (Fig.3) we were unable to provide any evidence consistent with insulin increasing the phosphorylation of the alpha subunit for G_i. We noted that under resting (basal) conditions, the immunoprecipitated alpha-subunit of G_i contained radioactivity. This was phosphoserine. There was no change in labelling or the type of phosphoamino acid present (data not shown) after exposure of the cells to insulin. Despite our failure to observe any insulin-mediated changes in G_i, we have been able to show previously [10], and would confirm here (data not shown) that both glucagon and phorbol esters can elicit the phosphorylation of G_i in intact hepatocytes.

Our studies indicate that a direct interaction of the occupied insulin receptor with G_i might be responsible for eliciting a change in the conformation of this G-protein rather than insulin eliciting any stable

(covalent) modification of G_i. Consistent with our theory that insulin causes no stable modification of G_i, we noted that pre treatment of intact hepatocytes for 5 min with insulin, prior to making washed membranes, had no effect on the subsequent ability of pertussis toxin to cause the ADP-ribosylation of G_i (Fig.1b).

The present study shows that the insulin receptor appears to interact with G_i and suggests that in doing so it does not promote G_i dissociation but, rather alters the conformation of its alpha subunit. This effect can be detected by alterations in the ability of pertussis toxin to elicit the ADP-ribosylation of alpha-G_i.

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