Islet-activating protein blocks glucagon desensitization in intact hepatocytes

Clare M. HEYWORTH,* Emmanuel HANSKI† and Miles D. HOUSLAY*†

*Department of Biochemistry and Applied Molecular Biology, U.M.I.S.T., P.O. Box 88, Manchester M60 1QD, U.K., and †Department of Hormone Research, Weizmann Institute of Science, Rehovot, Israel

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Treatment of intact hepatocytes with islet-activating protein, from *Bordatella pertussis*, led to a pronounced increase in the ability of glucagon to raise intracellular cyclic AMP concentrations. Islet-activating protein, however, caused no apparent increase in the intracellular concentration of cyclic AMP under basal conditions. These effects were attributed to an enhanced ability of adenylate cyclase, in membranes from hepatocytes treated with islet-activating protein, to be stimulated by glucagon. When forskolin was used to amplify the basal adenylate cyclase activity, elevated GTP concentrations were shown to inhibit adenylate cyclase activity in membranes from control hepatocytes. This inhibitory effect of GTP was abolished if the hepatocytes had been pre-treated with islet activating protein. In isolated liver plasma membranes, islet-activating protein caused the NAD-dependent ribosylation of a M_r -40000 protein, the putative inhibitory guanine nucleotide regulatory protein, N_i . This effect was inhibited if guanosine 5'-[β -thio]diphosphate rather than GTP was present in the ribosylation incubations. The ability of glucagon to uncouple or desensitize the activity of adenylate cyclase in intact hepatocytes was also blocked by pre-treating hepatocytes with islet-activating protein. Islet-activating protein thus heightens the response of hepatocytes to the stimulatory hormone glucagon. It achieves this by both inhibiting the expression of desensitization and also removing a residual inhibitory input expressed in the presence of glucagon.

Islet-activating protein is one of the pertussis toxins that can be isolated and purified to homogeneity from the culture medium of the whooping-cough bacterium *Bordatella pertussis* (Yajima *et al.*, 1978a,b). It is so named because it was noticed first to cause a sustained potentiation of the secretory response of pancreatic islet cells to a variety of stimuli (see, e.g., Katada & Ui, 1979, 1980). More recently it has become of intense interest owing to its ability to block the receptor-mediated inhibition of adenylate cyclase (see, e.g., Katada & Ui, 1981; Hazeki & Ui, 1981; Kurose *et al.*, 1983). It has also been demonstrated to potentiate β -adrenergic stimulation of adenylate cyclase in C6-glioma cells (Katada *et al.*, 1982). Islet-activating protein appears to exert these effects on adenylate cyclase by triggering the ADP-ribosylation of a specific guanine nucleotide

regulatory protein, called N_i (Katada & Ui, 1982; Hanski & Gilman, 1982; Murayama *et al.*, 1983; Kurose *et al.*, 1983; Codina *et al.*, 1983; Bokoch *et al.*, 1983). This guanine nucleotide regulatory protein is believed to mediate the action of inhibitory receptors on adenylate cyclase (see, e.g., Hildebrandt *et al.*, 1982, 1983; Jakobs & Schultz, 1983).

We have demonstrated that incubation of intact hepatocytes with glucagon leads to the dose-dependent desensitization or 'uncoupling' of plasma-membrane adenylate cyclase from stimulation by glucagon (Heyworth & Houslay, 1983a). This was suggested as being due to a lesion occurring at the level of the guanine nucleotide regulatory protein N_i , involved in mediating the stimulatory action of glucagon on adenylate cyclase, rather than on either the receptor itself or the catalytic unit of adenylate cyclase. Here we investigate the effect of exposure of intact hepatocytes to islet-activating protein on the ability of glucagon both to activate adenylate cyclase and to cause its 'uncoupling'.

† To whom reprint requests should be sent. Present address: Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Materials and methods

Islet-activating protein from *Bordetella pertussis* was purified by a modification (Sekura *et al.*, 1984) of the method of Yajima *et al.* (1978a,b). Hepatocytes were prepared from 200–300 g fed male Sprague–Dawley rats and incubated under conditions described previously in some detail (Heyworth *et al.*, 1983). All methods for determination of intracellular cyclic AMP and ATP concentrations were performed as described in that paper. Purified rat liver plasma membranes were prepared as in Marchmont *et al.* (1981). Their ribosylation, with islet-activating protein, was followed by using nicotinamide-[adenylate- ^{32}P] adenine dinucleotide. The condition for this used the methods described in detail by Hildebrandt *et al.* (1983). After incubation for 20 min, the samples were frozen rapidly in an acetone/solid- CO_2 mixture. They were then allowed to thaw out in the presence of sodium dodecyl sulphate sample buffer as defined by Laemmli (1970), and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as Laemmli (1970). In this instance 5%-acrylamide stacking gels were used, with a 12%-acrylamide running gel.

All materials were from sources as specified in the cited publications unless otherwise stated. Forskolin (7 β -acetoxy-9,13-epoxy-1 α ,6*N*,9 α -trihydroxyabd-14-en-11-one) was from Calbiochem. All radiochemicals, including radiolabelled marker proteins for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, were from Amersham International, Amersham, Bucks., U.K., except for nicotinamide-[adenylate- ^{32}P]adenine dinucleotide, which was from N.E.N., Southampton, U.K.

Results

Exposure of hepatocytes to glucagon elicits a dramatic elevation of the intracellular concentration of cyclic AMP. This is achieved through the activation of adenylate cyclase activity (see, e.g., Heyworth *et al.*, 1983). Fig. 1 shows that preincubation of hepatocytes with islet-activating protein (100 ng/ml) for 1 h led to a pronounced increase in the amount of intracellular cyclic AMP formed in response to such a challenge with glucagon (10 nM).

These experiments were performed in the presence of 3-isobutyl-1-methylxanthine (1 mM), which blocks the activity of cyclic AMP phosphodiesterase in these cells (Heyworth *et al.*, 1983). Indeed, after 10 min exposure to glucagon, cyclic AMP concentrations in hepatocytes treated with islet-activating protein were some 60–70% (range of three separate experiments) above those obser-

ved in control cells which had been preincubated for 1 h before challenge with glucagon. We noted, however, that preincubation of control cells for 1 h did not affect their capacity to respond to glucagon (<5% difference; also cf. data of Heyworth *et al.*, 1983).

Although such observations were made of the glucagon-stimulated increases in intracellular cyclic AMP production, we observed negligible (<10%) changes in the basal concentration of cyclic AMP on treatment with islet-activating protein (100 ng/ml) for 1 h.

Using a membrane fraction (Houslay & Elliott, 1979, 1981; Heyworth & Houslay, 1983a,b) derived from hepatocytes treated for 1 h with islet-activating protein (100 ng/ml), we observed that basal adenylate cyclase activity was little affected compared with the appropriate control ($-1 \pm 12\%$). However, in these membranes, the adenylate cyclase activity stimulated by GTP (0.1 mM) + forskolin (0.1 mM) was increased by some $36 \pm 7\%$, and that stimulated by glucagon (10 nM) + GTP (0.1 mM) was increased by $66.5 \pm 6.4\%$ over that of the adenylate cyclase activity expressed in membranes from the control cells. The specific activities for the adenylate cyclase activities under these various states were, in control cells for basal activity 0.43 ± 0.02 , for activity with GTP + forskolin 20.2 ± 2.02 and for activity with glucagon + GTP 14.8 ± 0.46 pmol/min per mg of protein respectively (results are means $\pm$ S.E.M. for three separate experiments using different cell preparations and triplicate determinations of adenylate cyclase activity).

Using forskolin (0.1 mM), which activates the adenylate cyclase activity in hepatocyte plasma membranes (Whetton *et al.*, 1983), we observed that, on increasing the GTP concentrations above about 50 nM, the activity of adenylate cyclase was actually inhibited (Fig. 2). However, this effect was abolished in membranes from hepatocytes that had been pre-treated with islet-activating protein (100 ng/ml) for 1 h.

Treatment of hepatocytes with glucagon has been shown to lead to the 'uncoupling' or desensitization of hormone-stimulated adenylate cyclase in these cells (Heyworth & Houslay, 1983a). This is readily shown by the time-dependent decrease in the (glucagon + GTP)-stimulated adenylate cyclase activity in membranes derived from glucagon-treated hepatocytes. We see here, however, that if cells were preincubated for 1 h with islet-activating protein (100 ng/ml) before being exposed to glucagon (10 nM), no such 'uncoupling' occurred (Fig. 3). Nevertheless, in control cells incubated for 1 h without islet-activating protein, 'uncoupling' occurred (Fig. 3), as reported previously (Heyworth & Houslay, 1983a).

Fig. 1. Treatment of hepatocytes with islet-activating protein heightens the ability of glucagon to raise intracellular cyclic AMP concentrations

Cells were preincubated for 1 h either with (●) or without (▲) islet-activating protein (100 ng/ml) before challenge with glucagon (10 nM) at zero time. Incubations were performed at 37°C in the presence of 1 mM-3-isobutyl-1-methylxanthine to inhibit cyclic AMP phosphodiesterase activity (Heyworth *et al.*, 1983). Intracellular cyclic AMP was determined as described in the Materials and methods section. Basal intracellular cyclic AMP concentrations were 1.75 ± 0.28 pmol/mg dry wt of cells in control hepatocytes and 2.05 ± 0.26 pmol/mg dry wt. of cells for hepatocytes pre-treated with islet activating protein (S.E.M., $n = 3$). Results are shown of a typical experiment which was performed three times with different cell preparations. Results are means

Fig. 2. Inhibition of adenylate cyclase caused by high concentrations of GTP

Hepatocyte plasma membranes were prepared from hepatocytes that either had (▲) or had not (●) been preincubated for 1 h with islet-activating protein (100 ng/ml). They were then assayed for adenylate cyclase activity in the presence of forskolin (0.1 mM) with increasing concentrations of GTP. Results are shown as a percentage change in activity in response to GTP for a typical experiment which was repeated three times with different cell preparations. The adenylate cyclase activity in membranes from control cells stimulated by forskolin was 36 ± 6 pmol/min per mg of protein and that in membranes from cells treated with islet-activating protein was 41 ± 3 pmol/min per mg of protein (means $\pm$ S.D. for three different membrane preparations).

If isolated plasma membranes were treated with islet-activating protein and [32 P]NAD, in the presence of GTP (1 mM), we were able to observe the labelling of a major band of $M_r 40000$ (Fig. 4, tracks *a* and *b*). Such labelling was, however, decreased markedly if guanosine 5'-[β -thio]diphosphate replaced GTP in the incubations (Fig. 4, tracks *c* and *d*).

The effect of islet-activating protein on inhibitory responses connected with adenylate cyclase is dependent on the concentration of islet-activating protein used, the time and temperature of exposure and the particular cell type under investigation

$\pm$ S.E.M. for triplicate determinations of intracellular cyclic AMP concentration. The intracellular cyclic AMP concentration after 5 min exposure to glucagon was increased by some 50–60% (range) in cells that had been pre-treated with islet-activating protein.

Fig. 3. Treatment of hepatocytes with islet-activating protein abolishes glucagon desensitization.

Hepatocytes which either had (▲) or had not (●) been pre-treated for 1 h with islet-activating protein (100 ng/ml) were challenged with glucagon (10 nM). At appropriate time intervals membranes were made from these cells and assayed for their (glucagon + GTP)-stimulated adenylate cyclase activity. The percentage decrease in this activity is plotted against time of exposure to glucagon. These results show a typical experiment which was repeated three times using different cell preparations. In control cells the specific adenylate cyclase activity was 14.8 ± 0.46 pmol/min per mg of protein. This was increased by some $66.5 \pm 6.4\%$ in membranes from hepatocytes treated with islet-activating protein ($n = 3$ for different cell preparations: means $\pm$ S.E.M.).

(see, e.g., Katada *et al.*, 1982; Hazeki & Ui, 1981; Murayama *et al.*, 1983). We observed little significant difference in the effects reported here by incubating either with islet-activating protein at $1 \mu\text{g/ml}$ instead of 100 ng/ml for 1 h or by incubating for up to 2 h with 100 ng of islet-activating protein/ml. In this respect, hepatocytes respond somewhat faster to islet-activating protein than do other cell types, as shown for cholera toxin (Houslay & Elliott, 1979, 1981).

We observed, however, that incubation of hepatocytes for 1 h with islet-activating protein (100 ng/ml) led to a decrease in the intracellular ATP concentration. This fell from 10.6 ± 1.0 to $5.5 \pm 0.5 \text{ nmol/mg dry wt. of cells}$ (means $\pm$ S.E.M. for three separate experiments, with duplicate determinations of ATP). Such a fall in intracellular ATP concentrations has, however, been shown

Fig. 4. Islet-activating-protein-catalysed ribosylation of liver plasma membranes

Isolated purified liver plasma membranes were ribosylated by using islet activating protein and labelled NAD^+ as described in the Materials and methods section. Labelling was performed in the presence of either GTP (1 mM), in tracks (a) and (b), or guanosine 5'-[β -thio]diphosphate (1 mM), in tracks (c) and (d). In the absence of islet-activating protein no labelling occurred (results not shown). This shows a typical experiment which was repeated three times using different membrane preparations.

previously by us not to affect the degree of 'uncoupling' of adenylate cyclase that is effected by treatment of hepatocytes with glucagon (Heyworth & Houslay, 1983a).

Discussion

Islet-activating protein catalyses the NAD^+ -dependent ADP-ribosylation of a protein of M_r about 40000 in various cell types. The ribosylation of this target protein, which has been called N_i , can be correlated with the ability of islet-activating protein to modify the activity of adenylate cyclase in these cells (Katada & Ui, 1982; Hildebrandt *et al.*, 1983; Murayama *et al.*, 1983). In particular, islet-activating protein reverses the receptor-mediated inhibition of adenylate cyclase, although it can also enhance the receptor mediated activation of this enzyme as well (see, e.g., Katada *et al.*, 1982).

More recently N_i has been purified to apparent homogeneity (Hanski & Gilman, 1982; Codina *et al.*, 1983; Bokoch *et al.*, 1983). It is a dimeric protein consisting of a M_r -40000 α -subunit, which acts as a substrate for islet-activating protein, and a M_r -35000 β -subunit. The β -subunit of N_i appears to be indistinguishable from the β -subunit of N_s (Manning & Gilman, 1983), a distinct guanine nucleotide regulatory protein (Ross & Gilman, 1980) which mediates the action of stimulatory receptors on adenylate cyclase (see Houslay, 1983). It is considered that both N_i and N_s are activated on dissociation of their respective subunits after interaction with an appropriate occupied receptor (see, e.g., Northup *et al.*, 1983a). For N_s , the activated α -subunit is believed to interact with adenylate cyclase, leading to its activation (Northup *et al.*, 1983a,b). However, the mechanism whereby N_i inhibits adenylate cyclase is unclear. It might achieve this either through the direct action of its α -subunit on adenylate cyclase or through the released β -subunit hindering the dissociation of N_s (Northup *et al.*, 1983a,b; Codina *et al.*, 1983; Houslay, 1984). These two guanine nucleotide regulatory proteins have, however, very different affinities for GTP (Rodbell, 1980; Cooper, 1982), which appears to be essential for the expression of their actions.

Here we observed that treatment of hepatocytes with islet-activating protein actually potentiated the cyclic AMP response of the cells to challenge by glucagon, but had no effect on basal concentrations of cyclic AMP (Fig. 1). Similar observations have been made for the effect of islet-activating protein on the stimulatory response caused by beta-adrenergic agents in C6-glioma and other cells (see Katada *et al.*, 1982). This response can be attributed to the increased activity exhibited by the (glucagon + GTP)-stimulated adenylate cyclase activity of plasma membranes isolated from hepatocytes treated with islet-activating protein. Such observations are in complete contrast with the action of cholera toxin, which elicits a dramatic activation of basal adenylate cyclase activity in hepatocytes (Martin *et al.*, 1977; Houslay & Elliott, 1979) and other cell systems (Van Heyningen, 1977; Vaughan & Moss, 1978). Thus the net hormonal response, if defined as a fold increase over the activity of the basal state, is actually enhanced in cells pre-treated with islet-activating protein, yet decreased in those pre-treated with cholera toxin.

The basis for this enhanced response to glucagon in hepatocytes pre-treated with islet-activating protein is unclear. However, it may be that islet-activating protein actually inactivates a residual input from the inhibitory N_i system. Hildebrandt *et al.* (1982) have demonstrated that the inhibitory

input, owing to N_i , occurs in cyc⁻ S49 mouse lymphoma cells, which are genetically deficient in N_s . They ascertained this by first amplifying the basal state of adenylate cyclase by using the diterpene forskolin. They were then able to show that high, but nevertheless physiological, concentrations of GTP could inhibit this activity of adenylate cyclase. Here, using hepatocyte plasma membranes we can demonstrate that GTP caused a similar inhibition of basal adenylate cyclase activity. Like Hildebrandt *et al.* (1982), we added forskolin to amplify the basal adenylate cyclase activity to a value sufficient to measure the inhibitory response accurately (Fig. 2). Furthermore, we now provide further evidence to support the existence of an active N_i in liver plasma membranes, as this inhibitory effect caused by high GTP concentrations was blocked by islet-activating protein. These data suggest that a functional N_i exists in rat hepatocyte plasma membranes. The presence of N_i in liver plasma membranes is also consistent with islet-activating protein causing the NAD-dependent ribosylation of a major protein band with a subunit M_r of around 40000. Additionally we show here that if, instead of GTP, the GDP analogue guanosine 5'-[β -thio]diphosphate is present in the ribosylation assay, the ribosylation of N_i is severely decreased (Fig. 4).

We (Heyworth & Houslay, 1983a) have demonstrated that glucagon treatment of hepatocytes leads to a functional desensitization or 'uncoupling' of hormone-stimulated adenylate cyclase activity. This is clearly shown by the fall in (glucagon + GTP)-stimulated adenylate cyclase activity observed in membranes from hepatocytes treated with glucagon (10 nM). Pre-treatment of hepatocytes, however, with islet-activating protein actually prevented this 'uncoupling' of adenylate cyclase activity from occurring. This may, in part, account for the enhanced glucagon-stimulated cyclic AMP response of hepatocytes pre-treated with islet-activating protein.

One could be tempted to conclude that the inhibition observed during uncoupling was due to the activation of N_i . However, N_i exerts its actions quite readily in broken membranes, as shown here (Fig. 2) and elsewhere (see Cooper, 1982, for review), whereas the 'uncoupling' response can only be elicited by the treatment of whole cells with glucagon and does not occur in broken membranes (Heyworth & Houslay, 1983a,b). Indeed, observations on the decreased lag for activation of adenylate cyclase by a non-hydrolysable analogue of GTP, in plasma membranes from 'uncoupled' hepatocytes, have led us to suggest that it is in fact N_s that is modified in these cells. If this were the case, islet-activating protein may have blocked the

process that elicited a modification in N_s , or alternatively enhanced the reversal of such a process. Further support for this contention comes from our observations that treatment of hepatocytes with N^6 -(phenylisopropyl)adenosine also blocked this 'uncoupling' process (Wallace *et al.*, 1984). In this instance N^6 -(phenylisopropyl)adenosine appears to be functioning via R-type receptors, which are not coupled into the adenylate cyclase system. As both N^6 -(phenylisopropyl)adenosine and islet-activating protein (Yajima *et al.*, 1978a,b; Fredholm, 1982; Endoh *et al.*, 1983) have been suggested to affect Ca^{2+} mobilization, it is possible that they are exerting effects on the 'uncoupling' reaction through such a means.

An alternative possibility is, of course, that glucagon, in intact cells, initiates a reaction that leads indirectly to the activation of N_i . This, of course, would also be expected (Cooper, 1982) to be abolished by replacing Mg^{2+} in the assay with Mn^{2+} , as we have shown previously (Heyworth & Houslay, 1983a).

At present we are not able to discriminate between these two possibilities. However, for reasons discussed elsewhere (Heyworth & Houslay, 1983a,b; Heyworth *et al.*, 1983), we tend to favour the former.

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