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The action of islet activating protein (pertussis toxin) on insulin's ability to inhibit adenylate cyclase and activate cyclic AMP phosphodiesterases in hepatocytes

Clare M. HEYWORTH,†‖ Anne-Marie GREY,† Sally R. WILSON,†¶ Emanuel HANSKI‡ and Miles D. HOUSLAY*§

*Molecular Pharmacology Group, Institute of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.; †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

Treatment of hepatocytes with islet activating protein (pertussis toxin) from *Bordatella pertussis* blocked the ability of insulin to inhibit adenylate cyclase activity both in broken plasma membranes and in intact hepatocytes. Such treatment of intact hepatocytes with pertussis toxin did not prevent insulin from activating the peripheral plasma membrane cyclic AMP phosphodiesterase although it did inhibit the ability of insulin to activate the 'dense-vesicle' cyclic AMP phosphodiesterase. The ability of glucagon pretreatment of hepatocytes to block insulin's activation of the plasma membrane cyclic AMP phosphodiesterase was abolished in pertussis toxin-treated hepatocytes. It is suggested that the ability of insulin to manipulate cyclic AMP concentrations by inhibiting adenylate cyclase and activating the plasma membrane and 'dense-vesicle' cyclic AMP phosphodiesterases involves interactions with the guanine nucleotide regulatory protein system occurring in liver plasma membranes.

INTRODUCTION

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.*, 1978). This toxin 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, 1980). More recently it has become of interest due to its ability to block the receptor-mediated inhibition of adenylate cyclase in various cells (see, e.g., Katada & Ui, 1981; Hazeki & Ui, 1981; Kurose *et al.*, 1983) including hepatocytes (Heyworth *et al.*, 1984*b*) as well as to potentiate the stimulation of adenylate cyclase by β-adrenergic agents in C6-glioma cells (Katada *et al.*, 1982) and by glucagon in hepatocytes (Heyworth *et al.*, 1984*b*).

Islet activating protein appears to exert these effects upon adenylate cyclase by triggering the ADP-ribosylation of a specific guanine nucleotide regulatory protein, called N_i (see, e.g., Bokoch *et al.*, 1983; Codina *et al.*, 1983; Hanski & Gilman, 1982; Heyworth *et al.*, 1984*b*; Katada *et al.*, 1982). N_i has been demonstrated to mediate the action of inhibitory receptors upon adenylate cyclase (see, e.g., Hildebrandt *et al.*, 1982, 1983; Jakobs & Schultz, 1983) and has recently been purified to apparent homogeneity (Bokoch *et al.*, 1983; Hanski & Gilman, 1982). It is a distinct species from the guanine nucleotide regulatory protein, N_s, which mediates the action of stimulatory hormone receptors upon adenylate cyclase (see, e.g., Gilman, 1984; Houslay, 1984). However, N_s, N_i and transducin, a guanine nucleotide regulatory protein that mediates the rhodopsin-catalysed activation of a cyclic GMP phosphodiesterase in retinal rods, all exhibit a similar trimeric structure: distinct α subunits, apparently identical β subunits and very similar γ subunits (Manning & Gilman, 1983; Gilman, 1984). This common basic structure is believed to play an inherent part in their activation mechanism which, in each case, appears to result as a consequence of their dissociation to release an α subunit from the complex. In the case of N_s and transducin it is the activated α subunit which now exerts a stimulatory effect on the adenylate cyclase and the cyclic GMP phosphodiesterase respectively. With N_i, however, it is considered that the β–γ complex released upon activation of N_i actually inhibits N_s dissociation by mass action and yields an inhibitory response (Gilman, 1984).

We have suggested that insulin might exert certain of its effects through interactions with the guanine nucleotide regulatory protein system in plasma membranes (see Houslay & Heyworth, 1983). This was suggested to account for the ability of insulin to (i) activate a plasma membrane cyclic AMP phosphodiesterase, in broken membranes (Heyworth *et al.*, 1983*b*) and intact cells (Heyworth *et al.*, 1983*a*), and (ii) inhibit adenylate cyclase in a GTP-dependent manner (Heyworth & Houslay, 1983*b*). It has been proposed by us that a specific species of guanine nucleotide regulatory protein, called N_{ins}, might mediate the action of insulin in stimulating the peripheral plasma membrane cyclic AMP phosphodi-

§ To whom reprint requests and correspondence should be sent at the Glasgow address.
‖ Present address: Paterson Laboratories, Christie Hospital, Wilmslow Road, Manchester, U.K.
¶ Present address: Department of Medicine, Medical School, Oxford Road, Manchester, U.K.

esterase. This regulatory protein has been tentatively identified as an M_r-25000 species (Heyworth et al., 1985*a*).

Here we examine the effects of islet activating protein upon the ability of insulin to activate both the peripheral plasma membrane and 'dense-vesicle' phosphodiesterases in intact hepatocytes and to inhibit adenylate cyclase both in intact hepatocytes and in a broken membrane system. In these studies we have chosen to pretreat the intact hepatocytes with islet activating protein rather than the whole animals. This is because treatment of animals with islet activating protein leads to an increase in insulin secretion (Katada & Ui, 1979) which might be expected to perturb insulin responsive processes perhaps by initiating some refractoriness in the hormonal response.

MATERIALS AND METHODS

Hepatocytes were prepared from fed, 200–300 g male Sprague–Dawley rats by the method of Elliott *et al.* (1976). They were then preincubated at 37 °C in Krebs–Henseleit (1932) buffer containing 2.5% bovine serum albumin and 2.5 mM-$CaCl_2$ for 20–30 min prior to their use. All subsequent incubations were performed at the same temperature with the same buffer, i.e. under conditions described by us previously in some detail (Heyworth *et al.*, 1983*a*). For the longer incubations employed with pertussis toxin then 10 mM-glucose was added to the incubation medium and cells were re-gassed every 15 min for 1 min with O_2/CO_2 (19:1). Intracellular cyclic AMP was determined as described in detail previously (Whetton *et al.*, 1983) using centrifugation of the cells through bromododecane into $HClO_4$/sucrose and determination of cyclic AMP in a neutralized sample by using the heart muscle binding protein assay of Rubin *et al.* (1975). ATP concentrations were ascertained by using the firefly luciferase assay as described before (Heyworth *et al.*, 1983*a*). Assessment of activation of the plasma membrane and 'dense-vesicle' cyclic AMP phosphodiesterase activity by insulin was made as before (Heyworth *et al.*, 1983*a*) by using the rapid Percoll fractionation procedure to separate out the various subcellular components of the hepatocytes. As detailed previously it was crucial to ensure a good separation of the plasma membranes from the endoplasmic reticulum fractions, otherwise the significant cyclic AMP phosphodiesterase activity in the endoplasmic reticulum fraction obscured the insulin-mediated activation of the plasma membrane enzyme. Adequate separation of these fractions was ensured by 'calibrating' each batch of Percoll to give marker enzyme separation profiles as detailed previously (Heyworth *et al.*, 1983*a*). Such 'calibration' involved the fine adjustment of the speed of centrifugation in order to obtain the required separation profile, as detailed in Heyworth *et al.* (1983*a*).

The effects of insulin action on the adenylate cyclase activity of a plasma membrane fraction prepared from hepatocytes (Houslay & Elliott, 1979, 1981) was performed as detailed previously by us (Heyworth & Houslay, 1983*b*). Briefly, adenylate cyclase was assayed in 0.1 ml final volume. The mixture had final concentration of 0.1 mM-GTP, 0.1 nM-glucagon, 5 mM-$MgSO_4$, 10 mM-theophylline, 1 mM-EDTA, 22 mM-disodium phosphocreatine, 1.5 mM-ATP, 1 mM-dithiothreitol, 1 mg of creatine kinase/ml, 25 mM-triethanolanine/HCl neutralized to a final pH 7.4 with KOH, and 0.1 mg of membrane protein. It was essential to use the highly purified phosphocreatine from Boehringer (Lewes, East Sussex, U.K.) rather than other sources, to add GTP to the assays and to employ low concentrations of glucagon (Heyworth & Houslay, 1983*b*) in order to observe such inhibitory actions of insulin on adenylate cyclase. All solutions were made up fresh every 2–3 days. Incubations were carried out over 10 min at 30 °C during which time assays were linear. After immersion in boiling water for 3 min, to stop the reaction, cyclic AMP produced in the assay was determined by using a purified cyclic AMP binding protein procedure as detailed above (Heyworth *et al.*, 1983*b*; Whetton *et al.*, 1983). Such an assay method obviates any requirement for a 'sink' of (unlabelled) cyclic AMP in the assay (see, e.g., Salomon *et al.*, 1974) which may inhibit insulin's effects, as elevated cyclic AMP concentrations have been shown to inhibit certain of insulin's actions, e.g. stimulation of glucose transport (see Houslay, 1985, for review). Similar results could be obtained with a liver plasma membrane preparation obtained as described previously by us (Heyworth *et al.*, 1983*b*).

Cyclic AMP phosphodiesterase activity was determined at a final substrate concentration of 10^{-7} M-cyclic AMP as detailed before (Heyworth *et al.*, 1983*a*). Samples derived from the Percoll gradients were treated as described by Heyworth *et al.* (1983*a*) prior to assay. Initial rates were taken from linear time courses.

All materials were as specified in the publications cited in this section. All radiochemicals were from Amersham International, Amersham, Bucks., U.K., except for [^{32}P]NAD^+, which was from New England Nuclear. Glucagon was kindly given by Dr. W. W. Bromer, Eli Lilly & Co., Indianapolis, IN, U.S.A. Pure (homogenous) islet activating protein, from *Bordatella pertussis*, was obtained from the PHLS Centre for Applied Microbiology and Research, Porton Down, Wilts, U.K.

RESULTS

We have previously demonstrated (Heyworth & Houslay, 1983*b*) that insulin can inhibit the adenylate cyclase activity of hepatocyte plasma membranes provided that only low, physiological glucagon concentrations (approx. 0.1 nM) were present together with GTP (0.1 mM) in the assays. However, if plasma membranes were prepared from hepatocytes treated with islet activating protein, then insulin (1.0 nM) failed to inhibit the activity of glucagon (+GTP)-stimulated adenylate cyclase (Table 1). In contrast, insulin still inhibited adenylate cyclase activity in membranes prepared from hepatocytes incubated similarly, but without islet activating protein (Table 1).

When intact hepatocytes were incubated with 3-isobutyl-1-methylxanthine (1 mM), to inhibit cyclic AMP phosphodiesterase activity (Heyworth & Houslay, 1983*b*) then, in the presence of low concentrations of glucagon (0.1 nM), insulin (10 nM) inhibited the rate of formation of cyclic AMP by adenylate cyclase. However, when the hepatocytes were pretreated with islet activating protein (100 ng/ml), for 1 h, this inhibitory effect of insulin on the adenylate cyclase activity of whole cells was abolished (Fig. 1).

Insulin (10 nM) treatment of intact hepatocytes leads to the activation of at least two specific cyclic AMP phosphodiesterases, the peripheral plasma membrane

Table 1. Pertussis toxin blocks the inhibitory action of insulin upon adenylate cyclase activity in rat liver plasma membranes

Hepatocytes were incubated for 1 h either with or without islet activating protein/pertussis toxin (100 ng/ml) prior to the preparation of a membrane fraction for adenylate cyclase assay. Inhibition of glucagon-stimulated adenylate cyclase by insulin (1.0 nM) was seen provided that physiological, low glucagon concentration (0.1 nM) are employed together with GTP (0.1 mM) (see Heyworth & Houslay, 1983*b*). Errors are given as S.E.M. for three separate experiments ($n = 3$) using different cell preparations with triplicate assays of adenylate cyclase activity.

	Inhibition of (glucagon+GTP)-stimulated adenylate cyclase activity (%)	Specific activity of (glucagon+GTP)-stimulated adenylate cyclase (pmol/min per mg of protein)
Membranes from control hepatocytes	23.5±3.8	14.8±0.46
Membranes from pertussis toxin-treated hepatocytes	−1.7±5	24.7±2.5

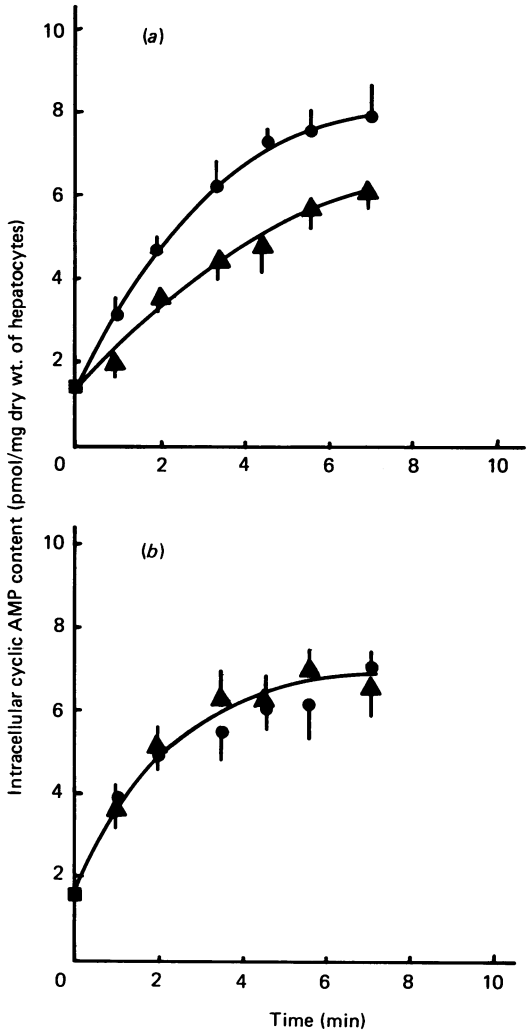

Fig. 1. Pertussis toxin blocks insulin's inhibition of adenylate cyclase activity in intact hepatocytes

Hepatocytes were incubated as described previously by us (Heyworth *et al.*, 1983*a*) and then, at the designated time interval, harvested for determination of intracellular cyclic AMP concentration (Whetton *et al.*, 1983). In this instance cells were preincubated for 10 min with 3-isobutyl-1-methylxanthine (1 mM) in order to inhibit cellular cyclic AMP phosphodiesterase activity. Additions of either glucagon (0.1 nM) alone (●) or both glucagon (0.1 nM) and insulin (10 nM) together (▲) were made. In (*a*) control cells were used which had been incubated for 1 h prior to exposure to the ligands, whereas in (*b*) the cells had been preincubated for 1 h with islet activating protein (100 ng/ml). The experiment shown is typical of one carried out on three different cell preparations. In each case cyclic AMP determinations were carried out in duplicate.

enzyme and the 'dense-vesicle' enzyme (Heyworth *et al.*, 1983*a*; Wilson *et al.*, 1983). The degree of activation of each species can be assessed after the resolution of cyclic AMP phosphodiesterase activity associated with various subcellular fractions upon Percoll gradient fractionation of hepatocyte homogenates. Here we observed (errors are S.E.M., for $n = 3$ separate experiments) that insulin's activation of the 'dense-vesicle' phosphodiesterase was in fact markedly reduced from 140% ±4% in control cells to 115% ±5% (significant activation at $P = 0.05$) in cells pre-treated for 1 h with islet activating protein (100 ng/ml).

Table 2 shows that the ability of insulin to activate the plasma membrane cyclic AMP phosphodiesterase was unaffected by prior exposure of the hepatocytes to islet activating protein (100 ng/ml for 1 h). However, we see here that the treatment of hepatocytes with islet activating protein actually prevented glucagon from blocking insulin's activation of the plasma membrane cyclic AMP phosphodiesterase (Table 2). We noted that treatment with glucagon alone did not activate this enzyme in hepatocytes that had been pretreated with islet activating protein (100 ng/ml for 1 h).

The effect of islet activating protein on inhibitory responses connected with adenylate cyclase is dependent upon the concentration of islet activating protein used, the time and temperature of exposure and the particular cell type under investigation (see, e.g., Hazeki & Ui, 1981; Katada *et al.*, 1982; Murayama *et al.*, 1983). We believe that, under the conditions employed here, maximal effects of islet activating protein were observed. This is because we noted little significant differences in the effects reported here by incubating either with islet activating protein at 1 μg/ml instead of 100 ng/ml for 1 h or by incubating for up to 2 h with 100 ng of islet activating

Table 2. Effect of islet activating protein on the peripheral, plasma membrane cyclic AMP phosphodiesterase

Hepatocytes were incubated, as described previously by us (Heyworth *et al.*, 1983*a*), for 1 h either without or with islet activating protein (100 ng/ml) as described in the Materials and methods section. Additions of hormones were then made to the hepatocyte incubations as shown in the Table. The total incubation time, with the ligands, is shown in parentheses. Cells were then harvested, by centrifugation, and the degree of activation of the peripheral plasma membrane cyclic AMP phosphodiesterase assessed from a Percoll gradient fractionation as detailed before by us (Heyworth *et al.*, 1983*a*). Errors are given as S.D. for three separate experiments using different cell preparations. Abbreviation used: IAP, islet activating protein/pertussis toxin.

	Activation (%) with respect to control (100%)	
Additions	Untreated cells (−IAP)	Cells pre-treated with IAP (+IAP)
None	100	106±10
Insulin (10nM) (5 min)	164±15	160±13
Glucagon (10 nM) and at 5 min insulin (10 nM) (10 min)	118±8	156±8
Glucagon (10 nM) (5 min)	106±5	100±10

protein/ml. In this respect hepatocytes respond somewhat faster to islet activating protein compared with other cell types, as has been shown to be the case for the action of cholera toxin on these cells (Houslay & Elliott, 1979, 1981).

DISCUSSION

We (Heyworth *et al.*, 1984*b*) and others (Itoh *et al.*, 1984) have demonstrated that there is a functional N_i in hepatocyte plasma membranes which can be inactivated by pretreating intact hepatocytes with islet activating protein. Such a treatment would appear to lead to the ribosylation of N_i *in situ* in the intact cell, as there was a reduced labelling of N_i in membranes prepared from such pretreated cells when incubated with [^{32}P]NAD$^+$ and pertussis toxin (Heyworth *et al.*, 1985*a*).

Here we see that exposure of hepatocytes to islet activating protein obliterates the inhibitory effect of insulin on adenylate cyclase (Table 1; Fig. 1). This inhibitory action of insulin has been shown previously by us to be dependent upon the presence of Na^+ in the assay mixture and to be abolished by replacing Mg^{2+} with Mn^{2+} in the assay mixture (Heyworth & Houslay, 1983*b*). Such evidence suggests that insulin exerts its inhibitory effect on adenylate cyclase through the inhibitory guanine nucleotide regulatory protein, N_i.

Prior exposure of hepatocytes to glucagon, before insulin, initiates the development of a selective 'insulin-resistant' state. This manifests itself as an inability of insulin to both inhibit adenylate cyclase and to activate the peripheral plasma membrane cyclic AMP phosphodiesterase in such glucagon pretreated cells (Heyworth & Houslay, 1983*b*; Heyworth *et al.*, 1983*a*, 1985*b*; Wallace *et al.*, 1984). This action of glucagon appears to be cyclic AMP-independent and its characteristics are remarkably similar to those of the process whereby glucagon elicits the desensitization of adenylate cyclase in intact hepatocytes (Heyworth & Houslay, 1983*a*). We (Heyworth *et al.*, 1984*b*) and others (Rich *et al.*, 1984) have shown previously that glucagon desensitization can be blocked by islet activating protein. One possibility is, therefore, that glucagon desensitization leads to the indirect activation of N_i. Certainly if N_i was switched on in such circumstances it would readily account for the fact that insulin can no longer inhibit adenylate cyclase in glucagon-desensitized cells.

Here we see (Table 2) that in hepatocytes, which have been pretreated with islet activating protein, insulin can still activate the peripheral plasma membrane cyclic AMP phosphodiesterase. This adds support to our contention (Heyworth *et al.*, 1983*a*, *b*) that insulin does not activate this enzyme by a process involving N_i. However, we also show here that the ability of glucagon pretreatment of hepatocytes to block insulin's activation of this enzyme is abolished in cells treated with islet activating protein (Table 2). Thus islet activating protein is like the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Heyworth *et al.*, 1985*b*) and adenosine (Wallace *et al.*, 1984) in both eliciting this action and also in blocking glucagon-desensitization of adenylate cyclase. One common factor that might link these observations is an action at the level of N_i.

Intriguingly the activation of the 'dense-vesicle' enzyme was inhibited in pertussis toxin-pretreated hepatocytes. This confirms studies (Elks *et al.*, 1983) performed on the equivalent enzyme found in adipocytes and supports our contention (Wilson *et al.*, 1983) that the plasma membrane and 'dense-vesicle' phosphodiesterase are activated by insulin through distinct routes. Whether activation of the 'dense-vesicle' enzyme involves a guanine nucleotide regulatory protein, such as either N_i or N_o (G_i/G_o), which are both pertussis toxin substrates (Sternweiss & Robishaw, 1984), remains to be seen. Our earlier evidence (Wilson *et al.*, 1983) suggested that the 'membrane processing' of either the internalized insulin receptor or a vesicle fraction containing the 'dense-vesicle' phosphodiesterase, was involved in eliciting the activation of the 'dense-vesicle' enzyme. It is possible, as pertussis toxin affects membrane-mediate secretory responses (see, e.g., Katada & Ui, 1979, 1980; Nakamura & Ui, 1985), that 'membrane traffic' phenomena within the cell are affected by this toxin.

We have accrued considerable evidence which, up until the present study, suggested that insulin might exert its actions on both adenylate cyclase and on the peripheral cyclic AMP phosphodiesterase through a similar mechanism involving a distinct guanine nucleotide regulatory protein called N_{ins} (see Houslay & Heyworth, 1983; Houslay, 1985). Our observations which show here that pertussis toxin can discriminate between these two insulin-mediated processes clearly casts doubt on such a hypothesis. However, whereas pertussis toxin clearly obliterates the inhibitory effect of insulin on adenylate cyclase (Table 1) we should take note that cholera toxin also achieves a similar result (Heyworth & Houslay, 1983*b*). As pertussis toxin can exert actions other than

just through N_i (Sternweiss & Robishaw, 1984) it might still be prudent to keep in mind that insulin could inhibit adenylate cyclase through the putative N_{ins}, with pertussis toxin eliciting its action through an indirect means. One possible mechanism is discussed elsewhere (Houslay, 1986).

We would like to suggest that these results add further support to our contention (see Houslay & Heyworth, 1983) that the insulin receptor can interact with the guanine nucleotide regulatory protein system in liver plasma membranes.

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