Insulin and glucagon attenuate the ability of cholera toxin to activate adenylate cyclase in intact hepatocytes

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Treatment of intact hepatocytes with cholera toxin at 37 °C caused a stable activation of adenylate cyclase activity after a lag period of around 10 min. The presence of either insulin (10 nM) or glucagon (10 nM) in the incubation medium had little effect on this lag period; however, these hormones markedly attenuated the maximal activation of adenylate cyclase activity that could be achieved by treatment with cholera toxin. Such actions of insulin and glucagon were dose-dependent, with EC₅₀ values (concn. giving 50% inhibition) of 0.20 nM for insulin and 0.49 nM for glucagon, and were not additive. Treatment of intact hepatocytes with either glucagon or insulin did not affect the ability of cholera toxin to cause the ADP-ribosylation of the 45 kDa α -subunit of the stimulatory guanine nucleotide regulatory protein, G_s, in intact hepatocytes. It is suggested that treatment of intact hepatocytes with either insulin or glucagon attenuates the stimulatory action of ADP-ribosylated G_s on adenylate cyclase.

INTRODUCTION

The receptor-mediated stimulation of adenylate cyclase is elicited via the guanine nucleotide regulatory protein G_s. Thus an occupied receptor interacts with the α -subunit of G_s, causing G_s to bind GTP and dissociate into its constitutive subunits. The GTP-bound G_s α -subunit subsequently interacts with and activates the catalytic unit of adenylate cyclase (Gilman, 1984; Houslay, 1984; Birnbaumer *et al.*, 1985; Northup, 1985; Levitzki, 1987). The stimulatory effect of G_s α -subunit on adenylate cyclase is terminated by the hydrolysis of GTP, making this activation process fully reversible.

In contrast with this, treatment of intact cells with cholera toxin causes the irreversible activation of adenylate cyclase. Such an effect is achieved as a consequence of cholera toxin causing the NAD⁺-dependent ADP-ribosylation of G_s α -subunit, which obliterates the GTPase activity normally associated with this subunit. This ADP-ribosylation, occurring in the presence of GTP, causes the dissociation of G_s to release a free G_s α -subunit, which is trapped in its active GTP-bound form. This species causes the constitutive activation of adenylate cyclase (Van Heyningen, 1977; Gilman, 1984; Birnbaumer *et al.*, 1985; Northup, 1985). Such an activation, however, occurs after a well-defined lag period, which has been observed in hepatocytes (Houslay & Elliott, 1979, 1981) and many other cell types (Van Heyningen, 1977; Vaughan & Moss, 1978). The molecular basis of this lag period remains to be defined, although it can be altered by changing temperature, cholera-toxin concentration and membrane fluidity (Van Heyningen, 1977; Vaughan & Moss, 1978; Houslay & Elliott, 1979). The lag time may, in part, be connected with a requirement for cholera toxin to undergo endocytosis (Houslay & Elliott, 1981) before it can act on G_s at the

cytosolic surface of the plasma membrane (Houslay *et al.*, 1977).

In this study we show that the presence of either insulin or glucagon in the incubation medium decreases the maximum activation of adenylate cyclase that can be achieved by cholera toxin without changing the lag time for the activation process.

MATERIALS AND METHODS

Collagenase, cholera toxin, insulin (pig), cyclic AMP and all nucleotides were purchased from Boehringer (U.K.) Ltd., Lewes, East Sussex, U.K. All other biochemicals were from Sigma Chemical Co., Poole, Dorset, U.K. Radiochemicals were obtained from Amersham International, Amersham, Bucks, U.K., except for [³²P]NAD⁺, which was from NEN (Stevenage, Herts., U.K.). All other chemicals were of AnalaR grade, from BDH Chemicals, Poole, Dorset, U.K. Glucagon was kindly given by Dr. W. W. Bromer of Eli Lilly and Co., Indianapolis, IN, U.S.A. TH-glucagon was prepared and purified as described by Bregman *et al.* (1980). It was kindly given by Professor V. Hruby and Dr. D. Trivedi, University of Arizona, Tucson, AZ, U.S.A. Forskolin (7 β -acetoxy-8,13-epoxy-1 α ,6 β ,9 α -trihydroxy-4-en-11-one) was from Calbiochem.

Isolation and incubation of hepatocytes

Isolated hepatocytes were prepared from fed 225–250 g male Sprague–Dawley rats and incubated essentially as in Smith *et al.* (1978) as described by Heyworth & Houslay (1983a). Cells (3–5 mg dry wt./ml) were preincubated at 37 °C for 20 min, with constant gassing (O₂/CO₂, 19:1), before use. Ligands were added to the reaction vessel in a volume which was less than

Abbreviations used: G-protein, guanine nucleotide regulatory protein; IBMX, 3-isobutyl-1-methylxanthine.

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1 % of the total incubation volume. After the appropriate time interval, samples were removed and the cells quenched by adding an equal volume of ice-cold 1 mM-KHCO₃, pH 7.2, and then placing them on ice. All further procedures were performed at 4 °C.

Determination of ATP content of hepatocytes

As previously (Heyworth *et al.*, 1983), the ATP content in the isolated hepatocytes was determined by the luciferase method on a neutralized HClO₄ extract (Stanley & Williams, 1969). Cells with an ATP concentration of 8.6 nmol/mg dry wt. were judged to be viable, as described previously (Smith *et al.*, 1978; Heyworth & Houslay, 1983a).

Isolation of a hepatocyte membrane fraction

A washed membrane fraction was obtained from isolated hepatocytes as previously described (Houslay & Elliott, 1979). In all cases membranes were used within 2 h of preparation.

Assay of adenylate cyclase and hepatocyte intracellular cyclic AMP content

Adenylate cyclase was assayed as described previously (Houslay *et al.*, 1976), in a mixture containing (final concns.) 1.5 mM-ATP, 5 mM-MgSO₄, 10 mM-theophylline, 1 mM-EDTA, 7.4 mg of phosphocreatine/ml, 1 mg of creatine kinase/ml and 25 mM-triethanolamine/KOH buffer, pH 7.4. The cyclic AMP produced was assessed in a binding assay using the cyclic AMP-binding subunit of protein kinase prepared from bovine heart (Whetton *et al.*, 1983). The intracellular cyclic AMP concentration was determined as described previously (Whetton *et al.*, 1983).

Cholera-toxin-mediated ribosylation of hepatocyte plasma membranes

The cholera-toxin-catalysed ribosylation of these membranes, with [³²P]NAD⁺, was performed by a modification of the conditions used by Hildebrandt *et al.* (1983), except that ATP was not present in the incubation and Ca²⁺ (10 µM) was added to the 'ribosylation mixture'. Briefly, membranes (50 µg of protein) were incubated in a final volume of 50 µl containing 15 mM-thymidine, 100 mM-potassium phosphate buffer (pH 7.5), 6 mM-dithiothreitol and 20 µM-[³²P]NAD⁺ (sp. radioactivity 500 mCi/mmol). Incubations were performed for 10 min at 30 °C. Ribosylation was elicited by the addition of cholera toxin (67.25 µg/assay) to the assay. This was thiol pre-activated (Johnson & Bourne, 1977) before its addition to the ribosylation assay. Briefly, this involved taking 50 µl of stock cholera toxin (1 mg/ml) and adding it to 50 µl of 50 mM-dithiothreitol. This was preincubated at 30 °C for 20 min before its addition (12.5 µl) to the ribosylation mixture (total volume 50 µl in all cases). After incubation of the membranes with cholera toxin, the samples were transferred to an ice bath and diluted 20-fold with ice-cold 50 mM-Tris/HCl buffer, pH 7.4. The sample was centrifuged at 14000 *g* for 10 min at 4 °C, and the membrane pellet was resuspended in Laemmli (1970) sample buffer. SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970). In this instance 5%-acrylamide stacking gels were used with 12%-acrylamide running gel (see Heyworth *et al.*, 1985). Autoradiography was performed with Kodak XAR-5 X-ray film by using Cronex-Dupont

intensifying screens. 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.

RESULTS

Treatment of intact hepatocytes with cholera toxin (1 µg/ml) led to the activation of basal adenylate cyclase activity assessed in an isolated washed membrane fraction with no added ligands (Fig. 1a). As described previously by us, this process occurred with a lag of approx. 10–15 min (Fig. 1a; Houslay & Elliott, 1979, 1981). However, the degree of persistent activation of basal adenylate cyclase, seen in isolated membranes from cholera-toxin-treated hepatocytes, was attenuated by some 40–50 % (range) if either insulin (10 nM) or glucagon (10 nM) was added together with cholera toxin to the intact hepatocytes (Figs. 1a and 1b). A similar effect to that observed with glucagon was also seen if the glucagon analogue TH-glucagon (10 nM) was added to hepatocytes incubated with cholera toxin (Fig. 1a).

Treatment of hepatocytes with insulin (10 nM), glucagon (10 nM), TH-glucagon (10 nM) or IBMX (1 mM) alone for periods of time between 0 and 40 min at 37 °C had no effect on the basal adenylate cyclase activity (less than 10 % change) exhibited by an isolated washed membrane fraction obtained from hepatocytes (the present study; Heyworth & Houslay, 1983a,b; Murphy *et al.*, 1987).

The action of insulin in attenuating the persistent stimulatory effect of cholera toxin on basal adenylate cyclase activity could also be observed by monitoring the increase in intracellular cyclic AMP accumulation elicited by cholera-toxin treatment (Fig. 2). In such cells, degradation of cyclic AMP was prevented (over 95 %) by inhibiting cyclic AMP phosphodiesterase activity with IBMX (1 mM; Heyworth *et al.*, 1983). Thus the increase in intracellular cyclic AMP elicited by cholera-toxin treatment (1 µg/ml) and the attenuation of this action by insulin (10 nM) could be attributed entirely to actions exerted through modulating adenylate cyclase activity (Fig. 2). Insulin (10 nM), however, had no effect (less than 10 % change) on the intracellular cyclic AMP concentration measured in the presence of IBMX (1 mM) over a 40 min period when cholera toxin was not added to the cells.

Both insulin and glucagon attenuated the ability of cholera-toxin treatment of intact hepatocytes to activate adenylate cyclase in a dose-dependent fashion (Fig. 3), with EC₅₀ values (concn. giving 50 % inhibition) of 0.20 ± 0.11 nM and 0.49 ± 0.15 nM respectively (means ± S.D., *n* = 3 cell preparations from different animals).

Treatment of an isolated hepatocyte membrane fraction with thiol-pre-activated cholera toxin and [³²P]-NAD⁺ led to the ribosylation of the 45 kDa α-subunit of G_s (Fig. 4, track a) and other species, as has been shown previously by us and others (Doberska *et al.*, 1980; Cooper *et al.*, 1981; Malbon & Greenberg, 1982; Gordon & Blecher, 1984; Heyworth *et al.*, 1985). The labelling of this species in isolated hepatocyte membranes can, however, be blocked if hepatocytes are pre-treated with cholera toxin (Fig. 4, track b) in order to cause the endogenous ribosylation and activation of G_s *in situ* in the intact hepatocyte (Heyworth *et al.*, 1985). Here

Fig. 1. The presence of insulin and glucagon attenuates the ability of cholera toxin to activate adenylate cyclase in intact hepatocytes

(a) Hepatocytes were treated at 37 °C with cholera toxin (1 μ g/ml) in the absence of any other ligand ($\square$), in the presence of 10 nM-insulin ($\blacksquare$) or in the presence of 10 nM-

Fig. 2. Insulin attenuates the ability of cholera toxin to increase intracellular hepatocyte cyclic AMP concentrations

Hepatocytes were treated with cholera toxin (1 μ g/ml) and incubated at 37 °C in either the absence ($\square$) or the presence ($\blacksquare$) of 10 nM-insulin. The degradation of cyclic AMP by phosphodiesterase activity was blocked with 1 mM-IBMX (Heyworth *et al.*, 1983). Intracellular cyclic AMP was determined as described in the Materials and methods section. Insulin did not alter (less than 10%) intracellular cyclic AMP concentrations seen with IBMX but without cholera toxin (see also Heyworth *et al.*, 1983). Results are means $\pm$ S.D. for $n = 3$ experiments using different cell preparations from different animals.

hepatocytes were preincubated for 40 min with cholera toxin (1 μ g/ml) before preparation of a membrane fraction. When cholera-toxin-pre-treated cells were used, the degree of labelling of G_s α -subunit (45 kDa) that could be achieved in isolated membranes treated with thiol-pre-activated cholera toxin and [32 P]NAD $^{+}$ was only some $21.7 \pm 4.4\%$ ($n = 4$; means $\pm$ S.D.) of the labelling that could be observed with isolated membranes

TH-glucagon ($\triangle$). Hepatocytes were also incubated in the absence ($\blacktriangle$) of any ligands over this time period. At the time points shown, cells were harvested and a washed membrane fraction was taken for assay of basal adenylate cyclase activity. The data shown are the results of six experiments performed on different cell preparations from different animals with adenylate cyclase assays done in duplicate ($\pm$ S.D., $n = 6$). (b) Hepatocytes were treated with cholera toxin as above in the absence of any other ligand ($\square$), in the presence of 10 nM-glucagon ($\blacksquare$) or in the presence of both 10 nM-glucagon and 10 nM-insulin ($\blacktriangle$) ($\pm$ S.D.; $n = 6$ as above).

Fig. 3. Insulin and glucagon attenuate the stimulatory action of cholera toxin in a dose-dependent fashion

(a) Cells were incubated with cholera toxin (1 $\mu\text{g/ml}$) and various concentrations of glucagon as shown. After a 30 min period, cells were harvested and basal adenylate cyclase activity was assessed. (b) Cells were treated similarly with cholera toxin but with various concentrations of insulin before being harvested for determination of intracellular cyclic AMP concentrations. These experi-

Fig. 4. ADP-ribosylation of G_s

Cells were preincubated for 40 min before being harvested for the preparation of a washed membrane fraction. This was treated with [^{32}P]NAD $^{+}$ and thiol-pre-activated cholera toxin to identify the 45 kDa α -subunit of G_s , as described in the Materials and methods section. Tracks: (a) no additions to the cell incubation; (b) cells preincubated with cholera toxin (1 $\mu\text{g/ml}$); (c) cells preincubated with cholera toxin and 10 nM-insulin; (d) cells preincubated with cholera toxin and 10 nM-glucagon; (e) cells preincubated with 10 nM-insulin only. This is a typical experiment of one done three times on cells from different animals.

from untreated hepatocytes (see Fig. 4, track b). This value indicates that approx. 68% of G_s α -subunit must have been ribosylated by the action of cholera toxin *in situ* in the intact hepatocytes. Similar experiments were then performed when either insulin (10 nM; track c) or glucagon (10 nM; track d) was added together with cholera toxin to the intact hepatocytes (Fig. 4), where we noted that the presence of either of these hormones in the hepatocyte incubations had little effect on the degree of ribosylation catalysed by cholera toxin in the intact cell (Fig. 4). Thus membranes from cells pre-treated with cholera toxin, together with either insulin or glucagon,

ments were done in the presence of 1 mM-IBMX. These data are from three experiments ($\pm$ S.D., $n = 3$) using different animals and cell preparations, with duplicate determinations of intracellular cyclic AMP/adenylate cyclase activity.

yielded labelling of approx. $23.1 \pm 4.5\%$ and $25.1 \pm 8.4\%$ respectively. These values are compared with that seen with membranes from control cells (100%) which had not been pre-treated with cholera toxin. This means that, in the presence of either insulin or glucagon, cholera-toxin treatment of intact cells modified some 75–77% of the G_s α -subunit, values that were very similar to those found by using cholera-toxin pre-treatment alone ($n = 4$; means $\pm$ s.d.). Treatment of cells with either insulin (10 nM; track *e*, Fig. 4) or glucagon (10 nM; results not shown) alone had no effect on the labelling of G_s α -subunit (compare track *a*).

In membranes from cholera-toxin-pre-treated cells, labelling of the 48 kDa band by cholera toxin was some $44 \pm 5\%$ of that for membranes from cells that had not been pre-treated with cholera toxin. This action was also unaffected by the presence of either 10 mM-insulin ($49.1 \pm 11.9\%$) or glucagon ($48.7 \pm 9.5\%$; $n = 4$; means $\pm$ s.d.; Fig. 4). Labelling of the 42 kDa band was unaffected by pre-treatment of the intact hepatocytes with cholera toxin, and presumably reflected a species which was only ribosylated in broken membranes and not in intact cells.

DISCUSSION

Cholera toxin causes the persistent activation of adenylate cyclase by eliciting the NAD^+ -dependent ADP-ribosylation of the α -subunit of G_s . This abolishes the GTPase activity associated with that subunit and releases the free G_s α -subunit locked in its activated GTP-bound form. It is this species which is capable of interacting with and activating adenylate cyclase. In intact cells it is the holotoxin which is active, and the process proceeds after a defined lag period. However, ribosylation of G_s α -subunit can be elicited in isolated membranes if thiol-pre-treated toxin is employed (Gilman, 1984; Birnbaumer *et al.*, 1985; Northup, 1985). In liver the α -subunit of G_s had been shown to have a molecular mass of 45 kDa, and can be readily identified by causing the ADP-ribosylation of isolated membranes with thiol-pre-treated cholera toxin and [^{32}P]NAD $^+$ (Birnbaumer *et al.*, 1985; Northup, 1985).

Here we make the novel observation that the presence of either glucagon or insulin in the hepatocyte incubation attenuated the ability of cholera toxin to activate basal adenylate cyclase activity (Figs. 1 and 2). These hormones exerted such an effect in a dose-dependent fashion (Fig. 3). One possible explanation for their action could be that these hormones inhibited the cholera-toxin-mediated ADP-ribosylation of G_s α -subunit in the intact hepatocytes. This, however, was clearly not the case, as we were able to show that, over a 40 min period of incubation of intact hepatocytes with cholera toxin, approx. 68–77% of G_s α -subunit became ADP-ribosylated *in situ* in the intact hepatocyte irrespective of whether either insulin or glucagon was present (Fig. 4). As both insulin and glucagon caused a fall of approx. 50% in the ability of cholera toxin to activate adenylate cyclase, we might have expected to see a parallel, marked, fall in the degree of ADP-ribosylation achieved *in situ* in the intact hepatocyte, to around 34–39% modification. This clearly did not occur (Fig. 4). Thus neither of these hormones appeared to attenuate the ability of cholera toxin to cause the ADP-ribosylation of G_s α -subunit in intact hepatocytes.

It is unlikely that glucagon could exert its action here via increasing intracellular cyclic AMP concentrations, as cholera toxin causes intracellular cyclic AMP concentrations to rise to greater values. However, we have previously provided evidence (Heyworth & Houslay, 1983a; Wakelam *et al.*, 1986; Murphy *et al.*, 1987) that glucagon can exert cyclic AMP-independent actions in hepatocytes. Indeed, the EC_{50} value for this attenuating action of glucagon is an order of magnitude lower than that observed for its ability to stimulate adenylate cyclase activity (see Heyworth & Houslay, 1983a). Furthermore, the value noted is identical with that observed for the ability of glucagon to cause the desensitization of adenylate cyclase in hepatocytes, which we have shown to be a cyclic AMP-independent process (Heyworth & Houslay, 1983a; Murphy *et al.*, 1987). Consistent with this conclusion is our observation that the glucagon analogue TH-glucagon, which does not activate adenylate cyclase but does cause desensitization (Wakelam *et al.*, 1986; Murphy *et al.*, 1987), was equally as effective as glucagon in attenuating the action of cholera toxin (Fig. 1). This suggests that glucagon also exerts its action here by a cyclic AMP-independent pathway, which may involve activation of protein kinase C (Murphy *et al.*, 1987). Indeed, we (Heyworth & Houslay 1983a) have presented evidence consistent with an alteration in G_s function occurring in the glucagon-desensitized state. It is possible that such a modification attenuates the stimulatory potency of ADP-ribosylated G_s on the catalytic unit of adenylate cyclase.

The action of insulin remains to be defined. However, the purified insulin receptor has been shown capable of phosphorylating a variety of G-proteins, namely transducin (Zick *et al.*, 1986), G_i and G_o (O'Brien *et al.*, 1987a) and p21 *ras* (O'Brien *et al.*, 1987b). It is thus possible that G_s could also act as a substrate for the insulin receptor and that such a modification attenuates the functioning of ADP-ribosylated G_s . However, it has been suggested (Graves & McDonald, 1985) that insulin might also activate protein kinase C or a similar serine kinase in cells. This could mimic the action of glucagon in this system and would explain the lack of additivity of their actions. In any event, these experiments lend further support to our contention (Heyworth & Houslay, 1983b; Houslay & Heyworth, 1983; Houslay, 1985, 1986a,b; Gawler & Houslay, 1987; Gawler *et al.*, 1987, 1988) that insulin can interact with the guanine nucleotide regulatory protein system in plasma membranes.

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