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The rapid desensitization of glucagon-stimulated adenylate cyclase is a cyclic AMP-independent process that can be mimicked by hormones which stimulate inositol phospholipid metabolism

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Treatment of intact hepatocytes with glucagon, TH-glucagon ([1-*N*-α-trinitrophenylhistidine, 12-homoarginine]glucagon), angiotensin or vasopressin led to a rapid time- and dose-dependent loss of the glucagon-stimulated response of the adenylate cyclase activity seen in membrane fractions isolated from these cells. Intracellular cyclic AMP concentrations were only elevated with glucagon. All ligands were capable of causing both desensitization/loss of glucagon-stimulated adenylate cyclase activity and stimulation of inositol phospholipid metabolism in the intact hepatocytes. Maximally effective doses of angiotensin precluded any further inhibition/desensitizing action when either glucagon or TH-glucagon was subsequently added to these intact cells, as has been shown previously for the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate) [Heyworth, Wilson, Gawler & Houslay (1985) FEBS Lett. **187**, 196–200]. Treatment of intact hepatocytes with these various ligands caused a selective loss of the glucagon-stimulated adenylate cyclase activity in a washed membrane fraction and did not alter the basal, GTP-, NaF- and forskolin-stimulated responses. Angiotensin failed to inhibit glucagon-stimulated adenylate cyclase activity when added directly to a washed membrane fraction from control cells. Glucagon GR2 receptor-stimulated adenylate cyclase is suggested to undergo desensitization/uncoupling through a cyclic AMP-independent process, which involves the stimulation of inositol phospholipid metabolism by glucagon acting through GR1 receptors. This action can be mimicked by other hormones which act on the liver to stimulate inositol phospholipid metabolism. As the phorbol ester TPA also mimics this process, it is proposed that protein kinase C activation plays a pivotal role in the molecular mechanism of desensitization of glucagon-stimulated adenylate cyclase. The site of the lesion in desensitization is shown to be at the level of coupling between the glucagon receptor and the stimulatory guanine nucleotide regulatory protein G_s, and it is suggested that one or both of these components may provide a target for phosphorylation by protein kinase C.

INTRODUCTION

Receptors, on occupancy by an appropriate ligand, generate an intracellular signal. However, in many instances, the functioning of such receptors, and their appropriate signal-generation system, is attenuated after stimulation. This gives rise to desensitization (see Harden, 1983; Perkins, 1983; Sibley & Lefkowitz, 1985). Such processes can be rapid, occurring in seconds/minutes, or may take many hours to develop. We (Heyworth & Houslay, 1983; Heyworth *et al.*, 1984*a*, 1985) and others (Plas & Nunez, 1975) have shown that the glucagon-stimulated adenylate cyclase activity of intact hepatocytes can be desensitized within a few minutes of exposure of hepatocytes to the stimulatory hormone glucagon. Although the molecular mechanism of desensitization is not as yet understood, it has been shown that the lesion lies at the point of coupling between the glucagon receptor and the stimulatory guanine nucleotide regulatory protein, G_s (Heyworth & Houslay, 1983). During this rapid phase of desensitization, which occurs within 2–5 min, no cell-surface glucagon receptors are internalized or lost (Santos & Blazquez, 1982; Heyworth & Houslay, 1983), and thus it has been suggested that desensitization leads to some modification of either the glucagon receptor or G_s (see Heyworth & Houslay, 1983; Houslay, 1986). However, although changes in G_s functioning were noted, it has also been observed that treatment of hepatocytes with pertussis toxin, which blocks the functioning of the inhibitory guanine nucleotide regulatory protein G_i, also prevented this rapid desensitization (Heyworth *et al.*, 1984*a*). Indeed, pertussis toxin has also been shown to prevent desensitization of vasopressin (V_2)-stimulated adenylate cyclase of renal epithelial cells (Wilson *et al.*, 1986). Nevertheless it remains to be seen whether the indirect activation of G_i plays a role in rapid desensitization or whether pertussis toxin, which is now known to exert effects other than just causing G_i inactivation, is achieving its action through another route (see, e.g., Heyworth *et al.*, 1984*a*; Milligan & Klee, 1985). For it has been shown (Wallace *et al.*, 1984) that there is a mechanism for blocking desensitization in hepatocytes which is controlled by A_3-type adenosine receptors, whose signal-generation system has yet to be identified (Ribeiro & Sebastiao, 1986). Such an adenosine-mediated mechanism for attenuating desensitization has also been noted (Dix *et al.*, 1985) with the lutropin-stimulated

Abbreviations used: TH-glucagon, [1-*N*-α-trinitrophenylhistidine,12-homoarginine]glucagon; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

adenylate cyclase in Leydig cells (Dix & Cooke, 1981, 1982), a system whose desensitization process appears to have many similarities with that responsible for modulating hepatocyte glucagon-stimulated adenylate cyclase activity.

The glucagon analogues TH-glucagon ([1-*N*-α-trinitrophenylhistidine, 12-homoarginine]glucagon; Corvera *et al.*, 1984; Wakelam *et al.*, 1986) and *N*-α-trinitrophenyl-glucagon (Cote & Epand, 1979) have been shown to mimic the ability of glucagon in stimulating a variety of metabolic processes, while not increasing intracellular cyclic AMP concentrations, suggesting that both of these glucagon analogues exert actions through a second-messenger system which does not involve the stimulation of cyclic AMP production. Indeed, we have demonstrated that both TH-glucagon and glucagon can stimulate inositol phospholipid metabolism in intact hepatocytes (Wakelam *et al.*, 1986). In this regard glucagon is akin to a variety of hormones (see Michell & Houslay, 1986) such as vasopressin, adrenaline (see, e.g., Creba *et al.*, 1983; Morgan *et al.*, 1983; Kunos *et al.*, 1985; Wilson *et al.*, 1986) and lutropin (Dix & Cooke, 1981; Lowitt *et al.*, 1982; Molcho *et al.*, 1984) which can exert metabolic effects by stimulating two second-messenger systems, one producing inositol 1,4,5-trisphosphate and diacylglycerol, and one producing cyclic AMP. We have suggested that glucagon might stimulate these two second-messenger systems in liver through two distinct populations of receptors: one set, which we have called GR1 receptors, being coupled to stimulate inositol phospholipid metabolism, and the other set, called GR2 receptors, being presumed to account for the stimulation of adenylate cyclase activity (Wakelam *et al.*, 1986).

We show here that the rapid desensitization of glucagon-stimulated adenylate cyclase is a cyclic AMP-independent process which can be mimicked by hormones, such as vasopressin and angiotensin, that activate inositol phospholipid metabolism. As TH-glucagon also causes desensitization of adenylate cyclase, while not increasing cyclic AMP concentrations, we suggest that glucagon causes the rapid desensitization of adenylate cyclase by stimulating inositol phospholipid metabolism.

MATERIALS AND METHODS

Collagenase, 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. All other chemicals were of AnalaR grade, from BDH Chemicals, Poole, Dorset, U.K. Homogeneous pertussis toxin was either from the PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts, U.K., or kindly given by Professor J. Freer, Department of Microbiology, University of Glasgow. Glucagon was kindly given by Dr. W. W. Brown of Eli Lilly and Co., Indianapolis, IN, U.S.A. TH-glucagon was prepared and purified as described previously by us (Bregman *et al.*, 1980). Vasopressin and angiotensin were obtained from CRB, Cambridge, U.K. Forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one) was from Calbiochem.

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 (1983). Cells (3–5 mg dry wt./ml) were preincubated at 37 °C for 20 min, with constant gassing (O_2/CO_2, 19:1), before use. Ligands were added to the reaction vessel in a volume which was less than 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-$KHCO_3$, pH 7.2, and then placing them on ice. All further procedures were performed at 4 °C.

As previously (Heyworth *et al.*, 1983), the ATP content in the isolated hepatocytes was determined by the luciferase method on a neutralized $HClO_4$ 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, 1983)].

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

The specific binding of glucagon to its receptors was performed by using ^{125}I-glucagon on isolated membranes, as described in detail previously (Houslay *et al.*, 1980).

Adenylate cyclase was assayed as described previously (Houslay *et al.*, 1976), in a mixture containing (final concns.) 1.5 mM-ATP, 5 mM-$MgSO_4$, 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).

The production of total inositol phosphates was measured as described previously (Wakelam *et al.*, 1986), by the methodology of Berridge *et al.* (1982). Hepatocytes were incubated at 37 °C, in Krebs–Henseleit buffer containing 1% (w/v) bovine serum albumin, 10 mM-glucose and 5 μCi of *myo*-[2-^{3}H]inositol/ml for 90 min with constant gassing with O_2/CO_2 (19:1). Cells were then washed and resuspended in the same buffer, but in the absence of inositol, and incubated for 10 min before addition of LiCl (final concn. 10 mM). Incubation then proceeded for 15 min, after which cells were dispensed into plasic vials containing ligands at the stated final concentrations, gassed (O_2/CO_2), capped and incubated for 30 min. All incubations were done in a shaking water bath. Experiments were terminated by addition of chloroform/methanol (1:2, v/v). Chloroform/water (1:1, v/v) was added, which generated two phases. The radioactivity in the total inositol phosphates was determined by batch chromatography of the upper phase on Dowex 1X8 (formate form) resins, with radioactivity in the lipids being measured on a portion of the lower chloroform phase (Berridge *et al.*, 1982).

RESULTS

As shown previously (Heyworth & Houslay, 1983), treatment of hepatocytes with glucagon (10 nM) led to the rapid loss (desensitization) of the glucagon-stimulated adenylate cyclase activity observed in a washed membrane fraction derived from these cells (Fig. 1*a*). This ability to cause desensitization was also elicited by the

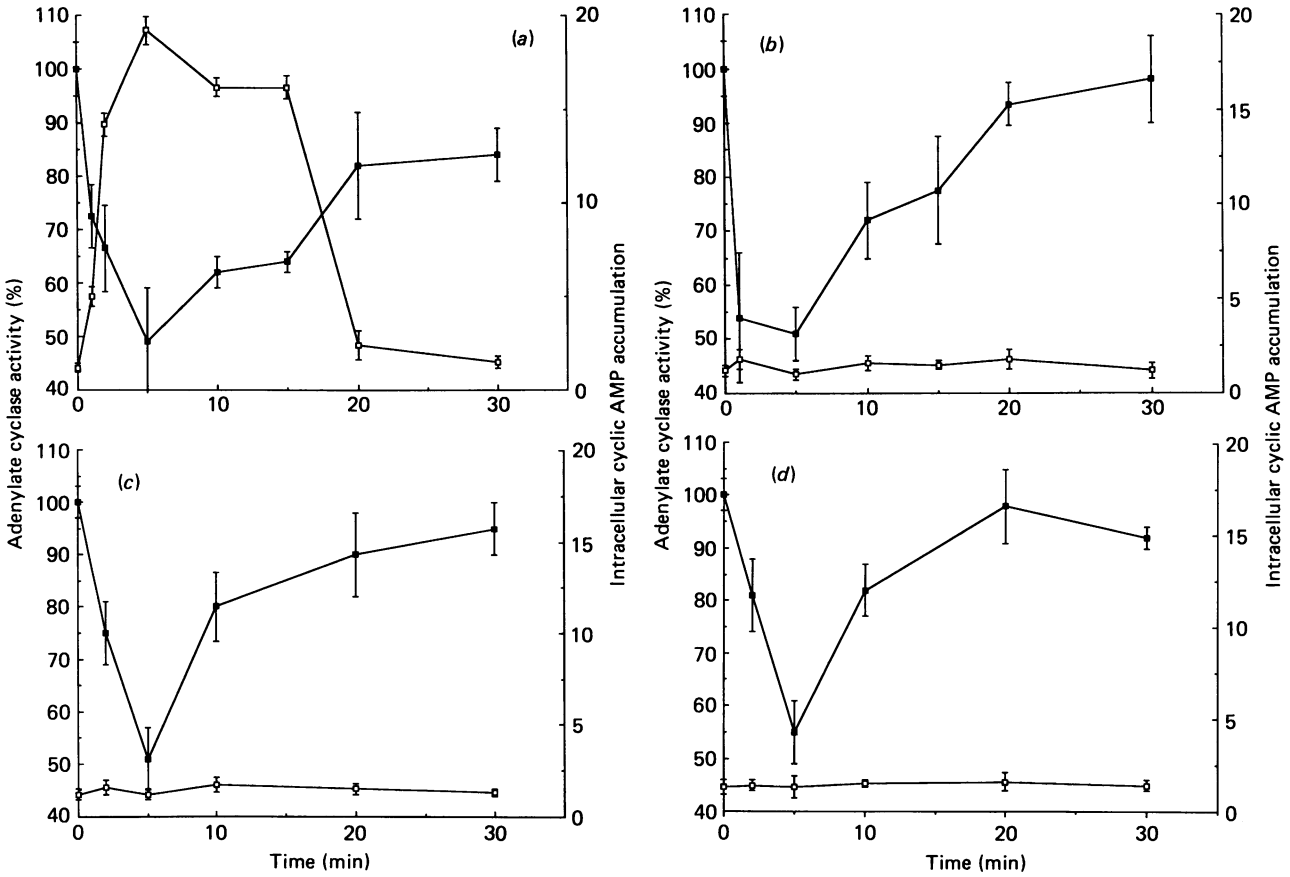

Fig. 1. Time course for the rapid uncoupling of glucagon-stimulated adenylate cyclase activity

After preincubation for 20 min, hepatocytes were challenged with 10 nM concentrations of glucagon (*a*), TH-glucagon (*b*), angiotensin (*c*) or vasopressin (*d*). Changes in both the intracellular cyclic AMP concentration (□) and desensitization of glucagon-stimulated adenylate cyclase activity (■) were noted. The loss of glucagon-stimulated adenylate cyclase activity in isolated membrane fractions is shown here as a percentage of control activity (100%) exhibited by a membrane fraction obtained from control cells. Thus, at appropriate time points, cells were harvested for the preparation of a washed-membrane fraction (Houslay & Elliott, 1979), which was used to assess adenylate cyclase activity (■) stimulated by glucagon (10 nM) in the presence of GTP (100 μM). In control cells intracellular cyclic AMP concentrations remained constant at 1.4 ± 0.2 pmol/mg dry wt. of cells, as did the activity of glucagon-stimulated adenylate cyclase observed in washed membrane fractions, at 27 ± 2 pmol/min per mg of protein over the time period studied in these experiments. Results are means $\pm$ S.D. for $n = 3$ separate experiments with different cells.

glucagon analogue, TH-glucagon. Such an action was not accompanied by any change in receptor number, as the specific binding of ^{125}I-labelled glucagon remained greater than 95% of that in control cells. Furthermore, we (Heyworth & Houslay, 1983) have noted previously that, in hepatocytes challenged with glucagon (10 nM), no internalization of glucagon receptors occurred over a 5 min period. Indeed, we (Houslay *et al.*, 1980) have also shown that glucagon, in the presence of GTP, activates adenylate cyclase through a collision coupling mechanism, which amplifies the stimulatory action of occupied receptors. Indeed, under such conditions of assay, we (Houslay *et al.*, 1980) were able to demonstrate that over 75% of glucagon receptors could be lost from the plasma membrane before any significant change in glucagon-stimulated adenylate cyclase activity ensued. Unlike glucagon, however, TH-glucagon did not cause any increase in the intracellular concentrations of cyclic AMP (Fig. 1*b*), nor any efflux of cyclic AMP from the hepatocytes (results not shown), which is consistent with its inability to activate adenylate cyclase in isolated membranes (Wakelam *et al.*, 1986).

Desensitization of glucagon-stimulated adenylate cyclase activity could, however, be mimicked by treatment of intact hepatocytes with either vasopressin or angiotensin (Fig. 1). As with TH-glucagon, these ligands also did not increase the intracellular concentration of cyclic AMP (Fig. 1).

In all of these instances we noted that this hormone-induced loss/desensitization of glucagon-stimulated adenylate cyclase activity was transitory, with a full recovery of the stimulatory response of adenylate cyclase to glucagon being achieved about 30 min after initial exposure to hormone. Indeed, the time courses for desensitization of glucagon-stimulated adenylate cyclase initiated by all these various ligands were very similar when maximally effective doses were employed (Fig. 1). With glucagon, we (Heyworth & Houslay, 1983) have demonstrated previously that this was not due to hormone degradation, as desensitization could not be overcome by making further additions of glucagon (10 nM) within the 20 min period investigated.

The ability of TH-glucagon and angiotensin to elicit this desensitization process was abolished in hepatocytes

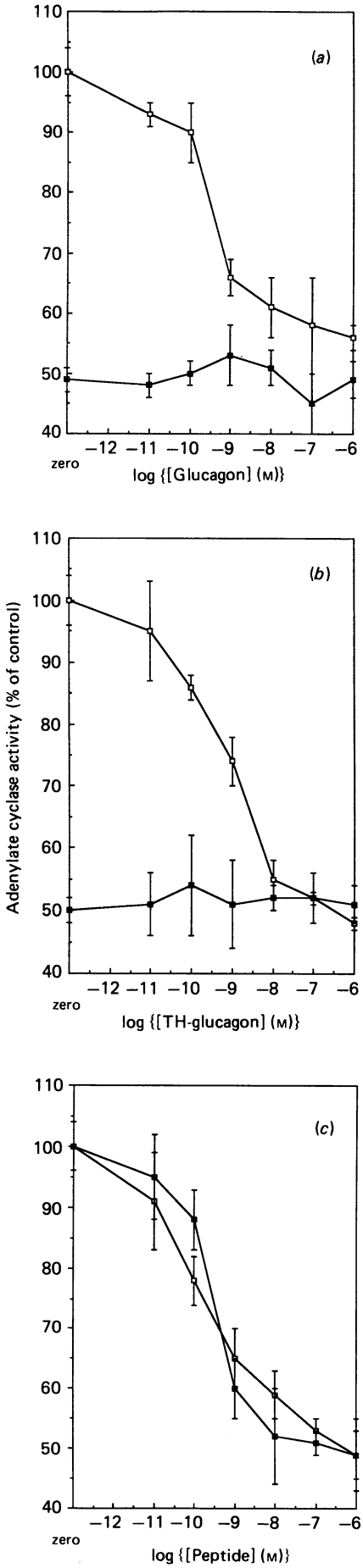

Table 1. Ligands stimulating the production of inositol phosphates and causing desensitization/loss of glucagon-stimulated adenylate cyclase activity

For assessment of desensitization of glucagon-stimulated adenylate cyclase activity, intact hepatocytes were challenged with the appropriate ligand for 5 min at 37 °C. Cells were harvested and a membrane fraction taken for assay as described in the legend to Fig. 1 and in the Materials and methods section. The stimulation of production of total inositol phosphates in response to the various hormones was done as described in the Materials and methods section. Experiments were all done at 1 nM concentration of hormone. Results are means ± S.D. for $n = 3$ separate experiments with different cell preparations.

Ligand	Desensitization/ 'loss' of glucagon-stimulated adenylate cyclase activity (%)	Stimulation of production of inositol phosphates (%)
Glucagon	31 ± 4	19 ± 2
TH-glucagon	25 ± 3	18 ± 1.5
Angiotensin	42 ± 5	72 ± 13
Vasopressin	37 ± 4	42 ± 5

which had been pretreated with pertussis toxin (100 ng/ml) for 1 h (results not shown), as has been demonstrated previously in some detail for glucagon (Heyworth *et al.*, 1984*a*).

Fig. 2 shows that treatment of intact hepatocytes with any of these ligands caused the dose-dependent loss/desensitization of glucagon-stimulated adenylate cyclase activity in membrane fractions prepared from such treated cells. The K_a values for the effects of glucagon, TH-glucagon, angiotensin and vasopressin were 0.45 ± 0.1 nM, 0.5 ± 0.2 nM, 0.3 ± 0.15 nM and 0.3 ± 0.2 nM (means ± S.D.; $n = 3$ separate experiments). Glucagon and TH-glucagon (Wakelam *et al.*, 1986), as well as angiotensin and vasopressin (Creba *et al.*, 1983; Thomas *et al.*, 1984), have all been demonstrated to cause a dose-dependent stimulation of the production of inositol phosphates in hepatocytes, and are shown to stimulate their production here (Table 1).

However, if hepatocytes were pre-treated with angiotensin (10 nM) for 5 min, then subsequent challenge with

Fig. 2. Dose-dependent attenuation of glucagon-stimulated adenylate cyclase

These show changes in the glucagon-stimulated adenylate cyclase activity of a washed membrane fraction prepared from intact hepatocytes: (*a*) challenged for 5 min with glucagon (10 nM) (□); (*b*) challenged for 5 min with TH-glucagon (10 nM) (□) [in some instances cells were pre-treated with angiotensin (10 nM) (■) for 5 min before challenge with glucagon in (*a*) or TH-glucagon in (*b*)]; (*c*) challenged for 5 min with either angiotensin (10 nM) (■) or vasopressin (10 nM) (□). The (glucagon + GTP)-stimulated adenylate cyclase activity observed in membrane fractions from control cells was 24.5 ± 2.5 pmol/min per mg of protein. Results are means ± S.D. for $n = 3$ separate experiments with different cell preparations.

Table 2. Selective loss/desensitization of glucagon-stimulated adenylate cyclase activity in membranes from hepatocytes treated with glucagon, TH-glucagon, angiotensin, vasopressin and TPA

Hepatocytes were incubated with or without the various 'cell pre-treatment ligands' for the time shown, before harvesting and the preparation of a washed membrane fraction as described by Houslay & Elliott (1979). This was then taken for assay of adenylate cyclase activity with the ligands shown as detailed in the legend to Fig. 1 and in the Materials and methods section.

Cell pre-treatment ligand	Assay ligand . . .	Adenylate cyclase activity (pmol/min per mg of protein) None (basal)	Forskolin (100 μM)	GTP (100 μM)	NaF (15 mM)	Glucagon (10 nM) +GTP
None (control) (5 min)		1.81±0.12	63.0±8.3	3.5±0.4	52.3±6.8	26.5±2.3
Glucagon (10 nM) for 5 min		3.0±0.12	61.3±3.1	5.2±0.5	46.3±3.9	14.1±1.6
TH-glucagon(10 nM) for 5 min		2.3±0.3	63.6±8.7	3.6±0.3	45.4±0.5	12.8±1.4
Angiotensin (10 nM) for 5 min		1.8±0.2	58.1±5.3	3.3±0.6	43.1±4.4	14.6±0.2
Vasopressin (10 nM) for 5 min		2.3±0.1	61.8±9.5	4.3±0.3	45.5±5.5	12.8±1.4
TPA (10 ng/ml) for 15 min		1.7±0.1	55.1±4.4	2.9±0.3	41.5±4.0	13.5±1.7

either glucagon or TH-glucagon failed to elicit any further loss/desensitization of glucagon-stimulated adenylate cyclase activity (Fig. 2), indicating that these ligands exerted their effects through a common mechanism of action.

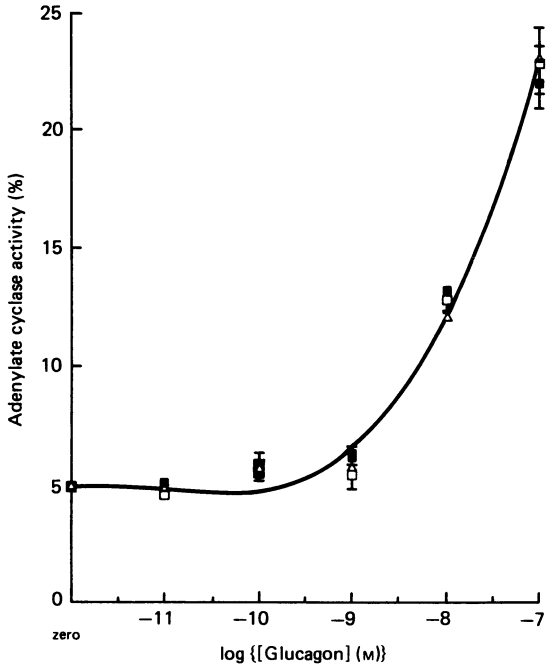

Fig. 3. Inability of angiotensin to inhibit adenylate cyclase activity in isolated membranes

This shows the dose-dependent stimulation of the adenylate cyclase activity of an hepatocyte membrane preparation. All assays contained 100 μM-GTP and they were performed in the absence (■) or presence of angiotensin at 10 nM (□) and 1 μM (△). Results are means±S.D. for $n = 3$ separate experiments.

When the various ligand-stimulated adenylate cyclase activities were monitored in hepatocytes treated with glucagon, TH-glucagon, angiotensin or vasopressin, it was apparent (Table 2) that, although the glucagon-stimulated activities were decreased, those for the basal and the GTP-, NaF- and forskolin-stimulated activities remained unaffected. A similar state to those noted here could be engendered (Table 2) by pre-treating hepatocytes with the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate), as has been shown previously by us (Heyworth *et al.*, 1984*b*).

The direct addition of angiotensin (0.1 nM–1 μM) to assays of glucagon-stimulated adenylate cyclase, employing broken membrane preparations, failed to show (Fig. 3) any direct inhibitory effect of angiotensin, under conditions where sufficient GTP (100 μM) was present to ensure the potential for G_i activation (Heyworth *et al.*, 1984*a*). Indeed, we failed to observe (less than 5% change) any inhibitory effect of a range of angiotensin concentrations (0.1 nM,/1 nM/10 nM/1 μM) on basal and NaF (15 mM)- or forskolin (100 μM)-stimulated adenylate cyclase activities in a washed membrane fraction (results not shown). Nevertheless, in such hepatocyte membranes we were, as previously (Heyworth *et al.*, 1984*a*), able to demonstrate a functional G_i activity, as the forskolin (100 μM)-stimulated adenylate cyclase activity, an indicator of the functioning of the catalytic unit of adenylate cyclase, could be inhibited by some 40±5% in the presence of low (100 nM) concentrations of guanosine 5′-[β,γ-imido]triphosphate, which have been shown to activate G_i, rather than G_s, selectively.

DISCUSSION

Glucagon treatment of intact hepatocytes caused a rapid time- and dose-dependent desensitization of glucagon-stimulated adenylate cyclase (Figs. 1 and 2) as has been described previously by us (Heyworth & Houslay, 1983). However, the glucagon analogue

TH-glucagon, which neither activates adenylate cyclase (Wakelam *et al.*, 1986), when used at concentrations up to 1 μM, nor causes any change (Fig. 1) in the intracellular concentrations of cyclic AMP, also elicited (Fig. 1) the desensitization of glucagon-stimulated adenylate cyclase. This clearly demonstrates that desensitization of glucagon-stimulated adenylate cyclase is elicited by a cyclic AMP-independent process, as has been suggested previously by us (Heyworth & Houslay, 1983). Such a conclusion would be in accord with our inability to mimic desensitization by treatment of intact hepatocytes with dibutyryl cyclic AMP (Heyworth & Houslay, 1983) and our observations that the rate of onset and the extent of desensitization were unaffected by the addition of the non-specific cyclic AMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, at concentrations (1 mM) which increased the rate and magnitude of glucagon-stimulated cyclic AMP accumulation in hepatocytes by over 4-fold (Heyworth *et al.*, 1983).

We (Wakelam *et al.*, 1986), and others (Blackmore & Exton, 1986), have demonstrated that both glucagon and TH-glucagon can stimulate inositol phospholipid metabolism in intact hepatocytes. This process exhibited a K_a for glucagon (0.25 nM) which was an order of magnitude lower than that (6 nM) observed for glucagon's ability both to activate adenylate cyclase and to increase hepatocyte intracellular cyclic AMP accumulation (see Wakelam *et al.*, 1986). However, the dose-dependence of the effect of glucagon (Heyworth & Houslay, 1983; Fig. 2) in eliciting desensitization (K_a 0.45 nM) paralleled that for stimulation of inositol phospholipid metabolism (0.25 nM; Wakelam *et al.*, 1986). This also appears to hold true for TH-glucagon, which exhibits a K_a of 0.3 nM for stimulation of inositol phospholipid metabolism (Wakelam *et al.*, 1986) and one of 0.5 nM for desensitization (Fig. 2). These K_a values, for actions of glucagon, are also similar to those observed (Sistare *et al.*, 1985; Mauger & Claret, 1986) for the ability of glucagon (K_a 0.2–0.4 nM) to increase the intracellular Ca^{2+} concentration of intact hepatocytes: a feature usually attributed as a consequence of inositol 1,4,5-trisphosphate production (see, e.g., Berridge & Irvine, 1984; Downes & Michell, 1985).

We therefore suggest that glucagon, by stimulating the inositol phospholipid pathway through GR1 receptors, causes the desensitization of the ability of GR2 glucagon receptors to stimulate adenylate cyclase. This would be in accord with our observations (Figs. 1 and 2; Table 2) which show that treatment of intact hepatocytes with either angiotensin or vasopressin, ligands known to stimulate inositol phospholipid metabolism in hepatocytes (Creba *et al.*, 1983; Downes & Michell, 1984; Table 1), mimic in full (Figs. 1 and 2; Table 2) this desensitized state of adenylate cyclase elicited by glucagon through GR1-receptor activation. Consistent with these ligands sharing a common mechanism of action were our observations that, after eliciting 'desensitization' of adenylate cyclase with angiotensin, neither glucagon nor TH-glucagon had any additional inhibitory effect on the system (Fig. 2).

It is of note that, at ligand concentrations close to the K_a values observed for the desensitization process triggered by either vasopressin (0.5 nM) or angiotensin (0.3 nM), stimulation of inositol phospholipid breakdown (Creba *et al.*, 1983) and production of inositol phosphates (M. J. O. Wakelam & M. D. Houslay, unpublished work; Thomas *et al.*, 1984) was only approx. 10% of the maximal effect that can be elicited by these ligands. However, the magnitude of the stimulation of production of inositol phosphates at such vasopressin or angiotensin concentrations is of an order comparable with that observed when using either glucagon or TH-glucagon (see Table 2; Wakelam *et al.*, 1986). Indeed, the K_a values for the activation of phosphorylase *a* by either vasopressin (0.3–0.5 nM; Creba *et al.*, 1983; Thomas *et al.*, 1984; Kunos *et al.*, 1985) or angiotensin (0.1–0.3 nM; Bocckino *et al.*, 1985), in intact hepatocytes, are very similar to those that we observe here for the ability of vasopressin (K_a 0.3 nM) and angiotensin (K_a 0.3 nM) to elicit desensitization of glucagon-stimulated adenylate cyclase.

The exposure of intact hepatocytes to either angiotensin or vasopressin yielded time courses for the desensitization of glucagon-stimulated adenylate cyclase which were very similar to those seen with either glucagon or TH-glucagon (Fig. 1). Intriguingly, the transient time course of this process mirrored that seen for the activation of phosphorylase *a*, the production of inositol 1,4,5-trisphosphate (Thomas *et al.*, 1984) and the depletion of phosphatidylinositol 4,5-bisphosphate (Creba *et al.*, 1983) by vasopressin in intact hepatocytes. This again suggests a close connection between stimulation of inositol phospholipid metabolism and the desensitization of glucagon-stimulated adenylate cyclase in hepatocytes.

We (Heyworth *et al.*, 1984*b*; Table 2) and others (Garcia-Sainz *et al.*, 1985) have shown that treatment of hepatocytes with the phorbol ester TPA also led to a selective decrease in glucagon-stimulated adenylate cyclase activity. Indeed, pre-treatment with TPA prevented any further desensitization/inhibitory effect that might be caused by addition of glucagon to the intact cells (Heyworth *et al.*, 1985). In this regard TPA, like angiotensin and vasopressin (Fig. 2), appears to mimic glucagon desensitization. Now, stimulation of inositol phospholipid metabolism also produces the second messenger diacylglycerol, which, like TPA, can activate protein kinase C (Nishizuka, 1983). These observations suggest that activation of protein kinase C by diacylglycerol may play a key role in the molecular mechanism of desensitization of glucagon-stimulated adenylate cyclase. It remains to be determined, however, as to which component of the hormone-regulated adenylate cyclase system provides the target for protein kinase C action.

We also point out, however, that it has been suggested (Pobiner *et al.*, 1985) that angiotensin might inhibit adenylate cyclase in hepatocytes by the direct activation of G_i, as it was observed that angiotensin's ability to inhibit glucagon-stimulated intracellular cyclic AMP accumulation could be blocked by treatment with pertussis toxin. However, as we have demonstrated (Heyworth *et al.*, 1984*a*) that glucagon desensitization can also be blocked by pertussis-toxin treatment of hepatocytes, a treatment that does not block ligand stimulation of inositol phospholipid metabolism (M. J. O. Wakelam & M. D. Houslay, unpublished work), this might offer the explanation for the loss of inhibitory action of angiotensin.

Indeed, in this study we failed to observe any direct inhibitory effect of angiotensin on the glucagon-stimulated adenylate cyclase of isolated hepatocyte membranes, which were shown to possess a functional

G_i. Pobiner *et al.* (1985) did, however, observe a small inhibitory effect on adenylate cyclase when angiotensin was added directly to isolated liver plasma membranes. However, as isolated liver membranes can exhibit a stimulated inositol phospholipid response (Uhing *et al.*, 1985) and have sufficient protein kinase C associated with them (Kiss & Mhina, 1982) for the direct treatment of plasma membranes with TPA to exert an ATP-dependent inhibitory effect on adenylate cyclase (Heyworth *et al.*, 1985), it is possible that the addition of angiotensin to isolated membranes could lead to diacylglycerol production, with concomitant inhibition of adenylate cyclase. However, in the isolated membrane preparations used in the present study, and under the assay conditions employed, we did not observe any ability of either angiotensin or glucagon to stimulate inositol phospholipid metabolism (results not shown). This would be in accord with our observations (results not shown; but see Houslay *et al.*, 1980) that glucagon stimulates the linear production of cyclic AMP, by broken membranes, over a period of at least 15 min. It is possible, however, that under other conditions the desensitization phenomenon could be achieved in a cell-free system by activation of the inositol phospholipid pathway.

We therefore suggest that desensitization of glucagon-stimulated adenylate cyclase can be elicited by hormones which activate protein kinase C by stimulating inositol phospholipid metabolism. The site(s) of action of protein kinase C on this system remain, however, to be elucidated. However, the resultant effect is an 'uncoupling' of stimulation of adenylate cyclase by glucagon, indicating that the lesion lies at the point of interaction between the glucagon receptor and the stimulatory guanine nucleotide regulatory protein, G_s. It is likely then that either or both of the glucagon receptor and G_s may provide a target for phosphorylation by protein kinase C.

Such an 'uncoupling' process, involving the activation of protein kinase C, as described here, may well be widespread and contribute to the overall processes of desensitization seen in many cells. Certainly this is implied by observations that a number of hormones, including glucagon, adrenaline and lutropin, can stimulate both the adenylate cyclase and inositol phospholipid pathways (see Michell & Houslay, 1986). Indeed, phorbol esters can mimic aspects of the densensitization process of β-adrenoceptors (Kelleher *et al.*, 1984; Sibley *et al.*, 1984; Nambi *et al.*, 1985) and can 'uncouple' adenylate cyclase from stimulation by various receptors in a wide range of cell types (Mukhopadhyay & Schumacher, 1985; Rebois & Patel, 1985; Quilliam *et al.*, 1985). However, one should note that phorbol esters have been seen to elicit net stimulatory effects in cell types which are subject to gross inhibition by G_i functioning (Bell *et al.*, 1985). This may result from the action of protein kinase C in causing the phosphorylation and inactivation of G_i (Katada *et al.*, 1985). Furthermore, in intact-cell studies, phorbol esters have been seen to inhibit cyclic AMP phosphodiesterase activity (Irvine *et al.*, 1986), hence augmenting cyclic AMP accumulation stimulated by hormones.

We therefore suggest that a complex interplay between the receptor-controlled cyclic AMP and inositol phospholipid signal-generation systems will be found in many cell types.

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