Challenge of hepatocytes by glucagon triggers a rapid modulation of adenylate cyclase activity in isolated membranes

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Membrane fractions obtained from hepatocytes treated with glucagon exhibited a decreased glucagon (with or without GTP)-stimulated adenylate cyclase activity. A maximum effect was seen in around 5 min. No change in the rate of cyclic AMP production was observed for the basal, NaF-, p[NH]ppG (guanosine 5'-[β,γ -imido]-triphosphate)- and GTP-stimulated states of the enzyme. The lag observed in the p[NH]ppG-stimulated adenylate cyclase activity of native membranes was abolished when membranes from glucagon-pretreated cells were used. When Mn^{2+} replaced Mg^{2+} in the assays, the magnitude of the apparent desensitization was decreased. Mn^{2+} abolished the lag of onset of p[NH]ppG-stimulated activity in native membranes. The desensitization process was dose-dependent on glucagon, which exhibited a K_a of 4×10^{-10} M. Depletion of intracellular ATP did not affect this process. It is suggested that this desensitization occurs at the level of the guanine nucleotide-regulatory protein.

Glucagon binds to specific receptors on the external surface of hepatocytes (Rodbell *et al.*, 1971; Sonne *et al.*, 1978). This leads to an increase in intracellular cyclic AMP accumulation (Johnson *et al.*, 1972; Christoffersen & Berg, 1974) through an activation of adenylate cyclase via a guanine nucleotide-regulated mechanism (Johnson & Mukku, 1979; Rodbell, 1980). The elevation of intracellular cyclic AMP concentrations is rapid, reaching a maximum value within minutes (Johnson *et al.*, 1972; Pilgis *et al.*, 1975).

It has been proposed that for β -receptors the desensitization or agonist-induced loss of hormonal response of adenylate cyclase activity is a two-phase process within the intact cell. Firstly, there is a rapid uncoupling of the β -adrenergic receptors from the guanine nucleotide-regulatory component of the adenylate cyclase complex, followed by a long-term induction of receptor loss from the cell surface (Harden *et al.*, 1979; Su *et al.*, 1980). We show here that the challenge of hepatocytes with glucagon achieves a rapid dose-dependent uncoupling of hormonal stimulation of adenylate cyclase in isolated membranes.

Materials and methods

Collagenase, cyclic AMP and ATP were purchased from Boehringer (U.K.) Ltd., Lewes, East

Abbreviation used: p[NH]ppG, guanosine 5'-[β,γ -imido]triphosphate.

Sussex, U.K. 3-Isobutyl-1-methylxanthine was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Glucagon was kindly given by Dr. W. W. Bromer of Eli Lilly and Co., Indianapolis, IN, U.S.A. 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 A.R. grade from BDH Chemicals, Poole, Dorset, U.K.

Isolated hepatocytes were prepared from fed 225–250 g male Sprague–Dawley rats (Elliott *et al.*, 1976) and incubated as previously described (Smith *et al.*, 1978). Cells (3–5 mg dry wt./ml) were preincubated (Smith *et al.*, 1978) at 37°C for 20–30 min before use. Glucagon was added to the reaction vessel in a volume that did not exceed 1% of the total incubation volume. After the appropriate time interval, samples were removed and the cells quenched by the adding of an equal volume of ice-cold 1 mM-KHCO₃, pH 7.2. All further procedures were performed at 4°C.

The ATP content in the isolated hepatocytes was determined on a HClO₄ extract. After neutralization, the ATP content of the extract was determined by the luciferase method (Stanley & Williams, 1969). Cells with an ATP concentration greater than 8.7 nmol/mg dry wt. were used as viable.

A washed membrane fraction was obtained as previously described (Houslay & Elliott, 1979). In

all cases membranes were used within 2 h of preparation.

Adenylate cyclase was assayed as described previously (Houslay *et al.*, 1976), in a mixture with final concentrations of 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 hydrochloride, adjusted to a final pH of 7.4 with KOH.

The preparation of ¹²⁵I-labelled glucagon and its use in specific binding assays was as detailed previously (Houslay *et al.*, 1977); identical protein concentrations of both control and treated membranes were used.

Protein was determined by the method of Warren *et al.* (1974).

Results

Membrane fractions obtained from hepatocytes treated with glucagon (10 nM) for various periods of time exhibited a decreased glucagon-stimulated adenylate cyclase activity. A maximum effect was achieved in around 5 min (Fig. 1). Table 1 shows the activity of various ligand-stimulated states of adenylate cyclase in membranes prepared from hepatocytes treated with glucagon (10 nM) for 5 min. No effect was observed on basal, NaF- and GTP-stimulated states; however, the glucagon- and glucagon + GTP-stimulated states exhibited activities some 30–40% below that of their appropriate controls.

In contrast, the p[NH]ppG-stimulated state exhibited an apparent activation (Table 1). Unlike the other ligand-stimulated states of adenylate cyclase, which exhibited linear time-courses over 15 min, (results not shown), in the presence of p[NH]ppG rates of cyclic AMP production were markedly non-linear, exhibiting a lag of about 3 min when native membranes were used (Fig. 2a). However,

after exposure of intact hepatocytes to either glucagon (Fig. 2a) or cholera toxin (C. M. Heyworth & M. D. Houslay, unpublished work) we noted that the p[NH]ppG-stimulated activity in the broken membranes from these cells did not exhibit a detectable lag period. Indeed, by taking rates from

Fig. 1. Time course for glucagon-induced loss of glucagon + GTP-stimulated adenylate cyclase activity

Hepatocytes were treated with glucagon (10 nM) for various time intervals at 37°C as in the Materials and methods section. The membrane fraction derived from the cells was washed once with 1 mM-KHCO₃, pH 7.2, before use. Glucagon (10 nM) + GTP (0.1 mM)-stimulated adenylate cyclase was determined during a 10 min incubation at 30°C. The enzymic activity of the glucagon-treated membranes is expressed as a percentage of that of untreated membranes at the same time point. Bars represent $\pm$ S.D. This shows a typical set of incubations with triplicate adenylate cyclase assays. The experiment was performed three times.

Table 1. Activation of adenylate cyclase, from control and glucagon-treated hepatocytes, by various ligands

For glucagon-treated hepatocytes, the cells were incubated with glucagon (10 nM) for 5 min at 37°C as in the Materials and methods section. The membrane fraction derived from the cells was washed once with 1 mM-KHCO₃, pH 7.2, before use. The activities expressed reflect the accumulation of cyclic AMP over a 10 min period of assay at 30°C (see the Results section). Results are means $\pm$ S.E.M., where *n* is the number of separate experiments with different cell/membrane preparations. In each instance assays were done in triplicate.

Ligand		Control hepatocytes (specific activity, pmol/min per mg of protein)	Glucagon-treated hepatocytes (activity as % of that of control hepatocytes)
Basal	(<i>n</i> = 3)	0.48 $\pm$ 0.14	107 $\pm$ 7
GTP (0.1 mM)	(<i>n</i> = 3)	1.02 $\pm$ 0.12	105 $\pm$ 15
p[NH]ppG (0.1 mM)	(<i>n</i> = 6)	5.30 $\pm$ 1.13	211 $\pm$ 30
Glucagon (10 mM)	(<i>n</i> = 6)	18.2 $\pm$ 0.53	72 $\pm$ 8
Glucagon (10 mM) + GTP (0.1 mM)	(<i>n</i> = 6)	24.7 $\pm$ 0.62	64 $\pm$ 10
NaF (15 mM)	(<i>n</i> = 6)	15.8 $\pm$ 3.2	92 $\pm$ 7

Fig. 2. Time courses of p[NH]ppG-stimulated cyclic AMP production by membrane fractions from control and glucagon-treated membranes

Hepatocytes were treated with glucagon (10nM) for 5 min at 37°C as in the Materials and methods section. The membrane fraction obtained from the cells was washed three times with 1mM-KHCO₃, pH 7.2, before use. p[NH]ppG (0.1 mM)-stimulated adenylate cyclase activity was assayed as in the Materials and methods section. (a) Assay includes 5 mM-MgSO₄ and (●) membranes from control cells or (■) membranes from glucagon-treated cells. (b) Assay includes 5 mM-MnSO₄ instead of MgSO₄ (●) and membranes from control cells or (■) membranes from glucagon-treated cells. The amount of cyclic AMP produced per mg of protein at various time intervals is given.

linear segments of these various plots one can demonstrate that the p[NH]ppG-stimulated activity was essentially unaffected by glucagon treatment of hepatocytes.

When Mn²⁺ (5 mM) replaced Mg²⁺ in the adeny-

Table 2. Activation of adenylate cyclase, from control and glucagon-treated hepatocytes, by various ligands when Mg²⁺ is replaced by Mn²⁺ (5 mM)

For details see the legend to Table 1, except that Mn²⁺ (5 mM) was substituted for Mg²⁺ in the adenylate cyclase assay: n = 6 for all experiments.

Ligand	Control hepatocytes (specific activity, pmol/min per mg)	Glucagon-treated hepatocytes (activity as % of that of control hepatocytes)
p[NH]ppG (0.1 mM)	13.8 ± 1.6	129 ± 12
Glucagon (10 mM)	17.6 ± 1.9	97 ± 4
Glucagon (10 mM) + GTP (0.1 mM)	27.6 ± 1.7	78 ± 5

ate cyclase assays (Table 2), we observed that after glucagon treatment of the hepatocytes the glucagon + GTP-stimulated state was still inhibited, albeit to a much smaller extent. However, with Mn²⁺, the glucagon-stimulated activity was the same in membranes from both glucagon-treated and untreated cells. If p[NH]ppG was used to stimulate adenylate cyclase, in the presence of Mn²⁺, then the activity in both the treated and the control membranes was very similar. Indeed, on examination of the rate of cyclic AMP production in the presence of both this stimulatory ligand and Mn²⁺ we noted that the time courses for both control and glucagon-treated membranes were linear (Fig. 2b). In contrast there was a marked lag period observed when Mg²⁺ and control membranes were used in the adenylate cyclase assay (Fig. 2a).

Washing the membranes in 1mM-KHCO₃, pH 7.2, by resuspension and centrifugation at 15000g for 10 min (4°C) had little affect on the inhibition of GTP + glucagon-stimulated activity that was elicited by glucagon pretreatment of hepatocytes. However, the inhibition of the glucagon-stimulated activity was dramatically decreased by this washing procedure (Table 3).

Fig. 3 demonstrates the dose-dependent inhibition of glucagon + GTP-stimulated adenylate cyclase activity in membranes from hepatocytes treated with various glucagon concentrations for 5 min. The K_a for this effect is 4.1 × 10⁻¹⁰ ± 2.2 × 10⁻¹⁰ M (n = 4, S.E.M.).

The K_a for glucagon activation of adenylate cyclase in the presence of GTP was very similar in control membranes and membranes from glucagon-treated cells, being 0.8 × 10⁻⁹ M and 1.6 × 10⁻⁹ M respectively.

Specific binding of ¹²⁵I-labelled glucagon in membranes from glucagon-pretreated hepatocytes was indistinguishable from that observed in control

Table 3. *Effect of washing on adenylate cyclase activity from glucagon-treated hepatocytes*

Hepatocytes were pretreated and membrane fractions were diluted with a 10-fold volume of 1 mM-KHCO₃, pH 7.2, and pelleted at 15000 g for 10 min. The pellet was either repeatedly washed a further twice before resuspension and use or resuspended in its original volume. The adenylate cyclase activity was measured over a 10 min period at 30°C, and is expressed as a percentage of that of control hepatocytes subjected to similar washing procedures. Results are means $\pm$ S.E.M., where *n* is the number of separate experiments with different hepatocyte preparations. Adenylate cyclase activities were assayed in triplicate.

Ligand	No. of further washings	Activity (%)	
		1	3
Glucagon (10 mM)	...	72 $\pm$ 8 (<i>n</i> = 6)	87 $\pm$ 5 (<i>n</i> = 3)
Glucagon (10 mM) + GTP (0.1 mM)		64 $\pm$ 10 (<i>n</i> = 6)	74 $\pm$ 8 (<i>n</i> = 3)
p[NH]ppG (0.1 mM)		211 $\pm$ 30 (<i>n</i> = 6)	191 $\pm$ 20 (<i>n</i> = 3)

Fig. 3. Dose-response curve to glucagon for hormone-induced loss of glucagon + GTP-stimulated adenylate cyclase activity

Hepatocytes were treated with various glucagon concentrations for 5 min at 37°C as in the Materials and methods section. The membrane fraction and adenylate cyclase activity were determined as described in the legend of Fig. 1. The enzymic activity of the glucagon-treated membranes is expressed as a percentage of that of untreated membranes. Bars represent $\pm$ S.D. This shows a typical set of incubations with triplicate adenylate cyclase assays. The experiment was performed four times.

membranes [$103 \pm 2\%$ of control in washed (once) membranes, and $99 \pm 1\%$ of control in extensively washed (three times) membranes (S.E.M.; *n* = 4)].

Glucagon (10 nM) treatment of hepatocytes still elicited the desensitization of adenylate cyclase, even when intracellular ATP concentrations were depleted by using 10 mM-fructose (Houslay & Elliott, 1979) from 9.1 to 3.1 nmol/mg dry wt. Such changes

in ATP concentrations have been demonstrated to diminish the effect of glucagon in elevating both intracellular cyclic AMP concentrations (C. M. Heyworth, unpublished work) and the activity of a particulate cyclic AMP phosphodiesterase (Boyes *et al.*, 1981). However, treatment of the hepatocytes with dibutyryl cyclic AMP (either 10 μ M or 100 μ M) failed to elicit any changes in glucagon-stimulated adenylate cyclase activity.

Discussion

Treatment of intact hepatocytes with glucagon causes a rapid and transient rise in intracellular cyclic AMP concentrations, which reach a peak within 5 min before returning to basal values within 30 min (Christoffersen & Berg, 1974; Heyworth *et al.*, 1983). Inclusion of 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, in the incubation led to a greatly enhanced intracellular cyclic AMP accumulation. However, this accumulation was also maximal 5 min after glucagon addition (Heyworth *et al.*, 1983).

The inclusion of 3-isobutyl-1-methylxanthine showed little or no difference for the time taken for the maximal intracellular cyclic AMP concentrations to be attained. This suggests that, although phosphodiesterase activation is involved in the rate of return to basal cyclic AMP concentrations, it is not concerned in the apparent cessation of cyclic AMP production occurring after 5 min of glucagon challenge. The activation of passive or active removal of cyclic AMP from the hepatocyte to the extracellular medium could also account for the observed transient maximal intracellular cyclic AMP accumulation. This, however, does not appear to be the case, as Pilkis *et al.* (1975) have shown that the rate of cyclic AMP exit from hepatocytes actually decreases after the observed maximum cyclic AMP accumulation.

These two factors can contribute to the decrease in intracellular cyclic AMP concentrations. However, our results imply that the main factor

determining the fall in the rate of cyclic AMP production is a rapid 'switching-off' or 'uncoupling' of the hormonal stimulation of hepatic adenylate cyclase. This is evident from experiments where hepatocytes were treated with glucagon, with the subsequent isolation of a membrane fraction. Here, it was found that the glucagon-stimulated adenylate cyclase activities were markedly decreased, but basal, NaF- and GTP-stimulated states were unaffected (Table 1). A comparison between the time-courses for this decrease in enzymic activity (Fig. 1) and that for hormonal stimulation of cyclic AMP accumulation in intact hepatocytes (Heyworth *et al.*, 1983) reveals that these two events reach apparent maxima at the same time.

The magnitude of the decrease in enzyme activity (Fig. 1) does not appear to account entirely for the apparent cessation of cyclic AMP production occurring after 5 min exposure to glucagon (Heyworth *et al.*, 1983). It may well be, however, that the activity of adenylate cyclase *in situ*, in hepatocytes, is considerably lower than the activity that we observed in membranes isolated from cells after such a treatment. This effect could be due to partial resensitization occurring during the membrane isolation procedure, as has been noted in other systems (Anderson & Jaworski, 1979).

Clearly, hormonal treatment of hepatocytes causes a rapid uncoupling of the glucagon receptor from the catalytic unit of adenylate cyclase, resulting in a decreased glucagon-stimulatable adenylate cyclase activity in isolated membranes. This process is dose-dependent and mediated via a high-affinity receptor ($K_a = 4.1 \times 10^{-10} \pm 2.2 \times 10^{-10} \text{ M}$). Indeed, this K_a value is apparently lower than that required for the glucagon-stimulated production of intracellular cyclic AMP in hepatocytes (Heyworth *et al.*, 1983; Sonne *et al.*, 1978). Although a heterogeneous population of glucagon receptors is known to exist (Sonne *et al.*, 1978; Lin *et al.*, 1977), a high-affinity glucagon-receptor fraction ($K_a = 7 \times 10^{-10} \text{ M}$) is thought to account for only 10% of the total population.

The diminished response to glucagon is unlikely to be due to a cell-surface receptor loss, as specific binding of ^{125}I -labelled glucagon in both control and treated membranes was identical in each case. Although the plasma-membrane fraction prepared was not highly purified, internal vesicles would not be expected to be sedimented under the conditions used during the washing procedure. Furthermore, after incubation of hepatocytes with ^{125}I -labelled glucagon for 5 min at 37°C we failed to observe the presence of any internalized ^{125}I -labelled glucagon after subcellular fractionation on Percoll gradients (S. R. Wilson & M. D. Houslay, unpublished work). Other studies using hepatocytes (Santos & Blazquez, 1982) have shown no immediate loss of

glucagon receptor from the cell surface after challenge with glucagon, but a slow loss over several hours can occur. Down-regulation, the slow loss of surface receptor, is an energy-dependent process (Terris *et al.*, 1979; Kosmakos & Roth, 1980), in contrast with the rapid uncoupling phenomenon that we observe here, which is not ATP-dependent. The loss of glucagon receptors is unlikely to account for this effect, as, in the presence of GTP, glucagon activates adenylate cyclase by a collision coupling mechanism (Martin *et al.*, 1979; Houslay *et al.*, 1980). Under such conditions we would need to inactivate some 70–80% of the total receptor population to account for the degree of uncoupling observed. Furthermore, we have shown that, in the absence of added GTP, glucagon activates adenylate cyclase by a mobile receptor mechanism (Houslay, 1981; Houslay *et al.*, 1977). Under such conditions, receptor loss causes a much greater fall in glucagon-stimulated activity compared with that observed when GTP is present as well (Houslay *et al.*, 1980). This is in complete contrast with the results that we observe here (Tables 1 and 3). Indeed the fall in glucagon-stimulated activity was abolished by extensive washing, although the glucagon + GTP-stimulated activity still showed a marked decrease under these conditions (Table 3). We consider that GTP is essential for the expression of the uncoupling of the glucagon receptor, where, presumably, the extensive washing of the membrane fractions depletes them of residual endogenous guanine nucleotides.

These observations imply that, rather than a loss of receptor or hormone binding occurring, there is a loss of functional coupling of the receptor through to the catalytic unit of adenylate cyclase. Such a coupling is mediated by guanine nucleotide-regulatory proteins (Rodbell, 1980; Ross & Gilman, 1980; Limbird, 1981). In a number of other systems it has been suggested that the rapid desensitization, giving rise to a functional uncoupling, could be due to a modification of the extent of interaction between the guanine nucleotide-regulatory protein and the receptor (Harden *et al.*, 1979; Su *et al.*, 1980; Pike & Lefkowitz, 1980).

Further evidence of a perturbation at the level of the guanine nucleotide-regulatory protein, caused by glucagon desensitization, comes from our observations with the non-hydrolysable analogue of GTP, p[NH]ppG, and also with Mn^{2+} . p[NH]ppG leads to an irreversible activation of adenylate cyclase (Salomon *et al.*, 1975; Rodbell, 1980). This, in the absence of hormone, proceeds with a marked lag phase (Salomon *et al.*, 1975; Iyengar & Birnbaumer, 1981; Fig. 2a). However, glucagon-desensitization clearly abolishes this lag (Fig. 2a), but does not alter the observed rate of p[NH]ppG-stimulated adenylate cyclase activity. This, together with our observ-

ations that the specific activities of basal, GTP- and fluoride-stimulated states (Table 1) are identical, in membranes from both control and glucagon-treated cells, suggests that the glucagon used in the treatment is removed during the washing and homogenization procedures.

Mn²⁺ ions have been demonstrated to exert potent effects on the adenylate cyclase system. In particular they perturb actions mediated by guanine nucleotide-regulatory proteins (see, e.g., Levinson & Blume, 1977; Hoffman *et al.*, 1981). When Mn²⁺ replaced Mg²⁺ in these assays, we observed a marked diminution of this desensitization response (Table 2). Indeed, in native membranes, Mn²⁺ abolished the lag seen in p[NH]ppG-stimulated adenylate cyclase activity, whereupon similar rates of p[NH]ppG-stimulated activity ensued with both native and glucagon-desensitized membranes (Fig. 2b).

The molecular mechanism by which this effect is achieved is at present unclear. Our results show, however, that occupancy of a glucagon receptor is essential for the response, as it cannot be elicited by dibutyryl cyclic AMP added at a sufficient concentration to mimic glucagon's action at intracellular sites (Heyworth *et al.*, 1983). Our contention is that the uncoupling/desensitization observed is due to a modification of the guanine nucleotide-regulatory protein(s) involved in regulating the hormonal response. This mechanism contrasts with that observed for the desensitization of catecholamine receptors, which does not appear to involve regulatory protein modification (see e.g. Green & Clark, 1981; Iyengar *et al.*, 1981), although it bears comparison with the desensitization of lutropin-stimulated adenylate cyclase (Ezra & Salomon, 1980; Dix & Cooke, 1982).

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