A role for protein kinase C-mediated phosphorylation in eliciting glucagon desensitization in rat hepatocytes

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An immobilized hepatocyte preparation was used to show that both vasopressin and glucagon could desensitize the ability of glucagon to increase intracellular cyclic AMP concentrations. This process was not dependent on any influx of extracellular Ca^{2+} and was not mediated by any rise in the intracellular level of Ca^{2+}. The protein kinase C-selective inhibitors chelerythrine, staurosporine and calphostin C acted as potent inhibitors of the desensitization process but with various degrees of selectivity regarding their ability to inhibit the desensitizing actions of glucagon and vasopressin. The protein phosphatase inhibitor okadaic acid was just as potent as vasopressin and glucagon in causing desensitization. Treatment of hepatocyte membranes with alkaline phosphatase restored to near control levels the ability of glucagon to stimulate adenylate cyclase activity in membranes from both glucagon- and vasopressin-treated (desensitized) hepatocytes. It is suggested that the desensitization of glucagon-stimulated adenylate cyclase activity involves a reversible phosphorylation reaction with the likely target being the glucagon receptor itself.

INTRODUCTION

A common feature seen following a hormone response is a desensitization process that allows a cell either to reduce its response to the continuing presence of agonist or to attenuate the effect of subsequent agonist challenge. This 'switching off' process can take many forms, including receptor down-regulation and uncoupling from the signal generation system (see [1] for a review).

The rat hepatic glucagon receptor provides an example of a hormone receptor that is subject to rapid desensitization subsequent to agonist challenge [2–4]. This plasma membrane receptor is coupled to the activation of adenylate cyclase through a process that is mediated by the stimulatory guanine nucleotide-binding protein G_s and leads to a profound, but transient, increase in the intracellular levels of cyclic AMP [2–5]. This is governed by the rapid desensitization of adenylate cyclase to glucagon stimulation, which has been attributed to an uncoupling of the glucagon receptor from the stimulatory G-protein G_s [2,5]. This desensitization process is, however, independent of any increase in cyclic AMP concentration [2] and it has been suggested that it may result from the action of protein kinase C [3–5]. In this regard, the hormones vasopressin and angiotensin, which do not increase intracellular cyclic AMP levels in hepatocytes but, rather, mediate their effects through phospholipid metabolism and protein kinase C activation, can also cause the rapid desensitization of glucagon-stimulated adenylate cyclase activity [3–5].

Here we develop a model system, using hepatocytes immobilized on collagen plates, which has allowed us to demonstrate that the glucagon-stimulated elevation of intracellular cyclic AMP levels can be desensitized. This was achieved by prior treatment with either glucagon itself or vasopressin, or the protein phosphatase inhibitor okadaic acid. We also show that desensitization elicited by glucagon and vasopressin can be inhibited differentially by protein kinase C-selective inhibitors and demonstrate that treatment of membranes from desensitized cells with alkaline phosphatase can reverse the desensitizing action of these hormones.

MATERIALS AND METHODS

Glucagon, [Arg]vasopressin, Ca^{2+} ionophore A23187, BSA, rat tail collagen, alkaline phosphatase, staurosporine, calphostin C and ATP were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. GTP, creatine phosphate, creatine kinase and cyclic AMP were supplied by Boehringer Mannheim, U.K. Ltd., Lewes, East Sussex, U.K. Okadaic acid and chelerythrine chloride were from Calbiochem Novabiochem Ltd., Nottingham, U.K. Collagenase was from Worthington Biochemical Corporation, Freehold, NJ, U.S.A.

Preparation of hepatocytes

Isolated hepatocytes were prepared from male Sprague–Dawley rats (200–250 g) as described previously [2,6], suspended in Krebs–Henseleit [7] buffer (2.5 mM Hepes, pH 7.4/2% BSA) [7] and pre-incubated at 37 °C in a shaking water bath for 30 min with gassing every 15 min using O_2/CO_2 (95:5, v/v). The cells were then centrifuged at 64 *g* in a Beckman MSE Centaur for 3 min and resuspended in the same buffer to give a cell density of approx. 1.5×10^6 cells/ml.

Attachment of hepatocytes to collagen plates

After the final wash, the cells were plated out by adding 1 ml of cell suspension (1.5×10^6 cells/ml) to collagen-coated Petri dishes (35 mm diam.) and placing them in an incubator for 40 min until the cells had adhered to the plates but not spread. After this period, cells that had not adhered were aspirated and the plates were washed once with Krebs–Henseleit buffer [7] (1 ml) before the addition of hormones.

Abbreviations used: G_s, stimulatory guanine nucleotide-binding protein acting upon adenylate cyclase; G_i, inhibitory guanine nucleotide-binding protein acting upon adenylate cyclase; cyclic AMP, cyclic 3′, 5′ adenosine monophosphate; PCA, perchloric acid.

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Assessing desensitization in immobilized cells

Hepatocytes were attached to collagen plates and incubated for a 40 min period to allow for adherence. Medium was aspirated from the immobilized hepatocytes before the addition of fresh medium (1 ml) containing the indicated ligand or with medium alone to determine the control state. They were then incubated for 6 min in this pre-incubation/desensitization period. Cells were then washed with Krebs buffer [7] (0.9 ml), to remove the ligand, and left for 6 min at 37 °C. Over this period, irrespective of the pre-incubation conditions, intracellular cyclic AMP levels stabilized at 0.4 ± 0.2 pmol/10^6 cells. This level approximated to that seen for the cells at the start of the experiment under basal conditions (0.3 ± 0.2 pmol/10^6 cells). These determinations showed that cyclic AMP levels in cells which had been pretreated with glucagon had returned to the resting/basal state over the recovery period. After the washing and stabilization period, glucagon desensitization was assessed by determining the ability of glucagon to stimulate an increase in intracellular cyclic AMP. This was done by treating the cells with 10 nM glucagon for 3 min and subsequently harvesting them for the determination of the intracellular concentration of cyclic AMP. Reactions were terminated and intracellular cyclic AMP levels determined as described below. The value of the glucagon-stimulated increase in the intracellular concentration of cyclic AMP in the absence of any hormone/ligand pretreatment was 11.7 ± 0.5 pmol/10^6 cells. In experiments where either chelerythrine or other protein kinase C inhibitors were added, then this was done at the attachment stage so that a 40 min pre-incubation with these ligands was achieved. The data obtained using okadaic acid used a final concentration of 0.1 % DMSO in the experiments due to a carry over of this solvent.

Assessing intracellular cyclic AMP concentrations

Incubations of immobilized cells with ligands were stopped by aspirating the medium from the plates and adding 250 μl of 2 % perchloric acid (PCA). The plates were then scraped and the precipitated protein pelleted by centrifuging the samples in a Beckman microfuge (13000 *g*) for 2 min. The supernatant was neutralized with KOH/triethanolamine/HCl (2 M/0.5 M) before centrifuging again to remove the potassium perchlorate precipitate. The cyclic AMP content of the supernatant was determined using a cyclic AMP-binding protein prepared from bovine adrenal glands as described in [8].

Assessing desensitization in a membrane fraction derived from intact hepatocytes

This was done exactly as described by Murphy et al. [2,5].

Treatment of hepatocyte membranes with alkaline phosphatase

An hepatocyte membrane fraction was prepared and suspended in the buffer described by Murphy et al. [5]. A sample (100 μl) of this, containing $\sim$ 200 μg of membrane protein, was treated for 10 min at 37 °C with 20 units of alkaline phosphatase. This was then used for assay of adenylate cyclase activity.

Preparation of a hepatocyte washed membrane fraction

In some cases a washed membrane fraction was prepared from desensitized cells as described by Heyworth and Houslay [2]. In these experiments the cells were incubated in suspension with the additions detailed in the Table legends. After the appropriate incubation period, the incubation vessels were placed on ice and 1 ml samples of cell suspension quenched by adding an equal volume of ice-cold 1 mM $KHCO_3$, pH 7.2. All further procedures were performed at 4 °C.

Adenylate cyclase assay

Adenylate cyclase activity was determined by a modification of an assay described previously [9]. Briefly, this involved using an assay mixture containing 10 μl of membranes (30–40 μg/μl protein), 10 μl of GTP (100 μM final concentration), 10 μl of glucagon (10 nM final concentration) and 50 μl of buffer/ATP-regenerating system with final concentrations of 1.5 mM ATP, 5 mM $MgSO_4$, 10 mM theophylline, 1 mM EDTA, 1 mM dithiothreitol, 25 mM triethanolamine hydrochloride/KOH (pH 7.4), phosphocreatine (7.4 mg/ml), creatine kinase (0.2 mg/ml) and BSA (0.8 mg/ml). The volume of each incubation mixture was made up to 100 μl with water. These were then incubated at 30 °C for 10 min before an equal volume of 4 % PCA was added to stop the reaction. The samples were centrifuged, the supernatants were neutralized and the cyclic AMP content measured as above.

Protein assay

Protein concentrations were assayed by the method of Petersen [10].

RESULTS AND DISCUSSION

Challenge of hepatocytes with glucagon leads to a transient rise in intracellular cyclic AMP concentrations (see, for example, [2]). We have suggested that this is primarily governed by the rapid desensitization of adenylate cyclase with the subsequent degradation of cyclic AMP through phosphodiesterase action [11,12]. Our criterion for desensitization was that in membranes isolated from cells which had been challenged with glucagon for various periods of time, we noted [2] a rapid attenuation in the ability of glucagon to stimulate adenylate cyclase activity, by approx. 50–60 %. However, we show here for the first time that treatment of intact hepatocytes with glucagon can desensitize the ability of a subsequent challenge with glucagon to elevate intracellular cyclic AMP levels (Table 1). We have done this using hepatocytes which were attached to collagen plates such that they could first be challenged with glucagon (10 nM; 6 min) then washed, both to remove the hormone and to allow intracellular cyclic AMP levels to re-attain those seen under basal (resting) conditions, before re-challenge with glucagon. Thus the intact-cell cyclic AMP response to glucagon itself became desensitized (Table 1). As with studies done observing the desensitization of glucagon-stimulated adenylate cyclase activity in membranes [5], desensitization assessed using the intact-cell cyclic AMP response could also be elicited by pretreating intact hepatocytes with the hormone vasopressin. This occurred in a manner that was just as effective as that elicited by glucagon in achieving the desensitization of the intact-cell cyclic AMP response (Table 1). Vasopressin does not, however, increase intracellular cyclic AMP levels in hepatocytes [2,12,13]. That it can elicit the desensitization of glucagon-stimulated adenylate cyclase activity (this study), as can angiotensin, tumour-promoting phorbol esters and synthetic diacylglycerols (see [3,4] for reviews), supports the concept that the desensitization of glucagon-stimulated adenylate cyclase is a cyclic AMP-independent process [2–5,14] that can be elicited through the activation of protein kinase C in rat hepatocytes [3–5].

Both glucagon and vasopressin [15–18] elicit major increases in intracellular Ca^{2+} levels. It is possible that such changes could be sufficient to trigger desensitization by stimulating the activity

Table 1 Desensitization of glucagon-stimulated cyclic AMP accumulation in intact hepatocytes

In these experiments desensitization of glucagon-stimulated cyclic AMP accumulation was studied in intact hepatocytes, attached to collagen plates, as described in the Materials and methods section. After the pre-incubation condition period, intracellular cyclic AMP levels stabilized at 0.4 ± 0.2 pmol/10^6 cells. This level approximated to that seen for the cells at the start of the experiment under basal conditions (0.3 ± 0.2 pmol/10^6 cells). These determinations showed that cyclic AMP levels in cells which had been pretreated with glucagon had returned to the resting/basal state over the recovery period. The value of the glucagon-stimulated increase in the intracellular concentration of cyclic AMP in the absence of any hormone/ligand pretreatment was 11.7 ± 0.5 pmol/10^6 cells. Chelerythrine and other protein kinase C inhibitors were added at the attachment stage so that a 40 min pre-incubation with these ligands was achieved. In experimental sets the appropriate control value was taken as the native stimulation (100%), with comparisons done using various ligands in the pre-incubation. The data obtained using okadaic acid used a final concentration of 0.1% DMSO in the experiments due to a carry over of this solvent. We noted that 0.1% DMSO itself had an effect upon subsequent challenge with glucagon in that the response was increased by 22 ± 9%. The effect of okadaic acid was thus assessed relative to this heightened glucagon response serving as the control. The experiments described below show data for $n = 5$ experiments.

Pre-incubation conditions	Cyclic AMP production by glucagon challenge (% of control response seen with no ligand pre-incubation)
None (control)	100
Glucagon (10 nM)	45 ± 3
Vasopressin (10 nM)	51 ± 3
Okadaic acid (100 nM)	49 ± 6
Chelerythrine (1 μM)	109 ± 7
Chelerythrine (5 μM)	91 ± 3
Staurosporine (10 nM)	98 ± 6
Calphostin C (10 nM)	98 ± 4
Calphostin C (100 nM)	96 ± 5
Vasopressin (10 nM) + chelerythrine (1 μM)	106 ± 5
Glucagon (10 nM) + chelerythrine (1 μM)	45 ± 5
Glucagon (10 nM) + chelerythrine (5 μM)	73 ± 4
Vasopressin (10 nM) + staurosporine (10 nM)	94 ± 2
Glucagon (10 nM) + staurosporine (10 nM)	58 ± 7
Vasopressin (10 nM) + calphostin C (10 nM)	71 ± 5
Vasopressin (10 nM) + calphostin C (100 nM)	103 ± 5
Glucagon (10 nM) + calphostin C (10 nM)	59 ± 5
Glucagon (10 nM) + calphostin C (100 nM)	101 ± 6

of either Ca^{2+}/calmodulin-dependent protein kinase activity or protein kinase C. We have investigated this in studies done where desensitization was elicited using both glucagon and vasopressin and analysed by following the ability of glucagon to stimulate either an increase in intracellular cyclic AMP concentrations or membrane adenylate cyclase activity. In these experiments we found that, under the incubation conditions used here, the addition of the Ca^{2+} ionophore A23187 (1 μM; 6 min) was unable (< 5% difference; n = three experiments) to elicit desensitization (data not shown). In accord with this we were also able to show that the addition of La^{2+} (0.5 μM), which blocks the entry of Ca^{2+} across the hepatocyte plasma membrane [19] was unable to block (< 5% difference; n = three experiments) the desensitization process (data not shown). Furthermore, treatment of cells with EGTA (Ca^{2+}-free medium; 1 μM for 15 min prior to the addition of glucagon), to chelate Ca^{2+}, failed to block (< 4% difference; n = three experiments) the desensitization process. Thus neither an increase in intracellular Ca^{2+} nor a flux of this bivalent cation across the plasma membrane is pivotal in mediating the desensitization of glucagon-stimulated adenylate cyclase that can be elicited by both vasopressin and by glucagon.

It has been suggested [3,4] that protein kinase C may play a pivotal role in mediating the desensitization of glucagon-stimulated adenylate cyclase. This was based upon observations which showed that not only did desensitization occur apparently independently of any increase in cyclic AMP but that it could be triggered by both phorbol esters [5] and synthetic diacylglycerols [20]. In addition, the hormones vasopressin and angiotensin, which are known to stimulate inositol phospholipid metabolism and produce diacylglycerol in hepatocytes [13,21], elicited a similar uncoupling of the glucagon receptor from adenylate cyclase [5]. Indeed, glucagon is regarded as utilizing such a mechanism, in that it too can elicit the production of diacylglycerol in hepatocytes [21] with the stimulation of phosphatidylcholine metabolism which is believed [22] to provide the major source of this lipid together with a contribution from the small stimulation of inositol phospholipid metabolism that it can achieve [23–26]. In this regard we [27] have demonstrated that both vasopressin and glucagon can cause a transient activation of hepatocyte protein kinase C activity, the kinetics of which parallel those seen for desensitization.

In hepatocytes, under resting conditions (absence of hormone challenge), the level of cyclic AMP is well below the threshold for activation of protein kinase A that remains in its inactive tetrameric state [2–4]. In contrast to this, however, it would seem that in hepatocytes, under resting conditions, a residual protein kinase C activity can be observed which is able to act on the α-subunit of the inhibitory G-protein G_i-2, leading to a small fraction of this protein being phosphorylated [28–30]. This G-protein appears to be the focus of an active phosphorylation/dephosphorylation cycle, in that its phosphorylation can be rapidly elicited by either activation of protein kinase C or inhibition of protein phosphatase activity [28–30]. This latter action can be demonstrated by treatment of intact hepatocytes with the protein phosphatase inhibitor okadaic acid [29]. This phosphorylative action on αG_i-2 was exclusive to the action of the protein kinase C site, as okadaic acid treatment did not effect phosphorylation at the protein kinase A site on this G-protein [28,30]. Here, then, we were able to show that treatment of hepatocytes, under basal conditions, with okadaic acid was able to mimic the degree of desensitization achieved by both glucagon and vasopressin (Table 1). In view of our previous studies done with αG_i-2 [28–30], this observation is consistent with protein kinase C being able to mediate the desensitization of glucagon-stimulated adenylate cyclase in intact hepatocytes. Presumably, the basal activity of protein kinase C is sufficient to allow this process to go to completion provided that protein phosphatase activity is ablated. Furthermore, it shows that desensitization can not only be achieved through the activation of protein kinase C, as with phorbol myristate acetate and diacylglycerols, but can also result as a consequence of protein phosphatase inhibition. This indicates that, in hepatocytes, the control of coupling of the glucagon receptor to adenylate cyclase is also at the centre of an active phosphorylation/dephosphorylation cycle which is determined by protein kinase C and protein phosphatase activity.

In order to explore further the possible role of protein kinase C in glucagon desensitization, we chose to investigate whether this process was affected by selective protein kinase C inhibitors. One such compound is chelerythrine, which has been shown to exhibit some selectivity for inhibition of protein kinase C at low concentrations [31]. Indeed, we found that a concentration of 1 mM chelerythrine was sufficient to obliterate completely the ability of vasopressin to cause desensitization (Table 1). However, intriguingly, such a concentration of chelerythrine had no effect upon the ability of glucagon to cause desensitization, with a higher concentration of 5 mM causing half maximal blockade (Table 1). This differential sensitivity to the action of a protein kinase C inhibitor was not limited to chelerythrine, however. For we also analysed the action (Table 1) of two other selective

Table 2 Treatment of hepatocyte membranes with alkaline phosphatase reverses the desensitization of glucagon-stimulated adenylate cyclase

Intact hepatocytes were treated with either glucagon (10 nM) or vasopressin (10 nM) or were untreated (control) for 6 min. After this time cells were harvested, washed, disrupted and a washed membrane fraction obtained as described previously [5]. The ability of glucagon (10 nM) to stimulate adenylate cyclase activity was then assessed in membranes from untreated cells (control; 100%) and hormone challenged cells (% of control response). In addition, a portion of the membranes were treated with alkaline phosphatase (see the Materials and methods section) prior to the determination of glucagon-stimulated adenylate cyclase activity. The % response is given here relative to the appropriate control. Values are given as means with errors as S.D. for $n = 5$ experiments. Alkaline phosphatase treatment of membranes from control cells, i.e. those not challenged with hormone, showed little difference in their glucagon-stimulated adenylate cyclase activity ($< 6\%$). Values in parentheses are data showing a typical experiment with glucagon-stimulated adenylate cyclase activity expressed in pmol of cyclic AMP produced/min per μg of membrane protein with values as means $\pm$ S.D. for three determinations of activity.

	Stimulation of adenylate cyclase activity (%)	
Pre-incubation conditions	No alkaline phosphatase	Plus alkaline phosphatase
Control	100 (23 $\pm$ 3)	100 (24 $\pm$ 2)
Glucagon (10 nM)	58 $\pm$ 4 (14 $\pm$ 1)	89 $\pm$ 7 (21 $\pm$ 2)
Vasopressin (10 nM)	54 $\pm$ 8 (11 $\pm$ 3)	85 $\pm$ 4 (20 $\pm$ 2)

protein kinase C inhibitors, namely staurosporine [31] and calphostin C [32,33]. Using these compounds we also noted that they expressed a greater susceptibility to block the vasopressin-, compared with the glucagon-induced desensitization of adenylate cyclase (Table 1). This was most apparent for staurosporine and, to a lesser extent, for calphostin C (Table 1). One possible explanation for these observations is that vasopressin and glucagon show very different abilities to stimulate lipid signalling pathways in hepatocytes (see above) and can thus be expected to produce diacylglycerol species with different spectra of acyl chains. This might well give rise to a different profile of activation of the various isoforms of protein kinase C that are found in hepatocytes. Consistent with such a hypothesis are our analyses showing marked differences [27] in the ability of both glucagon and vasopressin to increase protein kinase C activity in hepatocyte membrane and cytosolic compartments. If the spectrum of protein kinase C isoforms activated by these two hormones exhibited slightly different sensitivities to inhibition by chelerythrine then this might provide a basis for our observations. It is also possible, however, that additional regulatory effects may complicate the situation when glucagon is used to effect desensitization. For example, glucagon challenge will also lead to the activation of protein kinase A, which might affect the functioning of protein kinase C isoforms and of protein phosphatases. It is also possible, however, that in a similar fashion to that seen for the β-adrenoceptor [1], glucagon may elicit additional desensitizing contributions by activating a receptor-specific kinase which shows a susceptibility to inhibition by these protein kinase C inhibitors, albeit at slightly reduced sensitivity.

On the assumption that phosphorylation of a component of the glucagon-stimulated adenylate cyclase system becomes phosphorylated during the desensitization process, we have treated (Table 2) membranes isolated from hormone-challenged (desensitized) hepatocytes with alkaline phosphatase to try and provide conditions where dephosphorylation and re-activation might ensue. While such a treatment has little or no effect on the glucagon-stimulated activity seen in membranes from resting cells (basal conditions), we observed a profound increase in activity in that from cells that had been pretreated with either glucagon or vasopressin to achieve desensitization (Table 2). These data suggest that the phosphorylation of a membrane protein is pivotal to the desensitization process. They are also consistent with our contention that desensitization did not result from the internalization of receptor or G-protein [2–4].

By analysing various adenylate cyclase ligand-stimulated activities we have been able to show that the desensitization lesion takes the form of an inability of the glucagon receptor to couple functionally to G_s [2–5]. We do not believe that this occurs as a result of the phosphorylation of G_s as this G-protein can be immunoprecipitated from desensitized hepatocytes in a non-phosphorylated form [27,34]. On such a basis we believe that it is the glucagon receptor itself which becomes phosphorylated in the desensitization process through the action of protein kinase C. Desensitization of glucagon-stimulated adenylate cyclase can be achieved in both a homologous fashion by glucagon and also in a heterologous fashion by vasopressin, with protein kinase C-selective inhibitors identifying subtle differences in the processes utilized by these two hormones.

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