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Treatment of intact hepatocytes with synthetic diacyl glycerols mimics the ability of glucagon to cause the desensitization of adenylate cyclase

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Incubation of intact hepatocytes with either of the synthetic diacyl glycerols 1-oleoyl-2-acetyl glycerol (OAG) or dihexanoyl glycerol (DHG) caused the transient uncoupling of the ability of glucagon to stimulate adenylate cyclase in membranes prepared from those cells. No change occurred in either the activity of the catalytic unit of adenylate cyclase or the coupling of G_s to adenylate cyclase. Diacyl glycerol action appeared to mimic glucagon-mediated desensitization of adenylate cyclase, suggesting that protein kinase C activation may provide the molecular trigger for glucagon desensitization.

Desensitization; Adenylate cyclase; Diacyl glycerol; Protein kinase C; Glucagon; Guanine nucleotide regulatory protein

1. INTRODUCTION

In many systems the challenge of cells with an agonist for a specific receptor can lead to the desensitization of the biological response. This appears to play an important control in modulating cellular signal transduction processes and a variety of different molecules can be used to achieve such regulation [1–4].

We [4–6] and others [7–11] have shown that challenge of hepatocytes with glucagon gives rise to the rapid desensitization of adenylate cyclase activity. This is evident from both the transience of the increase in intracellular cyclic AMP concentration that ensues and also from the decreased ability of glucagon to stimulate adenylate cyclase in membranes derived from glucagon-treated cells. The ability of glucagon to elicit the desensitization of adenylate cyclase appears to be a cyclic AMP-independent phenomenon [4–6,10–12]. This is most clearly shown by the ability of TH-glucagon, an analogue of glucagon which is incapable of activating adenylate cyclase, to elicit desensitization in intact hepatocytes [6]. We have suggested [6] that the molecular mechanism underlying the ability of glucagon to desensitise adenylate cyclase might be related to the diacyl glycerol- catalysed activation of protein kinase C as glucagon can increase intracellular diacylglycerol concentrations [13] and both glucagon and TH-glucagon have been shown [14–17] to elicit a stimulation, albeit rather small, of inositol phospholipid metabolism.

In this study we examine the effect of treatment of intact hepatocytes with synthetic diacyl glycerols upon specific facets of adenylate cyclase activity and show that they closely mimic the ability of glucagon to effect the 'uncoupling' or desensitization of this key regulatory enzyme.

Abbreviations: OAG, 1-oleoyl-2-acetyl glycerol; DHG, dihexanoyl glycerol

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2. MATERIALS AND METHODS

Collagenase, cAMP and ATP and all other nucleotides were purchased from Boehringer (UK), Lewes, East Sussex, UK. 3-Isobutyl-1-methylxanthine (IBMX) was from Aldrich, Gillingham, Dorset, UK. Forskolin (7-β-acetoxy-8,13-epoxy-1α,6β,9α-trihydrolased-14-en-11-one) was from Calbiochem. Glucagon was kindly given by Dr W.W. Bromer of Eli Lilly and Co., Indianapolis, IN, USA. Dihexanoyl glycerol (DHG) was kindly given by Dr L. Garland, Wellcome Foundation Ltd., Bekenham, UK. 1-Oleoyl-2-acetyl-glycerol (OAG) was obtained from Sigma, Poole, Dorset, UK. All other biochemicals were from Sigma. Radiochemicals were obtained from Amersham International, Amersham, Bucks, UK. All other chemicals were of AnalaR grade, from BDH Chemicals, Poole, Dorset.

DGH was dissolved in chloroform to give a stock solution of 300 μg/ml with further dilutions being carried out in 10 mM glucose/2.5 g BSA/100 ml 2.5 mM Ca^{2+}-containing Krebs–Henseleit buffer. OAG was suspended in a 1% solution of dimethylsulfoxide (DMSO) to give a stock solution of 30 μg/ml which was sonicated for 30 min before any further dilutions were carried out using distilled water.

Isolated hepatocytes were prepared [18,19] from fed 225–250 g male Sprague–Dawley rats and pre-incubated as previously described [5] at 37°C for 20 min with gassing using CO_2/O_2 (19:1) and at 15 min intervals during the experiments. 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 as before [5] and the cells were quenched by adding an equal volume of ice-cold 1 mM $KHCO_3$, pH 7.2; and then placing them on ice. All subsequent procedures were performed at 4°C.

A washed membrane fraction was obtained as described before by

us [5]. In all cases membranes, which were kept on ice, were used within 2 h of their preparation. The cyclic AMP produced was assessed in a binding assay using the cyclic AMP-binding subunit of protein kinase prepared from bovine heart as before [5]. The activities expressed reflect the accumulation of cAMP over a 10 min period of assay at 30°C.

Adenylate cyclase assays were performed at 30°C as described in detail previously by us [20]. Final concentrations of stimulatory ligands were added as stated in the legends and text. Initial rates were taken from linear timecourses.

3. RESULTS AND DISCUSSION

Various investigators [10–12], including ourselves [4–6], have shown that glucagon elicits the desensitization of adenylate cyclase activity in intact rat hepatocytes through a cyclic AMP-independent process. This does not ensue as a result of receptor loss but, rather, is due to the uncoupling of the glucagon receptor from the stimulatory G-protein, G_s [4]. This process can be mimicked by hormones such as vasopressin and angiotensin [6], which stimulate inositol phospholipid metabolism. And, as glucagon has been shown capable of eliciting a small activation of this pathway [14–17] and the production of diacylglycerol [13], we have suggested [6] that desensitization might be caused through the action of protein kinase C. Certainly, this enzyme has been shown to attenuate the functioning of a variety of receptors, including G-protein-linked ones, by effecting their phosphorylation [4] and is activated upon an increase in intracellular diacylglycerol concentrations [21]. Indeed, we were able to demonstrate that incubation of intact hepatocytes with either OAG or DHG led to an apparent desensitization of adenylate cyclase (fig. 1a). This was readily apparent from the diminished ability of glucagon to stimulate adenylate cyclase activity in a washed membrane fraction derived from OAG-treated cells. The action of OAG was, as with glucagon treatment or hepatocytes [5,6], limited to the uncoupling of the receptor from stimulation of adenylate cyclase. This is clearly evident from data showing that the functioning of the catalytic unit of adenylate cyclase, as assessed by determining basal activity of that stimulated by forskolin, was unaltered when assessed in membranes from treated cells (Table I).

Similarly, coupling of G_s to adenylate cyclase, as assessed by stimulation with NaF, was unaltered (Table I). Similar results were obtained with DHG, although the rate of uncoupling (desensitization) and its magnitude were far greater (Fig. 1a). This may reflect differences in the rates of entry and metabolism of the two synthetic diacylglycerols as well as differences in their potency and selectivity in activation of protein kinase C isoforms. In both instances, however, maximal effects were exerted after around 20 min. This was followed by a subsequent reversal, with resensitization occurring after a further 20 min (Fig. 1a). We feel that this resensitization is unlikely to be due to the metabolism of diacylglycerol as the rate seen was unaltered using DAG concentrations some 10-fold greater than those employed to obtain the data shown on Fig. 1. Thus we

Table I

Adenylate cyclase activity in membranes from control and diacylglycerol-treated hepatocytes

	Ligand addition Adenylate cyclase activity (pmol/min/mg protein)			
Cell pre-treatment	None (basal)	Forskolin	NaF	Glucagon + GTP
Di-octanoyl glycerol (15 ng/ml for 20 min)	1.8 ± 0.3^{+}	63.8 ± 5.2^{+}	48.7 ± 6.3^{+}	19.2 ± 2.9^{++} (69.8%)
Di-hexanoyl glycerol (15 ng/ml for 20 min)	2.2 ± 0.2^{+}	56.4 ± 3.2^{+}	52.1 ± 3.6^{+}	8.4 ± 3.6^{+++} (30.5%)
None (control)	1.9 ± 0.1	58.2 ± 6.5	54.2 ± 6.2	27.5 ± 2.6 (100%)
Glucagon (10 nM for 5 min)	2.0 ± 0.2^{+}	61.0 ± 4.1^{+}	48.3 ± 5.8^{+}	8.6 ± 1.2^{+++} (31.3%)

Hepatocyte membranes were assayed for adenylate cyclase activity in either the absence of any stimulatory ligand (basal activity) or in the presence of forskolin (10^{-4} M), NaF (15 nM) or glucagon (10 nM) together with GTP (10 μM). Membranes were prepared from control cells or those pre-treated with either di-hexanoyl glycerol or di-octanoyl glycerol or glucagon as indicated above. Activity, in the presence of glucagon + GTP, is shown in parentheses also as a percentage of that found for the untreated cells. $^{+}$not significantly different from activities found in membranes from control (non pre-treated) cells; ^{++}P is less than 0.01; ^{+++}P is less than 0.001. Student's t-test for n=6 experiments using different membrane preparations (errors are SD of means).

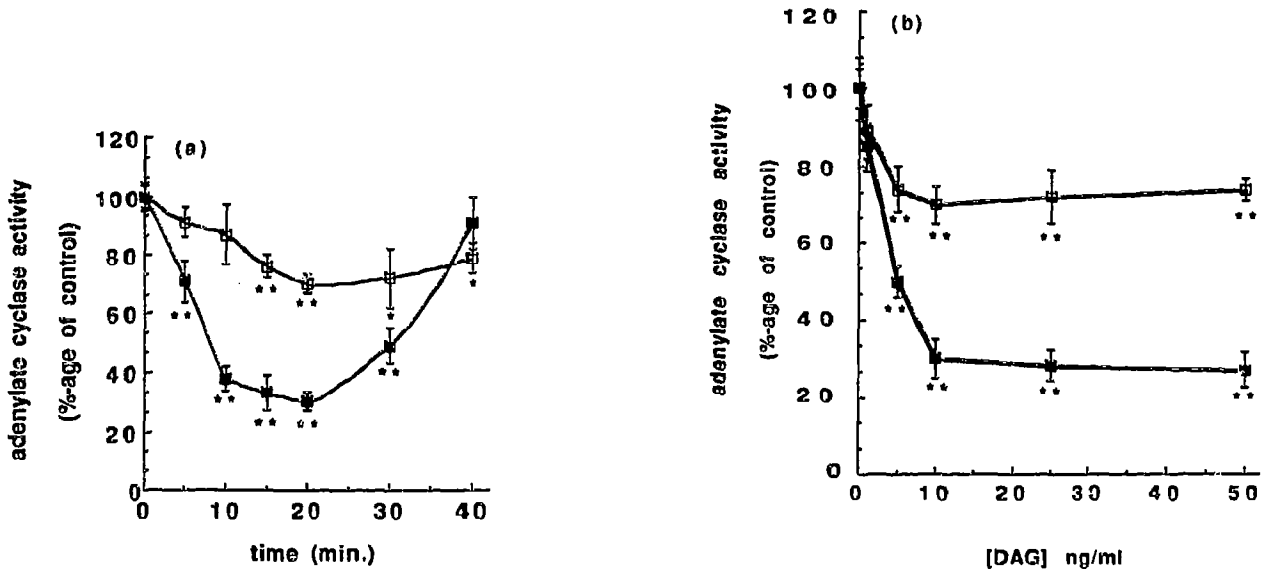

Fig. 1. Desensitization of glucagon-stimulated adenylate cyclase by synthetic diacylglycerols. (a) Shows a timecourse for the inactivation of (glucagon (10 nM) + GTP (10 μM))-stimulated adenylate cyclase activity in membranes from hepatocytes treated with either (■) di-hexanoyl glycerol (10 ng/ml) or (□) di-octanoyl glycerol (10 ng/ml). (b) Shows a dose–effect curve for the inactivation of (glucagon + GTP)-stimulated adenylate cyclase activity in membranes from hepatocytes treated for 10 min with either di-hexanoyl glycerol (■) or di-octanoyl glycerol (□). Experiments were done three times (n=3) using triplicate determinations of adenylate cyclase activity. Errors are SD *significant at P is less than 0.01 and **significant at P is less than 0.001 (Student's t-test).

believe that a separate resensitization process may be invoked as suggested before by us for glucagon [7]. Indeed, in the case of glucagon, desensitization was maximal after about 5 min, achieved levels comparable to that seen with DHG (Table I) and full resensitization was similarly obtained some 20 min after maximal desensitization [5–7].

The desensitization of glucagon-stimulated adenylate cyclase activity was dose-dependent with respect to both of the two synthetic diacylglycerols (Fig. 1b). In each instance, potencies were similar, yielding half-maximal effects at 2 ± 1 ng/ml OAG and 3 ± 1 ng/ml DHG (n=3 determinations; errors are SD).

These data are consistent with the notion [6] that it is the activation of protein kinase C by diacylglycerol that provides the trigger for glucagon desensitization in hepatocytes. Although this may be potentiated by the well-established rise in intracellular Ca^{2+} that glucagon elicits in hepatocytes by both cyclic AMP-independent and -dependent mechanism (see [22]). It is, unfortunately, not possible to test this by attempting to down-regulate protein kinase C using chronic exposure to the phorbol ester TPA as, in culture, primary hepatocytes begin to dedifferentiate and show alterations in and then loss of the ability of cyclase to be regulated normally by glucagon. The source of such diacylglycerol remains to be established: perhaps derived from the small stimulation of inositol phospholipid metabolism by glucagon, which has been noted, although there is the possibility that it could be sourced, in part, from the breakdown of phosphatidyl choline as has been noted recently for a variety of hormones [23]. It would appear from this and other studies (see [4]) that receptor-mediated diacylglycerol production and the activation of protein kinase C may play a pervasive role in modulating cellular responsiveness through altering adenylate cyclase functioning.

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