Resensitization of hepatocyte glucagon-stimulated adenylate cyclase can be inhibited when cyclic AMP phosphodiesterase inhibitors are used to elevate intracellular cyclic AMP concentrations to supraphysiological values

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Treatment of intact hepatocytes with glucagon led to the rapid desensitization of adenylate cyclase, which reached a maximum around 5 min after application of glucagon, after which resensitization ensued. Complete resensitization occurred some 20 min after the addition of glucagon. In hepatocytes which had been preincubated with the cyclic AMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), glucagon elicited a stable desensitized state where resensitization failed to occur even 20 min after exposure of hepatocytes to glucagon. Treatment with IBMX alone did not elicit desensitization. The action of IBMX in stabilizing the glucagon-mediated desensitized state was mimicked by the non-methylxanthine cyclic AMP phosphodiesterase inhibitor Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone]. IBMX inhibited the resensitization process in a dose-dependent fashion with an EC_{50} (concn. giving 50 % of maximal effect) of $26 \pm 5 \mu\text{M}$, which was similar to the EC_{50} value of $22 \pm 6 \mu\text{M}$ observed for the ability of IBMX to augment the glucagon-stimulated rise in intracellular cyclic AMP concentrations. Pre-treatment of hepatocytes with IBMX did not alter the ability of either angiotensin or the glucagon analogue TH-glucagon, ligands which did not increase intracellular cyclic AMP concentrations, to cause the rapid desensitization and subsequent resensitization of adenylate cyclase. It is suggested that, although desensitization of glucagon-stimulated adenylate cyclase is elicited by a cyclic AMP-independent process, the resensitization of adenylate cyclase can be inhibited by a process which is dependent on elevated cyclic AMP concentrations. This action can be detected by attenuating the degradation of cyclic AMP by using inhibitors of cyclic AMP phosphodiesterase.

INTRODUCTION

Hormones which exert their actions by stimulating adenylate cyclase activity in target cells often elicit a transient rise in the intracellular concentration of cyclic AMP (see, e.g., Heyworth *et al.*, 1983). The transient nature of such a response can be attributed both to the desensitization of adenylate cyclase and to the action and activation of specific cyclic AMP phosphodiesterases (Heyworth *et al.*, 1983; Houslay *et al.*, 1983; Houslay, 1986). Challenge of rat hepatocytes (Plas & Nunez, 1975; Heyworth & Houslay, 1983) and MDCK cells (Rich *et al.*, 1984) with glucagon has been shown to lead to desensitization of glucagon-stimulated adenylate cyclase. In hepatocytes this is an extremely rapid process, which is not caused by elevated intracellular cyclic AMP concentrations (Heyworth & Houslay, 1983). Indeed, desensitization can be elicited (Murphy *et al.*, 1987) by a glucagon analogue (TH-glucagon) which is incapable of both activating adenylate cyclase and increasing the intracellular concentration of cyclic AMP (Wakelam *et al.*, 1986). We (Murphy *et al.*, 1987) have suggested that desensitization is elicited as a result of stimulation of inositol phospholipid metabolism by both glucagon and TH-glucagon. This would be in accord with our

observations (Murphy *et al.*, 1987) that treatment of hepatocytes with either vasopressin or angiotensin can indeed mimic the glucagon desensitization process. The desensitization process may be mediated through the action of protein kinase C, as phorbol esters, which mimic the action of diacylglycerol in activating this enzyme (Nishizuka, 1983), can also similarly attenuate adenylate cyclase activity when added to intact hepatocytes (Heyworth *et al.*, 1984; Garcia-Sainz *et al.*, 1985; Murphy *et al.*, 1987).

The rapid desensitization of glucagon-stimulated adenylate cyclase activity is, however, itself transient, with resensitization of the system being achieved within a 20 min period (Heyworth & Houslay, 1983; Murphy *et al.*, 1987). The resensitization of glucagon-stimulated adenylate cyclase over this period is, however, difficult to reconcile with our previous observations that when hepatocytes were treated with IBMX (3-isobutyl-1-methylxanthine), to inhibit the degradation of cyclic AMP via phosphodiesterase action, maximal intracellular concentrations of cyclic AMP were achieved about 5 min after exposure to glucagon (Heyworth *et al.*, 1983). Such data implied that adenylate cyclase attained a desensitized state within 5 min and failed to undergo resensitization. This we explore in the study described

Abbreviations used: TH-glucagon, [1-*N*- α -trinitrophenylhistidine,12-homoarginine]glucagon; IBMX, 3-isobutyl-1-methylxanthine; EC_{50} , concn. giving 50 % of maximal effect.

Fig. 1. Desensitization and resensitization of adenylate cyclase activity in intact hepatocytes

(a) Elevation of intracellular cyclic AMP concentrations elicited by glucagon (10 nM) in the absence (□) or the presence of the phosphodiesterase inhibitors IBMX (0.1 mM; ■) or Ro-20-1724 (0.1 mM; △). (b) Desensitization of adenylate cyclase was assessed by measurement of the glucagon-stimulated adenylate cyclase activity observed in a washed membrane fraction isolated from intact hepatocytes which had been exposed to glucagon (10 nM) before harvest. Experiments were performed in the absence (□) or the presence of the phosphodiesterase inhibitors IBMX (0.1 mM; ■) or Ro-20-1724 (0.1 mM; △). (c) Activity of glucagon-stimulated adenylate cyclase was assessed in membranes from hepatocytes which had been treated with IBMX (0.1 mM) only. Results are means $\pm$ S.D. for experiments performed three times with different cell preparations from different animals ($n = 3$) and with triplicate assays of adenylate cyclase activity. The glucagon-stimulated adenylate cyclase activity in membrane fractions from control hepatocytes was in the range of 24–28 pmol/min per mg of protein for all experiments described herein.

here, making the observation that resensitization can be blocked by stabilizing intracellular cyclic AMP concentrations at elevated values.

MATERIALS AND METHODS

Collagenase, cyclic AMP and all nucleotides were purchased from Boehringer (U.K.), 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. Glucagon was kindly given by Dr. W. W. Brown of Eli Lilly and Co., Indianapolis, IN, U.S.A. TH-glucagon was kindly given by Professor V. J. Hruby and Dr. D. Trivedi (Department of Chemistry, University of Arizona, Tucson, AZ, U.S.A.); it was prepared and purified as described by Bregman *et al.* (1980). [8-Arginine]Vasopressin and angiotensin II were obtained from CRB, Cambridge, U.K. Forskolin (7 β -acetoxy-8,13-epoxy-1 α ,6 β ,9 α -tri-hydroxylab-14-en-11-one) was from Calbiochem. Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazo-

lidinone] was generously given by Roche Products, Welwyn Garden City, Herts., U.K.

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 pre-incubated at 37 °C for 20 min, with constant gassing (O₂/CO₂, 19:1), before use. Ligands were added to the reaction vessel in a volume which was less than 1% of the total incubation volume. In some experiments IBMX or Ro-20-1724 was added, in dimethyl sulphoxide, to the reaction vessel, with controls receiving dimethyl sulphoxide alone. The final concentration of dimethyl sulphoxide did not exceed 0.1%, and it did not exert actions on the processes described. IBMX and Ro-20-1724, when employed, were added 10 min ($t = -10$ min) before the addition of hormones ($t = 10$ min). After the appropriate time interval, samples were removed and the cells were quenched by adding an equal volume of ice-cold 1 mM-KHCO₃, pH 7.2, and then placing them on ice. All subsequent procedures were performed at 4 °C.

As described previously (Heyworth *et al.*, 1983), the

ATP content in isolated hepatocytes was determined by the luciferase method on a neutralized HClO_4 extract (Stanley & Williams, 1969). Cells with an ATP concentration of over 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 described by Houslay & Elliot (1979, 1981). In all cases membranes, which were kept on ice, were used within 2 h of their preparation.

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 hepatocyte intracellular cyclic AMP content was determined as described previously (Whetton *et al.*, 1983).

RESULTS

We (Heyworth *et al.*, 1983) and others (see, e.g., Christoffersen & Berg, 1974) have demonstrated that glucagon causes a transient increase in hepatocyte intracellular cyclic AMP concentrations (Fig. 1a). However, cells which had been preincubated with either IBMX (0.1 mM) or Ro-20-1724 (0.1 mM) for 10 min before the addition of glucagon (1 nM) showed a dramatic increase in the intracellular cyclic AMP concentrations that could be elicited upon exposure to glucagon (Fig. 1a). The rise in cyclic AMP concentrations attained maximal values around 5 min after the addition of glucagon. Such values slowly decreased after this point, and were not accompanied by any enhanced exit of cyclic AMP from the hepatocytes (Heyworth & Houslay, 1983).

As reported previously by us (Heyworth & Houslay, 1983; Murphy *et al.*, 1987), challenge of hepatocytes with glucagon caused a rapid, transient, desensitization of glucagon-stimulated adenylate cyclase activity. This was evaluated by treating intact cells with glucagon, harvesting them and preparing a washed membrane fraction for the determination of glucagon-stimulated adenylate cyclase activity. Desensitization was maximal approx. 5 min after challenge with glucagon, and resensitization was complete about 20 min after the time of addition of glucagon (Fig. 1b). However, if the hepatocytes were pretreated with IBMX (0.1 mM) for 10 min before addition of glucagon (1 nM), not only was the apparent desensitization greater, but it was also found to persist at a constant value even 30 min after exposure of the cells to glucagon (Fig. 1b). Similarly, treatment of cells with the non-methylxanthine cyclic AMP phosphodiesterase inhibitor Ro-20-1724 also allowed glucagon to elicit a stable desensitized state of adenylate cyclase (Fig. 1b). Treatment of hepatocytes with IBMX (0.1 mM) alone, however, had no effect on the glucagon-stimulated adenylate cyclase activity expressed by a membrane fraction isolated from such cells (Fig. 1c), i.e. did not elicit desensitization.

IBMX-treatment allowed glucagon to elicit a stable desensitized state in a dose-dependent fashion (Fig. 2). The EC_{50} (concn. giving 50% of maximal effect) for this

Fig. 2. IBMX blocks the resensitization of adenylate cyclase in a dose-dependent fashion

Cells were pre-treated with various concentrations of IBMX and then, 20 min after addition of glucagon (10 nM), harvested for analysis of desensitization (□) by assessing the glucagon-stimulated adenylate cyclase activity of a washed isolated membrane fraction. Hepatocyte intracellular cyclic AMP concentration (■) was determined in parallel. Results are means $\pm$ S.D. for an experiment performed three times with cell preparations from different animals ($n = 3$ as the number of cell preparations used at each data point), in each instance with duplicate assays of adenylate cyclase activity. Data obtained for desensitization and for determination of intracellular cyclic AMP concentrations were obtained on different days with different cell preparations.

process was $26 \pm 5 \mu\text{M}$. This paralleled the ability of IBMX to augment the action of glucagon in increasing intracellular cyclic AMP concentrations (Fig. 2), which yielded an EC_{50} value of $22 \pm 6 \mu\text{M}$. These values are not significantly different (t test, $P < 0.001$). Dose/effect curves were performed with IBMX, as it acts as a potent, non-selective, inhibitor of a variety of isoenzymes isolated from rat liver and rat hepatocytes (see Pyne *et al.*, 1987; M. D. Houslay & N. J. Pyne, unpublished work). Thus the use of this compound precludes the selective inhibition of specific phosphodiesterase species and, perhaps, the localization of augmentation of cyclic AMP concentrations to particular cellular compartments.

Treatment of hepatocytes with either TH-glucagon or angiotensin elicited, as described previously, the transient desensitization of adenylate cyclase (Fig. 3; Murphy *et al.*, 1987). However, this action was completely unaffected when cells were pre-treated with 0.1 mM-

Fig. 3. Glucagon desensitization triggered by either TH-glucagon or angiotensin was unaffected by the presence of IBMX

In (a), intact hepatocytes were either pre-treated (■) or not (□) with IBMX (1 mM) before challenge with 10 nM-angiotensin II. In (b), intact hepatocytes were either pre-treated (■) or not (□) with IBMX (0.1 mM) before

IBMX before addition of hormone (Fig. 3). Neither TH-glucagon nor angiotensin caused any change (less than 10% in this study) in the basal hepatocyte intracellular concentration of cyclic AMP in cells which had been either pre-treated or not with 0.1 mM-IBMX (see also Murphy *et al.*, 1987). The intracellular cyclic AMP concentration in the absence of added hormones (basal) was 1.4 ± 0.2 pmol/mg dry wt. of cells in the absence of IBMX and 4.0 ± 0.4 pmol/mg dry wt. of cells in its presence at time (t) = 0, with the addition of IBMX having been made at t = 10 min. At t = 20 min, intracellular cyclic AMP concentrations were 2.1 ± 0.3 and 8.2 ± 0.2 pmol/mg dry wt. of cells in the absence and presence of IBMX respectively.

DISCUSSION

Challenge of hepatocytes with glucagon leads to the rapid desensitization of adenylate cyclase (Heyworth & Houslay, 1983). The site of the lesion appears to be at the level of coupling between the glucagon receptor and the stimulatory guanine nucleotide regulatory protein, G_s (Heyworth & Houslay, 1983; Murphy *et al.*, 1987). The molecular basis of the lesion remains to be ascertained. We have, however, presented evidence suggesting that the lesion may be due to modification of G_s (Heyworth & Houslay, 1983), although there is evidence which suggests that it may involve the indirect activation of the inhibitory guanine nucleotide regulatory protein, G_i (Heyworth *et al.*, 1984). What is clear, however, is that elevation of intracellular cyclic AMP concentrations is not responsible for triggering desensitization. Thus, not only did treatment of cells with dibutyl cyclic AMP fail to elicit desensitization (Heyworth & Houslay, 1983), but desensitization could be elicited by TH-glucagon, a glucagon analogue which neither activated adenylate cyclase nor increased intracellular cyclic AMP concentrations (Wakelam *et al.*, 1986; Murphy *et al.*, 1987). Glucagon desensitization could also be mimicked by treatment of cells with either angiotensin or vasopressin (Murphy *et al.*, 1987), which serve only to stimulate inositol phospholipid metabolism in hepatocytes (Creba *et al.*, 1983; Downes & Michell, 1984) and do not increase the intracellular concentration of cyclic AMP (Murphy *et al.*, 1987). This, together with the fact that both glucagon and TH-glucagon can give a small stimulation of inositol phospholipid metabolism (Wakelam *et al.*, 1986; Williamson *et al.*, 1987; Blackmore & Exton, 1986) led us to suggest that glucagon desensitization was elicited by activation of the inositol phospholipid pathway (Murphy *et al.*, 1987). This may involve phosphorylation of the signal-transduction system, perhaps either the glucagon receptor or G_s , by protein kinase C, as treatment of cells for a short period with low concentrations of the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate can elicit an uncoupled state similar to that seen in glucagon desensitization (Heyworth *et al.*, 1984, 1985; Murphy *et al.*, 1987).

challenge with 10 nM-TH-glucagon. After the denoted time intervals, cells were harvested and a washed membrane fraction isolated for the determination of desensitization by analysis of glucagon-stimulated adenylate cyclase activity. Results are means $\pm$ s.d. for an experiment performed on three cell preparations obtained from different animals with triplicate adenylate cyclase assays ($n = 3$).

Desensitization of glucagon-stimulated adenylate cyclase is, however, a transient event, which attains a maximal effect some 3–5 min after exposure of cells to glucagon, after which resensitization occurs (Fig. 1b; Heyworth & Houslay, 1983; Murphy *et al.*, 1987). However, we see here that the resensitization process can be dramatically inhibited if the degradation of cyclic AMP was prevented by incubating hepatocytes with a cyclic AMP phosphodiesterase inhibitor (Fig. 1b). This appears to provide the explanation for the cessation in the glucagon-stimulated rise in intracellular cyclic AMP concentrations which occurs some 5 min after exposure of IBMX-pre-treated hepatocytes to glucagon (Heyworth *et al.*, 1983; Fig. 1a). Now, methylxanthines such as IBMX can not only inhibit cyclic AMP phosphodiesterase activity, but they can also act as antagonists at certain adenosine receptors (Fain & Malbon, 1979; Londos *et al.*, 1983). However, as the action of IBMX, in blocking resensitization, was mimicked by the cyclic AMP phosphodiesterase inhibitor Ro-20-1724 (Fig. 1b), which does not exert actions at adenosine receptors (Fain & Malbon, 1979; Londos *et al.*, 1983), it would seem that IBMX is acting solely by attenuating the degradation of cyclic AMP, whose intracellular concentration had been elevated by glucagon. The close correlation between the dose/response curves for the ability of IBMX to sustain both the glucagon-desensitized state of adenylate cyclase and the glucagon-elevated cyclic AMP concentrations (Fig. 2) again supports the notion that elevated cyclic AMP concentrations inhibit the resensitization of adenylate cyclase. Further evidence for such a cyclic AMP-dependent effect comes from our observation that IBMX completely failed to alter the desensitization/resensitization response engendered by both TH-glucagon and angiotensin, agents which are not capable of increasing the intracellular concentrations of cyclic AMP (Murphy *et al.*, 1987).

We suggest that, although the desensitization of glucagon-stimulated adenylate cyclase is brought about by a cyclic AMP-independent process, the resensitization response can be attenuated by elevated cyclic AMP concentrations. Such an action can be identified if the hydrolysis of cyclic AMP by phosphodiesterase action is inhibited in order to sustain elevated intracellular concentrations of cyclic AMP. Whether this observation has physiological relevance remains to be seen. It may be that high transient cyclic AMP concentrations occur, or that the process may be triggered to elicit hormone-resistant states, e.g. in lactation or pathophysiologically. The use of phosphodiesterase inhibitors therapeutically may also affect liver function through this route.

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