An assessment of the ability of insulin-stimulated cyclic AMP phosphodiesterase to decrease hepatocyte intracellular cyclic AMP concentrations

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Treatment of hepatocytes with either NH_4Cl (10mM) or fructose (10mM) blocks insulin's activation of the 'dense-vesicle' cyclic AMP phosphodiesterase. The ability of insulin (10nM) to decrease intracellular cyclic AMP concentrations raised by glucagon (10nM) was unaffected by pre-treatment with either NH_4Cl (10mM) or fructose (10mM). It is concluded that the 'dense-vesicle' enzyme does not play a significant role in this action of insulin and that as yet unidentified cyclic AMP phosphodiesterase(s) must be activated by insulin. Treatment of hepatocytes with either NH_4Cl or fructose appeared to increase, reversibly, cyclic AMP phosphodiesterase activity. When N^6 -(phenylisopropyl)adenosine was used to prevent glucagon from blocking insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase activity, insulin's ability to decrease intracellular cyclic AMP concentrations in glucagon-treated hepatocytes was increased markedly. Insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase activity can exert a potent effect in decreasing intracellular cyclic AMP concentrations elevated by glucagon.

Stimulation of adenylate cyclase activity by glucagon and adrenaline causes an increase in intracellular cyclic AMP concentrations in liver and in fat. It has been suggested that insulin antagonizes this effect by causing the activation of cyclic AMP phosphodiesterase activity within the cell (see Butcher *et al.*, 1968; Exton *et al.*, 1971).

Loten & Sneyd (1970), using adipocytes, were the first investigators to demonstrate that insulin could indeed increase the activity of cyclic AMP phosphodiesterase. The activity of a similar particulate enzyme was later found to be activated by insulin treatment of hepatocytes (Loten *et al.*, 1978). Subsequently, it was demonstrated in hepatocytes that intracellular cyclic AMP concentrations are modulated by insulin exerting effects both on cyclic AMP phosphodiesterase activity and on that of adenylate cyclase (Heyworth *et al.*, 1983a; Heyworth & Houslay, 1983b). However, the relative contributions of the control exerted

through these two enzyme systems by insulin is, intriguingly, determined by the glucagon concentration (Heyworth & Houslay, 1983a).

At least two distinct membrane-bound cyclic AMP phosphodiesterases are activated in hepatocytes on treatment with insulin (Heyworth *et al.*, 1983a). One is a plasma-membrane enzyme, which has been purified to homogeneity (Marchmont *et al.*, 1981) and shown to be activated by a phosphorylation mechanism (Marchmont & Houslay, 1980, 1981; Houslay, 1981; Houslay *et al.*, 1983a,b). This enzyme has properties which are quite different from those of the enzyme described by Loten *et al.* (1978). Indeed, we (Heyworth *et al.*, 1983a) have now located the enzyme described by Loten *et al.* (1978) in an intracellular-vesicle fraction and called this enzyme the 'dense-vesicle' cyclic AMP phosphodiesterase. Unlike the plasma-membrane cyclic AMP phosphodiesterase, the 'dense-vesicle' enzyme is activated by both glucagon and insulin. The mechanism by which this occurs is at present unclear (Francis & Kono, 1982), although evidence has been presented which suggests that a membrane-recycling event might be involved (Wilson *et al.*, 1983).

Abbreviations used: PIA, N^6 -(phenylisopropyl)-adenosine.

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In liver, as in all other tissues studied to date, multiple forms of cyclic AMP phosphodiesterase are found (see, e.g., Thompson & Strada, 1978). This poses the problem of understanding their relative contributions to the metabolism of cyclic AMP within the cell. We (Heyworth *et al.*, 1983a) have demonstrated that two distinct cyclic AMP phosphodiesterases are activated by insulin in intact hepatocytes. In the present study we attempt to evaluate the ability of each of these enzymes, on stimulation by insulin, to lower intracellular cyclic AMP concentrations that have been elevated by glucagon.

Materials and methods

All methods, including the preparation of hepatocytes, incubation conditions for hepatocytes, assay of cyclic AMP phosphodiesterase, Percoll fractionation of hepatocyte homogenates and determination of hepatocyte intracellular cyclic AMP concentrations and intracellular ATP content, were as described in some detail by Heyworth *et al.* (1983a). Brief descriptions of the procedures used are given in the preceding paper (Wallace *et al.*, 1984). Treatment of hepatocytes with NH_4Cl and fructose, and the determination of the degree of activation by insulin of the 'dense-vesicle' enzyme by using a 'hypo-osmotic method' were as described previously (Wilson *et al.*, 1983). Briefly, cells were preincubated for 15 min at 37°C with either the lysosomotropic agent NH_4Cl (10 mM) or fructose (10 mM) before initiation of the experiment by the addition of glucagon (10 nM). Incubation of hepatocytes with PIA was per-

formed as in the preceding paper (Wallace *et al.*, 1984). The materials used were as detailed explicitly in the papers cited above. Ro-20-1724 [(+)-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was kindly given by Roche Products, Welwyn Garden City, Herts., U.K.

Results and discussion

The addition of glucagon (10 nM) to intact hepatocytes causes a rapid rise in intracellular cyclic AMP concentrations. These reach a peak after about 5–6 min and subsequently fall slowly back to basal values after about 30 min. If, however, insulin (10 nM) is added 5 min after exposure to glucagon, the intracellular cyclic AMP concentrations fall much more rapidly back to basal values (Heyworth *et al.*, 1983a). Under these conditions, with glucagon concentrations at the upper end of the physiological range (see Montague, 1983), this effect of insulin has been shown to be almost entirely due to cyclic AMP phosphodiesterase activation and not to adenylate cyclase inhibition (Heyworth *et al.*, 1983a). Exposure of hepatocytes to glucagon before addition of insulin, however, blocks the ability of insulin to activate the plasma-membrane phosphodiesterase (Heyworth *et al.*, 1983a; Table 1). So under these conditions we only observed the activation of the 'dense-vesicle' phosphodiesterase by insulin (Heyworth *et al.*, 1983a; Table 1). If insulin's activation of the 'dense-vesicle' phosphodiesterase can be blocked, it is possible for us to evaluate the contribution of this enzyme to the increased breakdown of cyclic AMP. Here (Table 1) we show

Table 1. Effect of glucagon and insulin on the activation of the 'dense-vesicle' (DV) and plasma-membrane (PM) cyclic AMP phosphodiesterases in hepatocytes pre-treated with either fructose or NH_4Cl

Abbreviation: n.d., not detected (< 5%); results are means $\pm$ S.D. for three separate experiments using different cell preparations. Cells were preincubated and analysis of cyclic AMP phosphodiesterase activity was performed as described in the Materials and methods section. Incubation with glucagon or insulin alone was for 5 min, otherwise cells were exposed to glucagon for 5 min and then to insulin for 5 min before harvest. Data analysis was performed by Student's two-tailed *t* test, where data was taken as significant if $P < 0.05$: *1 not significant, **1 significant at $P < 0.01$ and **2 significant at $P < 0.005$, when compared with the degree of activation obtained in the untreated state. Activation is presented relative to the appropriate control (100%) gradient. This is obtained by assessment of the cyclic AMP phosphodiesterase activity in the peak fractions as described in detail in Heyworth *et al.* (1983b). A control gradient was performed for every experiment. Assayed at 1 μM -cyclic AMP at 30°C, the specific activities of these phosphodiesterases were 12–18 and 31–40 pmol/min per mg of protein for the 'dense-vesicle' and plasma-membrane fractions respectively (range for different cell preparations).

	Activity (% of control)					
	Untreated		NH_4Cl -treated		Fructose-treated	
	PM	DV	PM	DV	PM	DV
Insulin (10 nM)	159 $\pm$ 3	140 $\pm$ 9	161 $\pm$ 5*1	107 $\pm$ 8**1	160 $\pm$ 9*1	106 $\pm$ 5**2
Glucagon (10 nM)	n.d.	153 $\pm$ 12	n.d.	135 $\pm$ 6*1	n.d.	111 $\pm$ 6**1
Glucagon (10 nM) + insulin (10 nM)	108 $\pm$ 8	198 $\pm$ 12	107 $\pm$ 2*1	138 $\pm$ 9**2	104 $\pm$ 6*1	127 $\pm$ 9**2

that it is possible to prevent insulin activating the 'dense-vesicle' phosphodiesterase in glucagon-treated hepatocytes. This was achieved by pre-treating the hepatocytes with either NH_4Cl (10mM) or fructose (10mM).

A variety of lysosomotropic agents have been shown by us to block the ability of insulin to activate the 'dense-vesicle' phosphodiesterase (Wilson *et al.*, 1983). In contrast, the activation of this enzyme by glucagon is not significantly affected by NH_4Cl pretreatment of the hepatocytes (Table 1). Indeed, when NH_4Cl -pretreated hepatocytes were exposed to both glucagon and insulin, the degree of activation of the 'dense-vesicle' cyclic AMP phosphodiesterase that was observed reflected that seen in the presence of glucagon alone (Table 1). This provides further support for the suggestion (Heyworth *et al.*, 1983b; Houslay *et al.*, 1983a; Wilson *et al.*, 1983) that insulin and glucagon activate this enzyme through distinct routes.

Fig. 1. Insulin-induced changes in intracellular cyclic AMP concentrations in hepatocytes treated with NH_4Cl . Hepatocytes were preincubated for 20min in the presence of NH_4Cl (10mM) and then challenged with glucagon (10nM) at zero time. After 5min some samples (○) were treated with insulin (10nM) and others (●) acted as controls. Samples were then taken for analysis of intracellular cyclic AMP. This shows the result of a typical experiment repeated on three different cell preparations.

Depletion of intracellular ATP by using, for example, fructose has also been demonstrated (Boyes *et al.*, 1981) to block the hormonal activation of the 'dense-vesicle' phosphodiesterase, which we confirm here (Table 1). ATP depletion, however, has no effect on the ability of insulin to activate the plasma-membrane phosphodiesterase or on the ability of glucagon to block insulin's activation of this enzyme (Table 1; Wilson *et al.*, 1983).

After pretreatment of hepatocytes with either NH_4Cl or fructose, before exposure to glucagon, neither the plasma-membrane phosphodiesterase nor the 'dense-vesicle' phosphodiesterase was activated by insulin. Nevertheless, insulin still managed to lower intracellular cyclic AMP concentrations markedly (Figs. 1 and 2; Table 2); it decreased intracellular cyclic AMP concentrations by some 60–70% over a 5min period whether or not NH_4Cl or fructose was present (Table 2). This suggests that the 'dense-vesicle' phosphodiesterase contributes little, if anything, to the increased metabolism of cyclic AMP that occurred under these conditions.

The ability of insulin to decrease the intra-

Fig. 2. Insulin-induced changes in intracellular cyclic AMP concentrations in hepatocytes treated with fructose. Hepatocytes were preincubated for 20min in the presence of fructose (10mM) and then challenged with glucagon (10nM) at zero time. After 5min some samples (○) were treated with insulin (10nM) and others acted as controls (●). Samples were then taken for analysis of intracellular cyclic AMP. This shows the result of a typical experiment repeated on three different cell preparations.

Table 2. Effect of pre-treating hepatocytes with either fructose or NH_4Cl on changes in the intracellular cyclic AMP concentrations elicited by insulin and glucagon

Results are means $\pm$ S.D. for three separate cell preparations ($n = 3$) with triplicate determinations of cyclic AMP. Incubations and the determination of intracellular cyclic AMP were performed as described in the Materials and methods section. Insulin and glucagon were both added at 10 nM concentrations. Abbreviation: IBMX, 3-isobutyl-1-methylxanthine (used at 1 mM final concn.). Cyclic AMP concentrations are given as pmol/mg dry wt. of cells. Data analysis was performed by Student's two-tailed t test; data were taken as significant if $P < 0.05$: $^{*1}P < 0.001$ compared with appropriate control state; *1 not significant (N.S.) compared with control state with 'glucagon'; *2 N.S. compared with control state with 'insulin + glucagon'; $^{*2}P < 0.001$ compared with 'plus glucagon' alone state; *3 N.S. compared with 'plus glucagon' alone state.

	+ Glucagon for 5 min		+ Glucagon for 5 min, then + insulin for 5 min		+ IBMX	
		(as % of control state)		(as % of ' + glucagon alone' state)	+ Glucagon for 5 min	+ Glucagon for 5 min, then + insulin for 5 min
Control	17.1 ± 0.51	(100%)	6.79 ± 0.82**2	(40%)	94.1 ± 3.95	87.4 ± 3.34*1
+ NH ₄ Cl (10mM)	13.6 ± 0.34**1	(80%)	5.9 ± 0.91*2,**2	(43%)	95.7 ± 4.4*1	88.5 ± 5.47*2,*3
+ fructose (10mM)	8.76 ± 0.66**1	(51%)	2.98 ± 0.24**1,*2	(34%)	90.5 ± 3.34*1	84.2 ± 5.47*2,*3

cellular cyclic AMP concentrations elevated by glucagon was, however, still blocked by the cyclic AMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine when either NH_4Cl or fructose was present (Table 2). This occurred under conditions where 3-isobutyl-1-methylxanthine has been shown to inhibit over 90% of the hepatocyte cyclic AMP phosphodiesterase activity (Heyworth *et al.*, 1983a). It seems, therefore, that this effect of insulin is mediated by one of the other multiple forms of cyclic AMP phosphodiesterase that are found in the hepatocyte (Thompson & Strada, 1978; Beavo *et al.*, 1982). Insulin's activation of such a phosphodiesterase is presumably either extremely labile or, alternatively, reversible in nature. An obvious candidate for such a reversibly activated cyclic AMP phosphodiesterase would be the soluble calmodulin-dependent enzyme, which occurs in significant concentrations in the cytosol (Beavo *et al.*, 1982). An alternative possibility is that the purported small increase in cyclic GMP that occurs on exposure of hepatocytes to insulin (see Czech, 1977), might activate the cyclic-GMP-stimulated cyclic AMP phosphodiesterase that occurs in cytosol (Beavo *et al.*, 1982; Martins *et al.*, 1982).

We noted that both NH_4Cl and fructose decreased the increase in the intracellular cyclic AMP concentration caused by glucagon treatment of hepatocytes (Table 2). Although this effect was small with NH_4Cl (approx. 20%), it was appreciable with fructose (approx. 50%). Yet, in contrast with this, when the cyclic AMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was present in the incubations, glucagon caused an identical increase in the intracellular cyclic AMP concentra-

tion whether or not fructose or NH_4Cl was present (Table 2). This suggests that certainly fructose, and probably NH_4Cl , treatment of hepatocytes caused an increase in hepatocyte cyclic AMP phosphodiesterase activity. This effect was presumably reversible or very labile, as pre-treatment with either fructose or NH_4Cl did not lead to any enhancement of phosphodiesterase activity on Percoll fractionation of glucagon-treated hepatocytes (A. V. Wallace & M. D. Houslay, unpublished work).

In the preceding paper we demonstrated (Wallace *et al.*, 1984) that PIA prevented glucagon from blocking insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase. Furthermore, PIA did not affect the increase in intracellular cyclic AMP concentrations caused by glucagon. Thus by pre-treating cells with PIA we can hope to evaluate the ability of the plasma-membrane cyclic AMP phosphodiesterase, on stimulation by insulin, to increase the degradation of cyclic AMP. Fig. 3 shows that in cells pre-treated with PIA the rate at which cyclic AMP concentrations decrease was roughly doubled. This is consistent with the notion that the plasma-membrane cyclic AMP phosphodiesterase, when stimulated by insulin, can indeed increase the degradation of intracellular cyclic AMP. Such an interpretation is in accord with estimates that, after stimulation by insulin, its activity forms a significant fraction (approx. 20%) of the total cyclic AMP phosphodiesterase activity exhibited by homogenates (Marchmont & Houslay, 1980b; Heyworth *et al.*, 1983a). It is also consistent with the magnitude of the effect predicted from the simulation studies performed on this system by Reynolds (1982).

Fig. 3. Insulin-induced changes in intracellular cyclic AMP concentrations in hepatocytes treated with N^6 -(phenylisopropyl)adenosine

Hepatocytes were either exposed (■) or not exposed (○) to PIA (100nM) for 1 min and then challenged with glucagon (10nM) zero time. After 5 min all samples were treated with insulin (10nM) and samples taken for analysis of intracellular cyclic AMP. This shows the result of a typical experiment repeated on three different cell preparations.

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