Insulin activates the plasma-membrane and dense-vesicle cyclic AMP phosphodiesterase in hepatocytes by distinct routes

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1. Insulin elicits the activation of two distinct membrane-bound cyclic AMP phosphodiesterases when incubated at 37°C for 5 min with intact hepatocytes: the 'dense-vesicle' enzyme and the peripheral-plasma-membrane enzyme. 2. In hepatocytes the lysosomotropic agents chloroquine, methylamine and NH_4Cl , as well as intracellular ATP depletion elicited by fructose or incubation with insulin at 22°C, blocks selectively the activation of the 'dense-vesicle' enzyme. 3. Incubation of hepatocytes with bacitracin, leupeptin and a variety of proteinase inhibitors failed to affect insulin's activation of these two cyclic AMP phosphodiesterases by distinct routes. It is suggested that activation of the 'dense-vesicle' enzyme occurs through a pathway triggered by the endocytosis, processing and recycling of the insulin receptor. This might involve the delivery, with subsequent activation, of a latent phosphodiesterase into this fraction.

Although over the past few years there have been significant advances in our understanding of the structure of the insulin receptor (see, e.g., Czech, 1981), the molecular events that occur immediately upon occupancy of the receptor by insulin are ill-understood (see, e.g., Czech, 1977; Houslay, 1981; Denton *et al.*, 1981). More recently, however, evidence has been provided to suggest two molecular mechanisms whereby insulin might initiate events at the plasma membrane that could affect intracellular processes. The first is the observation that the insulin receptor itself is a protein kinase (Kasuga *et al.*, 1981). This kinase activity is highly unusual in that it specifically transfers phosphate groups to tyrosine residues on target proteins and also autophosphorylates the insulin receptor. In this respect it bears great resemblance to the epidermal-growth-factor receptor and to the kinase gene products of transforming viruses, such as pp60^{src} from Rous sarcoma virus (see Houslay, 1981). This might imply that such an activity reflects the long-term growth-promoting properties of insulin. Secondly, evidence has been presented (Heyworth *et al.*, 1983a,b; Heyworth & Houslay, 1983) which suggests that insulin exerts effects through a specific type of guanine nucleotide regulatory protein and associated protein kinases. This regulatory protein has been demonstrated to mediate insulin's activation of a plasma-membrane cyclic AMP phosphodiesterase and inhibition of adenylate cyclase. The activation of membrane-bound kinases is

undoubtedly a credible mechanism for initiating a variety of intracellular events (see Denton *et al.*, 1981; Houslay, 1981; Cohen 1982). However, upon occupancy by insulin the receptor is rapidly internalized into the cell (see, e.g., Fehlmann *et al.*, 1982), which might provide a further route by which insulin could initiate intracellular processes.

In hepatocytes there are two distinct cyclic AMP phosphodiesterases which are activated by insulin (Heyworth *et al.*, 1983a). The first is a plasma-membrane enzyme, which is activated through a phosphorylation mechanism (Marchmont & Houslay, 1980b; Houslay *et al.*, 1983a,b) mediated by a specific guanine nucleotide regulatory protein and associated protein kinase (Heyworth *et al.*, 1983a, b). The second is the 'dense-vesicle' enzyme, which is associated with a unique intracellular vesicle fraction, resolved by us on Percoll gradients. This enzyme does not appear to be activated by insulin through a route involving the proposed guanine nucleotide regulatory protein (Heyworth *et al.*, 1983a). Here we provide further evidence to show that insulin's activation of these enzymes is achieved by distinct routes.

Materials and methods

All methods, including the preparation of hepatocytes, incubation conditions for hepatocytes, Percoll fractionation of a hepatocyte homogenate, assay of cyclic AMP phosphodiesterase and determina-

tion of intracellular ATP content, were performed precisely as described in some detail by Heyworth *et al.* (1983a).

In some experiments the 'dense-vesicle' phosphodiesterase that is activated by insulin (Heyworth *et al.*, 1983a) was selectively solubilized from a hepatocyte homogenate by the procedure characterized and developed by Loten *et al.* (1978). Briefly, this involved homogenizing, at 4°C, hepatocytes in 0.25 M-sucrose/2 mM-EDTA/20 mM-Tris/HCl, final pH 7.4, with 30 strokes of a ground-glass homogenizer. A particulate fraction was obtained by centrifugation at 20 000 g_{av} for 20 min. This fraction was extracted with 1 mM-EDTA/20 mM-Tris/HCl, final pH 7.4, with four strokes of the homogenizer before centrifugation as above to yield a supernatant, which was used for phosphodiesterase assay. This hypo-osmotic-shock procedure has been demonstrated to release selectively a single insulin-activated phosphodiesterase (Loten *et al.*, 1978), whose properties and conditions of activation define it as being the 'dense-vesicle' enzyme (see Heyworth *et al.*, 1983a, for discussion).

Results

Treatment of hepatocytes with 10 nM-insulin for 5 min led to the activation of two distinct, membrane-bound, cyclic AMP phosphodiesterases, namely the 'dense-vesicle' enzyme and the plasma-membrane enzyme (Heyworth *et al.*, 1983a). The activation of these enzymes was assessed from Percoll gradients of hepatocyte homogenates (Table 1). A similar activation of the 'dense-vesicle' enzyme was also observed (Table 1) when followed by a hypo-osmotic-shock procedure (see the Materials and methods section) to release the 'dense-vesicle' enzyme selectively from a crude membrane fraction

as described by Loten *et al.* (1978). On the basis of several criteria this enzyme has been shown (Heyworth *et al.*, 1983a) to be that designated by us as the 'dense-vesicle' enzyme.

Cells were also preincubated with the lysosomotropic agents NH_4Cl (10 mM), chloroquine (0.2 mM) and methylamine (10 mM) for 15 min at 37°C before exposure to insulin (10 nM) for 5 min. After such a treatment, insulin's activation of the 'dense-vesicle' enzyme, whether assessed directly by Percoll-gradient analysis or indirectly by hypo-osmotic release, was almost completely abolished. In contrast, insulin's activation of the plasma-membrane enzyme was essentially unchanged (Table 1). A similar selective effect (Table 1) could be achieved by using fructose to deplete specifically the intracellular ATP concentration (Boyes *et al.*, 1981) to about 40% of its native (control) value.

All activations for the enzymes are expressed relative to control incubations where no insulin was added. The lysosomotropic agents had no effect on the activity of the plasma-membrane enzyme in control gradients. Although NH_4Cl had no effect on the 'dense-vesicle' enzyme in control gradients ($99 \pm 3\%$ of native), activity was decreased by either methylamine ($78 \pm 5\%$ of native) or chloroquine ($63 \pm 3\%$ of native; means $\pm$ S.E.M., $n=3$). However, at the concentrations used in the incubations, the addition of these agents directly to the membrane fraction, used for hypo-osmotic extraction, derived from control cells affected neither the extent of release of the enzyme by hypo-osmotic shock nor the total cyclic AMP phosphodiesterase activity displayed.

If the incubation of the hepatocytes with insulin was performed at 22°C, rather than 37°C, then the activation of the 'dense-vesicle' enzyme was markedly decreased ($<20\%$ of that at 37°C), whereas

Table 1. *Effects of lysosomotropic agents, cellular ATP depletion and lowered temperature on the activation by insulin of hepatocyte 'dense-vesicle' and peripheral-plasma-membrane cyclic AMP phosphodiesterase*

Unless otherwise specified, incubations were performed at 37°C as described in detail in the Materials and methods section. Experiments were performed with three different cell preparations (results are means $\pm$ S.E.M.). Cells were preincubated as described in the Materials and methods section with ligands before exposure to insulin for 5 min. Activation of the 'dense-vesicle' enzyme was monitored either directly by Percoll-gradient analysis or indirectly by selective extraction by using hypo-osmotic shock.

	Activation (% of the appropriate* control value)			Cellular ATP content (nmol/mg dry wt.)
	'Dense-vesicle' enzyme (hypo-osmotic)	'Dense-vesicle' enzyme (Percoll)	Plasma-membrane enzyme (Percoll)	
Insulin (10 nM)	144 $\pm$ 4	135 $\pm$ 2	159 $\pm$ 2	10.9 $\pm$ 0.4
Insulin + NH_4Cl (10 mM)	107 $\pm$ 6	106 $\pm$ 8	163 $\pm$ 2	9.7 $\pm$ 0.2
Insulin + chloroquine (0.2 mM)	106 $\pm$ 6	105 $\pm$ 8	146 $\pm$ 3	10.4 $\pm$ 0.2
Insulin + methylamine (10 mM)	97 $\pm$ 5	112 $\pm$ 5	152 $\pm$ 5	10.3 $\pm$ 0.2
Fructose (10 mM)	107 $\pm$ 3	105 $\pm$ 3	155 $\pm$ 6	3.9 $\pm$ 0.4
Insulin (10 nM) at 22°C	107 $\pm$ 3	112 $\pm$ 5	128 $\pm$ 6	10.3 $\pm$ 0.3

* Cells incubated without insulin but under the conditions specified, i.e. with lysosomotropic agent or fructose present, or incubated at 22°C.

the degree of activation of the plasma-membrane enzyme fell by only about one-half (Table 1).

Intracellular ATP concentrations were unaffected either by the lysosomotropic agents or by incubation at 22°C (Table 1).

Before exposure of the cells to insulin (10 nM) for 5 min at 37°C, incubation for 15 min at 37°C with bacitracin (1 mM), *p*-tosyl-L-arginine methyl ester (2 mM), α -*N*-benzoyl-L-arginine methyl ester (2 mM), *N*- α -acetyl-L-lysine methyl ester (2 mM), soya-bean trypsin inhibitor (0.1 mg/ml) and leupeptin (0.1 mM) failed to exert any effect (<10%) on insulin's activation of the 'dense-vesicle' enzyme. These experiments were performed by the hypo-osmotic-shock release method, except for leupeptin, which was assessed by the Percoll-gradient procedure. This was because the hypo-osmotic-shock release of the 'dense-vesicle' enzyme was potentially inhibited by leupeptin at the concentrations used. Leupeptin also failed to exert any inhibitory effect (<10%) on insulin's activation of the plasma-membrane enzyme.

Discussion

Insulin exerts its effects on hepatocyte intracellular cyclic AMP concentrations by activating cyclic AMP phosphodiesterases (see, e.g., Houslay *et al.*, 1983a,b; Heyworth *et al.*, 1983a) and inhibiting adenylate cyclase (Heyworth & Houslay, 1983). The activity of at least two distinct cyclic AMP phosphodiesterases is augmented by the exposure of hepatocytes to insulin. These high-affinity enzymes are associated with the plasma membrane and an internal 'dense-vesicle' fraction respectively (Heyworth *et al.*, 1983a). Such enzymes have been used by us as probes to investigate the molecular action of insulin.

Here we see that depletion of intracellular ATP concentrations (confirming the observation by Boyes *et al.*, 1981), preincubation of hepatocytes with lysosomotropic agents, and lowering the incubation temperature to 22°C, all blocked the activation by insulin of the 'dense-vesicle' cyclic AMP phosphodiesterase selectively (Table 1). This supports our contention (Heyworth *et al.*, 1983a) that insulin activates the dense-vesicle enzyme through a distinct route from that employed to activate the plasma-membrane enzyme.

Insulin binds to its receptors at the cell surface. Then, like a number of ligands, the receptor-insulin complex is recruited into coated pits (Le Cam *et al.*, 1979; Fan *et al.*, 1982; Pilch *et al.*, 1983) and internalized within minutes by receptor-mediated endocytosis into vesicle fractions (Khan *et al.*, 1982; Caro *et al.*, 1982). The receptor complex may then be eventually degraded within the lysosomes or re-cycled back to the plasma membrane (Green & Olefsky, 1982; Fehlmann *et al.*, 1982; Marshall *et al.*, 1981).

Lysosomotropic amines have been shown to block both the recycling of receptors to the plasma membrane and also lysosomal processing of the internalized insulin receptors (Marshall & Olefsky, 1979; Marshall *et al.*, 1981; Draznin *et al.*, 1981; Whittaker *et al.*, 1981; Green & Olefsky, 1982; Posner *et al.*, 1982). Such studies demonstrated that, both in hepatocytes and in adipocytes, insulin internalization does occur in the presence of lysosomotropic amines, although the rate of internalization might well be decreased (Le Cam *et al.*, 1979). Receptor-mediated endocytosis and so-called membrane processing/traffic phenomena are also both strongly inhibited upon ATP depletion of cells and by lowering the temperature to around 20°C or below (Marshall & Olefsky, 1979; Carpentier *et al.*, 1979; Draznin *et al.*, 1981; Besterman & Low, 1983). It is thus possible that insulin might activate the dense-vesicle enzyme through an event triggered by the internalization and subsequent processing of the insulin-receptor complex. Certainly, insulin is known to cause the redistribution and activation of glucose-transporter molecules from an internal-vesicle pool to the plasma membrane of adipocytes (Wardzala *et al.*, 1978; Suzuki & Kono, 1980; Karnieli *et al.*, 1981). This process is also blocked upon ATP depletion and by lowering the incubation temperature (Siegel & Olefsky, 1980). Applied to the 'dense-vesicle' enzyme, such a mechanism might be expected to involve the recruitment of a latent phosphodiesterase enzyme into the vesicle fraction, with subsequent activation, as insulin causes a net increase in cyclic AMP phosphodiesterase activity in hepatocytes (Heyworth *et al.*, 1983a). Furthermore, none of the expressed cyclic AMP phosphodiesterase activities associated with other subcellular membranes (Marchmont & Houslay, 1980a; Cercek & Houslay, 1982; Cercek *et al.*, 1983) have characteristics similar to those of the integral 'dense-vesicle' enzyme.

We cannot exclude the possibility that a proteolytic event is involved with the activation of this enzyme. Certainly a number of phosphodiesterases are activated by limited proteolysis (see, e.g., Makino *et al.*, 1982; Wilson & Houslay, 1983). Indeed, Makino *et al.* (1982) have suggested that, in adipocytes, the phosphodiesterase corresponding to our 'dense-vesicle' enzyme might be activated by a thiol-dependent proteinase, which was specifically inhibited by leupeptin. However, although leupeptin is known to be taken up into hepatocytes, where it inhibits protein degradation (Grinde & Seglen, 1980), it failed to block insulin's activation of the dense-vesicle enzyme (Table 1). Furthermore, a variety of other proteinase inhibitors failed to exert any effect on insulin's activation of the 'dense-vesicle' enzyme, although we have not determined their accessibility to the cell interior. Chloroquine has been demonstrated to inhibit the rate of insulin

degradation (Dennis & Aronson, 1981), presumably by blocking endocytosis. In order to investigate the possibility that degradation of insulin at the cell surface or extracellularly was not involved in the activation of the 'dense-vesicle' enzyme, we added bacitracin to block this process (Carpentier *et al.*, 1979). Under the conditions of our experiment (10 nM-insulin) bacitracin does not impair insulin's internalization (Carpentier *et al.*, 1979) and was shown to exert no effect on insulin's activation of the 'dense-vesicle' enzyme. We thus consider a proteolytic event involved in the activation of the 'dense-vesicle' enzyme by insulin as unlikely, on the basis of the current evidence.

Activation of the plasma-membrane enzyme by insulin is known to involve phosphorylation by a membrane-bound kinase. This event can occur in broken membranes (Marchmont & Houslay, 1980b; Houslay *et al.*, 1983a) and therefore would not be expected to be affected by receptor internalization. Furthermore, the kinase involved expresses a K_m for MgATP of around 30 μ M (Houslay *et al.*, 1983b), which would, again, not be affected by the magnitude of ATP depletion elicited in the present studies. The decrease in the degree of activation elicited on lowering the temperature to 22°C (Table 1) is thus quite in accord with an enzyme-mediated phenomenon. Our present study thus provides further support for our contention that the activation of the plasma-membrane enzyme by insulin, which has been suggested to involve a specific species of guanine nucleotide regulatory protein (Heyworth *et al.*, 1983a,b) and associated protein kinase(s), occurs through a route distinct from that whereby insulin activates the 'dense-vesicle' enzyme.

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