Insulin regulation of pyruvate kinase activity in cultured rat hepatocytes, in the presence of vasopressin, ionophore A23187 or 4 β -phorbol 12 β -myristate 13 α -acetate

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The short-term interactions of insulin and vasopressin on pyruvate kinase (PK) activity were studied in primary cultures of rat hepatocytes. (1) Vasopressin inhibited PK activity by approx. 30% within 15 s, but activity returned to control values by 5 min. The transient inhibition by vasopressin was mimicked by either 4 β -phorbol 12 β -myristate 13 α -acetate (PMA) or ionophore A23187. (2) Insulin alone transiently inhibited PK activity at 1 min, but stimulated PK activity at 5 and 15 min. (3) Insulin completely antagonized the early inhibition by vasopressin, PMA or A23187 of PK activity at 15 s. (4) Insulin inhibited PK activity in the presence of vasopressin, PMA or A23187 at 5 min. (5) 8-Bromo cyclic AMP inhibited PK activity within 15 s, and this inhibition was maintained for at least 5 min. Insulin did not antagonize the inhibition by the cyclic AMP analogue. These results show that insulin under appropriate conditions can act as an inhibitor or activator of PK.

INTRODUCTION

The short-term regulation of liver L-type pyruvate kinase (PK; EC 2.7.1.40) appears to play an important role in the control of the hepatic glycolytic and gluconeogenic pathways (Hers & Hue, 1983). Glucagon or cyclic AMP analogues inhibit PK activity as a result of phosphorylation of the enzyme by cyclic-AMP-dependent protein kinase, leading to a decrease in the affinity of the enzyme for its substrate, PEP (Feliu *et al.*, 1976, 1977; Claus *et al.*, 1979; Garrison *et al.*, 1979, 1984; Garrison & Wagner, 1982). Insulin has been shown to reverse these effects (Feliu *et al.*, 1976; Claus *et al.*, 1979), and insulin alone stimulates PK activity (Fleig *et al.*, 1984).

Phenylephrine and vasopressin inhibit PK activity (Chan & Exton, 1978; Claus *et al.*, 1979; Garrison *et al.*, 1979; Blair *et al.*, 1979; Staddon & Hansford, 1987) and phosphorylate the enzyme (Garrison *et al.*, 1979, 1984; Garrison & Wagner, 1982; Knowles & Hems, 1983; Hsu *et al.*, 1987). Direct phosphorylation of PK by Ca²⁺/calmodulin-dependent protein kinase has been demonstrated at a serine residue that is also phosphorylated by the cyclic-AMP-dependent protein kinase (Schworer *et al.*, 1985) as well as a threonine residue that is five residues from the serine (Soderling *et al.*, 1986). The extent of inhibition of PK activity and the degree of phosphorylation of PK induced by these agents is usually more smaller than those effects observed with either glucagon or cyclic AMP (Garrison *et al.*, 1979, 1984; Garrison & Wagner, 1982; Knowles & Hems, 1983).

The effects of phenylephrine on Ca²⁺ mobilization, glycogen phosphorylase, glycogen synthase and glucose output are similar to those seen with vasopressin. However, insulin appears to antagonize the effects of phenylephrine, but not those of vasopressin, at the level

of intracellular Ca²⁺ mobilization, in freshly isolated hepatocytes (Blackmore *et al.*, 1979; Dehay *et al.*, 1981; Thomas *et al.*, 1985). The phenylephrine-mediated inhibition of PK activity is antagonized by insulin (Blackmore *et al.*, 1979; Claus *et al.*, 1979). The experiments presented here were designed to investigate, in primary cultures of rat hepatocytes, the interactions of insulin with vasopressin, 4 β -phorbol 12 β -myristate 13 α -acetate (PMA) and ionophore A23187.

METHODS

Preparation of hepatocytes

Hepatocytes were prepared from male 150–250 g Sprague–Dawley rats as described by Pittner *et al.* (1985). The hepatocytes attached to 50 mm-diam. Falcon tissue-culture plates after a 1 h incubation in modified Liebowitz L15 medium containing 10% (v/v) newborn-calf serum. Non-attached cells were removed by replacing this medium. After 6–8 h the hepatocytes were incubated in serum-free medium containing 0.2% (w/v) fatty-acid-poor bovine serum albumin for 18–24 h in the absence of any added hormones. This medium was replaced with fresh medium 2 h before each experiment. After the appropriate incubations in serum-free media at 37 °C, the plates were placed on ice and cells were scraped from the plates in 1 ml of ice-cold 0.25 M-sucrose containing 0.5 mM-dithiothreitol and 10 mM-Hepes (adjusted to pH 7.4 with NaOH). Homogenates were prepared by sonication on ice and stored at –20 °C (Pittner *et al.*, 1985). PMA and A23187 were added in dimethyl sulphoxide (0.5%, v/v). Control plates received an equivalent volume of dimethyl sulphoxide, which was found to have no significant effect on PK activity.

Abbreviations used: LDH, lactate dehydrogenase; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PMA, 4 β -phorbol 12 β -myristate 13 α -acetate.

Determination of enzyme activities

The activity of PK was determined essentially as described by Bucher & Pfeleiderer (1955). Each assay contained, in a final volume of 1 ml, 50 mM-Tris/HCl (adjusted to pH 7.4), 25 mM-MgSO₄, 100 mM-KCl, 2 mM-ADP, 1 mM-PEP, 0.15 mM-NADH, 2.5 μ l of a LDH preparation (approx. 20 units) and up to 50 μ l of sample. The reaction mixture was preincubated at 30 °C and was started by the addition of PEP. Enzyme activity was calculated by measuring the difference in the rate of change of A_{340} in the presence and absence of added PEP; 1 mM-PEP was used routinely, as this gave the best measurement for changes in PK activity. Similar changes were seen with 0.15–1.5 mM-PEP. In early experiments, 50 mM-NaF was added to the sucrose medium, to inhibit any phosphatase activity which may act on PK. However, it was found that NaF had no significant effect on the activity of PK measured in the presence or absence of any agent, even if 20 mM-NaF was also added to the assay cocktail; therefore NaF was not included in subsequent experiments.

The activity of LDH was determined as described by Saggerson & Greenbaum (1969). PK activity was expressed relative to total cellular LDH to compensate for any variability in cell number from plate to plate. There was no effect of any of the agents tested on the recovery of LDH activity (results not shown). LDH activity, rather than protein, was used to express enzyme activity, as the recovery of LDH activity is also a good indicator of cell viability (Berg *et al.*, 1972).

Materials

Tissue-culture reagents were from Flow Laboratories; Falcon tissue-culture plates were from Becton Dickinson; bovine serum albumin was from Armour Pharmaceutical Co.; collagenase, LDH and other reagents were from Sigma Chemical Co.

RESULTS

Effects of 8-bromo cyclic AMP

The addition of 8-bromo cyclic AMP to hepatocytes inhibited PK activity at 15 s in a dose-dependent manner (Fig. 1), as expected (Feliu *et al.*, 1976, 1977; Claus *et al.*, 1979), and the inhibition was maintained for 5 min (Fig. 1b). Insulin did not antagonize the effects of the cyclic AMP analogue either at 15 s or at 5 min (Fig. 1).

Effects of vasopressin and insulin

The addition of 100 nM-vasopressin inhibited PK activity by 30 % at 15 s (Fig. 2), as previously described (Chan & Exton, 1978; Claus *et al.*, 1979; Garrison *et al.*, 1979; Staddon & Hansford, 1987). This inhibition was also seen at 1 min, but PK activity returned to control values by 5 min. No effect could be detected at 5 s (results not shown). The inhibition by vasopressin was dose-dependent (Fig. 3a), requiring only 1 nM-vasopressin ($P < 0.05$ for $n = 3$).

Insulin (50 nM) initially inhibited PK activity slightly at 1 min ($P < 0.05$ for $n = 3$), but was stimulatory by 5 min (Fig. 2, Table 1), as expected (Fleig *et al.*, 1984). This stimulation was sustained for 15 min. Insulin completely antagonized the vasopressin-mediated inhibition of PK activity seen at 15 s (Figs. 2 and 3a). The antagonism of the vasopressin response was also seen with 5 nM-insulin (Fig. 4a).

Paradoxically, at 5 min in the presence of vasopressin there was an inhibition of PK activity by insulin (Figs. 2, 3b and 4b). This inhibition was dependent on the concentration of vasopressin (Fig. 3b) as well as that of insulin (Fig. 4b). At 5 min vasopressin alone was without effect (Fig. 3b), and insulin (50 nM) alone slightly stimulated enzymic activity (Fig. 2). The inhibition by insulin of PK activity in the presence of vasopressin was also transient, and returned to control values by 15 min (Fig. 2).

Fig. 1. Interactions of 8-bromo cyclic AMP and insulin on PK activity

Hepatocytes were incubated for (a) 15 s or (b) 5 min with 10 μ M- or 100 μ M-8-bromo cyclic AMP, in the absence (○) or presence (●) of 50 nM-insulin. Results are expressed as percentages of control activities, which were 29 ± 9 and 29 ± 8 nmol of PEP converted/unit of LDH for (a) and (b) respectively. Results are means $\pm$ S.E.M. of duplicate plates from four independent experiments.

Fig. 2. Time course for the effects of vasopressin and insulin on PK activity

Hepatocytes were incubated in the presence of 100 nM-vasopressin (●) or 50 nM-insulin (■) alone or in combination (▲) for the times indicated. The basal PK activity was 31 ± 6 nmol of PEP converted/unit of LDH. Results are means $\pm$ S.E.M. for three independent experiments for 1 min and 15 min, and of six experiments for 15 s and 5 min incubations.

Fig. 3. Effects of vasopressin on PK activity in the presence or absence of insulin

Hepatocytes were incubated for (a) 15 s or (b) 5 min with vasopressin at the concentrations indicated in the absence (○) or presence (●) of 50 nM-insulin. The basal PK activity was 33 ± 11 and 29 ± 8 nmol of PEP converted/unit of LDH for (a) and (b) respectively. Results are means $\pm$ S.E.M. of duplicate plates for (a) three and (b) four independent experiments.

The inhibition of PK activity by vasopressin at 15 s, as well as that by insulin in the presence of vasopressin at 5 min, was also seen at a range of substrate concentrations from 0.15 to 1.5 mM, but was smaller at the two extremes. No effect was seen at a substrate concentration of 2 mM (results not shown), as has been shown previously with glucagon (Feliu *et al.*, 1977). PEP was routinely used at 1 mM, as this gave the best changes in PK activity in response to added agents (results not shown).

Effects of PMA and A23187

The actions of vasopressin are thought to be mediated through stimulation of phosphoinositide turnover, producing inositol 1,4,5-trisphosphate, which is responsible for raising intracellular $[Ca^{2+}]$, and diacylglycerol, which activates protein kinase C (Berridge, 1984; Nishizuka *et al.*, 1984). PMA and A23187 were tested to see whether the effects of vasopressin could be mimicked by an activator of protein kinase C such as PMA or by increasing intracellular $[Ca^{2+}]$, and possibly diacylglycerol as well, by using the Ca^{2+} ionophore A23187.

At 15 s low doses of PMA (10 nM) or A23187 (1 μ M) had no significant effect on PK activity (Table 1). However, at higher concentrations PMA (100 nM) or A23187 (5 μ M) both inhibited PK activity (Table 1). Insulin completely antagonized the inhibition by both A23187 and PMA (Table 1). At 5 min neither PMA or A23187 inhibited PK activity, at the concentrations tested (Table 1).

Insulin inhibited PK activity at 5 min in the presence of either PMA (10 nM) or A23187 (1 μ M), which had no effect on PK activity at 15 s (Table 1). There was no synergism between 10 nM-PMA and 1 μ M-A23187 with regard to effects on PK (Table 1). The degree of inhibition of PK activity by insulin in the presence of A23187 or PMA at 5 min was the same as that seen in the presence of vasopressin (Table 1).

DISCUSSION

The inability of insulin to affect the inhibition of PK by 8-bromo cyclic AMP in these experiments (Fig. 1) is paradoxical. This cyclic AMP analogue is a poor substrate for the cyclic-AMP-dependent phosphodiesterase (Miller *et al.*, 1975). However, Gabbay & Lardy (1984, 1986) found that insulin could antagonize the effects of low concentrations of 8-bromo cyclic AMP on both glycogenolysis and gluconeogenesis, indicating a non-obligatory role of phosphodiesterase activation in these effects of insulin (Gabbay & Lardy, 1984, 1986). The negative results in our experiments suggest that insulin does not modify cyclic-AMP-dependent protein kinase activity towards PK.

Claus *et al.* (1979) reported that the phenylephrine-mediated inhibition of PK was reversed by insulin with no apparent lag period, and is not due to changes in the concentration of the allosteric activator fructose 1,6-bisphosphate. Figs. 2–4 show that vasopressin transiently inhibited PK activity, and this early inhibition was also reversed by insulin. In contrast, although insulin reversed the effects of phenylephrine on glycogen synthase, glycogen phosphorylase and gluconeogenesis, it did not affect those of vasopressin in freshly isolated hepatocytes (Blackmore *et al.*, 1979; Dehay *et al.*, 1981; Thomas *et al.*, 1985). The insulin reversal did not involve

Table 1. Effects of PMA, A23187 and vasopressin on PK activity in the presence or absence of insulin

Hepatocytes were incubated for 15 s or 5 min with PMA, A23187 or vasopressin at the concentrations indicated, in the absence or presence of 50 nM-insulin. Results are expressed as the percentage change in activity from either control incubations or incubations performed in the presence of insulin, and are means $\pm$ S.E.M. for *n* experiments. The basal PK activity was 30–32 nmol of PEP converted/unit of LDH for all groups at either time point. The significance of difference between the groups, calculated by using a paired *t* test, is indicated by: **P* < 0.05, ***P* < 0.025, ****P* < 0.01, †*P* < 0.0025 and ††*P* < 0.001.

Incubation time ...	PK activity (% of control)			
	15 s		5 min	
	Without insulin	With insulin	Without insulin	With insulin
(a) <i>n</i> = 3				
Control	100	98 $\pm$ 5	100	115 $\pm$ 4*
10 nM-PMA	-7 $\pm$ 3	-6 $\pm$ 14	-1 $\pm$ 4	-30 $\pm$ 9**
1 μ M-A23187	-12 $\pm$ 10	-7 $\pm$ 5	-12 $\pm$ 8	-33 $\pm$ 10**
(b) <i>n</i> = 4				
Control	100	97 $\pm$ 4	100	115 $\pm$ 5**
100 nM-PMA	-22 $\pm$ 7**	-4 $\pm$ 10	-7 $\pm$ 8	-24 $\pm$ 11*
5 μ M-A23187	-21 $\pm$ 4***	0 $\pm$ 6	-8 $\pm$ 8	-32 $\pm$ 2††
(c) <i>n</i> = 6				
Control	100	95 $\pm$ 3*	100	109 $\pm$ 2††
100 nM-Vasopressin	-31 $\pm$ 5†	-2 $\pm$ 2	-3 $\pm$ 3	-29 $\pm$ 5†

Fig. 4. Effects of insulin on PK activity in the presence or absence of vasopressin

Hepatocytes were incubated for (a) 15 s or (b) 5 min with insulin at the concentrations indicated, in the absence (○) or presence (●) of 100 nM-vasopressin. The basal PK activity was 16 $\pm$ 2 and 21 $\pm$ 1 nmol of PEP converted/unit of LDH for (a) and (b) respectively. Results are means $\pm$ ranges of duplicate plates from a representative experiment.

The reversal by insulin of the 15 s inhibition of PK activity by either PMA or A23187 suggests that the insulin inhibition is not at the level of the vasopressin receptor. A23187, but not PMA, has been reported to increase the phosphorylation of PK (Garrison & Wagner, 1982; Knowles & Hems, 1983). However, in our experiments both A23187 and PMA inhibited PK activity (Table 1). Whether the phosphorylation of PK is increased by PMA in our experiments is unknown. The effects of the Ca²⁺ ionophore A23187 might also involve activation of protein kinase C, since diacylglycerol accumulation is also increased by high concentrations of A23187 (Takenawa *et al.*, 1982; Bocckino *et al.*, 1985).

There was no synergism between low concentrations of A23187 and PMA with respect to PK (Table 1). Vaartjes *et al.* (1986) also found no synergism between effects of PMA and a Ca²⁺ ionophore on fatty acid synthesis. This is in contrast with the regulation of glycogen phosphorylase, where synergism was noted by Fain *et al.* (1984) and Van de Werve *et al.* (1985), but not by Cooper *et al.* (1985), in isolated hepatocytes.

There may be three separate effects of insulin on cultured rat hepatocytes in these experiments. The first is the ability of insulin to block the rapid inactivation of PK activity by vasopressin, PMA or A23187. This effect is clearly not on coupling of the vasopressin receptor to phosphoinositide breakdown, since insulin also blocks the effects of agents that mimic or increase diacylglycerol and Ca²⁺, the products of phosphoinositide breakdown. However, insulin does block the vasopressin-mediated stimulation of phospholipase C in cultured rat hepatocytes (R. A. Pittner & J. N. Fain, unpublished work). The second effect of insulin is a small activation of PK activity at 5 or 15 min in the absence of vasopressin.

The third effect of insulin, to inhibit PK activity, clearly involves a different mechanism, since the effect on PK is exactly the opposite to the first two effects. Conceivably insulin could increase the phosphorylation

inhibition of phenylephrine-stimulated phosphoinositide turnover (Thomas & Williamson, 1983; Taylor *et al.*, 1985), but was apparently secondary to decreases in intracellular Ca²⁺.

of PK at sites distinct from those phosphorylated by cyclic AMP-, diacylglycerol- or Ca^{2+} -activated protein kinases, or inhibit a PK-specific protein phosphatase. From the data in Fig. 2, it might be argued that insulin inhibition of PK activity at 5 min is due to a delay in the onset of vasopressin inhibition. However, the data in Table 1 indicate that with the lower concentrations of PMA or A23187 there is little inhibition of PK activity at 15 s. However, at 5 min, insulin inhibited PK activity in the presence of these agents, whereas in the absence of these agents there was a stimulation of PK activity by insulin. Further studies on the phosphorylation patterns of PK may provide some indication as to the mechanism of action of these agents.

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