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STIMULATION OF PYRUVATE KINASE PHOSPHATASE ACTIVITY BY INSULIN IN ISOLATED RAT HEPATOCYTES

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SUMMARY: Addition of insulin (10^{-8} M) to hepatocytes, incubated either in the absence or in the presence of a suboptimal concentration of glucagon, caused the reactivation of pyruvate kinase and simultaneously provoked a transient stimulation of pyruvate kinase phosphatase activity (40-70% over control values). The stimulatory effect of insulin on pyruvate kinase phosphatase activity was dose-dependent (ED_{50} = 1 to 2 x 10^{-11} M) and persisted after Sephadex G-25 filtration or ammonium sulfate precipitation of hepatocyte extracts. Our results demonstrate that insulin exerts a short-term regulation on hepatic pyruvate kinase phosphatase activity.

Hepatic pyruvate kinase-L can be phosphorylated and inactivated by a cyclic AMP-dependent protein kinase (1). This phosphorylation and the resultant inactivation also occur after administration of glucagon "in vivo" (2) or as a result of treatment of isolated hepatocytes with this hormone (3,4).

Phosphorylated pyruvate kinase can be dephosphorylated and reactivated by protein phosphatases (1,5,6). It has been reported that native pyruvate kinase phosphatase activity of liver extracts is markedly activated by Mg^{++} ions and is completely insensitive to the thermostable inhibitor-2 of protein phosphatases (7). However, little is known about the hormonal control of this protein phosphatase activity. Addition of insulin to isolated rat hepatocytes accelerated the reactivation of pyruvate kinase previously inactivated by a suboptimal concentration of glucagon (8). Furthermore, addition of this hormone to rat hepatocytes incubated under basal conditions, in the absence of glucagon, caused a small but statistically significant increase of pyruvate kinase activity (9). At least one of the possible explanations for these results could be the stimulation by insulin of the protein phosphatase(s) responsible for the dephosphorylation and reactivation of hepatocyte pyruvate kinase.

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Abbreviation used: PK, pyruvate kinase.

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In this work, we have studied the influence of insulin on both pyruvate kinase and pyruvate kinase phosphatase activities in isolated rat hepatocytes. Our results indicate that insulin exerts a short-term regulation on hepatic pyruvate kinase phosphatase activity.

MATERIALS AND METHODS

Materials.- Pork insulin and beef-pork glucagon were obtained from Novo Industri A/S (Copenhagen, Denmark). Lactate dehydrogenase and collagenase were purchased from Boehringer Mannheim GmbH (Mannheim, West Germany).

Isolation and incubation of hepatocytes.- Hepatocytes were isolated from fed Wistar rats (200-300 g) by perfusion of the liver with collagenase (10). Cells were incubated in Krebs-Henseleit medium with 10 mM glucose, as described elsewhere (9). For the determination of enzyme activities, 0.1-0.2 ml aliquots of the cell suspensions were taken at the selected times. These aliquots were immediately frozen in liquid N_2 and kept in these conditions until enzyme assays were carried out.

Measurement of enzyme activities.- Hepatocyte pyruvate kinase phosphatase activity was assayed by estimating the reactivation caused in a preparation of pyruvate kinase partially purified in its inactive form from livers of rats treated with glucagon (11). With minor changes, the enzymatic assay was similar to that described by Jett et al. (7). Frozen aliquots of cell suspensions were thawed and homogenized by vigorous shaking in the presence of an adequate volume of medium A, (50 mM glycylglycine, 100 mM KCl, 0.1 mM DTT, 2.5 mM EDTA and 5% glycerol (v/v), at pH 7.4) to reach a final protein concentration of 3.4 mg/ml. Aliquots (20 µl) of these homogenates were used to assay the phosphatase activity at 36°C. Control incubations without extracts were carried out in parallel. The reaction mixture contained, in a final volume of 120 µl, 50 mM glycylglycine, 100 mM KCl, 0.1 mM DTT, 5% glycerol (v/v), BSA (1 mg/ml), purified pyruvate kinase-L (10 U/ml) and different concentrations of magnesium acetate, at pH 7.4. The reaction was started by addition of hepatocyte extract; 20 minutes later, aliquots (20 µl) of the assay mixture were taken by duplicate, mixed with 180 µl of a solution containing 50 mM glycylglycine, 70 mM KCl, 30 mM KF, 0.1 mM DTT, 5% glycerol (v/v) and BSA (1 mg/ml), at pH 7.4, and immediately frozen in liquid N_2. These samples were kept at -30°C until pyruvate kinase activity was assayed (12). Pyruvate kinase phosphatase activity was expressed as mU of pyruvate kinase reactivated per minute and mg of protein. It is important to mention that the amount of pyruvate kinase-L incorporated with the hepatocyte extracts into the phosphatase assay corresponded to less than 5% of the activity of the partially purified pyruvate kinase-L present as substrate in the reaction mixture.

For the preparation of Sephadex G-25 filtrated extracts, 2 ml aliquots of cell suspension were taken by duplicate four minutes after saline or insulin addition, and pipetted into centrifuge tubes containing 200 µl of 27.5 mM EDTA. After centrifugation (1500 g x 1 min), the supernatants were discarded and the cell pellets were frozen in liquid N_2. They were thawed in medium A and then homogenized in a conical sintered glass tissue grinder. Homogenates were centrifuged at 12,000 g x 15 minutes at 4°C. Aliquots (2 ml) of the corresponding supernatants were filtrated through a column (20 x 1 cm) of Sephadex G-25, equilibrated with medium A. In some experiments, aliquots of these supernatants were mixed with three volumes of saturated ammonium sulfate. After keeping the samples in ice for thirty minutes, they were centrifuged (12,000 g x 15 min), and the resulting pellets dissolved in medium A. Pyruvate kinase phosphatase activity of these fractions was assayed as indicated before.

RESULTS

According to previous reports (7,12), Mg^{++} ions markedly stimulated pyruvate kinase phosphatase activity of hepatocyte extracts (Fig. 1), the half-maximal stimulation corresponding to about 4.3 mM magnesium acetate. Treatment of hepatocytes with 10^{-8}M insulin, without significantly affecting either basal or maximally stimulated pyruvate kinase phosphatase activity, increased the apparent affinity for Mg^{++} ions, lowering the concentration of magnesium acetate responsible for the half-maximal stimulation to 1.9 mM. As a result of these kinetic changes, insulin caused a clear-cut stimulation (about 70% over control value) of hepatic pyruvate kinase phosphatase activity when it was measured in the presence of 1 mM magnesium acetate.

As expected, glucagon (3×10^{-10} M) provoked a marked decrease of hepatocyte pyruvate kinase (Fig. 2A) which was reduced to 20% of its initial value five minutes after the incorporation of the hormone to the incubation medium. In the following minutes, pyruvate kinase was spontaneously reactivated. The addition of insulin (10^{-8} M) to glucagon-treated hepatocytes accelerated the reactivation of pyruvate kinase (Fig. 2A) as previously described by Felíu et al. (8). Simultaneously, insulin provoked a transient stimulation of pyruvate kinase phosphatase activity (Fig. 2B) which was almost doubled two minutes after insulin addition. As shown in Fig. 2B, glucagon did not affect the phosphatase activity.

Similarly, addition of insulin (10^{-8} M) to hepatocytes incubated under basal conditions, in the absence of glucagon, resulted in a transient stimulation of pyruvate kinase phosphatase activity (Fig. 2D). This stimulation

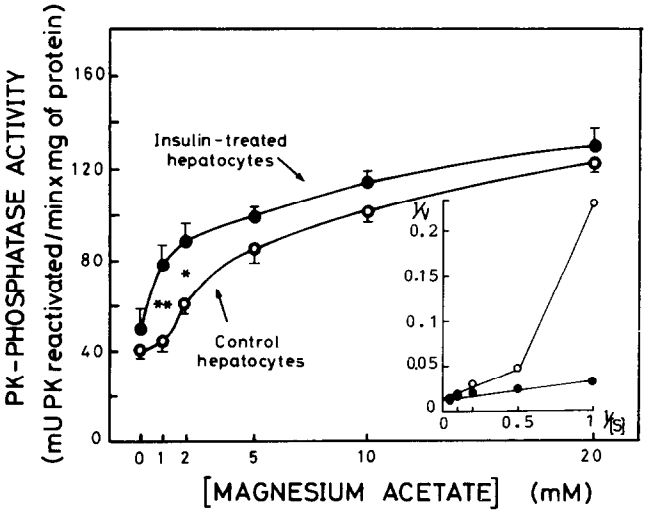

Figure 1.- Effect of different magnesium acetate concentrations on pyruvate kinase phosphatase activity of control and insulin-treated hepatocytes. Aliquots of cell incubations were taken four minutes after saline or insulin (10^{-8} M) addition. Values are the mean ± SEM of three experiments. Paired Student's t test versus control hepatocytes: *p = 0.1, **p < 0.05.

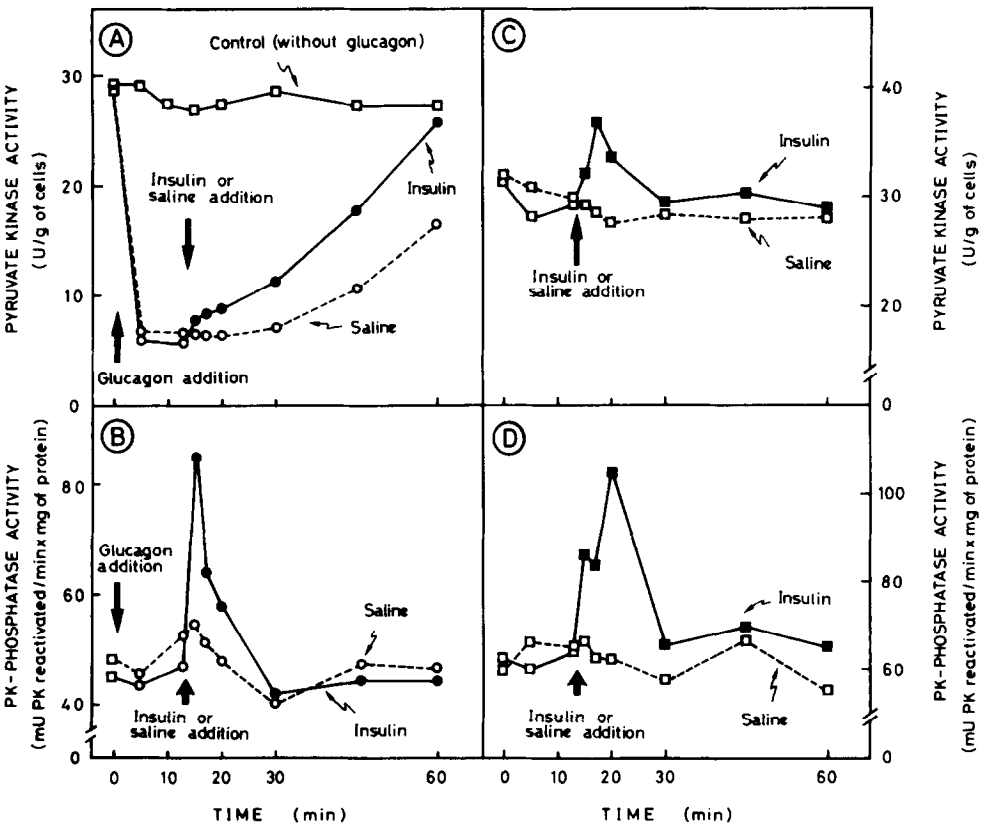

Figure 2.- Time-course effect of insulin (10^{-8} M) on both pyruvate kinase and pyruvate kinase phosphatase activities of hepatocytes incubated in the presence of 3 x 10^{-10} M glucagon (A and B) or under basal conditions (C and D). Full symbols represent insulin-treated hepatocytes.

was accompanied by an also transient reactivation of pyruvate kinase (Fig. 2C).

As shown in Fig. 3, the stimulation of hepatocyte pyruvate kinase phosphatase activity elicited by insulin was dose-dependent. The hormone concentration corresponding to the half-maximal stimulation was 1 to 2 x 10^{-11} M. It is of note that other biological effects of insulin, like stimulation of glucose oxidation in adipocytes, show a very similar ED_{50} concentration (13).

Parallel to the stimulation of the phosphatase activity, insulin caused a dose-dependent reactivation of pyruvate kinase (Fig. 3). However, in this case the half-maximal effect corresponded to 1 to 3 x 10^{-10} M insulin, concentration which is about 10 times higher than that calculated for the half-maximal insulin effect on the phosphatase activity. A possible explanation for this different sensitivity could be that in order for the reactivation of pyruvate kinase to take place, pyruvate kinase phosphatase activity had to be almost completely stimulated by the hormone.

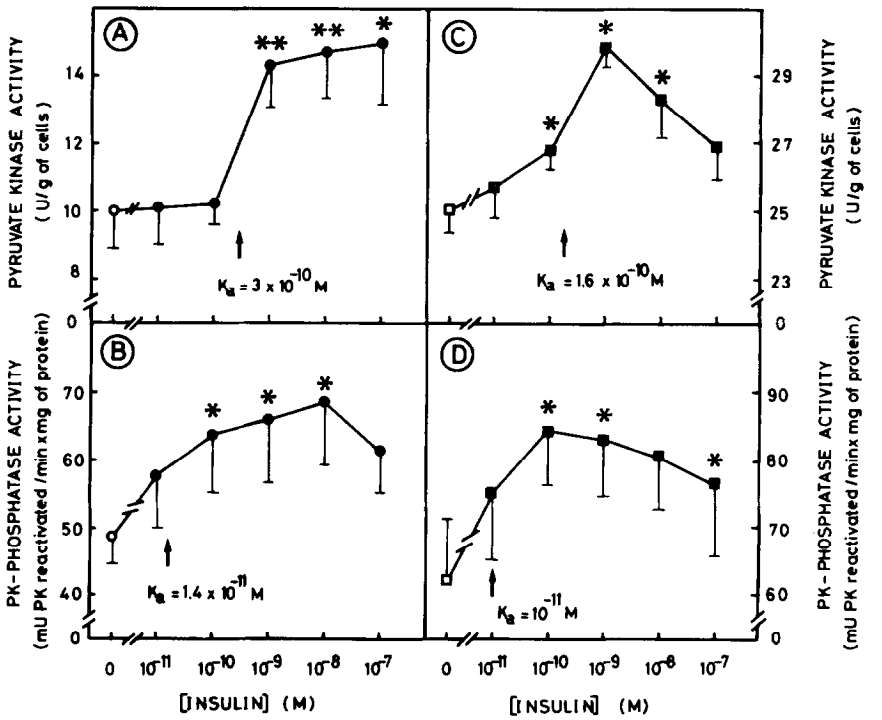

Figure 3.- Effect of different concentrations of insulin on both pyruvate kinase and pyruvate kinase phosphatase activities of hepatocytes incubated in the presence of 10^{-10} M glucagon (A and B) or under basal conditions (C and D). Enzyme activities were measured four minutes after insulin or saline addition. Paired Student's t test versus control (open symbols): *p < 0.05; **p < 0.01).

As shown in Table 1, the ratio between pyruvate kinase phosphatase activity measured at 1 mM magnesium acetate and that measured in its absence (v_1/v_0), reflects in a simple way the stimulatory effect of insulin on the

Table 1.- v_1/v_0 activity ratio of pyruvate kinase phosphatase activity of hepatocytes incubated in the absence or in the presence of 10^{-8} M insulin

	Control hepatocytes	Insulin-treated hepatocytes
Homogenates (n=5)	1.23 ± 0.07	1.86 ± 0.19[a]
Sephadex G-25-filtrated extracts (n=7)	1.41 ± 0.08	1.84 ± 0.10[b]
Ammonium sulfate-precipitated extracts (n=5)	1.20 ± 0.15	1.70 ± 0.15[a]

v_1/v_0 is the ratio between pyruvate kinase phosphatase activity measured at 1 mM magnesium acetate and that measured in its absence. Values are the mean ± S.E.M. of five or seven different experiments. Paired Student's t test versus control hepatocytes: [a]p < 0.05, [b]p < 0.01.

phosphatase activity. Table 1 also shows that the insulin effect persisted after Sephadex G-25 filtration or ammonium sulfate precipitation of hepatocyte extracts.

DISCUSSION

It has been shown that insulin can promote the phosphorylation of some cellular proteins as well as the dephosphorylation of others (14,15). In general, insulin-induced dephosphorylation can be mediated by inhibition of protein kinases and/or stimulation of protein phosphatases. In some instances, insulin affects the activity of different interconvertible enzymes through direct activation of specific protein phosphatases (16,17). Our results indicate that this seems to be the case for pyruvate kinase through modulation by insulin of a specific pyruvate kinase phosphatase activity.

In our assay conditions, two pyruvate kinase phosphatase activities have been evidenced in liver extracts. One of them seems to be independent of Mg^{++} ions for activity while the other is markedly stimulated by this cation. Our data indicates that only the Mg^{++}-dependent phosphatase activity is influenced by insulin.

As indicated, several protein phosphatases acting on phosphorylated pyruvate kinase have been detected in liver (1,5,6). Cohen and co-workers (5, 18) have identified in mammalian tissues four enzymes that appear to account for many of the protein phosphatase activities involved in regulating the major pathways of intermediary metabolism. According to this group (18), two of these enzymes, termed protein phosphatase 2C and 2A, represent the major dephosphorylating activities against pyruvate kinase in rat liver. Protein phosphatase 2C is a Mg^{++}-dependent enzyme, while this cation does not significantly affect protein phosphatase 2A activity (18,19). More recently, Mieskes et al. (6) have reported the purification to homogeneity of a rat liver protein phosphatase able to effectively dephosphorylate the key enzymes of glucose metabolism 6-phosphofructo-1-kinase, fructose-1,6-bisphosphatase, 6-phosphofructo-2-kinase and pyruvate kinase. This protein phosphatase, completely dependent on Mg^{++} ions for activity and insensitive to heat-stable inhibitor-2 (6), has been identified as protein phosphatase 2C (20). Whether or not protein phosphatase 2C accounts for the insulin-stimulated pyruvate kinase phosphatase activity reported in this work remains a possibility to be demonstrated.

The fact that the stimulatory effect of insulin on pyruvate kinase phosphatase activity persisted after Sephadex G-25 filtration or ammonium sulfate precipitation of hepatocyte extracts suggests that a covalent modification of the protein phosphatase molecule (or of a potential high molecular weight regulator) could be responsible for this increased activity.

At this point, it must be mentioned that insulin receptor has an intrinsic tyrosine protein kinase activity which is thought to play a role in the transmission of the hormonal signal to the cell (21). In connection with this, it has recently been shown (22) that protein phosphatase 1 can be phosphorylated and inactivated "in vitro" by $pp60^{src}$, a product of the src oncogen which is a member of a unique class of protein kinases which specifically phosphorylate target proteins in tyrosine residues.

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