CONTROL OF PYRUVATE KINASE ACTIVITY BY GLUCAGON IN ISOLATED HEPATOCYTES

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SUMMARY: Incubation of hepatocytes with 10 nM glucagon led to an increase in the $K_{0.5}$ for phosphoenolpyruvate for pyruvate kinase measured in homogenates of the cells. Incubation of partially purified rat liver pyruvate kinase with protein kinase and ATP led to a similar result. In addition, both the phosphorylated enzyme and homogenates prepared from cells incubated with glucagon exhibited an apparently decreased sensitivity to stimulation by fructose diphosphate when activity was measured in the presence of physiological concentrations of ATP and alanine. These similarities suggest that the effect of glucagon to inhibit hepatocyte pyruvate kinase may be mediated at least in part by a phosphorylation-dephosphorylation mechanism.

INTRODUCTION

Recent studies on dihydroxyacetone gluconeogenesis in isolated hepatocytes have suggested involvement of pyruvate kinase in the acute control of glucose synthesis by glucagon (1,2). Inhibition of pyruvate kinase activity by glucagon has been shown in crude extracts of whole liver and of isolated hepatocytes (1-5). The mechanism of the effect of glucagon is unknown. An attractive hypothesis is that it is mediated by phosphorylation since the enzyme can be phosphorylated by a cyclic AMP-dependent protein kinase. This phosphorylation alters the response of the enzyme ot its substrate and to various allosteric effectors (6,7). However, it has not yet been demonstrated that phosphorylation-dephosphorylation is a relevant control mechanism in vivo. The present studies were undertaken to characterize the enzyme obtained from hepatocytes incubated with glucagon and to compare its properties with those of the pyruvate kinase that has been phosphorylated in vitro.

METHODS

Hepatocyte Preparation and Incubation Conditions. Male Sprague-Dawley rats (200-250 gm) were fed ad libitium on Purina Laboratory Chow. Hormonally responsive rat hepatocytes were

prepared as previously described (8, 9). Aliquots (10 ml) of the cells were then transferred to 125 ml plastic flasks, gassed for 1 min, stoppered, and incubated for 10 min in the presence or absence of 10 nM glucagon. The cells were then rapidly sedimented, washed with 5 ml of ice cold Krebs-Henseleit buffer minus albumin and immediately homogenized in 1.2 ml of ice cold 20 mM sodium phosphate buffer - 40% glycerol - 1 mM mercaptoethanol, pH 7.8. The cells were broken with an Ultraturrax homogenizer (30 sec x 3 at full speed) and a 30,000 x g supernatant fraction prepared. This supernatant (10-20 µl) was used for the assay of pyruvate kinase. In the presence of glycerol the enzyme activity was stable for at least 5 days.

Pyruvate Kinase Activity. Pyruvate kinase was estimated by the method of Llorente et al. (10). The assay mixture contained 50 mM imidazole pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM ADP, 0.5 U lactate dehydrogenase, 0.15 mM NADH, and various concentrations of phosphoenolpyruvate (PEP) and fructose diphosphate (FDP), in a final volume of 1 ml. The reaction, run at 25° C, was started by the addition of the enzyme or phosphoenolpyruvate and the decrease in absorbance at 340 nm was followed. The initial rate of the reaction was measured during the first 2-3 minutes of the reaction. The reaction rate was linear with protein concentration in the range 0.05-1 mg/ml. Pyruvate kinase activity was expressed as nmol pyruvate formed from PEP per min per mg of protein or as units/mg protein where 1 unit equals 1 µmol of pyruvate formed from PEP per min. Protein was determined by the method of Lowry et al. with bovine serum albumin as standard (11). The phosphoenolpyruvate concentration was determined enzymatically (12).

Preparation and Source of Pyruvate Kinase and Protein Kinase. Rat liver pyruvate kinase was purified by the method of Ljungstrom et al. (13) through the second DEAE-cellulose step. The enzyme had a specific activity of 40-50 U/mg and was stored at 0-4° C in a 20 mM phosphate - 30% glycerol buffer, pH 7.2, containing 0.1 mM fructose diphosphate. The protein kinase used in these studies was a homogenous preparation (14) of the catalytic subunit from bovine liver which was kindly supplied to us by Dr. P. Sugden and Dr. J.D. Corbin (Department of Physiology, Vanderbilt University). It was capable of incorporating 3×10^6 pmol of γ-^{32}P-ATP into histone per min per mg protein (3×10^6 units/mg protein).

Results are expressed as the mean ± SEM of triplicate aliquots of cells from a single preparation. Each experiment was repeated at least three times to ensure that results were reproducible.

RESULTS

Characterization of the Effect of Glucagon on Hepatocyte Pyruvate Kinase.

The inhibition of pyruvate kinase activity in homogenates of cells incubated with a maximally effective concentration (10 nM) of glucagon was seen after 2 minutes and was maximal by 10 minutes (Fig. 1). The activity in homogenates of cells incubated without hormone was unchanged during the 20 min incubation. The inhibition by glucagon was dependent on the PEP concentration used in the assay (Fig. 2). The hormone shifted the apparent $K_{0.5}$ for PEP from about 0.025 mM to 0.1 mM. Glucagon had no effect in the presence of saturating PEP concentrations or in the presence of maximally effective concen-

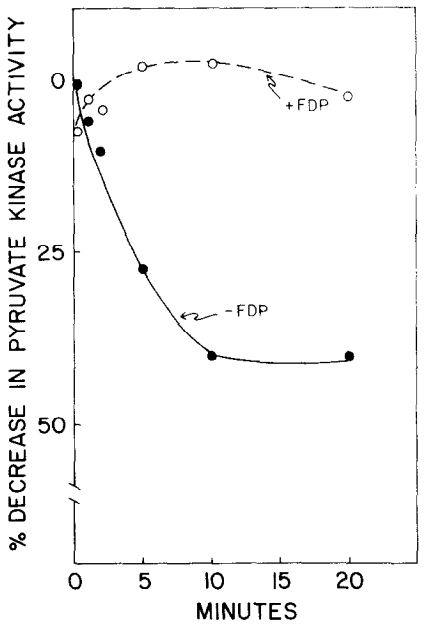

Fig. 1. Time Course of the Effect of Glucagon on Hepatocyte Pyruvate Kinase Activity. Ten ml of hepatocytes (39 μg DNA/ml) prepared from fed rats were incubated for different times in the presence and absence of 10 nM glucagon. Pyruvate kinase activity was determined in homogenates of these cells as described in Methods. The assay was conducted with 0.125 mM phosphoenolpyruvate, in the presence (O) and absence (●) of 20 μM fructose diphosphate. The results are expressed as the % decrease in activity seen in the presence of glucagon relative to that seen in its absence. Each point represents the average of duplicate flasks.

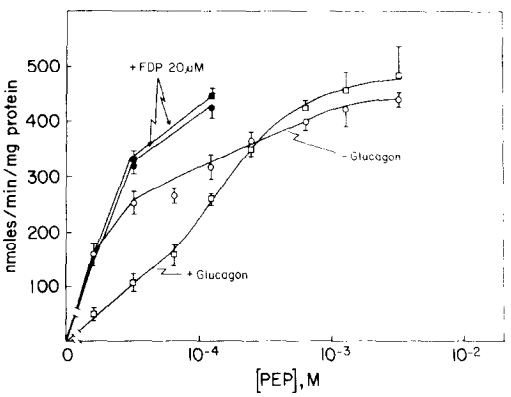

Fig. 2. Substrate Dependence of the Glucagon Effect on Hepatocyte Pyruvate Kinase Activity. Ten ml of hepatocytes (46 μg DNA/ml) from fed rats were incubated for 10 min as described in Methods in the presence (□ , ■) and absence (O, ●) of 10 nM glucagon. Preparation of hepatocyte homogenates and assay of pyruvate kinase were performed as described in the Methods. Enzyme activity was determined with various phosphoenolpyruvate concentrations (0-3.2 mM) in the presence (solid symbols) and absence of 20 μM fructose diphosphate (open symbols).

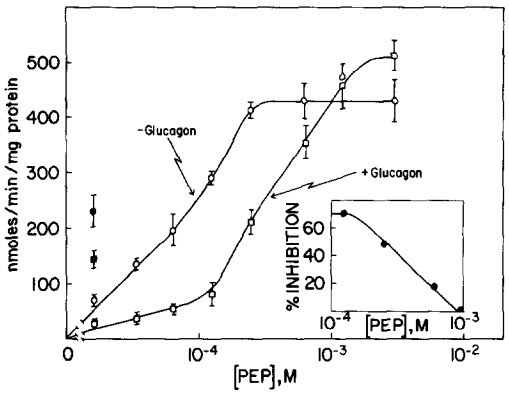

Fig. 3. Substrate Dependence of the Glucagon Effect on Pyruvate Kinase Activity Assayed in the Presence of ATP and Alanine. The incubation conditions were the same as those given in Fig. 2. Pyruvate kinase activity was assayed with (open symbols) and without (solid symbols) alanine (0.125 mM) and ATP (4 mM). Phosphoenolpyruvate concentration was varied from 0-3 mM. The inset shows the percent inhibition of pyruvate kinase activity by glucagon as a function of the phosphoenolpyruvate concentration in the assay.

trations of FDP (20 μM). It should be noted that FDP had a very small stimulatory effect in the absence of glucagon. This is probably related to the fact that the predominant form of pyruvate kinase in fresh tissue homogenates has FDP bound to it (15). When the enzyme was precipitated with 30-45% ammonium sulfate the suppression of pyruvate kinase activity by glucagon was still observed but the apparent $K_{0.5}$ for PEP was increased 30-40 times, probably as a result of removing the bound FDP. Consistent with this idea was the observation that the stimulation by FDP in ammonium sulfate fractions was 10-fold greater than in the fresh homogenates (data not shown).

The effect of glucagon on the substrate dependence of hepatocyte pyruvate kinase activity was examined when physiological concentrations of the allosteric inhibitors ATP (4 mM) and L-alanine (0.15 mM) were added to the assay mixture (Fig. 3). ATP and alanine were potent inhibitors when tested with low PEP concentrations. Moreover, when ATP and alanine were present, the glucagon effect was even more evident than in their absence (cf. Fig. 2). As much as a 70% decrease in activity was observed at 0.1 mM PEP. The

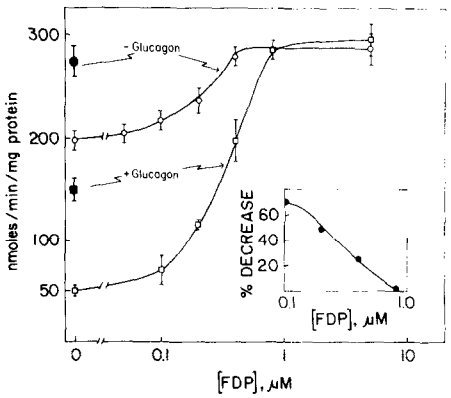

Fig. 4. The Ability of Glucagon to Inhibit Pyruvate Kinase in Hepatocytes from Fed Rats as a Function of Fructose Diphosphate Concentration. The incubation conditions for the hepatocytes were the same as given in Fig. 2. Pyruvate kinase activity in homogenates of the cells was measured with a substrate concentration of 0.06 mM phosphoenolpyruvate in the presence (open symbols) and absence (solid symbols) of ATP (4 mM) and alanine (0.125 mM) with various concentrations of fructose diphosphate (0-5 μM). The inset shows the percent decrease in pyruvate kinase activity from glucagon-treated cells versus control as a function of fructose diphosphate concentration.

apparent $K_{0.5}$ for PEP was about 0.08 mM in the absence of glucagon and 0.3 mM in its presence.

Fig. 4 shows that FDP stimulation of pyruvate kinase, assayed in the presence of L-alanine and ATP, was also modified in homogenates from hepatocytes treated with glucagon. Maximal pyruvate kinase activity was observed with 0.4 μM FDP in the absence of hormone and at 0.8 μM in its presence. Maximal stimulation of enzyme activity by FDP was 1.4 -fold in the control and 6-fold in the glucagon-treated case. Similar differences were also noted in the absence of alanine and ATP and in 30-45% ammonium sulfate extracts of the cells (data not shown). The effect of glucagon could be overcome by addition of as little as 1 μM FDP to the assay (inset, Fig. 4).

The Effect of in vitro Phosphorylation of Partially Purified Liver Pyruvate Kinase.

Incubation of pyruvate kinase with bovine liver protein kinase and ATP led to a 70% inhibition of pyruvate kinase activity in 5 min (Fig. 5). Enzyme incubated with

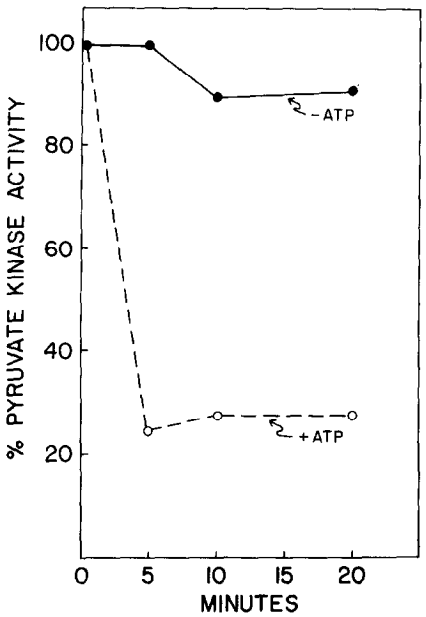

Fig. 5. The Time Course of the Effect of Incubation With Protein Kinase on the Activity of Partially Purified Liver Pyruvate Kinase. The L-form of pyruvate kinase was purified as described in the Methods. Aliquots of this preparation (20-30 Units) were incubated with 0.1 mM ATP in the presence of 10 mM $MgSO_4$, 40 mM imidazole buffer, pH 7.5, 15% glycerol, 0.2 mM dithiothreitol, and 5 μg of a homogenous preparation of the catalytic subunit of bovine liver protein kinase (specific activity 3×10^6 U/mg protein) (14). As a control, the pyruvate kinase was incubated in the absence of ATP (solid symbols). After various times of incubation at 30° C an aliquot of the phosphorylation mixture was diluted 10-fold with the following buffer: 30 mM imidazole, pH 7.5, 30% glycerol, 2 mM EDTA, 20 mM NaF, and 0.2 mM dithiothreitol. Aliquots of this solution were assayed for pyruvate kinase activity as described in Methods except that 10 mM deoxyglucose and 3 units of hexokinase were added to the assay mixture to act as an ATP trap.

protein kinase but without ATP showed no appreciable loss of activity. Incubation of the enzyme with ATP alone also had no effect on activity (data not shown).

Fig. 6A shows that incubation with protein kinase and ATP led to almost complete inhibition of activity[1] when low concentrations (<0.4 mM) of PEP were used. With concentrations of PEP greater than 1.6 mM, or with 20 μM FDP, there was no inhibition of activity. The apparent $K_{0.5}$ for PEP increased from about 0.6 mM to 1.2 mM as a result of phosphorylation.

Fig. 6B compares the FDP dependence of the phosphorylated and nonphosphorylated

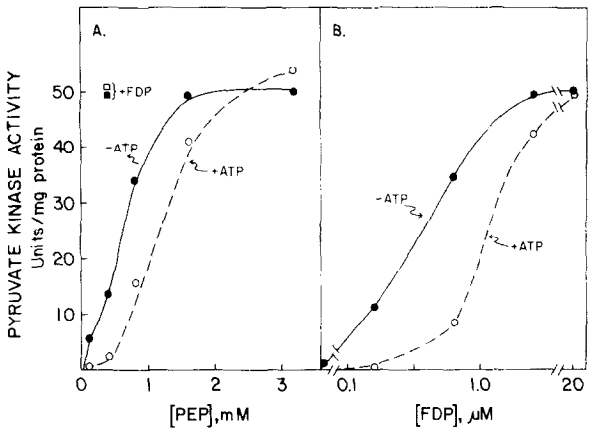

Fig. 6. The Effect of Phosphorylation on the Substrate Dependence and Response to Fructose Diphosphate of Partially Purified Hepatic Pyruvate Kinase. Partially purified pyruvate kinase was incubated for 5 minutes with protein kinase in the presence (-O-) and absence of (-●-) of ATP as described in Fig. 5. In part A, pyruvate kinase was assayed with increasing concentrations of phosphoenolpyruvate (0-3.2 mM). Fructose diphosphate (square symbols) were present at 20 μM. In part B, the assay medium contained 0.4 mM phosphoenolpyruvate, 4 mM ATP, and 0.125 mM alanine.

enzyme assayed in the presence of 4 mM ATP and 0.15 mM L-alanine. In the absence of FDP, addition of the inhibitors led to almost complete inhibition of activity in both cases. Phosphorylation resulted in a decrease in the sensitivity of pyruvate kinase activity to FDP. Half maximally effective stimulation by FDP occurred at about 0.5 μM with the nonphosphorylated enzyme and at about 3 μM with the phosphorylated enzyme.

DISCUSSION

Several reports (1, 2, 5) have shown that incubation of hepatocytes with glucagon alters pyruvate kinase activity in hepatocyte homogenates. Others (6, 7) have reported that in vitro phosphorylation of pyruvate kinase by protein kinase also modifies its activity. This report demonstrates that the glucagon induced alteration in pyruvate kinase activity in hepatocyte homogenates and the phosphorylation induced changes in the purified enzyme

[1]Under these conditions, we can demonstrate incorporation of ^{32}P from γ-^{32}P-ATP into the enzyme protein after isolation of the pyruvate kinase by immunoprecipitation. This confirms the findings of Engstrom and coworkers (6,7).

in vitro have several common features. In both cases the apparent $K_{0.5}$ for PEP is increased but enzyme activity is essentially unaffected when the assays are conducted with high substrate concentrations or in the presence of saturating concentrations of FDP. Furthermore, when the enzyme is assayed in the presence of physiological concentrations of ATP and alanine the sensitivity to FDP stimulation is decreased in both cases. These data suggest that glucagon may alter the phosphorylation state of the enzyme in intact cells.

It should be emphasized that the changes in enzyme activity induced by glucagon in intact cells occur when the enzyme is assayed with concentrations of substrate (inset, Fig. 3) which are in the physiological range (approx. 0.2 mM). Moreover, a small variation in the intracellular concentration of free FDP in the presence of low PEP concentrations could appreciably change enzyme activity as suggested by the inset to Fig. 4. Several studies have demonstrated that glucagon does lower FDP levels in hepatocytes from fed rats (1, 16). The decreased sensitivity of the pyruvate kinase to FDP observed in this study raises the possibility that glucagon treatment of intact cells and/or phosphorylation alter the binding of FDP to the enzyme. It seems reasonable to postulate that the effect of glucagon on hepatocyte pyruvate kinase activity may be mediated by both a lowering of FDP levels and by phosphorylation of the enzyme.

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