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THE EFFECT OF FRUCTOSE DIPHOSPHATE AND PHOSPHOENOLPYRUVATE ON CYCLIC AMP-MEDIATED INACTIVATION OF RAT HEPATIC PYRUVATE KINASE

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SUMMARY

The addition of cyclic AMP and Mg-ATP to Sephadex-treated hepatocyte homogenates produced a time dependent inactivation of pyruvate kinase. The concentration of cyclic AMP giving half-maximal inhibition was 0.16 μM. The cyclic AMP-induced inactivation of pyruvate kinase was characterized by an increase in the $K_{0.5}$ for phosphoenolpyruvate from 0.56 to 1.15 mM and could be completely blocked by the addition of the protein kinase inhibitor. These experiments provide clear evidence that the cyclic AMP induced inactivation is a result of enzyme phosphorylation. Fructose-diphosphate and phosphoenolpyruvate, at physiological concentrations, suppressed inactivation induced by submaximal concentrations of cyclic AMP. It is suggested that hormonal induced changes in the levels of fructose diphosphate and phosphoenolpyruvate may influence the phosphorylation state of the enzyme in intact cells.

INTRODUCTION

Pyruvate kinase has been identified as an important site of glucagon and insulin action in the gluconeogenic pathway (1-4). The hormones presumably act by changing the phosphorylation state of the enzyme (5-7). However, little is known about the nature of the kinase(s) and phosphatase(s) responsible for phosphorylation and dephosphorylation of the enzyme. Partially purified preparations of cyclic AMP-dependent protein kinase from rat liver cytoplasm are capable of phosphorylating the enzyme with concomitant alterations in enzyme activity (6,8). In this report, we have developed an in vitro system for studying the regulation of inactivation of hepatic pyruvate kinase in rat hepatocyte homogenates.

METHODS

Preparation of Sephadex-treated hepatocyte homogenates. Isolated rat hepatocytes were prepared as previously described (9). The cells were suspended in Krebs-Henseleit buffer that contained 1% bovine albumin and incubated without any additions for 20 min at 37°. Five ml aliquots (200-300 μg DNA) of the suspen-

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sion were centrifuged through 5 ml of ice-cold buffer without albumin, and the pellet frozen in liquid nitrogen and stored at -70°. Each cell pellet was suspended in 1.5 ml of a buffer that contained 20 mM potassium phosphate pH 7.2, 30% glycerol, 0.1 mM EDTA, 10 mM NaF, and 0.1 mM dithiothreitol and homogenized for 90 sec (30 sec, 3 times) with an Ultraturrax homogenizer (Tekmar Co., Cincinnati, Ohio) at full speed. The homogenate was then centrifuged at 12,000 x g for 45 minutes. The supernatant fraction from 2 or 3 aliquots of cells were combined and subjected to gel filtration on a Sephadex G-25 column (1.5 x 90 cm) that was equilibrated with the homogenizing buffer. The pyruvate kinase fraction that emerged in the void volume was free of ATP, cyclic AMP, fructose diphosphate, and phosphoenolpyruvate.

Inactivation of hepatocyte homogenate pyruvate kinase. Aliquots (0.6 ml) of the Sephadex-treated hepatocyte homogenate were incubated at 30° with 20 mM potassium phosphate, pH 7.4, 30% glycerol, 0.1 mM EDTA, 10 mM NaF, 0.1 mM dithiothreitol, 1 mM theophylline, 5 mM Mg-ATP, and various effectors in a total volume of 1 ml. The reaction was stopped by the addition of 1.5 ml of cold, saturated $(NH_4)_2SO_4$ and the tube placed on ice. In order to completely remove all low molecular weight effectors, the $(NH_4)_2SO_4$ pellet was washed once with 2.5 ml of 60% $(NH_4)_2SO_4$. The washed pellet was dissolved in 0.2 ml of a buffer that contained 20 mM potassium phosphate, pH 7.2, 40% (v/v) glycerol, and 1 mM mercaptoethanol and assayed for pyruvate kinase activity.

Assay procedures. Pyruvate kinase was assayed by previously described Methods (6,10). The activity of the enzyme is expressed as the ratio of activity measured at 0.4 mM phosphoenolpyruvate to that at 4 mM phosphoenolpyruvate. Protein was estimated by the method of Lowry (11). Hepatocyte DNA was estimated as previously described (9).

RESULTS

Characterization of the cyclic AMP-dependent inactivation of pyruvate kinase in hepatocyte homogenates. Incubation of a Sephadex-treated extract with 5 mM Mg-ATP and 50 μM cyclic AMP resulted in a time-dependent inhibition of pyruvate kinase activity which reached a maximum of 70% at 20 min (Fig. 1). The inhibition was almost completely dependent upon the addition of cyclic AMP and ATP since the addition of ATP, Mg^{2+}, or cyclic AMP alone had no effect and the addition of Mg-ATP produced only a small inhibition (Table I). Fig. 2 shows that the cyclic AMP-dependent inactivation shifted the substrate curve to the right and increased the $K_{0.5}$ for phosphoenolpyruvate from 0.56 mM to 1.15 mM without changing the maximum velocity. Identical changes were seen when the partially purified enzyme was phosphorylated by the catalytic subunit of the cyclic AMP-dependent protein kinase (6,8).

The inactivation of pyruvate-kinase was dependent upon the concentration of cyclic AMP that was added to the homogenate. Fig. 3 shows that maximal inhibition was observed with as little as 1 μM concentration of the nucleotide. Half-maxima-

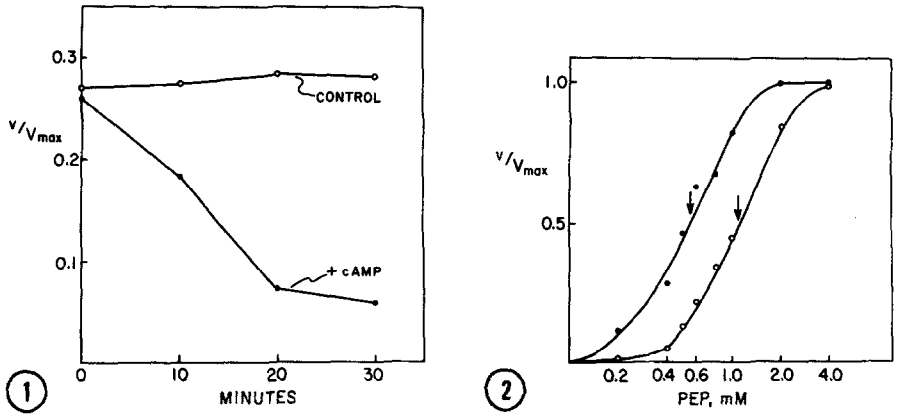

Fig. 1. Time Course of the Effect of Cyclic AMP on Inactivation of Pyruvate Kinase in Hepatocyte Homogenates. Sephadex-treated hepatocyte homogenates were prepared as described in Methods. The extract was incubated with 5 mM Mg-ATP, 1 mM EDTA and 10 mM NaF in the presence and absence of 50 μM cyclic AMP. The reaction was terminated by addition of saturated $(NH_4)_2SO_4$ and the resulting pellets washed, suspended, and assayed as described in Methods. v/V_{max} represents the ratio of activity measured at 0.4 mM to that at 4 mM phosphoenolpyruvate. Cyclic AMP addition had no effect on activity measured at saturating phosphoenolpyruvate concentration (4 mM). The experiment was repeated four times with identical results.

Fig. 2. Phosphoenolpyruvate Concentration Curves for Control and Inactivated Pyruvate Kinase from Hepatocyte Homogenates. Sephadex-treated extracts were incubated for 30 min with and without cyclic AMP and washed $(NH_4)_2SO_4$ fractions prepared. Pyruvate kinase activity was assayed as a function of phosphoenolpyruvate concentration. v/V_{max} represents the ratio of activity measured at any given substrate concentration to that measured at 4 mM phosphoenolpyruvate.

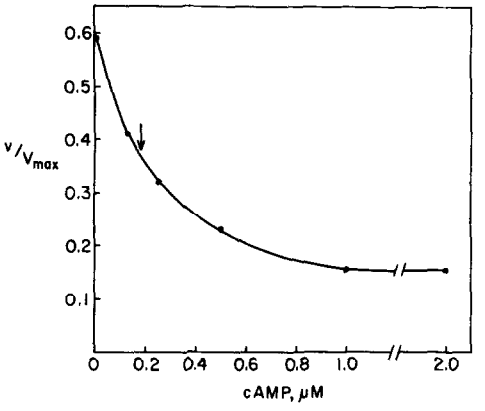

Fig. 3. Effect of Cyclic AMP Concentration on the Inactivation of Pyruvate Kinase in Hepatocyte Homgenates. Sephadex-treated extracts were incubated in the presence of increasing concentrations of cyclic AMP as described in Methods. The reaction was terminated and pyruvate kinase activity assayed as described in Methods except that v/V_{max} represents the ratio of activity measured at 0.6 mM and 4 mM phosphoenolpyruvate.

TABLE I

Effect of Protein Kinase Inhibitor on cyclic AMP Induced Inactivation of Pyruvate Kinase in Hepatocyte Homogenates

Sephadex treated extracts were prepared from rat hepatocytes as described in Methods. The extracts were incubated for 25 min at 30° with the additions listed below. The incubations were terminated by addition of saturated $(NH_4)_2SO_4$. The resulting pellets were washed and dissolved in homogenizing buffer as described in Methods. Pyruvate kinase activity was assayed at 0.4 mM and 4.0 mM phosphoenolpyruvate in the absence of fructose diphosphate. v/V_{max} represents the ratio of activity at 0.4 and 4.0 mM substrate. No agent had any effect on the activity of the enzyme determined with high (4 mM) phosphoenolpyruvate. Five hundred units of protein kinase inhibitor were added per incubation. One unit of protein kinase inhibitor is that amount which will completely inhibit one unit of activity of the catalytic subunit of cyclic-dependent protein kinase (14). Suppression of inactivation by protein kinase inhibitor was dose dependent and 400 units gave maximal inhibition under the conditions employed.

	Additions	Concentration	v/V_{max}
1.	None (6)*	5 mM	0.40
2.	Mg-ATP (6) + cAMP	5 mM 1 μM	0.16
3.	Mg^{2+} (3)	5 mM	0.41
4.	ATP (3)	5 mM	0.40
5.	Mg-ATP (3)	5 mM	0.40
6.	cAMP (3)	1 μM	0.40
7.	Mg-ATP + cAMP + Protein kinase inhibitor (3)	5 mM 1 μM	0.41
8.	Protein kinase inhibitor (3)		0.43
9.	Mg-ATP + Protein kinase inhibitor (3)	5 mM	0.40

* The number in parenthesis is the number of hepatocyte preparations tested.

inhibition by cyclic AMP was completely prevented by the addition of protein kinase inhibitor (Table I). The inhibitor had no effect by itself or when just Mg-ATP was added to the homogenate.

Influence of allosteric effectors on the cyclic AMP-dependent inactivation of puruvate kinase in hepatocyte homogenates. Pyruvate kinase activity is influenced

by the concentration of fructose diphosphate, ATP, alanine, and phosphoenolpyruvate and possibly by pyruvate as well (12,13). It is not known whether any of these compounds affect the phosphorylation of the enzyme. We have used the in vitro hepatocyte homogenate system to study the influence of these effectors on the cyclic AMP-dependent inactivation of the enzyme (Table II). Physiological concentrations of fructose diphosphate (10-50 μM) were able to prevent the inactivation of pyruvate kinase by a submaximal concentration (0.2 μM) of cyclic AMP. Physiological concentrations of phosphoenolpyruvate (0.1-0.4 mM) were also able to suppress the inactivation of the enzyme but they were not as effective as fructose diphosphate. Alanine (0.1-10 mM) had little or no effect as did physiological concentrations of pyruvate. A small inhibitory effect was seen with high concentrations of pyruvate (1-10 mM). When a maximally effective concentration (2 μM) of cyclic AMP was used to induce inactivation of pyruvate kinase, alanine, pyruvate, and phosphoenolpyruvate had no effect. Fructose diphosphate had a slight inhibitory effect, but it was not nearly as great as that seen when a submaximal concentration of cyclic AMP was used to inactivate the enzyme.

The effects were not due to carry over of the effector into the assay media. The addition of these agents had no effect on the homogenate activity in the absence of added cyclic AMP (Table II)or on the phosphoenolpyruvate concentration curve for the enzyme (data not shown). The lack of effect under these conditions illustrates that the $(NH_4)_2SO_4$ precipitation and wash procedures effectively removed all low molecular weight effectors.

DISCUSSION

Several lines of evidence suggest that the cyclic AMP induced inactivation of pyruvate kinase in hepatocyte homogenates is due to phosphorylation of the enzyme. First, the effect requires the presence of ATP and it is time dependent. Second, the effect is observed even though all low molecular weight effectors have been removed from the enzyme by the $(NH_4)_2SO_4$ procedure. Third, the inactivation led to an increase in the $K_{0.5}$ for phosphoenolpyruvate for the enzyme and the change is identical to that induced by phosphorylation of the purified enzyme by the catalytic

TABLE II

Effect of Various Agents on the Cyclic AMP Induced Inactivation of Pyruvate Kinase in Hepatocyte Homogenates

Sephadex-treated extracts were prepared and incubated as described in Methods. The incubation time was 25 min. The activity is expressed as the ratio of activity measured at 0.4 mM/4.0 mM (v/V_{max}). The results are expressed as the average ± standard deviation of four experiments.

Additions	v/V_{max} -cAMP	+ 0.2 μM cAMP	+ 2 μM cAMP
Mg-ATP (5 mM)	0.30 ± 0.02	0.17 ± 0.01	0.11 ± 0.02
+ FDP (10 μM)		0.15 ± 0.02	
(20 μM)		0.25 ± 0.01	
(50 μM)	0.30 ± 0.01	0.24 ± 0.01	0.15 ± 0.02
(100 μM)	0.30 ± 0.02	0.27 ± 0.02	
+ PEP (0.1 mM)		0.18 ± 0.01	
(0.2 mM)		0.22 ± 0.01	
(0.5 mM)	0.31 ± 0.02	0.24 ± 0.01	0.13 ± 0.02
+ Alanine (0.1 mM)		0.19 ± 0.02	
(1 mM)		0.20 ± 0. 02	
(10 mM)	0.30 ± 0.02	0.18 ± 0.01	0.11 ± 0.02
+ Pyruvate (0.1 mM)		0.17 ± 0.02	
(1 mM)	0.29 ± 0.02	0.20 ± 0.02	
(10 mM)	0.28 ± 0.03	0.20 ± 0.02	0.14 ± 0.02

FDP: fructose diphosphate; PEP: phosphoenolpyruvate; cAMP: cyclic AMP.

subunit of the cyclic AMP-dependent protein kinase (6). Fourth, the cyclic AMP induced inactivation of pyruvate kinase is blocked by addition of protein kinase inhibitor.

The inactivation of pyruvate kinase in hepatocyte homogenates occurs primarily by a cyclic AMP-dependent mechanism. Addition of Mg-ATP alone had essentially no effect whereas the addition of protein kinase inhibitor, which is specific for the cyclic AMP-dependent protein kinase (14) completely prevented the inactivation by cyclic AMP. Thus, no direct evidence for any cyclic AMP-independent phosphorylation of the enzyme was obtained in the present study. However, the concentration

of cyclic AMP-dependent protein kinase in the Sephadex-treated homogenates is at least an order of magnitude less than that concentration of purified catalytic subunit (0.3-1 μM) that is needed to phosphorylate purified pyruvate kinase (15). Thus, it is possible that there is an intermediate kinase present in these crude extracts whose activity is independent of cyclic AMP, but which is dependent upon prior activation by the cyclic AMP-dependent protein kinase. A cyclic AMP-independent kinase which phosphorylates pyruvate kinase has been isolated from chicken liver (16), but it is not known whether it can be phosphorylated by the cyclic AMP-dependent protein kinase. A similar enzyme has not been found in rat liver.

The demonstration that physiological concentrations of fructose diphosphate and phosphoenolpyruvate can affect the inactivation of pyruvate kinase is of potential importance for control of the phosphorylation state of the enzyme in vivo. Glucagon addition to hepatocytes produces a decrease in the level of fructose diphosphate (1), an inhibition of pyruvate kinase activity (1-2), and an elevation in phosphoenolpyruvate levels (1). The decrease in fructose diphosphate, a potent allosteric activator of pyruvate kinase, would decrease pyruvate kinase activity directory (1,6) and also increase the degree of phosphorylation of the enzyme. The decrease in pyruvate kinase activity leads to an elevation in phosphoenolpyruvate levels which in turn may act as a negative feedback inhibitor of the phosphorylation of the enzyme. Preliminary experiments in this laboratory suggest that alterations in fructose diphosphate and phosphoenolpyruvate can affect the phosphorylation state of the enzyme in intact cells (17). The mechanism whereby these intermediates affect the inactivation of pyruvate kinase is unknown. Eigenbrodt and Schoner (18) have reported that fructose diphosphate and alanine influence the cyclic AMP-independent phosphorylation of chicken liver pyruvate kinase by affecting the dimer to tetramer equilibrium of the enzyme. Fructose diphosphate inhibited and alanine enhanced the cyclic AMP-independent phosphorylation of chicken liver enzyme (18). Berglund et al. (19) have reported that alanine increases the rate of phosphorylation of purified pig liver pyruvate kinase by a partially purified preparation of cyclic AMP-dependent protein kinase although alanine had no effect in the present study. In the extract system reported here it is

not clear whether the effects are on phosphorylation or dephosphorylation. If indeed fructose diphosphate and phosphoenolpyruvate affect phosphorylation of pyruvate kinase in vivo, it will be necessary to determine whether these substances affect phosphorylation by the cyclic AMP-dependent kinase or by a possible intermediate kinase. Studies on these questions are now in progress.

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