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CYCLIC AMP STIMULATED PHOSPHORYLATION OF LIVER PYRUVATE KINASE IN HEPATOCYTES

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Summary. The addition of glucagon (10^{-6} M) to an incubation mixture containing ^{32}Pi and hepatocytes isolated from livers of rats fed ad libitum results in both a 3-fold increased incorporation of ^{32}P into L-type pyruvate kinase and a decreased catalytic activity. The ^{32}P incorporated into pyruvate kinase was covalently bound to the enzyme as evidenced by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In addition, exogenous cyclic AMP (10^{-3} M) stimulated the phosphorylation and the suppression of catalytic activity to a similar extent. On the other hand, insulin (10^{-7} M) had essentially no effect on the incorporation of ^{32}P into pyruvate kinase or on its catalytic activity under the conditions used in this study. These results suggest that phosphorylation of pyruvate kinase in vivo is stimulated by glucagon via cyclic AMP and cyclic AMP-dependent protein kinase and that the activity of the enzyme is, at least in part, regulated by a phosphorylation-dephosphorylation mechanism.

Introduction. The purified L-type isozyme of pyruvate kinase (EC 2.7.1.40) is phosphorylated by a cyclic AMP-dependent protein kinase and ATP (1,2). Phosphorylated pyruvate kinase has a lower affinity for the substrate, phosphoenolpyruvate, and the activator, fructose-1,6-bisphosphate (2). Administration of glucagon to intact animals (3,4), perfused livers (5), and isolated hepatocytes (6-11) results in a rapid decrease in the liver pyruvate kinase activity. Furthermore, the kinetics obtained from pyruvate kinase after glucagon administration are similar to kinetics observed with the phosphorylated enzyme. Thus, it was suggested that the phosphorylation-dephosphorylation of pyruvate kinase is a rapid acting regulatory mechanism for liver pyruvate kinase in vivo.

Recently, a phosphorylated pyruvate kinase has been isolated from liver slices (12), livers of intact rats (13), and from hepatocytes (11) incubated

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with ^{32}Pi. In each instance, glucagon rapidly stimulated the incorporation of ^{32}P into L-type pyruvate kinase. The next question is whether the phosphorylation of the enzyme occurs *in vivo* via a cyclic AMP-dependent or independent mechanism. It has been previously reported that cyclic AMP exogenously added to hepatocytes results in a lower catalytic activity and modified kinetics for pyruvate kinase which suggests that a cyclic AMP mediated process may be involved (4,9).

In this communication, a phosphorylated pyruvate kinase is isolated from hepatocytes after administration of cyclic AMP or glucagon. Furthermore, these agents suppress the catalytic activity of the enzyme to similar extents. In contrast, addition of insulin does not result in an increased phosphorylation of pyruvate kinase or a decrease in its catalytic activity.

Materials and Methods. Hepatocytes were isolated essentially as described by Berry and Friend (14) and Ingebretsen and Wagle (15) from livers of male Sprague-Dawley rats (340-400 g) fed a standard laboratory chow and water *ad libitum* as described previously (11). About 2.5 g of isolated hepatocytes were suspended in 25 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 mM glucose in 125 ml Erlenmeyer flask. ^{32}Pi (5 mCi/ml, ICN Pharmaceutical Inc., Irvine, Calif.) was added to a concentration of 50 μCi/ml to a prewarmed cell suspension, gassed with 95% O_2-5% CO_2 for 1 min, stoppered and incubated at 37° with shaking at 120 cycles per min. After incubation for 40 min, samples (3 ml) of the cell suspension were transferred to 50 ml centrifuge tubes and subsequently incubated 10 min at 37° with either 10^{-6} M glucagon, 10^{-3} M cyclic AMP, 10^{-7} M insulin or a similar volume of saline (30 μl). After the hepatocytes were incubated in the presence or absence of hormones, an equal volume of cold 0.1 M Tris buffer, pH 7.4, containing 0.1 M KF and 15 mM EGTA was added and the tubes were placed in ice. The zero time control was taken at the time the hormone incubations were started.

The hepatocytes were immediately sonicated for 30 sec with a Sonifier Cell Disruptor Model W185D from Branson Sonic Power Co. and centrifuged at 10,000 x g for 15 min. After adjusting the supernatant to 50% ammonium sulfate saturation, the precipitate was collected by centrifugation at 10,000 x g for 15 min. The pellet was dissolved in 3 ml of 0.1 M Tris-HCl, pH 7.4, containing 30% glycerol, 50 mM KF and 7.5 mM EGTA and centrifuged at 100,000 x g for 60 min to obtain a clear supernatant solution. The pyruvate kinase in this supernatant fraction is stable as reported previously (11). Total pyruvate kinase activity in this fraction was assayed using 5 mM phosphoenolpyruvate. A sample of this fraction which contained 1 E.U. of pyruvate kinase, with 3 E.U. of purified enzyme added as a carrier, was incubated overnight at 4° with 200 μl of anti-liver pyruvate kinase immunoglobulin which quantitatively precipitates the enzyme activity (11). The antigen-antibody complex was collected by centrifugation at 20,000 x g for 15 min, resuspended in 0.15 M NaCl and washed 3 times through 1 M sucrose which contained 0.15 M NaCl, 1% deoxycholate and 1% Triton X-100. The precipitate was resuspended in 5 ml of 0.15 M NaCl and collected by filtration on a 0.45 μ Millipore filter. The filter was washed 5 times with 5 ml of 0.15 M NaCl before the filter was transferred to a counting vial and

dried at 80° for 15 min. After cooling, 10 ml of Aquasol-II (New England Nuclear) were added and the radioactivity in the immune complex was determined using a Beckman Scintillation Counter. Net ^{32}P incoporation is expressed as dpm per one enzyme unit (E.U) of total pyruvate kinase activity (assayed with 5 mM phosphoenolpyruvate) after subtracting the non-specific (approximately 100 dpm) incorporation of ^{32}P into immunoprecipitates obtained by incubation of similar volumes of hepatocyte extracts with γ-globulin obtained from a pre-immune serum sample. The immune complex was electrophoresed in the presence of sodium dodecyl sulfate (SDS) with purified liver pyruvate kinase and the isolated IgG fraction used as references. The gels were then subjected to autoradiography or sliced and analyzed for radioactivity. The immuno-precipitate from hepatocytes incubated with ^{32}Pi resulted in only one major phosphorylated component, the position of which corresponded to that of the reference pyruvate kinase subunits (11). The ^{32}P incorporation into total soluble protein is expressed as dpm per mg of protein precipitated with 5% trichloroacetic acid after the 10,000 x g centrifugation (11).

Pyruvate kinase activity was assayed spectrophotometrically as reported previously (16). The protein concentration was determined by the method of Lowry et al (17) using bovine serum albumin as a standard. Specific antisera against liver pyruvate kinase was elicited in goats and the γ-globulin fraction was used for this study. This preparation of immunoglobulin was specific for L-type pyruvate kinase and 1 ml would neutralize 120 E.U. of liver pyruvate kinase (11).

Results and Discussion. Table I summarizes the ^{32}P incorporation into pyruvate kinase and the enzyme activity elicited upon the addition of exogenous hormone to hepatocyte preparations. Incubation of hepatocytes in the presence of glucagon, at concentrations giving maximal gluconeogenesis (10^{-6} M) (10,18), results in a 3-fold increase in ^{32}P incorporation into pyruvate kinase. The incubation of hepatocytes with exogenous cyclic AMP at a concentration giving maximal gluconeogenesis (10^{-3}M) (18) results in a similar incorporation of ^{32}P into pyruvate kinase. Furthermore, the addition of either glucagon or cyclic AMP results in suppression of the enzyme activity by at least 40% when assayed in the absence of fructose-1,6-bisphosphate and at a suboptimal concentration of the substrate, phosphoenolpyruvate. However, there is no decrease of enzyme activity when assayed in the presence of fructose-1,6-bisphosphate at a saturating concentration of phosphenolpyruvate. These results are in good agreement with previous suggestions that glucagon decreases the enzyme activity through a mechanism involving cyclic AMP and the cyclic AMP-dependent protein kinase phosphorylating system.

As seen in Table I, insulin had essentially no effect on the incorporation of phosphate into pyruvate kinase or on the activity of pyruvate

TABLE I

Incorporation of ^{32}P Into Pyruvate Kinase[a]

	Net ^{32}P Incorporation Into Pyruvate Kinase[b] (dpm/EU)	(% of Saline)	^{32}P Incorporation Into Total Soluble Protein[c] (dpm/mg of protein)	Pyruvate Kinase Activity[b] (EU/mg or protein) [phosphoenolpyruvate] (1 mM)	(5 mM)
Zero Time Control (After 40 min preincubation with ^{32}Pi)	381 ± 68		18157 ± 5128	0.143 ± .023	0.552 ± .114
10 min after addition of					
Saline	339 ± 37	100	20251 ± 5397	0.141 ± .014	0.549 ± .107
Glucagon (10^{-6}M)	1032 ± 258[d]	304	25065 ± 4660	0.087 ± .017[d]	0.560 ± .108
Cyclic AMP (10^{-3}M)	1054 ± 196[d]	311	23886 ± 7510	0.079 ± .003[d]	0.522 ± .108
Insulin (10^{-7}M)	377 ± 143	111	21729 ± 5607	0.123 ± .027	0.523 ± .109

About 2.5 g of hepatocytes isolated from a rat fed ad libitum were incubated at 37° with ^{32}Pi (50 µCi/ml) in 25 ml of Krebs-Ringer bicarbonate buffer, pH 7.4 containing 10 mM glucose. After incubation for 40 minutes, three ml samples were transferred to 50 ml-centrifuge tubes which contained either one of the hormones or only the saline carrier solution and the incubation was continued for 10 more minutes. The incubation was stopped by the addition of an equal volume of ice-cold buffer containing 0.1 M Tris-HCl, pH 7.4, 0.1 M KF and 15 mM EGTA and the hepatocytes were immediately sonicated. Following centrifugation at 10,000 x g for 15 min, the supernatant fractions (Fraction I) were adjusted to 50% ammonium sulfate. The precipitates were collected by centrifugation, dissolved in 0.1 M Tris-HCl, pH 7.4, containing 30% glycerol, 50 mM KF and 7.5 mM EGTA, and centrifuged at 100,000 x g for 60 min. Pyruvate kinase was quantitatively precipitated from the supernatant fraction (Fraction II) by addition of the γ-globulin fraction of antiserum elicited against liver pyruvate kinase. The incorporation of ^{32}P into pyruvate kinase was obtained by measuring the radioactivity in the immune complex after extensive washing and collection on a 0.45 µ Millipore filter. Incorporation is expressed as dpm/E.U. of pyruvate kinase assayed using 5 mM phosphoenolpyruvate after subtracting the dpm incorporated into an immunoprecipitate obtained by using preimmune serum. Pyruvate kinase activity was determined spectrophotometrically with the phosphoenolpyruvate concentration as indicated. Protein concentration was determined by the method of Lowry et al. (17), with bovine serum albumin as a standard.

[a] All values were obtained from four different batches of hepatocytes and expressed as mean ± S.D. Results were subjected to statistical evaluation by means of the t-test for small samples.

[b] Obtained from Fraction II extracts.

[c] Obtained from Fraction I extracts.

[d] $p < 0.01$ compared with the value for saline.

kinase under the conditions used in this study. Avruch et al (19) previously reported that when insulin was added to hepatocytes as the sole hormone, there was a selective stimulation of ^{32}P incorporation into a single ^{32}P-phosphopeptide (molecular weight 46,000) without altering ^{32}P content of the other major phosphopeptides. Our results demonstrate that addition of insulin to a hepatocyte suspension does not result in a phosphorylation of pyruvate kinase. Insulin also has no effect on the basal level of cyclic AMP in the isolated liver cell (18,19).

The present study demonstrates a marked effect of glucagon and cyclic AMP on the phosphorylation of L-type pyruvate kinase and the suppression of its catalytic activity. Engström and coworkers (1,2,20) have reported that phosphorylation of rat liver pyruvate kinase in vitro using a cyclic AMP-dependent protein kinase results in a decreased catalytic activity. They have also reported that full catalytic activity can be regained upon dephosphorylation of the enzyme. All these results suggest that the activity of pyruvate kinase in vivo is regulated, at least in part, by a phosphorylation-dephosphorylation mechanism. Furthermore, it is suggested that the glucagon effect on the enzyme is mediated by cyclic AMP and a cyclic AMP-dependent protein kinase.

Walsh and Ashby (21) outlined six modified criteria, originally defined by Krebs (22), that must be satisfied before concluding that a protein phosphorylation is mediated by cyclic AMP and a cyclic AMP activated protein kinase. Liver pyruvate kinase appears to satisfy all six criteria: a) the cell type involved contains a cyclic AMP-dependent protein kinase (23), b) protein that bears a functional relationship to a cyclic AMP-mediated process is phosphorylated in vitro (1,2), c) in vitro phosphorylation of protein leads to modified function (2), d) stoichiometric correlation exists between in vitro phosphorylation and modified function (20), e) phosphorylation in vivo can be demonstrated in response to cyclic AMP (present paper), and f) modified function in vivo can be demonstrated in response to cyclic AMP (4,6, present paper).

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