CYCLIC NUCLEOTIDE-, PYRUVATE- AND HORMONE-INDUCED CHANGES IN PYRUVATE KINASE ACTIVITY IN ISOLATED RAT HEPATOCYTES

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SUMMARY. Incubation of isolated rat hepatocytes with glucagon (10^{-6} M), db-cAMP (0.1 mM) and db-cGMP (0.1 mM) causes a decrease in pyruvate kinase activity of 46, 49 and 34% respectively, when measured at 1 mM Mg^{2+}_{free} and suboptimal substrate (P-enolpyruvate) concentrations, while the V_{max} is uninfluenced. An increase in activity (25%) is noticed when the cells are incubated with 1 mM pyruvate. The glucagon inactivated enzyme (L_b) shows a decreased affinity for the substrate P-enolpyruvate and for the allosteric activator Fru-1,6-P_2 as compared to the activated form (L_a). The nature of the hormone and cyclic nucleotide-induced changes in pyruvate kinase is discussed. It is concluded that the P-enolpyruvate cycle is under comparable acute hormonal control as the FDPase-PFK cycle. Both cycles are linked by the common effector Fru-1,6-P_2 making not only direct but also indirect hormonal control of pyruvate kinase flux possible.

Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) catalyses the last step of glycolysis. Its activity is important in the regulation of the balance between gluconeogenesis and glycolysis. Although liver contains two types of pyruvate kinase (1), isolated hepatocytes only contain the L-type (2). This enzyme possesses many regulatory properties but the mechanism to inactivate the enzyme under gluconeogenic conditions is still uncertain (3-6). From kinetic studies *in vitro* it was concluded that the known properties of L-type pyruvate kinase could not lead to the presence of an inactive enzyme during gluconeogenesis. This was mainly the consequence of the high affinity of the enzyme for its allosteric activator Fru-1,6-P_2 (6). It was suggested that "A change in the enzyme itself can influence its affinity for Fru-1,6-P_2" and the use of isolated parenchymal cells to test this possibility was therefore proposed. Taunton *et al.* (7,8) showed recently that short term changes in V_{max} of pyruvate kinase may be induced by alteration of

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the hormonal state of the intact animal, while Rognstad (9) showed that cyclic-AMP induces inhibition of the pyruvate kinase flux rate in the intact liver cell. However, a direct hormonal effect on pyruvate kinase was questioned by Clark (10) who suggested an indirect effect on the pyruvate kinase flux rate as a consequence of an alteration of the ratio of FDPase and PFK activities. In the present paper we report cyclic nucleotide- and hormone-induced effects on the kinetic properties of pyruvate kinase which suggests that pyruvate kinase flux can be under both direct and indirect hormonal control.

MATERIALS AND METHODS. Isolated rat liver hepatocytes were prepared from fed animals essentially as described by Berry and Friend (11), modified as described previously (12). The parenchymal cells were incubated at 37°C in 50 ml Erlenmeyer flasks stoppered with rubber caps. The incubation medium was 10 ml of Krebs-Ringer buffer containing the additions as indicated in the figures. At the indicated times 2 ml samples were withdrawn, homogenized immediately and put in ice-water. Zero time controls contained all the additions but no incubation at 37°C was performed. After termination of the incubation (the total time did not exceed 50 min) the homogenized cells were centrifuged for 10 min at 10.000 g and the supernatant was immediately tested for pyruvate kinase activity by adding 0.5 ml supernatant to a cuvette containing 24 mM Tris-HCl pH 7.5, 200 mM KCl, 1 mM ADP 0.1 mg lactate dehydrogenase, 0.4 mM NADH and 1 mM free Mg^{2+}. The free Mg^{2+} concentration was calculated as described earlier (13). The reaction was started by adding phosphoenolpyruvate (final concentration 2.5 mM) after 5 min preincubation. This sequence of additions is essential because bound Fru-1,6-P_2 will be split off under the applied high salt concentration in the absence of P-enolpyruvate (12,14). Linear curves were obtained by this addition sequence throughout this study. When indicated Fru-1,6-P_2 (0.5 mM final concentration) was added as second addition.

RESULTS

Fig. 1 shows the effect of cyclic nucleotides on the activity of pyruvate kinase when the isolated liver cells are incubated with 1 mM pyruvate. It can be seen that pyruvate alone raises the pyruvate kinase activity at 10 min. This effect, however, disappears on further incubation. The presence of 1 mM pyruvate + 0.1 mM dibutyryl-cyclic-AMP causes a 50% decrease of pyruvate kinase activity. The addition of dibutyryl-cyclic-GMP does not alter the effect of db-cAMP. When V_{max} values are measured at saturating Fru-1,6-P_2 concentrations neither db-cAMP alone nor its combination with db-cGMP have an effect on the maximal enzymatic activity.

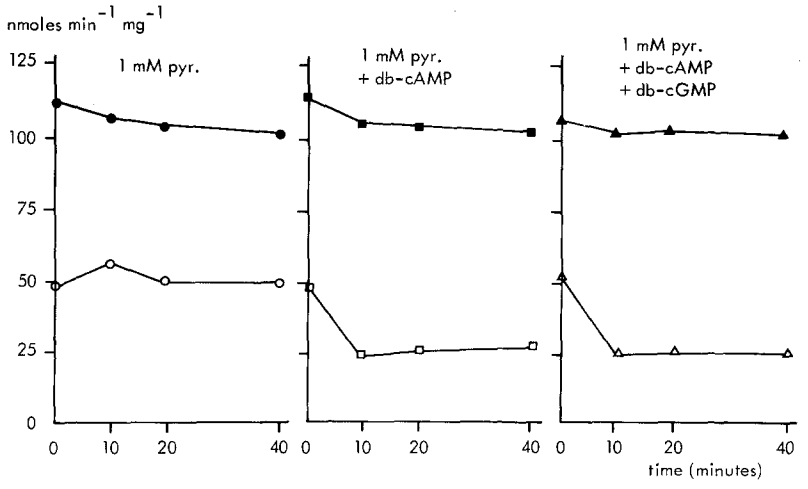

Fig. 1 Influence of pyruvate, pyruvate+db-cAMP (0.1 mM) and pyruvate+db-cAMP (0.1 mM) + db-cGMP (0.1 mM) on the pyruvate kinase activity of isolated hepatocytes. The additions to incubations of intact cells are indicated in the figure. For further incubations see MATERIALS AND METHODS. The open symbols represent the pyruvate kinase activity at 2.5 mM P-enolpyruvate and 1 mM Mg^{2+}_{free}. The closed symbols represent the activity after subsequent addition of Fru-1,6-P_2 (0.5 mM).

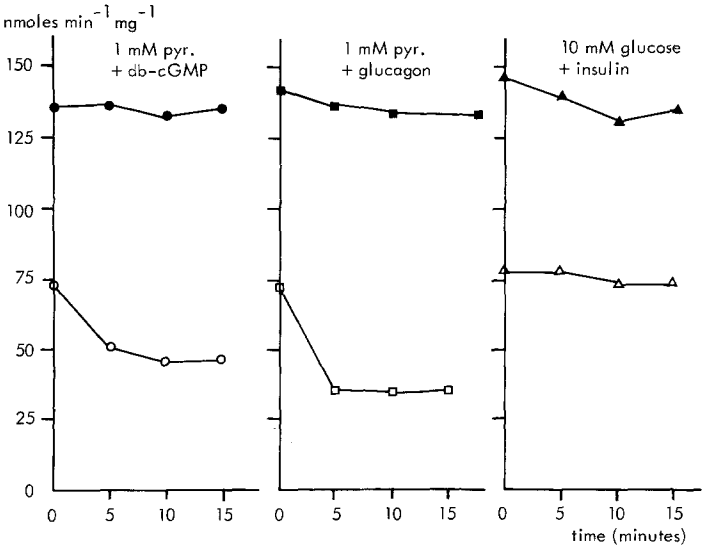

Fig. 2 Influence of pyruvate + db-cGMP (0.1 mM), pyruvate + glucagon (10^{-6} M) and glucose + insulin (4 mU/ml) on the pyruvate kinase activity of isolated hepatocytes. The additions to incubations of intact cells are indicated in the figure. For further incubation conditions see MATERIALS AND METHODS. The open symbols represent the pyruvate kinase activity at 2.5 mM P-enolpyruvate + 1 mM Mg^{2+}_{free}. The closed symbols represent the activity after subsequent addition of Fru-1,6-P_2 (0.5 mM).

TABLE I

EFFECT OF Mg^{2+} AND FRU-1,6-P_2 ON THE CYCLIC NUCLEOTIDE- AND HORMONE-INDUCED CHANGES IN PYRUVATE KINASE ACTIVITY

Additions to incubations of intact cells	Activity at 1 mM Mg^{2+}_{free}	+10 mM Mg^{2+}	+10 mM Mg^{2+} +0.5 mM Fru-1,6-P_2
1 mM pyr.	72.8	138.4	164.3
1 mM pyr. + 0.1 mM db-cAMP	33.7	103.0	160.9
1 mM pyr. + 0.1 mM db-cGMP	42.6	109.7	158.9
1 mM pyr. + 10^{-6} M glucagon	37.7	99.5	162.4
10 mM glucose	62.4	129.1	159.6
10 mM glucose + insulin (4 mU/ml)	64.6	133.4	158.7

When shown to be present 10 mM Mg^{2+} and/or 0.5 mM Fru-1,6-P_2 were added to the cell homogenates prepared after the intact cells had been incubated for 10 min with the substances mentioned in the first column. Activity expressed in nmoles min^{-1}. mg^{-1} protein.

Fig. 2 shows that 0.1 mM db-cGMP itself also decreases the pyruvate kinase activity. However, its effect is smaller than that of an equimolar concentration of db-cAMP (see also TABLE I); Fig. 2 also shows that the addition of glucagon instead of the cyclic nucleotides causes a decrease in activity as well. Insulin in the presence of glucose, on the other hand, is ineffective.

In an attempt to explore the nature of the changes that had taken place in the intact cell we added several compounds to the liver cell homogenate. We reported earlier that interconvertible forms of pyruvate kinase exist depending upon the redox state of the enzymes thiol groups (5,14). However, the effects of the cyclic nucleotides and of glucagon on intact cells were unaltered whether the homogenates were prepared in the absence or presence of 10 mM mercaptoethanol, suggesting that oxidation of essential -SH groups is not involved in the observed effects. Recently Ljungström *et al.* (15) and Titanji *et al.* (16) have shown that under certain condi-

tions liver pyruvate kinase can be phosphorylated, which is accompanied by a decrease of activity, especially when measured at suboptimal P-enolpyruvate concentrations (16). The phosphorylation of other enzymes, such as pyruvate dehydrogenase is reversed by incubation at high Mg^{2+} concentrations (17,18) which activates phosphoprotein phosphatases. TABLE I illustrates the effect of high Mg^{2+} concentrations on the enzyme activity. At 1 mM Mg^{2+}_{free} the effects of cyclic nucleotides and hormones are maximal, addition of 10 mM Mg^{2+} causes a prompt (within 10 sec) stimulation of pyruvate kinase activity. However, even at high Mg^{2+} concentrations the effects of cyclic nucleotides and hormones may be observed. Addition of Fru-1,6-P_2 relieves these differences.

TABLE II summarizes the changes of kinetic properties of pyruvate kinase by glucagon treatment of the liver cells. L_b stands for the enzyme obtained after incubation of the cells for 10 min with glucagon while L_a is the enzyme obtained from cells incubated with pyruvate (1 mM) alone. It can be seen that besides the difference in affinity for the substrate P-enolpyruvate, the L_b form also

TABLE II

COMPARISON OF THE KINETIC PARAMETERS OF PYRUVATE KINASE TYPES L_a AND L_b

	L_a	L_b
$K_{0.5}$ P-enolpyruvate	2.2 mM	4.1 mM
$K_{0.5a}$ Fru-1,6-P_2	0.6 µM	1.0 µM
$K_{0.5a}$ Fru-1,6-P_2 in presence of ATP (2 mM) and alanine (1 mM)	4.2 µM	9.8 µM

L_b was prepared by incubation of the cells with 1 mM pyruvate + glucagon (10^{-6} M) for 10 min

L_a was prepared by incubation of the cells for 10 min with 1 mM pyruvate alone.

possesses a lower apparent affinity for the allosteric activator Fru-1,6-P_2 especially in the presence of physiological concentrations of the allosteric inhibitors ATP (2 mM) and alanine (1 mM) (6,19).

DISCUSSION

Taunton et al. (7,8) were the first to observe rapid effects on the enzymatic activities of phosphofructokinase, FDPase and pyruvate kinase by the administration of hormones. This was confirmed for pyruvate kinase by Cimbala and Blair (20) and Friedrichs (21). The present experiments show that isolated hepatocytes can serve, as suggested earlier (6), as a model to study the regulation of pyruvate kinase. From our earlier studies with isolated L-type pyruvate kinase (2,5,6,19) we concluded by extrapolation from the in vitro data to the in vivo situation that the enzyme should be fully activated under both glycolytic and gluconeogenic conditions. We suggested (6) that interconvertible forms should be present in vivo. The reported kinetic parameters of the enzyme inactivated by glucagon, as determined at the physiological Mg^{2+}_{free} concentration of 1 mM (22), show that in the liver cell an enzyme form (L_b) can be present with properties distinct from the purified enzyme. The described properties of the L_b form of pyruvate kinase, especially the lower affinity for the allosteric activator Fru-1,6-P_2, will enable a decrease of pyruvate kinase activity under gluconeogenic conditions. Incubation of liver cells with pyruvate (1 mM) surprisingly leads to an activation of the enzyme. This L_a form possesses kinetic properties identical to the purified enzyme (6). The conversions may be summarized as follows:

$$L_a \underset{\text{pyruvate}}{\overset{\text{glucagon, db-cAMP, (db-cGMP)}}{\rightleftharpoons}} L_b$$

(active form) (inactive form)

The mechanism of the transition between L_b and L_a form is not clear at the moment. Regulation by means of the reduction state of the thiol groups of the enzyme seems less probable as incubation of the L_b form with 10 mM mercaptoethanol did not convert this form into the L_a form. Recently it was shown that pyruvate kinase from

rat liver (15,16) and chicken liver (23) may be converted *in vitro* into a phosphorylated, less active, form. This phosphorylation is catalysed by a cAMP dependent protein kinase. The findings of the present paper that in intact cells the activity of pyruvate kinase is diminished by glucagon and db-cAMP then could indeed be based on enzyme phosphorylation. Whether db-cGMP, which has a lower efficiency than db-cAMP, represents an effect of cGMP remains to be determined, since it is possible that db-cGMP added will prevent the breakdown of endogenous cAMP by inhibition of phosphodiesterase. Besides phosphorylation, hormones may also exert their action by influencing the binding of other low molecular weight compounds. Recently Pilkis *et al*. (24) showed that glucagon promotes Ca^{2+} transport inside the cell. This cation is an inhibitor of pyruvate kinase, although *in vitro* it is only effective at relatively high concentrations (25). A further possibility is the binding of free fatty acids to the enzyme. This has been suggested for phosphofructokinase (26). The occurrence of such a mechanism for pyruvate kinase could possibly link hormone actions to both the FDPase-PFK cycle and the pyruvate kinase activity cycle.

The possibility to influence the kinetic properties of pyruvate kinase by hormones and/or cyclic nucleotides is attractive in relation to the regulation of liver metabolism. Rognstad (9) showed recently that under gluconeogenic conditions cAMP inhibited the flux through pyruvate kinase at high pyruvate concentrations. With lactate as substrate for gluconeogenesis, however, the pyruvate kinase flux was low and consequently no effect of cAMP is noticed. Katz and Rognstad (27) state that with pyruvate the P-enolpyruvate cycle is necessary for the transfer of reducing equivalents to the cytosol (28). The occurrence of such a useful cycle is strengthened by our observation that with pyruvate as substrate for gluconeogenesis the activity of pyruvate kinase increases.

The direct effect of hormones on pyruvate kinase flux is questioned by Clark (10) who suggests an indirect influence, mediated by the FDPase-PFK couple, on pyruvate kinase. Our data show that pyruvate kinase can be influenced directly by glucagon and/or cyclic nucleotides, which lead to an enzyme form with a lowered affinity for Fru-1,6-P_2, the product of PFK and the substrate for FDPase. We showed earlier that under physiological conditions the pyruvate kinase activity is largely dependent upon this compound. This in-

dicates that a relation exists between the two cycles, in which glucagon can regulate the pyruvate kinase flux both directly, by influencing the affinity for Fru-1,6-P_2, and indirectly by influencing the Fru-1,6-P_2 concentration itself through the balance of FDPase-PFK activities.

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