Hormonal regulation of L-type pyruvate kinase in hepatocytes from phosphorylase kinase-deficient (*gsd/gsd*) rats

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The hormonal regulation of L-type pyruvate kinase in hepatocytes from phosphorylase *b* kinase-deficient (*gsd/gsd*) rats was investigated. Adrenaline (10 μ M) and glucagon (10 nM) each led to an inactivation and phosphorylation of pyruvate kinase. Dose-response curves for adrenaline-mediated inactivation of pyruvate kinase, phosphorylation of pyruvate kinase and the stimulation of gluconeogenesis from 1.8 mM-lactate were similar for hepatocytes from control and *gsd/gsd* rats. Time-course studies indicated that adrenaline-mediated inactivation and phosphorylation of pyruvate kinase proceeded more slowly in phosphorylase kinase-deficient hepatocytes than in control hepatocytes. The age-dependent change in the adrenergic control of pyruvate kinase was similar between control and phosphorylase kinase-deficient hepatocytes. Adrenaline, glucagon and noradrenaline activated the cyclic AMP-dependent protein kinase and inhibited pyruvate kinase in phosphorylase kinase-deficient hepatocytes. Vasopressin (0.2–2 nM), angiotensin (10 nM) and A23187 (10 μ M) had no effect on the activity ratio of the cyclic AMP-dependent protein kinase or pyruvate kinase in these cells. It is concluded that phosphorylase kinase plays no significant role in the hormonal control of pyruvate kinase and that phosphorylation and inactivation of this enzyme results predominantly from the action of the cyclic AMP-dependent protein kinase.

Adrenaline-mediated inhibition of pyruvate kinase may involve either α -adrenergic (cyclic AMP-independent) or β -adrenergic mechanisms. Both α -adrenergic and β -adrenergic activations by adrenaline of the cyclic AMP-dependent protein kinase have been demonstrated (Kemp & Clark, 1978). The α -adrenergic cyclic AMP-dependent mechanism appears to be a function of the age and sex of the animal (Blair *et al.*, 1979a; Morgan *et al.*, 1983a,b). Despite these complexities, several groups have proposed that adrenaline-mediated inhibition of L-type pyruvate kinase predominantly involves an α -adrenergic cyclic AMP-independent mechanism (Foster & Blair, 1978; Chan & Exton, 1978; Garrison & Borland, 1979; Steiner *et al.*, 1980). Such a mechanism may be similar to that regulating glycogenolysis, where there is evidence to indicate that phosphorylase kinase is activated by an increase in cytosolic Ca^{2+} (Assimakopoulos-Jeannet *et al.*, 1977). This enzyme, in turn, is believed to phosphorylate and activate phosphorylase *b* (Assimakopoulos-Jeannet *et al.*, 1977). As hepatic tissue from rats with glycogen storage

disease (*gsd/gsd*) is deficient in phosphorylase *b* kinase (Malthus *et al.*, 1980), we have used hepatocytes from these animals to assess the role of phosphorylase kinase in the regulation of L-type pyruvate kinase by hormones that operate with and without the involvement of cyclic AMP. A preliminary report of some of these findings has appeared elsewhere (Clark *et al.*, 1981).

Materials and methods

Animals

Fed male rats with glycogen storage disease (*gsd/gsd*) were maintained in the Division (Clark *et al.*, 1982). Control rats were of similar body weight, age, sex and nutritional status and were of the Hooded Wistar strain. The animals were fed on a standard diet (Milling Industries Ltd., Adelaide, S.A., Australia; Rattigan *et al.*, 1983). Animals were anaesthetized by intraperitoneal injection (1.0 ml/kg body wt.) of sodium pentobarbitone (60 mg/ml). Rats were from three groups representing three different ages: post-weaning rats were

30–35 days old and weighed 80–120 g, immature rats were 35–45 days old and weighed 150–180 g, and mature rats were 60–105 days old and weighed 210–420 g.

Hepatocyte incubations

Hepatocytes were prepared and incubated as described previously (Kemp & Clark, 1978; Clark *et al.*, 1981).

For phosphorylation studies [^{32}P]P_i was added to each vial to give a final concentration of 1.2 mM. Hepatocytes were preincubated for 40 min with [^{32}P]P_i (Blair *et al.*, 1982) before addition of the hormones. Incubations were then terminated at the times shown by adding 2.6 M-(NH₄)₂SO₄ (Foster & Blair, 1978). Hepatocytes were separated from the medium by centrifugation (1000 g for 2 min) and extracted with 2 ml of 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol, 100 mM-NaF, 1% (w/v) bovine serum albumin, 1 mM-EDTA and 0.1% (v/v) Triton X-100. The insoluble membrane fraction was removed by centrifugation (1000 g for 20 min), and 1.5 ml of the clear supernatant containing pyruvate kinase was incubated for 17 h at 0°C with 0.25 ml of a goat anti-(pyruvate kinase) preparation (100 mg of protein/ml), containing 3 mM-NaN₃. The immunoprecipitate was centrifuged (8000 g for 10 min), washed three times, solubilized, and resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Patten *et al.*, 1981). The region of the gel corresponding to the pyruvate kinase subunit was located by comparison with a standard [rabbit liver pyruvate kinase (Sigma type V)], cut out and counted for radioactivity (Patten *et al.*, 1981).

Anti-(pyruvate kinase) serum

Goat antiserum to rabbit liver pyruvate kinase was prepared, fractionated and stored as a powder at -20°C (Hopgood *et al.*, 1973; Philippidis *et al.*, 1972). Approx. 2.0 mg of antiserum inactivated all of the pyruvate kinase activity in 0.1 ml of a 1:10 (w/v) extract of rat liver.

Enzyme assays

These were conducted on samples of the hepatocyte suspension that had been mixed with cold extraction buffer and stored on ice. The cyclic AMP-dependent protein kinase was measured with the synthetic peptide substrate (Kemp & Clark, 1978). Hormones were present with the hepatocytes for 3 min before samples were removed for assay of the protein kinase. Assays were conducted 10–20 min after extraction. These conditions were optimal for adrenaline-mediated effects (Kemp & Clark, 1978) and were therefore used in the present study. Pyruvate kinase activity was determined at a sub-saturating concentration

of 0.15 mM-phosphoenolpyruvate ($v_{0.15}$) (Kemp & Clark, 1978). Total activity was determined at 1.5 mM-phosphoenolpyruvate ($v_{1.5}$). The phosphorylase $a/(a+b)$ activity ratio was determined as described by Birnbaum & Fain (1977).

Materials

Collagenase (CLS type II) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Bovine serum albumin, glucagon, adrenaline hydrochloride, noradrenaline hydrochloride, phenylephrine hydrochloride, DL-propranolol hydrochloride, [arginine]vasopressin, angiotensin and A23187 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Phenoxybenzamine hydrochloride was given by Rohm Pharma G.m.b.H., Weiterstadt, Germany. Ionophore A23187 was prepared as a concentrated solution in dimethyl sulphoxide. A dimethyl sulphoxide control accompanied all experiments in which the ionophore was used. The other agents that were added to hepatocyte incubations were freshly prepared in iso-osmotic saline (0.9% NaCl).

Results and discussion

Although the identity of the receptor involved in adrenaline-mediated control of pyruvate kinase appears to be predominantly α (Foster & Blair, 1978; Chan & Exton, 1978; Garrison & Borland, 1979), the identity of the protein kinase responsible for the inactivation and phosphorylation of pyruvate kinase remains unclear. For the other catecholamines and for the peptide hormones which mobilize Ca²⁺, the mechanisms controlling pyruvate kinase are equally unclear. Proposals have been made for exclusive involvement of the cyclic AMP-dependent protein kinase (Kemp & Clark, 1978) and for Ca²⁺-mediated phosphorylation of pyruvate kinase independent of the cyclic AMP-dependent protein kinase (Garrison *et al.*, 1979; Steiner *et al.*, 1980). Thus the use of phosphorylase kinase-deficient hepatocytes permits the assessment of the hormonal control of pyruvate kinase in the absence of a major Ca²⁺-dependent protein kinase.

The effects of phosphorylase kinase deficiency on the phosphorylation and inactivation of pyruvate kinase by adrenaline were examined by comparing dose and time curves. Fig. 1 shows that half-maximal inactivation and phosphorylation of pyruvate kinase as well as the stimulation of gluconeogenesis from 1.8 mM-[U-¹⁴C]lactate each occurred at approx. 0.1 nM-adrenaline. The shape of the dose-response curves did not differ significantly between the two hepatocyte preparations. Thus a deficiency in phosphorylase kinase did not appear to alter the sensitivity of the cells to

Fig. 1. Dose-response curves for adrenaline-mediated changes in gluconeogenesis and the activity and phosphorylation of pyruvate kinase in control (a) and *gsd/gsd* (b) hepatocytes

Hepatocytes were prepared from mature rats and incubated as described in the Materials and methods section. Cells were preincubated for 40 min before adrenaline addition. Adrenaline was present for 5 min for pyruvate kinase activity (●) and phosphorylation (△) and for 30 min for gluconeogenesis (○). Means $\pm$ s.e.m. are shown for three hepatocyte preparations; the data in (a) for $[{}^{32}\text{P}]\text{P}_i$ incorporation are from a representative experiment of three experiments.

adrenaline with respect to the three parameters assessed.

Time-course studies (Fig. 2) indicated that adrenaline-mediated inactivation and phosphorylation of pyruvate kinase occurred more slowly in phosphorylase kinase-deficient cells (Fig. 2b) than

in control cells (Fig. 2a). Thus, as noted by Blair *et al.* (1979b), inactivation of pyruvate kinase occurred within 1 min in control hepatocytes (Fig. 2a); inactivation and phosphorylation required at least 4 min in phosphorylase kinase-deficient cells. Glucagon-mediated phosphorylation of pyruvate

Fig. 2. Time-course curves for adrenaline-mediated changes in the activity and phosphorylation of pyruvate kinase in control (a) and *gsd/bsd* (b) hepatocytes. Details are as for Fig. 1. Adrenaline ($1 \mu\text{M}$) was added at zero time. Data for the activity of pyruvate kinase ($\bullet$) are from three hepatocyte preparations and are shown as means $\pm$ S.E.M.; the data for $[{}^{32}\text{P}]\text{P}_i$ incorporation (Δ) are from a representative experiment of three experiments.

kinase did not differ significantly between the two cell preparations. Thus exposure for 5 min to 10 nM-glucagon increased ${}^{32}\text{P}$ incorporation from 6107 ± 1387 (6) to 24959 ± 1893 (6) c.p.m./mg of pyruvate kinase in control hepatocytes and from 7679 ± 1520 (3) to 22453 ± 4827 (3) c.p.m./mg in phosphorylase kinase-deficient hepatocytes.

To assess further the role for phosphorylase kinase in the phosphorylation and inactivation of pyruvate kinase, other hormones and agents that activate phosphorylase in a cyclic AMP-independent manner were used. Animals of three different age groups were compared, since age-dependent alterations in adrenergic-receptor-mediated events

have been reported (Blair *et al.*, 1979b; Morgan *et al.*, 1983a). The data from these experiments are shown in Table 1. For the post-weaning rats (80–120 g) the only hormones to give a significant effect on pyruvate kinase were glucagon (10 nM), adrenaline (10 μM) and noradrenaline (10 μM). The responses of the two hepatocyte preparations (control and *gsd/bsd*) to each hormone were similar (Table 1). As noted by Blair *et al.* (1979b), adrenaline-mediated inactivation of pyruvate kinase in hepatocytes from post-weaned animals was preferentially blocked by propranolol and unaffected by phenoxybenzamine. The data of Table 1 confirm this observation. For immature rats there were differences between the two hepatocyte preparations. Whereas glucagon, adrenaline, angiotensin, phenylephrine and noradrenaline each resulted in a significant inactivation of pyruvate kinase in control hepatocytes, angiotensin and phenylephrine had no effect in phosphorylase kinase-deficient cells. In neither cell preparation was the effect of adrenaline blocked by phenoxybenzamine or propranolol. For animals older than 60 days ('mature' rats), the responses of the two hepatocyte preparations (control and *gsd/bsd*) to each hormone were similar (Table 1). In both preparations pyruvate kinase was inactivated by glucagon, adrenaline and noradrenaline. In addition, the effect of adrenaline was significantly, but not totally, blocked by phenoxybenzamine and propranolol ($P < 0.05$). It is not clear why hepatocytes from control immature rats should show significant inactivation of pyruvate kinase by angiotensin and phenylephrine.

Table 1 shows that the total activity of pyruvate kinase increased with increasing age of the control animal, from 47.6 ± 9.0 (3) (post-weaned) to 84.2 ± 18.3 (3) (mature age) $\mu\text{mol}/\text{min}$ per 10^8 cells. This was not evident when the results were expressed as a function of cell dry weight. Indeed, maturation of the control rat resulted in a marked increase in cell size, so that the cell-number/g dry wt. ratio decreased from $(757 \pm 51) \times 10^6$ (3) at 30 days of age to $(438 \pm 57) \times 10^6$ (5) at 100 days of age. This was less pronounced with hepatocytes from *gsd/bsd* animals, where this ratio decreased from $(408 \pm 49) \times 10^6$ (3) to $(349 \pm 51) \times 10^6$ (7) and the total pyruvate kinase activity changed from 82.4 ± 18.8 (3) to 110.8 ± 6.1 (3) $\mu\text{mol}/\text{min}$ per 10^8 cells over the same period. The difference in gluconeogenic rate between the control and phosphorylase kinase-deficient hepatocytes (Fig. 1) may also be explained in part by the difference in cell size, which is particularly evident in young animals. There is no obvious explanation for the difference between the pyruvate kinase activity ratios of control and *gsd/bsd* animals. Since hepatic lipogenesis is decreased in *gsd/bsd* rats (Clark *et*

Table 1. *Hormonal control of pyruvate kinase in control and phosphorylase kinase-deficient hepatocytes of post-weaned, immature and mature rats*
Hepatocytes from control and *gsd/gsd* rats were prepared and incubated as described in the Materials and methods section. Agonists and antagonists were added after a preincubation period of 15 min and were present with the cells for 3 min. Pyruvate kinase activity at 0.15 mM- and 1.5 mM-phosphoenolpyruvate was determined on cell-free extracts. Data are means $\pm$ S.E.M. for the numbers of hepatocyte preparations shown in parentheses. A statistically significant difference between hormone and saline effects is indicated by * $P < 0.05$ (Student's *t*-test).

Additions	Pyruvate kinase activity ratio ($v_{0.15}/v_{1.5}$)					
	Control rats			<i>gsd/gsd</i> rats		
	Post-weaned (97 $\pm$ 12 g)	Immature (169 $\pm$ 10 g)	Mature (283 $\pm$ 35 g)	Post-weaned (98 $\pm$ 14 g)	Immature (164 $\pm$ 11 g)	Mature (280 $\pm$ 40 g)
Saline	0.21 $\pm$ 0.02 (3)	0.41 $\pm$ 0.01 (3)	0.27 $\pm$ 0.001 (8)	0.42 $\pm$ 0.02 (3)	0.42 $\pm$ 0.02 (3)	0.40 $\pm$ 0.01 (8)
10 nM-Glucagon	0.11 $\pm$ 0.01 (3)*	0.15 $\pm$ 0.03 (3)*	0.09 $\pm$ 0.02 (6)*	0.22 $\pm$ 0.02 (3)*	0.19 $\pm$ 0.03 (3)*	0.10 $\pm$ 0.02 (7)*
10 μ M-Adrenaline	0.13 $\pm$ 0.01 (3)*	0.21 $\pm$ 0.01 (3)*	0.18 $\pm$ 0.015 (7)*	0.28 $\pm$ 0.01 (3)*	0.27 $\pm$ 0.02 (3)*	0.25 $\pm$ 0.02 (7)*
10 μ M-Phenoxylbenzamine	0.22 $\pm$ 0.03 (3)	0.35 $\pm$ 0.01 (3)	0.29 $\pm$ 0.03 (6)	0.41 $\pm$ 0.03 (3)	0.43 $\pm$ 0.01 (3)	0.41 $\pm$ 0.01 (6)
10 μ M-Propranolol	0.21 $\pm$ 0.03 (3)	0.33 $\pm$ 0.02 (3)	0.29 $\pm$ 0.03 (6)	0.43 $\pm$ 0.03 (3)	0.42 $\pm$ 0.01 (3)	0.40 $\pm$ 0.02 (6)
10 μ M-Adrenaline + 10 μ M-phenoxylbenzamine	0.12 $\pm$ 0.01 (3)*	0.24 $\pm$ 0.02 (3)*	0.23 $\pm$ 0.02 (6)	0.26 $\pm$ 0.02 (3)*	0.26 $\pm$ 0.03 (3)*	0.32 $\pm$ 0.02 (6)*
10 μ M-Adrenaline + 10 μ M-propranolol	0.16 $\pm$ 0.03 (3)	0.24 $\pm$ 0.03 (3)*	0.26 $\pm$ 0.02 (6)	0.33 $\pm$ 0.02 (3)	0.30 $\pm$ 0.02 (3)*	0.32 $\pm$ 0.03 (6)*
2 nM-Vasopressin	0.19 $\pm$ 0.02 (3)	0.31 $\pm$ 0.04 (3)	0.27 $\pm$ 0.02 (3)	0.41 $\pm$ 0.03 (3)	0.38 $\pm$ 0.03 (3)	0.36 $\pm$ 0.05 (3)
10 nM-Angiotensin	0.20 $\pm$ 0.02 (3)	0.33 $\pm$ 0.01 (3)*	0.26 $\pm$ 0.03 (3)	0.43 $\pm$ 0.03 (3)	0.42 $\pm$ 0.04 (3)	0.34 $\pm$ 0.04 (3)
10 μ M-Phenylephrine	0.17 $\pm$ 0.02 (3)	0.27 $\pm$ 0.01 (3)*	0.23 $\pm$ 0.02 (3)	0.38 $\pm$ 0.01 (3)	0.31 $\pm$ 0.03 (3)	0.33 $\pm$ 0.05 (3)
10 μ M-Noradrenaline	0.13 $\pm$ 0.01 (3)*	0.21 $\pm$ 0.02 (3)*	0.19 $\pm$ 0.02 (3)*	0.25 $\pm$ 0.03 (3)*	0.27 $\pm$ 0.01 (3)*	0.28 $\pm$ 0.03 (3)*
10 μ M-A23187	0.20 $\pm$ 0.01 (3)	0.35 $\pm$ 0.02 (3)	0.27 $\pm$ 0.02 (3)	0.39 $\pm$ 0.03 (3)	0.37 $\pm$ 0.01 (3)	0.40 $\pm$ 0.05 (3)
Dimethyl sulphoxide (3.7%)	0.21 $\pm$ 0.01 (3)	0.36 $\pm$ 0.01 (3)	0.29 $\pm$ 0.01 (3)	0.43 $\pm$ 0.02 (3)	0.44 $\pm$ 0.02 (3)	0.41 $\pm$ 0.05 (3)
$v_{1.5}$ (μ mol/min per 10^8 cells)	47.6 $\pm$ 9.0 (3)	39.2 $\pm$ 9.4 (3)	84.2 $\pm$ 18.3 (3)	82.4 $\pm$ 18.8 (3)	106.6 $\pm$ 32.1 (3)	110.8 $\pm$ 6.1 (3)

al., 1980), a decreased activity ratio of pyruvate kinase would be expected.

In an attempt to identify the protein kinase(s) responsible for phosphorylation and inactivation of pyruvate kinase in phosphorylase kinase-deficient hepatocytes (Table 1 and Figs. 1 and 2), the activity ratio of the cyclic AMP-dependent protein kinase was determined. The assay procedure used was the same as that developed previously (Kemp & Clark, 1978) and employed the synthetic peptide analogue of the pig hepatic pyruvate kinase phosphorylation-site sequence as phosphate acceptor. In that work (Kemp & Clark, 1978) it was shown that both total activity and adrenaline-stimulated activity of hepatocyte extracts was inhibited by the specific heat-stable inhibitor of the cyclic AMP-dependent protein kinase. Those results were assumed to indicate that essentially all of the synthetic peptide phosphorylation measured was catalysed by the cyclic AMP-dependent protein kinase (Kemp & Clark, 1978). This was not tested in the present experiments, but was assumed to be valid.

Fig. 3 shows that 10 μ M- and 1 μ M-adrenaline as well as 10 nM-glucagon activated the protein kinase and inactivated pyruvate kinase. Phenoxybenzamine (10 μ M) blocked the activation of the protein kinase by 10 μ M-adrenaline and the inactivation of pyruvate kinase by 1 μ M-adrenaline. The blocking effects of 10 μ M-propranolol were essentially the same as those of 10 μ M-phenoxybenzamine. Thus these observations are comparable with those reported previously for control cells, where both phenoxybenzamine and propranolol blocked the activation of the cyclic AMP-dependent protein kinase (Kemp & Clark, 1978) and the inactivation of pyruvate kinase (Kemp & Clark, 1978; Clark *et al.*, 1981).

Vasopressin (0.2–2 nM), angiotensin (10 nM) and A23187 did not significantly increase the ATP-dependent phosphorylation of the synthetic peptide (measured in the absence of added cyclic AMP) or the inactivation of pyruvate kinase of *gsd/gsd*-hepatocyte extracts (Fig. 3). Thus it appeared unlikely that Ca^{2+} -dependent cyclic AMP-independent protein kinases capable of phosphorylating pyruvate kinase were activated by the Ca^{2+} -mobilizing hormones and agents. In addition, none of the hormones used in Fig. 3 significantly altered the phosphorylase $a/(a+b)$ activity ratio in phosphorylase kinase-deficient hepatocytes (M. G. Clark, G. S. Patten & D. G. Clark, unpublished work).

The question of whether rat liver pyruvate kinase can be phosphorylated by protein kinases other than the cyclic AMP-dependent protein kinase has received surprisingly little attention. Titanji *et al.* (1976) showed that rat liver cytosol

Fig. 3. Relationship between pyruvate kinase and cyclic AMP-dependent protein kinase activity ratios in phosphorylase kinase-deficient hepatocytes

Hepatocytes from mature *gsd/gsd* rats were prepared and incubated as described in the Materials and methods section. Agonists and antagonists were added after a preincubation period of 15 min and were present with the cells for 3 min. Additions were: saline (1), 10 nM-glucagon (2), 10 μ M-adrenaline (3), 10 μ M-phenoxybenzamine (4), 10 μ M-propranolol (5), 10 μ M-adrenaline + 10 μ M-phenoxybenzamine (6), 10 μ M-adrenaline + 10 μ M-propranolol (7), 1 μ M-adrenaline (8), 1 μ M-adrenaline + 10 μ M-phenoxybenzamine (9), 1 μ M-adrenaline + 10 μ M-propranolol (10), 1 μ M-adrenaline + 10 μ M-phenoxybenzamine + 10 μ M-propranolol (11), 2 nM-vasopressin (12), 0.2 nM-vasopressin (13), 10 nM-angiotensin (14), 10 μ M-phenylephrine (15), 10 μ M-noradrenaline (16), 10 μ M-A23187 (17), 3.7% dimethyl sulphoxide (18). Mean values are given from a minimum of three hepatocyte preparations. For clarity, S.E.M. bars have been omitted. Protein kinase activity ratio is activity without cyclic AMP/activity with cyclic AMP.

contained three (or possibly four) protein kinases resolved on DEAE-cellulose which were all capable of phosphorylating rat liver pyruvate kinase. However, each of the three protein kinases was stimulated by cyclic AMP, and was thus con-

sidered by Titanji *et al.* (1976) to be cyclic AMP-dependent. Attempts to phosphorylate pyruvate kinase in a liver extract in the absence of cyclic AMP (Feliu *et al.*, 1977) or on addition of Ca^{2+} (Van den Berg *et al.*, 1980) have been so far unsuccessful. A paper (Garrison *et al.*, 1984) which appeared while the present manuscript was in preparation shows that vasopressin and angiotensin combined led to a 2-fold increase in the phosphorylation of pyruvate kinase and that the extent of phosphorylation of this enzyme was unaffected by phosphorylase kinase deficiency. In addition, 4β -phorbol 12-myristate 13-acetate, which is believed to activate protein kinase C, led to the phosphorylation of protein substrates unique for Ca^{2+} -linked hormones, but did not increase the phosphorylation of pyruvate kinase (Garrison *et al.*, 1984). Phosphorylation of pyruvate kinase was increased 2-fold by treatment of the cells with A23187, and the enzyme was thus concluded to be a substrate for Ca^{2+} -calmodulin-requiring protein kinases other than phosphorylase kinase and protein kinase C (Garrison *et al.*, 1984). Finally, it is not entirely clear whether phosphorylation of pyruvate kinase and inactivation of the enzyme are always coupled. Data from several laboratories indicate that the treatment of hepatocytes with α -adrenergic agonists and vasopressin produces varying effects, with significant (Garrison *et al.*, 1979), intermediate (Chan & Exton, 1978; Claus *et al.*, 1979; Steiner *et al.*, 1980) or minor (Thomas & Halestrap, 1981; Hue *et al.*, 1978; Blair *et al.*, 1979b) change in the activity of pyruvate kinase, although phosphorylation of the enzyme is stimulated at least 2-fold (Garrison & Borland, 1979; Steiner *et al.*, 1980; Nagano *et al.*, 1980; Garrison *et al.*, 1984). Although it is clear from the data of Blair *et al.* (1982) that there is a correlation between the activity ratio of pyruvate kinase and phosphorylation state, a doubling of the basal phosphate content of the enzyme produces no more than 11% decrease in the activity ratio (Fig. 7 of Blair *et al.*, 1982). Thus the 2-fold change in extent of phosphorylation of pyruvate kinase reported for the Ca^{2+} -mobilizing hormones and agents (Garrison *et al.*, 1984) may not be sufficient to affect significantly the activity ratio of the enzyme. Our present findings would support the view that regulation of pyruvate kinase by phosphorylation is mediated predominantly by the cyclic AMP-dependent protein kinase.

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