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# ADRENERGIC REGULATION OF PYRUVATE KINASE AND GLUCONEOGENESIS IN HEPATOCYTES FROM PHOSPHORYLASE KINASE-DEFICIENT (gsd/gsd) RATS

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SUMMARY : Hepatocytes were prepared from a strain of rats deficient in hepatic phosphorylase b kinase and were used to assess the role of this enzyme in the adrenergic regulation of pyruvate kinase and gluconeogenesis. Epinephrine (10 μM) stimulated glucose output and gluconeogenesis from 1.8 mM lactate but did not significantly affect the concentration of hepatocyte glycogen. In addition epinephrine treatment led to an inhibition of pyruvate kinase. The stimulation of gluconeogenesis and the inhibition of pyruvate kinase by epinephrine were blocked by both α- and β-antagonists: similar effects with epinephrine were observed in cells from control animals. It is concluded that mechanisms for the adrenergic regulation of pyruvate kinase and gluconeogenesis are similar in hepatocytes from both phosphorylase kinase-deficient and normal rats.

Catecholamines (1), glucagon (2) and cyclic AMP (3) stimulate hepatic gluconeogenesis. Each of these agents also leads to an inhibition of the activity of pyruvate kinase (4). With glucagon and cyclic AMP a relationship appears to exist between the extent of inactivation of pyruvate kinase and the stimulation of gluconeogenesis (4). However, for epinephrine and the α-agonist, phenylephrine, the stimulation of gluconeogenesis is not always accompanied by a corresponding proportional inhibition of pyruvate kinase (5-7).

Although epinephrine-mediated inactivation of pyruvate kinase may involve phosphorylation by a β-receptor-mediated activation of the cyclic AMP-dependent protein kinase, there is considerable evidence to indicate that an α-receptor cyclic AMP-independent mechanism predominates (8-11). 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 $Ca^{2+}$ (12). This enzyme, in turn, is believed to

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phosphorylate and activate phosphorylase (12). As hepatic tissue from the glycogen storage disease (gsd/gsd) rat lacks phosphorylase b kinase (13) we have used hepatocytes from these animals to assess the role of this enzyme in the regulation of gluconeogenesis and pyruvate kinase by catecholamines.

MATERIALS AND METHODS : Glycogen storage disease (gsd/gsd) rats, 210 g body wt. (fed, ad libitum) were maintained in the Division (14). Control rats were of similar body weight and nutritional status and were of the Hooded Wistar strain. Hepatocytes were prepared essentially as described by Berry and Friend (15), with the omission of hyaluronidase and the substitution of $Ca^{2+}$-free Krebs-Henseleit saline buffer, pH 7.4 (16). Livers were removed for perfusion and the concentration of collagenase (Worthington, type II) was 0.25 mg/ml. At the end of the perfusion the liver was dispersed into $Ca^{2+}$ containing Krebs-Henseleit buffer, washed twice and resuspended in ice-cold Krebs-Henseleit saline buffer containing 2.5% (w/v) bovine serum albumin (Calbiochem, fatty acid poor). The cell suspensions were adjusted to approx 6.5 x $10^6$ cells per ml and kept on ice. Cells (1 ml) were incubated, in triplicate, in the albumin containing buffer (2.65 mM free $Ca^{2+}$) at 37°C in stoppered 20 ml glass vials that had been gassed with $O_2$+$CO_2$ (19+1). After a 15 min preincubation, 37 µl of a freshly prepared solution of hormone, agonist and/or antagonist, was added together with 25 µl of 83 mM [U-$^{14}$C]lactate. The vials were regassed and stoppered. Samples (0.2 ml) were removed after a further 1 min incubation for pyruvate kinase analyses (7). The incubation was allowed to continue for a total of 45 min at which time 0.4 ml of 12% (v/v) $HClO_4$ was added. A sample (0.3 ml) of the acidified cell suspension was adjusted to pH 7 using 5M-KOH. Denatured protein and potassium perchlorate were removed by centrifugation. Gluconeogenesis or [$^{14}$C] glucose synthesis was estimated by measuring the incorporation of [U-$^{14}$C] lactate into glucose and glycogen (17). Lactate was determined on neutralized perchlorate extracts (18). Glucose in the deproteinized extracts and that liberated by amyloglucosidase treatment of the purified hepatocyte glycogen was determined using a glucose oxidase method (14).

RESULTS

The glycogen storage disease (gsd/gsd) rats were discovered during liver perfusion studies with food deprived rats (Clark, D.G. and Watts, C., unpublished observations). Subsequent work (13,14) has demonstrated that liver from these animals contained large deposits of glycogen (12.5% by weight) which were not degraded even after 24 h of starvation. The inability to break down this polysaccharide has been attributed to a deficiency of hepatic phosphorylase b kinase (13). The data of Table 1 demonstrate that the glycogen levels in hepatocytes from the phosphorylase kinase-deficient hepatocytes were about 6-fold higher than those found in

TABLE 1

Effect of epinephrine on glucose output and glycogen levels in phosphorylase kinase-deficient and control hepatocytes.

Isolated hepatocytes were prepared and incubated as described in the Materials and Methods section. Samples were taken after 15 and 45 min incubation at 37°C for the determination of glycogen and glucose. Individual values are shown for cell preparations from 3 phosphorylase kinase-deficient animals and 3 control animals. The estimated standard error from triplicate determinations of glycogen concentration was ± 2%.

| Animal | Additions to hepatocytes | Time (min) | Glycogen content* | Glucose output |
|---|---|---|---|---|
| | | | (μmoles per $10^8$ cells) | |
| Phosphorylase kinase-deficient | None | 15 | 659, 615, 408 | 8.1, 12.5, 12.5 |
| | None | 45 | 618, 602, 386 | 25.0, 30.0, 29.0 |
| | $10^{-5}$M-Epinephrine | 45 | 628, 588, 401 | 33.3, 35.3, 32.8 |
| Control | None | 15 | 171, 29.3, 86.6 | 21.9, 14.3, 19.0 |
| | None | 45 | 132, 10.4, 64.8 | 44.8, 30.8, 33.3 |
| | $10^{-5}$M-Epinephrine | 45 | 113, 3.8, 53.8 | 73.1, 41.1, 50.3 |

* Glycogen content is expressed as μmoles of glucose equivalents

control hepatocytes. Although the glycogen levels in hepatocytes from the phosphorylase kinase-deficient rats were depleted by about 5% by incubation for 30 min, the addition of $10^{-5}$M epinephrine did not increase the rate of glycogenolysis (Table 1). This suggests that the loss of glycogen in these cells resulted from autolysis rather than from phosphorolytic degradation by phosphorylase *a*. In the same incubations, however, epinephrine increased glucose output by 34% (Table 1), indicating that this hormone increased glucose synthesis from endogenous and added precursors (see below). Basal glucose output (15-45 min) was similar for both control and phosphorylase kinase-deficient hepatocytes (Table 1) but with the normal cells epinephrine increased glycogenolysis by 46% and glucose production by over 100% (Table 1).

In Fig. 1 the adrenergic control of gluconeogenesis from 1.8 mM [U-$^{14}$C] lactate in phosphorylase kinase-deficient and control hepatocytes was com-

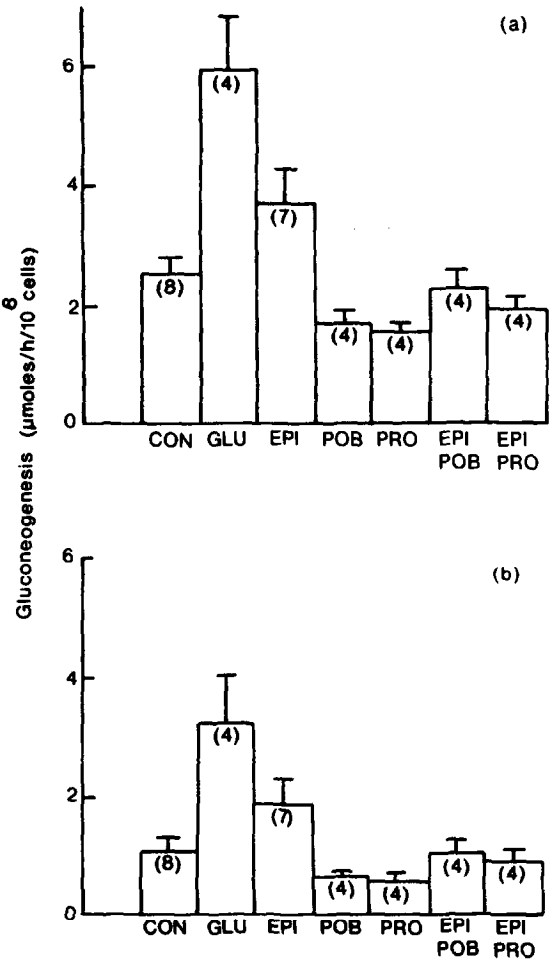


Fig. 1. Adrenergic regulation of gluconeogenesis in phosphorylase kinase-deficient (a) and control (b) hepatocytes. Details were as given in the Materials and Methods section. The initial concentration of substrate [U-$^{14}$C]lactate was 1.8 mM. Mean values are given. Bars indicate the SEM and the number of cell preparations are shown in parentheses. Abbreviations: CON, no additions; GLU, $10^{-8}$M-glucagon; EPI, $10^{-5}$M-epinephrine; POB, $10^{-5}$M-phenoxybenzamine; PRO, $10^{-5}$M-propranolol.

pared. In the former $10^{-8}$M glucagon and $10^{-5}$M epinephrine each stimulated glucose production. The stimulatory effect of epinephrine was partly blocked by both the α-blocker, phenoxybenzamine and the β-blocker, propranolol. Overall, propranolol (at $10^{-5}$M), was the more effective. For control hepatocytes the rate of gluconeogenesis was similarly affected; the stimulation due to $10^{-5}$M epinephrine was partly blocked by both α and

β blockers. Again propranolol was the most effective. The unstimulated rate of gluconeogenesis from 1.8 mM [U-$^{14}$C] lactate was 2.4-fold greater in hepatocytes from phosphorylase kinase-deficient hepatocytes (Table 2). This was less pronounced however when the results were expressed as a function of cell dry weight; in this case the basal rates were 4.7±0.3 (4) and 3.0±0.6 (5) nmoles/h/mg dry wt. for phosphorylase kinase-deficient and control cells, respectively. These isotopic results do not correlate with those for total glucose production in the two cell types (see above and Table 1). The 'additional' glucose produced in the control hepatocytes probably resulted from glycogenolysis and gluconeogenesis from unlabelled endogenous precursors. The difference did not result from different intracellular concentrations of lactate as this was approximately 0.7 mM in both the control and phosphorylase kinase-deficient hepatocytes.

In Fig. 2 the adrenergic control of pyruvate kinase in phosphorylase kinase-deficient and control hepatocytes was compared. Epinephrine treatment of hepatocytes from phosphorylase kinase-deficient animals led to an inactivation of pyruvate kinase equal to approx 50% of that obtained with glucagon. These effects were similar to those reported for control (fed) hepatocytes by other groups (4). The inactivating effect produced by epinephrine was antagonized by both phenoxybenzamine and propranolol. Neither antagonist produced significant effects on their own.

## DISCUSSION

Considerable evidence has accumulated to show that hepatic glycogenolysis is controlled by epinephrine acting through an α-adrenergic receptor mechanism independent of cyclic AMP [see review, 19]. The data are also consistent with the possibility that this mechanism involves an increase in the intracellular cytosolic concentration of $Ca^{2+}$. This increase is believed to then activate the $Ca^{2+}$ ion-dependent phosphorylase kinase which in turn phosphorylates phosphorylase b, thereby converting it to the active

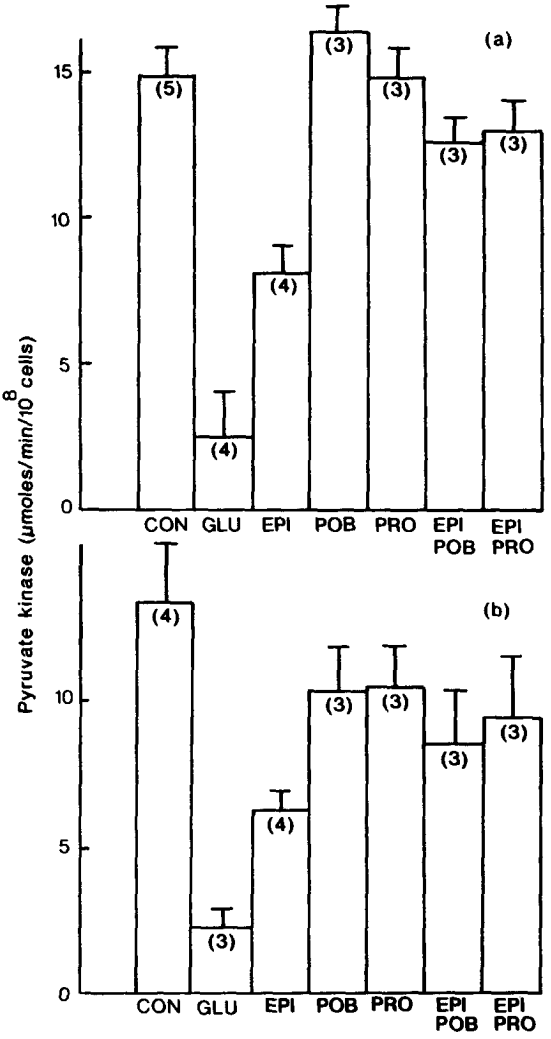


Fig. 2. Adrenergic regulation of pyruvate kinase in phosphorylase kinase-deficient (a) and control (b) hepatocytes. Details were as given in the Materials and Methods section or in Fig. 1.

form, phosphorylase a (19). In addition gluconeogenesis is stimulated by epinephrine and this effect is probably α-mediated (8-10, 20-22) but it is not always accompanied by an inhibition of pyruvate kinase (5-7). Although the identity of the receptor involved in controlling pyruvate kinase appears to be predominantly α (8-10), it is uncertain whether inactivation and phosphorylation is mediated by the cyclic AMP-dependent protein kinase (7,9,11).

To date phosphorylation has been the only mechanism reported for the stable inactivation of pyruvate kinase. The site of phosphorylation on the enzyme has been determined. Humble et al (23) showed that alkali-inactivated pig liver pyruvate kinase and a cyanogen bromide peptide from the same enzyme could be phosphorylated by [$\gamma$-$^{32}$P]ATP in the presence of cyclic AMP-dependent protein kinase. These workers (24) also showed that the minimum structural requirements for phosphorylation were met by the pentapeptide Arg-Arg-Ala-Ser-Val. Evidence from other laboratories suggest that this or very closely related sequences are at the sites of phosphorylation in other proteins that serve as substrates for cyclic AMP-dependent protein kinases (25-27). In addition, the rat and pig liver pyruvate kinases appear to be phosphorylated only by a cyclic AMP-dependent protein kinase (28-31). Nevertheless, a strong argument has been made for an $\alpha$-receptor mediated phosphorylation and inactivation of rat hepatic pyruvate kinase that does not involve significant activation of the cyclic AMP-dependent protein kinase (11). Thus if conservation of the 'second messenger' for the $\alpha$-receptor-mediated increase in glycogenolysis occurs then it appears reasonable to predict that phosphorylase kinase is involved. Several possibilities emerge: (i) phosphorylase kinase itself phosphorylates pyruvate kinase in vivo under conditions that, as yet, have not been duplicated in vitro; (ii) phosphorylase kinase phosphorylates and activates an as yet unknown protein kinase; (iii) phosphorylase kinase phosphorylates and inactivates the phosphoprotein phosphatase responsible for dephosphorylating pyruvate kinase, etc. Despite these possibilities, it was noted in the present study that adrenergic mechanisms for the regulation of pyruvate kinase and gluconeogenesis were similar in phosphorylase kinase-deficient and normal hepatocytes. Thus it is concluded that phosphorylase kinase is not involved in these mechanisms. These findings may lend support to the view (7) that both $\alpha$ and $\beta$ receptor-mediated inactivation of pyruvate kinase involves the cyclic AMP-dependent protein kinase. Alternatively a mechanism

involving $Ca^{2+}$-mediated phosphorylation of pyruvate kinase independent of both phosphorylase kinase and the cyclic AMP dependent protein kinase cannot be ruled out.

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