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The hepatic glycogenolysis induced by reversible ischaemia or KCN is exclusively catalysed by phosphorylase *a*

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1. Ischaemia was applied for 30 min to the liver of Wistar rats and of *gsd/gsd* rats, which have a genetic deficiency of phosphorylase kinase. The rate of glycogenolysis corresponded closely to the concentration of phosphorylase *a*. The loss of glycogen from Wistar livers was accounted for by the intrahepatic increase in glucose plus lactate. Further, the accumulation of oligosaccharides was negligible in the *gsd/gsd* liver. 2. Isolated hepatocytes from Wistar and *gsd/gsd* rats were incubated for 40 min in the presence of either KCN or glucagon. Again, the production of glucose plus lactate was strictly dependent on the presence of phosphorylase *a*. However, the catalytic efficiency of phosphorylase *a* was about 2-fold higher in the presence of KCN. 3. We conclude that the hepatic glycogenolysis induced by anoxia and by KCN is solely mediated by phosphorylase *a*. The higher catalytic activity of phosphorylase *a* under these circumstances could be due to an increased concentration of the substrate P_i.

INTRODUCTION

A massive glycogenolysis occurs in the liver when the mitochondrial generation of ATP becomes insufficient, as a result of either a shortage of oxygen [1–10] or the presence of inhibitors [9,11–13] or uncouplers [12] of the respiratory chain. The glycogenolytic rate in these conditions is higher than expected from the concentration of the active *a* form of phosphorylase [2,7–9,13]. Therefore alternative mechanisms have been proposed for the breakdown of glycogen. For example, anoxia or a malfunction of the respiratory chain could induce the generation of a stimulator of phosphorylase *b* [8,9,13]. However, several lines of evidence indicate that the well-known ligand AMP does not qualify as a candidate [10,14]. Also, hepatic glycogen might be degraded by a hydrolytic pathway involving α-amylase and/or α-glucosidases [9,13].

gsd/gsd rats have an inherited deficiency of hepatic phosphorylase kinase [15]. As a consequence, they maintain a very low amount of phosphorylase *a*, even after administration of glucagon. The use of these rats has allowed us to demonstrate the exclusive role of phosphorylase *a* in the hepatic glycogenolysis induced by phlorrhizin, vinblastin and CCl_4 [16]. However, inconsistent results have been obtained when the same approach was applied to the glycogenolysis induced by ischaemia [8,10], anoxia [9] and KCN [9]. In the present work we have reinvestigated this problem. Part of this work has been published in abstract form [17].

EXPERIMENTAL

For the experiments on ischaemia, rats aged 7–12 weeks were anaesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg) about 15 min before the abdomen was opened. A liver sample was frozen between aluminium tongs precooled in liquid nitrogen. The remainder of the liver was wrapped in cloth moistened with 0.15 M-NaCl, and incubated for up to 30 min in water-saturated air at 37 °C. At 5 min intervals samples were cut and quick-frozen. Part of each frozen specimen was homogenized in a Potter–Elvehjem tube in 4 vol. of 1.2 M-$HClO_4$. After sedimentation of the denatured proteins, the supernatant was neutralized and assayed for glycogen, glucose, lactate and, exceptionally, oligosaccharides. For the assay of phosphorylase, another part of the frozen liver samples was homogenized in 4 vol. of 50 mM-glycylglycine, pH 7.4, containing 0.5% shellfish glycogen plus 25 mM-EDTA and 125 mM-NaF as inhibitors of protein kinases and phosphatases [16].

Hepatocytes were isolated from rats of various age and incubated as described in ref. [13]. The incubation medium was a Krebs–Henseleit bicarbonate buffer (pH 7.4), supplemented with 10 mM-glucose, bacitracin (1 mg/ml) and the α-amylase inhibitor BAY e4609 (0.1 mg/ml). The last compound was routinely added to prevent the artifactual degradation of extracellular glycogen from broken cells [16]. After preincubation for 15 min, buffer, glucagon (50 nM) or KCN (1 mM) was added, and the incubation was continued for 40 min. At regular times samples were taken for the assay of glucose, lactate and lactate dehydrogenase in the medium, and for phosphorylase and lactate dehydrogenase in the total cell suspension [16].

The assays are described in ref. [16]. One enzyme unit converts 1 μmol of substrate/min under the appropriate assay conditions.

Results are expressed as means ± S.E.M. for the indicated numbers of observations. Vertical and horizontal bars in the graphs represent ± S.E.M. Statistical treatment of the data was by Student's *t* test for paired samples or by linear-regression analysis.

RESULTS

Glycogenolysis induced by ischaemia

Table 1 illustrates the effect of ischaemia (30 min at 37 °C) on the concentrations of glycogen, glucose and lactate in livers from Wistar and *gsd/gsd* rats. Glycogen was lost from Wistar livers during the 30 min period at an average rate of 3.3 μmol of C_6 units/min per g of liver.

Table 1. Glycogen loss and accumulation of glucose and of lactate in Wistar and *gsd/gsd* livers during 30 min of ischaemia

Each result is based on ten observations. The initial concentrations of glycogen were 287±36 and 567±28 μmol/g of liver for Wistar and *gsd/gsd* rats respectively.

	Glucose equivalents (μmol/30 min per g of liver)	
	Wistar	*gsd/gsd*
Glycogen lost	99.3±31.5	—
Glucose plus lactate produced	88.2±9.3	11.4±1.8
Glucose produced	80.7±9.0	8.4±1.8
Lactate produced	7.5±0.9	3.0±0.6

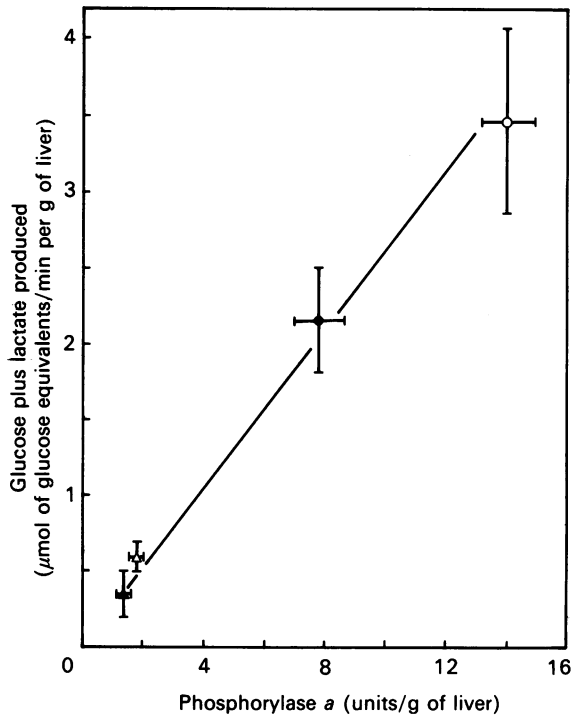

Fig. 1. Relationship between the concentration of phosphorylase *a* and the rate of production of glucose plus lactate in ischaemic livers of Wistar and *gsd/gsd* rats

Liver pieces from ten Wistar rats (○, ●) and ten *gsd/gsd* rats (△, ▲) were quick-frozen at 5 min intervals during incubation at 37 °C and assayed for glucose, lactate and phosphorylase activity. The production of glucose plus lactate and the mean concentration of phosphorylase *a* are shown during the early ischaemic period (5–15 min: ○, △) and during late ischaemia (20–30 min: ●, ▲). Correlation coefficient $r = 0.996$. The total concentration of phosphorylase ($a+b$) was 17.3±1.4 units/g of Wistar liver and 19.7±1.6 units/g of *gsd/gsd* liver.

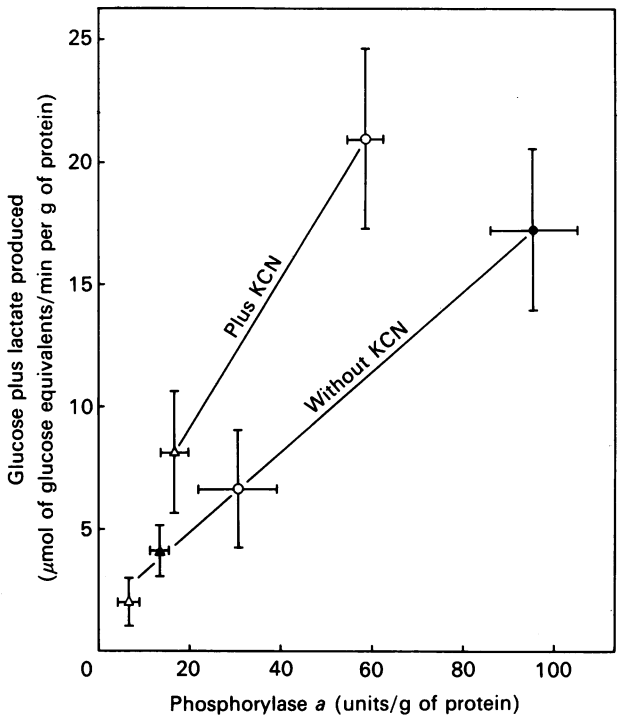

Fig. 2. Relationship between the concentration of phosphorylase *a* and the rate of production of glucose plus lactate during glycogenolysis in isolated hepatocytes from Wistar and *gsd/gsd* rats

Hepatocytes from four Wistar rats (○, ●) and four *gsd/gsd* rats (△, ▲) were incubated for 40 min in the presence (●, ▲) or absence (○, △) of 50 nM-glucagon. In the latter condition, 1 mM-KCN was added as indicated. The average concentration of phosphorylase *a* (assayed after 10, 20, 30 and 40 min) was plotted against the mean production of glucose plus lactate, calculated from the regression line fitted to the values determined between 0 and 40 min.

About 90% of this loss was accounted for by the accumulation of glucose and lactate; a small amount of metabolite may have leaked out of the liver. The production of glucose plus lactate was about 8-fold lower in ischaemic *gsd/gsd* livers. This difference was mostly due to the 10-fold lower rate of glucose production. In contrast, the lactate production was still 40% of the value noted for Wistar livers. The high initial glycogen concentration and the low rate of glycogenolysis in *gsd/gsd* livers precluded an accurate quantification of the glycogen loss. In one *gsd/gsd* liver, the oligosaccharide concentration at the end of the ischaemic period amounted to 2.1 μmol of C_6 units/g, i.e. about twice the amount measured in pre-ischaemic livers [16]. Thus the production of oligosaccharides (barely 10% of glucose plus lactate) remained an insignificant pathway of glycogen breakdown during ischaemia.

In the Wistar livers 50% of the total phosphorylase was initially in the *a* form. This value rose to 87% after 5 min of ischaemia ($P = 0.011$). In the *gsd/gsd* livers the corresponding values were 5 and 10% ($P = 0.018$). In agreement with other reports [8,18], the concentration of phosphorylase *a* started to decline again in either liver from 15 min onwards. Therefore the pattern of glycogenolysis was examined separately during the early period of ischaemia (5–15 min) and during the span between 20 and 30 min. Fig. 1 shows that, in either type of liver, the rate of production of glucose plus lactate was linearly correlated with the mean concentration of phosphorylase *a* during each period.

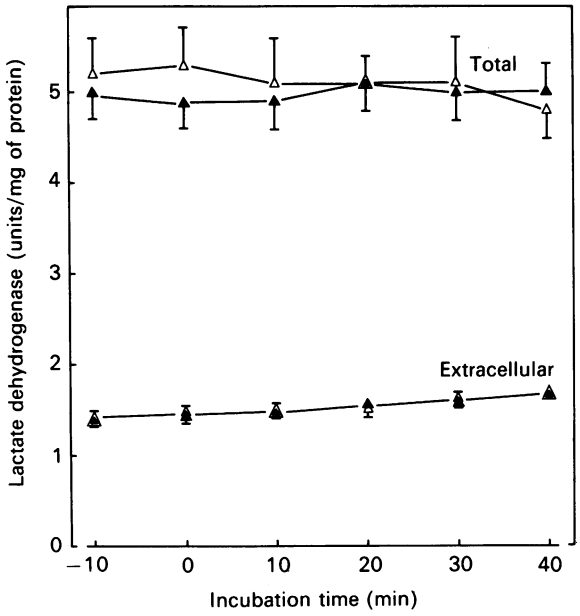

Fig. 3. Effect of KCN on the extracellular concentration of lactate dehydrogenase in hepatocyte suspensions from *gsd/gsd* rats

Freshly isolated hepatocytes from *gsd/gsd* rats were pre-incubated for 15 min. Then (0 min) either buffer (△) or 1 mM-KCN (▲) was added. Samples were taken at the indicated times for the assay of total and extracellular enzyme.

Glycogenolysis induced by KCN

We have also compared the patterns of glycogenolysis induced by glucagon and by KCN in isolated hepatocytes from Wistar and *gsd/gsd* rats (Fig. 2). As shown previously [13,16], the rate of production of glucose plus lactate in the absence of KCN was linearly related to the concentration of phosphorylase *a*. In the conditions with KCN a similar relationship was observed, but the catalytic efficiency of phosphorylase *a* was about 2-fold higher.

Hepatocytes from *gsd/gsd* rats are more fragile than those from Wistar rats. For instance, the extracellular concentration of lactate dehydrogenase was 2.5-fold higher in freshly prepared suspensions of *gsd/gsd* hepatocytes [16]. Disruption of the cell structure triggers glycogen breakdown to oligosaccharides by α-amylase (see [16]). The operation of such a hydrolytic pathway was blocked in our experiments by the presence of an extracellular inhibitor of α-amylase. We therefore wondered whether KCN might affect the survival of the hepatocytes. However, as judged from the leakage of lactate dehydrogenase into the extracellular medium, the integrity of the *gsd/gsd* cells was not affected (Fig. 3).

DISCUSSION

Glycogenolysis in anoxic liver requires phosphorylase *a*

In the present investigation ischaemia was limited to 30 min and contact with KCN to 40 min. In those conditions, vital parameters such as the ATP concentration can still largely be restored [19,20]. Our basic conclusion is that glycogenolysis in these conditions is essentially catalysed by phosphorylase *a*, with minimal contributions of phosphorylase *b* and of glycogen hydrolases. Livers of *gsd/gsd* rats have little potential for activation of phosphorylase, and they respond to anoxia or KCN with an accordingly lower rate of glycogenolysis. The quantitatively unimportant role of α-amylase is further substantiated by our findings that oligosaccharides hardly accumulate in the anoxic *gsd/gsd* liver and that, in ischaemic Wistar livers, nearly all the degraded glycogen was recovered as glucose and lactate.

Previous experiments on *gsd/gsd* livers [8–10] have yielded rather contradictory results. We cannot fully explain these discrepancies. However, one element that should be taken into account is the report by Clark *et al.* [21] that the ability of the *gsd/gsd* rats to mobilize hepatic glycogen increases with age. This in turn was explained by an increase in the activity of phosphorylase kinase and higher concentrations of phosphorylase *a* [21]. We have therefore selected very young *gsd/gsd* rats for the studies on ischaemia. On the other hand, non-phosphorolytic glycogenolysis could conceivably occur in lengthy experiments with irreversible cell damage. It appears to account for the loss of liver glycogen in *gsd/gsd* pups *post mortem* [22].

Our conclusions on the mechanism of glycogenolysis in ischaemic livers are consonant with those of Irvine [10], based on similar work in Griffiths' laboratory, but are at variance with previous observations in the same laboratory [8]. Indeed, Lutaya *et al.* [8] noted an equally large glycogenolysis during ischaemia of *gsd/gsd* and Wistar livers, in spite of large differences in the concentration of phosphorylase *a*, which was, however, measured in other liver samples.

Other results [9] agree with our findings inasmuch as the effect of KCN on isolated hepatocytes of Wistar and *gsd/gsd* rats is concerned. However, Conaglen *et al.* [9] measured an unusually high glucose production during perfusions of *gsd/gsd* livers with KCN or without oxygen. Instead of a transient burst of glycogenolysis, the production of glucose occurred after a lag and increased during the later stages of perfusion. Also, the extent of phosphorylase activation was not negligible in the cyanide-perfused *gsd/gsd* livers. Conaglen *et al.* [9] suggested that an activator of phosphorylase *b* would be generated in the anoxic perfused liver, whereas such a substance might diffuse out of KCN-treated isolated hepatocytes. This hypothesis can now be discarded, since such a putative effector should also have accumulated in the ischaemic *gsd/gsd* livers that we examined.

In the light of the present work, we have to withdraw our previous proposal that the glycogenolysis induced by anoxia or KCN in hepatocytes from normal rats was not mediated by phosphorylase *a* [13]. In those experiments the hepatocytes were incubated with tagatose and accumulated tagatose 1-phosphate, a presumed competitive inhibitor of phosphorylase *a*. At similar concentrations of tagatose 1-phosphate, the glucagon-induced glycogenolysis was completely blocked, but the glycogenolysis caused by anoxia or KCN was not affected. However, these experiments should certainly be re-evaluated in the light of recent data concerning the effect of anoxia on the intrahepatic free concentration of P_i (see below).

Hypoxia increases the catalytic efficiency of phosphorylase *a*

We can now address the question why the glycogenolytic rate in the anoxic liver is higher than expected

from the concentration of phosphorylase *a* [2,7–9,13]. Indeed, the data in Fig. 2 suggest that anoxic conditions increase the catalytic efficiency of phosphorylase *a* about 2-fold. Which metabolite(s) are responsible for the increased activity of phosphorylase *a*? AMP increases sufficiently, but its maximal effect on phosphorylase *a* seems simply too small [14]. Hems & Whitton [23] have proposed a role for the increase in P_i, which could provide more substrate for phosphorolysis. It appeared previously that such a role was doubtful, because the published K_m values of phosphorylase *a* (1–3.5 mM; see ref. [14]) were lower than the apparent concentration of P_i in well-oxygenated liver. However, this view should be corrected on both accounts. First, the K_m value of phosphorylase *a* is higher in assay conditions that are closer to the intracellular environment [14]. Second, n.m.r. measurements indicate that the free intrahepatic concentration of P_i *in vivo* is at most 1 μmol/ml of cell water [24], and that this concentration increases 4–5-fold after a few minutes of ischaemia [19]. Thus anoxia and inhibitors of the respiratory chain may induce high rates of hepatic glycogenolysis by increasing the concentration of phosphorylase *a* and its substrate P_i. Of course, our experiments do not exclude the generation of another metabolite that could increase the catalytic efficiency of phosphorylase *a*.

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