The cytosolic concentration of phosphate determines the maximal rate of glycogenolysis in perfused rat liver

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Glycogenolysis was studied in glycogen-rich perfused livers in which glycogen phosphorylase was fully converted into the *a* form by exposure of the livers to dibutyl cyclic AMP. We monitored intracellular P_i by ^{31}P n.m.r. Perfusion with P_i -free medium during 30 min caused a progressive decrease of the P_i signal to 50 % of its initial value. In contrast, exposure of the livers to KCN and/or 2,4-dinitrophenol resulted in a rapid doubling of the P_i signal. Alterations in the intracellular P_i coincided with proportional changes in the rate of hepatic glycogenolysis (measured as the output of glucose plus lactate). The results indicate that the rate of glycogenolysis catalysed by phosphorylase *a* depends linearly on the hepatic P_i concentration. Hence the K_m of phosphorylase *a* for its substrate P_i must be considerably higher than the concentrations that occur in the cytosol, even during hypoxia.

INTRODUCTION

In the liver, glycogenolysis is exclusively catalysed by the phosphorylated *a* form of glycogen phosphorylase (Vandebroeck *et al.*, 1985, 1988). Reported K_m values of the enzyme *in vitro* for its substrate, P_i , range from as low as 1 mM (Van den Berghe *et al.*, 1973) to as high as 20 mM, depending on the anionic composition of the medium (Stalmans & Gevers, 1981).

^{31}P -n.m.r. data suggest that the free P_i concentration in liver is only a fraction of the amount of P_i recovered in acid tissue extracts (Iles *et al.*, 1985; Malloy *et al.*, 1986). The excess of P_i in acid extracts may stem from hydrolysis of labile phosphate compounds, or from a P_i pool which is n.m.r.-invisible, because only the signal from molecules which freely tumble in solution gives rise to a sharp resonance peak in the n.m.r. spectrum. Many hold that the n.m.r.-observed *o*-phosphate signal stems mainly from the cytosolic compartment, since it resonates at a frequency typical for the pH of that compartment, and it responds with a corresponding high-field shift to manipulations believed to acidify the cytosol (Cohen *et al.*, 1978; Desmoulin *et al.*, 1987).

Thus free cytosolic P_i under basal aerobic conditions would amount to about 1 mM (Iles *et al.*, 1985). When a substrate is present at concentrations near or below its K_m value for a given reaction, it can exert control on that step. Thus it has been suggested that metabolic control of glycogen phosphorylase *a* by P_i is likely to occur (Iles *et al.*, 1985). We devised an experimental protocol to assess the effect of variations of intracellular P_i on the kinetic efficiency of phosphorylase *a* in the intact organ.

MATERIALS AND METHODS

Materials

Dibutyl cyclic AMP was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), 2,4-dinitro-

phenol from Koch–Light Laboratories (Colnbrook, Bucks., U.K.), and KCN from Janssen Chimica (Beerse, Belgium). All reagents were of analytical grade, and were purchased from various suppliers.

Liver perfusion

Male Wistar-derived Pfd rats, weighing about 250 g, were maintained on a 12 h-dark/12 h-light cycle (starting at 21:00 h), with free access to regular rat chow and water. During the night before the experiment, they were offered additional sucrose. Surgery was started at 09:00 h. The animals were anaesthetized by intraperitoneal injection of 6 mg of sodium pentobarbital/100 g body wt. The operative technique (Hems *et al.*, 1966) was slightly modified to accommodate the n.m.r. experiments. The livers were suspended with the portal hilus facing upwards in a vertical cylindrical cup (diameter 50 mm) which slid into the cavity of the resonator used for the n.m.r. observations. A hooked catheter (3 mm diameter), inserted into the vena cava inferior, functioned as a syphon draining the liver with a negative pressure of about 2 cmH₂O. The perfusion was essentially leak-free.

The perfusion medium consisted of a balanced salt solution, modified from that of Krebs & Henseleit (1932), containing 129 mM-Cl⁻, 24.9 mM-HCO₃⁻, 2.37 mM-PO₄³⁻, 1.19 mM-SO₄²⁻, 145 mM-Na⁺, 5.9 mM-K⁺, 1.27 mM-Ca²⁺ and 1.19 mM-Mg²⁺, and was supplemented with 10 mM-Hepes (44.3 % Na salt; pH 7.45), 10 µg of oxytetracycline and ampicillin each/ml, and 1.25 units of heparin/ml. The buffer (pH 7.45), kept at 37 °C, was gassed with humidified O₂/CO₂ (19:1) at 1 atm (101 kPa).

The perfusion apparatus was designed as described by Meijer *et al.* (1981). The apparatus consisted of two parallel circuits. The experimental design called for a 30 min pre-perfusion phase needed to mount the organ in the magnet, and to adjust the magnet shims for optimal field homogeneity. To prevent glycogen degra-

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dation during this phase, the perfusate was supplemented iso-osmotically with 15 mM-glucose, 2.7 mM-sodium lactate, 0.3 mM-sodium pyruvate and 40 μ M-glycerol. During this first phase the perfusate (200 ml) was recirculated and the hydrostatic pressure (approx. 10 cmH₂O net pressure over the liver) was adjusted to sustain a flow of about 50 ml/min. After 30 min pre-perfusion, the liver was switched to the second circuit, operated in single-pass mode. During this experimental phase nutrient-free salt solution, containing 100 μ M-dibutyl cyclic AMP, was pumped at a constant rate of 50 ml/min. After an initial control phase (15 min), the medium was exchanged for an identical one, but either with omission of P_i (30 min) or with addition of 1 mM-KCN and/or 100 μ M-2,4-dinitrophenol (up to 15 min).

³¹P-n.m.r. spectroscopy

Spectra were obtained with a Biospec instrument equipped with a 4.7 T 30 cm-bore magnet (Bruker, Karlsruhe, Germany). The liver was inserted in a perfusion cell, which was mounted in a cylindrical resonator described elsewhere (Van Hecke *et al.*, 1989). The lid of the perfusion cell was perforated for the indwelling portal catheter and the draining caval syphon. The perfusate was collected in a funnel, which was drained by suction. The cell was continuously flushed with 154 mM-NaCl (100 ml/min). This served several purposes. (i) Immersion of the liver in a volume with regular geometry was necessary to improve shimming over the volume of interest. Field homogeneity improved from worse than 1 p.p.m. to better than 0.5 p.p.m. (ii) The use of a P_i-free solution excluded spectral contribution from outside the liver under all conditions. (iii) Furthermore, the rinsing solution served as a thermostatically controlled bath (37 °C) for the suspended organ.

We acquired ³¹P-n.m.r. spectra at 81.1 MHz in blocks of 128 scans by using 60 μ s (72°) pulses repeated every 0.70 s. Hexachlorocyclotriphosphazene (phosphonitrilic chloride trimer, equivalent to 37.5 μ mol of ³¹P), relaxed by the addition of a Cr(III) complex, was used as an external standard (Gard & Ackerman, 1983). The accumulated FIDs (free induction decays) were weighted with a 10 Hz exponential decay function to improve the signal-to-noise ratio. In order to determine the saturation factor, which scales each of the peaks, we measured longitudinal relaxation times (*T*₁) of intracellular metabolites on two livers by the inversion-recovery method (Vold *et al.*, 1968). The livers were perfused in recirculating mode with 500 ml of nutrient-enriched medium. Under these conditions, metabolic homeostasis of the organ, as judged from the stability of the relaxed ³¹P spectrum, was maintained for up to 4.5 h, the timespan needed for the *T*₁ measurement. For the *T*₁ measurement, we accumulated eight groups of 32 scans corresponding to time intervals ranging between 0.2 and 15 s between the 180° inversion-pulse and the 90° read-pulse. A recovery time of 50 s was allowed after each 90° pulse.

Relative changes in metabolite levels were estimated from changes in peak heights. Correct measurement of the height of individual metabolite peaks in liver spectra is hampered by the presence of a broad underlying hump. The contribution of this homogeneously broadened peak can be largely removed during the acquisition by saturation of the latter peak through selective irradiation (Thoma *et al.*, 1985; Iles *et al.*, 1985), or during the data processing by a deconvolution technique (Campbell

et al., 1973), or by manual fitting of the baseline. We found that, after saturation of the broad peak by selective irradiation between the γ -NTP and the phosphodiester peaks, the *o*-phosphate region could not be freed of an apparent edge to the hump. This remaining hump appears to be an unresolved cluster of peaks. Owing to their inhomogeneous character, these are not saturated to the same extent as the underlying broad hump by the irradiation. Partial saturation of some of these peaks (as we also observed for the γ -NTP peak) was likely to occur. The latter effect was a function of the spectral position of the peaks. Because KCN and dinitrophenol treatments acidify the cytosol and provoke a rightward shift of the P_i peak towards the selective saturation frequency (see Fig. 1), the degree of saturation of the P_i peak could not be considered to be constant. For this reason, and because selective irradiation insufficiently improved spectral resolution around the P_i peak, we made no further use of this method. In the post-acquisition deconvolution method, the FID was multiplied by an exponential function with short time constant [approximately the inverse of the width of the broad peak (500 Hz)] and the result was subtracted from the original FID, removing the large component. The peaks in the *o*-phosphate region still emerged as a cluster. Therefore, all metabolite peak heights reported in this study were measured from a baseline which was manually drawn and accounted for the broad hump and the cluster in the phosphate region.

Assays

Phosphate (Ames, 1966), glucose (Dahlqvist, 1961) and lactate (Noll, 1981) were measured in caval samples of the perfusate, stored on ice. The hepatic output of hexose equivalents was calculated from the measured porto-caval difference in glucose and lactate and the imposed perfusion flux. It was used as an estimate of the rate of glycogenolysis.

At the end of the experiment, the liver was disconnected, immediately freeze-clamped with a pair of liquid-N₂-cooled aluminium tongs, and weighed. Residual tissue glycogen at the end of the experiment was measured (Chan & Extton, 1976) in KOH extracts of the tissue. To check the degree of activation of glycogen phosphorylase, the activity of the *a* form and the total activity (*a* + *b*) were measured (Vandebroek *et al.*, 1985).

Statistics

Data are presented as means $\pm$ S.E.M., with the numbers of observations in parentheses. Occasionally ranges are given.

RESULTS

Design and validation of the experimental model

Conditions of glycogenolysis. The experiments were designed to compare the maximal rate of output of hexose equivalents by the liver during a control period with the output during a subsequent phase in which the cytosolic P_i concentration was manipulated. Conversion of phosphorylase into its *a* form was achieved by continuous treatment of the livers with dibutyl cyclic AMP; 100 μ M-dibutyl cyclic AMP suffices to elicit an immediate, maximal and sustained stimulation of hepatic glycogenolysis (Kimura *et al.*, 1982). Sampling of liver

tissue was impossible during the n.m.r. experiments. However, we established (outside the magnet) that this amount of dibutyl cyclic AMP sufficed to activate phosphorylase fully, within 5–10 min (result not shown). A steady state of glycogenolysis was obtained during the original control phase soon after addition of the cyclic nucleotide (see Fig. 2). Completeness of the effect was checked at the end of each experiment. Activation of the enzyme was $89 \pm 3\%$ ($n = 13$).

Glycogen breakdown was monitored for up to 45 min. We used livers from well-fed rats to maintain sufficient glycogen stores throughout the observation period. In the experiments reported here, residual hepatic glycogen determined at the end of the experiments was never lower than $155 \mu\text{mol}$ of glucose equivalents/g of liver.

Observation of intracellular P_i by n.m.r. In most published n.m.r. experiments on perfused liver, the organ is immersed in the caval effluent, which is allowed to fill the perfusion cell. Overflow is prevented by suction from the top. Since the n.m.r. probe observes the whole volume of the cell, a contribution of P_i from the perfusion buffer to the P_i signal is unavoidable. In our experiments, the interference by extrahepatic P_i was minimized by caval cannulation, and by flushing the cell with a non-recirculating 154 mM-NaCl solution (Iles *et al.*, 1980).

Under these conditions, the vascular and extracellular space within the liver still makes up about 30% of the total liver volume (Iles *et al.*, 1985). The longitudinal relaxation time (T_1) of intracellular P_i is much shorter than that of extracellular P_i (McLaughlin *et al.*, 1979). Proper selection of pulse repetition times allows one to saturate selectively the extracellular contribution with minimal alteration of the intracellular P_i signal (Iles *et al.*, 1980). As described above, we estimated T_1 times of intracellular metabolites on two livers (results not shown). Relaxation times of intracellular metabolites varied between 0.3 s for ATP and approx. 7 s for phosphomonoesters. The relaxation behaviour of P_i was biphasic, suggesting the simultaneous observation of two compartments, one with a short relaxation time comparable with that of ATP, and one with a long relaxation time, presumably stemming from the extracellular compartment. The relaxation time of P_i in the perfusion medium was independently determined to be 12 s at pH 7.4 and 37 °C.

Ultimately, we settled on the pulse parameters described in the Materials and methods section. Scanning at short repetition times served a double purpose. (i) It allowed us to achieve reasonable signal-to-noise ratios in time spans sufficiently short to study whether the effects of the various treatments on the ^{31}P spectra and on the rate of glycogenolysis correlated in time (see Fig. 2). (ii) With the selected pulse parameters, and assuming an extracellular volume of 3 ml for a 10 ml liver (Iles *et al.*, 1985), the apparent contribution of extracellular P_i to the P_i signal was expected to amount to approx. $0.37 \mu\text{mol}$ of ^{31}P . This is only approx. 1% of the added external phosphonitrilic trimer standard, and therefore is negligible compared with the observed P_i peak (Fig. 1). The estimated T_1 value of intracellular P_i corresponds to a partial saturation of 10%. Because of the limited accuracy with which the latter factor can be known, we did not attempt to give precise estimates for the intracellular P_i concentrations. However, since the contribution of extracellular P_i is minimized, valid estimates for relative

Fig. 1. ^{31}P -n.m.r. spectra of a perfused rat liver

The liver was perfused and the n.m.r. spectrum recorded as described in the Materials and methods section. Peak A arises from P_i , peaks B and C from phosphomono- and phosphodi-esters respectively, peaks D, E and F from the γ -, α - and β -phosphate groups of NTP, and peak G from the external standard, hexachlorocyclotriphosphazene. Panel (a) corresponds to a control spectrum of the liver 10 min after addition of $100 \mu\text{M}$ -dibutyl cyclic AMP to the perfusion buffer. Panel (b) shows the result 15 min after the subsequent addition of 1 mM-KCN and $100 \mu\text{M}$ -2,4-dinitrophenol to the perfusate.

changes in the intracellular P_i can be obtained. These changes were correlated with relative changes in the glycogenolytic rate in individual livers.

Dependence of glycogenolysis on intracellular P_i

Effect of perfusion with P_i -free buffer. In preliminary experiments we intended to avoid a contribution of extracellular P_i to the spectra by omission of P_i from the perfusion buffer. However, this resulted in a large loss of P_i into the perfusate, at a rate of $2.61 \pm 0.12 \mu\text{mol}$ of $P_i/30$ min per g of liver ($n = 2$). The P_i peak in the ^{31}P spectra (Fig. 2a) slowly decreased over this period to $59 \pm 2\%$ ($n = 4$) of its original size. If the amount of P_i lost were to stem entirely from the n.m.r.-observable P_i pool, then the original size of that pool could be estimated as 6 mM, which is an unrealistically high value (Iles *et al.*, 1985). The evolution of the spectrum provided no obvious indications for redistribution of P_i over the various P_i -

Fig. 2. Time course of changes of intracellular P_i and glycogenolysis in perfused livers

Livers were initially (time < 0) perfused with buffer to which 100 μ M-dibutyl cyclic AMP was added. At the zero time point, perfusion continued in the presence of dibutyl cyclic AMP, either in a P_i -free buffer (panel a) or in regular buffer to which 1 mM-KCN and 100 μ M-2,4-dinitrophenol were added (panel b). The n.m.r. signals of P_i ($\circ$) and of β -NTP ($\square$), and the rate of hepatic output of hexose equivalents ($\triangle$), were expressed for each liver relative to the corresponding value measured over the last 5 min before zero time. Means are shown for four experiments in each panel. Where possible, vertical bars mark the $\pm$ S.E.M. range.

containing metabolites. The signal from the β -phosphate of NTP remained essentially constant (Fig. 2a). Therefore, it appears that the main part of the P_i lost by the liver is mobilized from an n.m.r.-invisible pool.

Concomitantly with the decline of the P_i peak, the hepatic output of hexose equivalents into the perfusate decreased to a similar extent (Fig. 2a).

Models functionally mimicking anoxia. As shown in Fig. 3, cytosolic P_i levels increased when the oxidative phosphorylation was blocked with KCN (1.3–1.6-fold; $n = 4$), or when the mitochondria were uncoupled with the protonophore 2,4-dinitrophenol (1.6-fold; $n = 1$), or when the two treatments were combined (1.6–2.0-fold; $n = 4$). The addition of either KCN or 2,4-dinitrophenol resulted in a rapid increase in P_i at the expense of ATP, which decreased to about one-third of the control level when the two agents were combined (Fig. 2b).

The increase in P_i coincided with a proportional enhancement of output of hexose equivalents by the liver (Fig. 2b). The presence of these metabolic inhibitors markedly stimulated anaerobic glycolysis in these perfused livers (Table 1). Anaerobic glycolysis was of minor importance during the basal control conditions, indicating a sufficient O_2 supply. However, in the presence of KCN with or without 2,4-dinitrophenol, about half of the hexose units mobilized in excess were converted into lactate.

Relationship between P_i concentration and maximal glycogenolysis. When the results of the omission of P_i and of the addition of mitochondrial poisons were combined ($n = 13$), we observed a linear correlation

between variations in the P_i signal and the hepatic output of hexose equivalents ($r^2 = 0.86$). A linear correlation between the maximal glycogenolytic rate and the hepatic P_i signal would indicate that the K_m of phosphorylase a for P_i is well above the P_i concentrations that occur in the cytosol, even during hypoxia.

DISCUSSION

Anoxia and metabolite control of phosphorylase activity

A number of manipulations are known to induce enhanced hepatic glycogenolysis (Vandebroeck *et al.*, 1985, 1988). These include anoxia, or functional equivalents thereof, such as the administration of mitochondrial poisons. The effects depend on the availability of phosphorylase in its active a form, since they hardly occur in the liver of the *gsd/gsd* rat, which has a deficiency of glycogen phosphorylase kinase.

The mechanism by which anoxia induces glycogenolysis encompasses the conversion of at least a fraction of phosphorylase into the a form. However, the rate of glycogen breakdown under anaerobic conditions exceeds considerably that expected from the same concentration of phosphorylase a under aerobic conditions (see Vandebroeck *et al.*, 1988). A block of the mitochondrial resynthesis of ATP results in an increase in P_i , AMP and IMP. AMP stimulates phosphorylase a *in vitro*, but the effect is small, and the enzyme should already be substantially saturated with AMP in the well-oxygenated liver (Stalmans & Gevers, 1981). It is possible that an increase in the substrate P_i is responsible for the endowment of phosphorylase a with extra activity (Hems & Whitton, 1980; Vandebroeck *et al.*, 1988).

Fig. 3. Relationship between relative changes in hepatic P_i and hepatic output of hexose equivalents

The points in the scatter-plot represent results obtained on individual livers, and show the relative change in hepatic P_i and the corresponding change in the glycogenolytic response. The Figure comprises the experiments shown in Fig. 2 on the effects of perfusion with P_i -free buffer (○) or of addition of 1 mM-KCN and 100 μ M-2,4-dinitrophenol (□) and the result of additional experiments in which the effects of KCN (△) or 2,4-dinitrophenol (●) were studied separately. Values for hepatic P_i and glycogenolytic efficiency were scaled as defined in the legend to Fig. 2. Here we correlate the average relative changes recorded over the last 5 min of the individual experiments.

Before studying the effects of KCN and 2,4-dinitrophenol, we converted phosphorylase into its *a* form by treatment of the liver with dibutyl cyclic AMP. Hence, the mitochondrial poisons cannot have caused their effects by generating extra phosphorylase *a*, or by enhancing the catalytic activity of phosphorylase *b*. Thus the observed effects of these metabolic inhibitors must stem directly from changes in metabolite levels, which affect the activity of phosphorylase *a*. Changes in the rate of glycogenolysis correlated directly with changes in the P_i concentration.

The regulation of the phosphorylation of glycogen by P_i may be more widespread than suggested by the present experiments. Sympathetic-nerve stimulation and administration of vasoactive compounds also stimulate glycogenolysis in perfused livers (Iwai & Jungermann, 1989). These treatments also perturb the perfusion of the organ, resulting in decreased O_2 extraction by the liver. Inasmuch as this corresponds to a sizeable hypoxic effect, an increase in the intracellular P_i concentration may contribute to the observed amplification of hepatic glycogenolysis, by enhancing the kinetic efficiency of phosphorylase *a*.

Table 1. Hepatic glucose and lactate production before and after induction of anoxia

The Table corresponds to experiments summarized in Figs. 2 and 3. The values correspond to the mean absolute rates of glucose and lactate production by the individual livers, over the last 5 min of the control phase preceding the experimental manipulation, and over the last 5 min of the latter phase.

Experimental manipulation (<i>n</i> = 4)	Glucose and lactate produced (μ mol of hexose equivalents/min per g)	
	Control	Experiment
1 mM-KCN		
Glucose	5.34 ± 1.209	6.82 ± 1.053
Lactate	0.45 ± 0.090	1.67 ± 0.249
Sum	5.80 ± 1.298	8.49 ± 1.313
100 μ M-2,4-dinitrophenol + 1 mM-KCN		
Glucose	5.07 ± 0.867	6.67 ± 0.816
Lactate	0.35 ± 0.035	2.46 ± 0.212
Sum	5.42 ± 1.092	9.13 ± 1.228

On the cytosolic origin of the P_i signal

The P_i signal from the intact organ as observed by n.m.r. corresponds to only a fraction of the signal gathered from acid tissue extracts (Iles *et al.*, 1985; Malloy *et al.*, 1986). It has been suggested that the P_i that is present in excess in the extracts stems from hydrolysis of labile phosphate-containing compounds, or, alternatively, that the P_i that is lacking in the spectrum of the intact organ is present in an n.m.r.-invisible compartment. We found that perfusion with P_i -free medium resulted in a large loss of P_i from the liver, which could not be accounted for by shifts in other phosphate-containing compounds. If the lost P_i stemmed entirely from the n.m.r.-visible pool, then the original size of that pool would have had to be unexpectedly large. This implies the existence of an n.m.r.-invisible phosphate pool from which P_i can be mobilized.

The P_i signal from the intact liver stems potentially from the cytosol as well as the mitochondria, the two main P_i -containing compartments in the cell. Studies on perfused livers and yeast indicate that many intramitochondrial metabolites may only be partially n.m.r.-visible (Unkefer & London, 1984; Murphy *et al.*, 1988; Hutson *et al.*, 1989). However, isolated mitochondria contain a large amount of P_i , which is at least partially n.m.r.-visible (Ogawa *et al.*, 1978). Respiring mitochondria maintain a pH gradient with the surrounding cytosol. Because the resonance of P_i is sensitive to physiological pH changes, knowledge of and manipulations of this pH gradient have allowed one to assign the P_i signal to a specific compartment. The resonance of the P_i signal appears to correspond to the pH of the cytosolic compartment, as estimated by other methods (Cohen *et al.*, 1978; Desmoulin *et al.*, 1987).

However, some authors have occasionally reported a splitting of the P_i signal in 31 P spectra of perfused liver (Thoma & Ugurbil, 1988) or isolated hepatocytes (Cohen *et al.*, 1978), suggesting that the P_i signal of intact livers

might comprise a poorly and often only partially resolved signal from the mitochondrial compartment. The nature of the correlation between the rate of glycogenolysis and the n.m.r.-observable P_i signal in our experiments indicates that changes in the P_i signal from whole liver reflected variations in the P_i concentration in the cytosolic compartment which houses the phosphorylase.

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