Effect of phenylephrine on the compartmentation of inorganic phosphate in perfused rat liver during gluconeogenesis and urea synthesis: a ^{31}P-n.m.r.-spectroscopic study

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The transport of P_i between the cytosol and the mitochondria was investigated in perfused rat liver stimulated with phenylephrine and metabolic precursors of glucose and urea: pyruvate, lactate, NH_4^+ and ornithine. The relative concentrations of phosphorus metabolites in the liver were measured by ^{31}P-n.m.r. spectroscopy. When added simultaneously, phenylephrine and the precursors induced a decrease in the P_i level which in 4–5 min reached a new steady state at 73% of the control level. After 5 min or more of stimulation the ATP level had also decreased. When the stimulation ended, P_i and ATP returned to their initial levels within 15 min. In mitochondria isolated after 5 min of stimulation, P_i was increased more than 2-fold as compared with control mitochondria and, in addition, an accumulation of P_i from the perfusion buffer into the liver was observed. Phenylephrine by itself did not cause any significant changes in the ATP or P_i levels, whereas the glucose and urea precursors in the absence of phenylephrine induced a 9% decrease in P_i, while ATP remained constant. The P_i content of mitochondria isolated under these conditions was not significantly increased as compared with control mitochondria. These results showed that P_i accumulated into the mitochondria by a mechanism possibly involving exchange for malate, and that a major part of the intramitochondrial P_i was invisible by n.m.r.

INTRODUCTION

P_i takes part in several important processes of energy metabolism and the biosynthesis of nucleotides and phospholipids. The transport of P_i through the plasma membrane has been studied in detail in the kidney and the intestine, but little is known about the intracellular distribution and transport of P_i (Wehrle and Pedersen, 1989). Best studied in this respect are the mitochondria, where two well-defined components, the phosphate carrier protein and the dicarboxylate carrier protein, transport phosphate across the inner membrane (LaNoue and Schoolwerth, 1984; Wohlrab, 1986). The electroneutral phosphate carrier exchanges P_i for OH^- (or co-transporting P_i with H^+) and transports the major part of P_i needed for ATP synthesis. The dicarboxylate carrier protein catalyses the exchange of dicarboxylates or P_i with a capacity that is one order of magnitude lower than that of the phosphate carrier and may play a role in regulating the distribution of citric-acid-cycle intermediates and P_i across the mitochondrial membrane.

^{31}P-n.m.r. spectroscopy has been applied to study the phosphorus metabolites of a number of cells and tissues (Radda, 1992) with the aim of determining the bulk concentration of these metabolites. Under normoxic conditions the amount of ATP detected by n.m.r. is generally considered close to 100% of the total tissue ATP, whereas a considerable portion of the P_i cannot be detected by n.m.r. (Stubbs et al., 1984; Iles et al., 1985; Cunningham et al., 1986; Desmoulin et al., 1987) and this P_i pool has traditionally been thought to occur in mitochondria (Bailey et al., 1981; Adler et al., 1984). However, some authors have observed additional P_i peaks assigned to mitochondrial P_i (Thoma and Ugurbil, 1988; Garlick et al., 1992).

It was therefore of interest to investigate whether the ^{31}P-n.m.r. technique could be used to assess the exchange of P_i for dicarboxylates in perfused rat liver during coupled gluconeogenesis and ureogenesis. The result shows that the amount of P_i in the mitochondria increases 2-fold under the conditions employed, by a mechanism possibly involving exchange for malate. The data also support the notion that the cellular P_i n.m.r. signal is of mainly cytosolic origin.

EXPERIMENTAL

Liver perfusion

Fed Wistar rats (130–150 g body wt. each) were anaesthesized by intraperitoneal injection of pentobarbital (60 mg/kg). The livers were cannulated and perfused with standard buffer containing 119 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.3 mM $CaCl_2$, 0.65 mM $MgSO_4$, 1.5 mM NaH_2PO_4, 5.6 mM glucose and 10 mM Hepes/NaOH, pH 7.40±0.05, and equilibrated with O_2/CO_2 (19:1) at a temperature of 32 °C and at a flow rate of 6–7 ml/min per g wet weight. The portal-vein branches going to the median, left and caudate lobes were occluded by ligatures, the corresponding lobes were removed and the remaining right lateral lobe was inserted into an n.m.r.-tube. The effluent buffer was removed with a suction pump, and the O_2 concentration was measured using a Clark electrode placed in the effluent buffer stream. The P_i of the effluent buffer was measured as described by Lanzetta et al. (1979). A stabilization period of 20–30 min prior to data acquisition was used.

Preparation of mitochondria

The liver was perfused with ice-cooled perfusion buffer for 2 min and subsequently for 30 s with a buffer containing 225 mM mannitol, 75 mM sucrose, 5 μM Ruthenium Red, 1 μM cyclosporin A, 2 μM BSA, 5 mM EGTA and 10 mM Hepes/KOH, pH 7.0, that had been treated with a Chelex (Bio-Rad, Richmond, VA, U.S.A.) cation-exchange resin. The lobe was excised and homogenized in the presence of 0.5 mM N-ethylmaleimide and the mitochondria were isolated as described by Allshire et al. (1985). The P_i content of the mitochondria was analysed after extraction with $HClO_4$ as described by Williamsson and Corkey

Table 1 Effect of phenylephrine and precursors of urea and glucose on ATP, P_i, mitochondrial P_i and respiration

The effects were measured as described in the Experimental section. For each liver the values of the P_i and ATP concentration obtained prior to stimulation were taken as 100%. The P_i content of control mitochondria was 11.6±2.0 nmol/mg of protein. The effect on respiration is expressed as the ratio of the activated respiration to the resting-state respiration at 5 min of stimulation. The number of experiments performed under each set of conditions are shown in parentheses (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Stimulation	Content as measured by n.m.r. (%)		Mitochondrial P_i (nmol/mg of protein)	Respiration ratio
	ATP	P_i		
(a) Phenylephrine	103±2 (5)	96±4	13.3±4.2 (5)	1.5±0.1*** (10)
(b) Lactate, pyruvate, NH_4^+ and ornithine	92±4 (3)	91±1*	15.5±4.0 (4)	1.6±0.1** (7)
(c) Phenylephrine, lactate, pyruvate, NH_4^+ and ornithine	86±3** (5)	73±2***	25.3±3.7** (4)	2.1±0.2*** (9)

(1969), and mitochondrial Ca^{2+} was determined by atomic-absorption spectrometry. The control values for livers perfused with standard buffer for 30 min before isolation was 11.6±2.0 nmol of P_i/mg of protein and 8.0±1.0 nmol of Ca^{2+}/mg of protein ($n = 5$) respectively. To test the sensitivity of the method to hormonal stimuli, controls were performed by stimulating the liver with 20 nM glucagon and 2 μM phenylephrine; under these conditions isolated mitochondria contained 27.0±4.0 nmol of Ca^{2+}/mg of protein and 43.0±8.5 nmol of P_i/mg of protein, in agreement with the findings of Bygrave et al. (1990).

^{31}P-n.m.r. spectroscopy

^{31}P-n.m.r. spectra were obtained on a Varian 500 MHz n.m.r. spectrometer (Varian, Rheinstetten, Germany) at 202.4 MHz. For each spectrum, 64, 128 or 256 free induction decays were accumulated using pulses corresponding to a flip angle of 72°, with a repetition time of 0.5 or 1.0 s. An exponential multiplication that corresponded to a further line broadening of 15 Hz was applied before Fourier transformation. The spin-lattice relaxation time (T_1) of the P_i of the perfusion buffer was determined to 11.0±1.5 s and at 0.5 s repetition time the contribution of the external phosphate to the P_i peak could be estimated to be 10±5%, the uncertainty being due to individual differences in the size of the lobe. This was further confirmed in one control experiment by adding 1.5 mM PP_i to the perfusion buffer showing that the contribution of the buffer P_i to the total P_i peak was 8%.

The area of the P_i and ATP peaks was calculated from manually drawn baselines, and the cytosolic pH was calculated from the difference in the chemical shift between the P_i and the α-phosphate of ATP as described by Bailey et al. (1981). The part of the P_i peak arising from the P_i of the perfusion buffer was subtracted before calculations. Since the resulting peak may be composed of several resonances with different T_1 values, changes in peak area may not be strictly proportional to changes in the concentration of P_i in the visible pool(s).

Experimental protocol and statistics

The livers were stimulated for a period of 4–10 min with (a) 2 μM phenylephrine or (b) 2 mM ornithine/5 mM NH_4Cl/2 mM lactate/1 mM pyruvate, omitting glucose from the buffer, or (c) 2 μM phenylephrine/2 mM ornithine/5 mM NH_4Cl/2 mM lactate/1 mM pyruvate, omitting glucose from the buffer. The stimulation was followed by a 15 min relaxation period during which the liver was perfused with standard buffer. During the relaxation period the concentration of ATP and P_i returned to their initial values, whereupon a new stimulation–relaxation cycle was started; this protocol was repeated up to four times per liver. Isolation of mitochondria was carried out in parallel experiments at the end of the first stimulation period.

The area of the β-ATP and P_i peaks acquired immediately before a stimulation period was taken as 100%. For each liver the mean values of the β-ATP and P_i peak areas obtained during all the stimulation periods were calculated and used to assess the statistical significance by a Student's t test. In the control experiments ($n = 5$), carried out in the absence of any metabolic stimuli, the levels of ATP and P_i remained stable for at least 3 h. All values are expressed as means±S.E.M.

Chemicals

The chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) and cyclosporin A was kindly given by Dr. P. Juutilainen of Sandoz OY, Helsinki, Finland.

RESULTS

The main results of the experiments are summarized in Table 1.

When the liver was stimulated with phenylephrine alone, ATP and P_i stayed almost unchanged, as measured by n.m.r.; the ATP/P_i ratio was possibly slightly increased, in spite of a markedly activated respiration. In the presence of the metabolites alone, a significant decrease in the P_i level was observed, whereas the ATP level remained virtually constant.

When used together, phenylephrine and the metabolites activated respiration more than either of them separately, and there was a pronounced decrease in the level of P_i as well as of ATP. In mitochondria isolated from stimulated livers the amount of P_i was increased more than 2-fold in comparison with mitochondria isolated from control livers. Figure 1 shows the complete ^{31}P-n.m.r. spectrum of a liver before stimulation (spectrum A) and during the stimulation period (spectrum B); the decrease in the P_i peak (labelled b) was not accompanied by an increase in any of the other resonances. In the beginning of the stimulation period an acidification of 0.2 pH unit of the environment of the P_i took place. The kinetics of the decrease in P_i was investigated in more detail by acquiring spectra in 30 s blocks, and the result of one such experiment is shown in Figure 2. The P_i decreased immediately upon stimulation, and a new equilibrium in the P_i level was essentially reached within 4–5 min; it did not decrease any further during longer stimulation periods (results not shown). The stimulation also induced an uptake of P_i from the perfusion

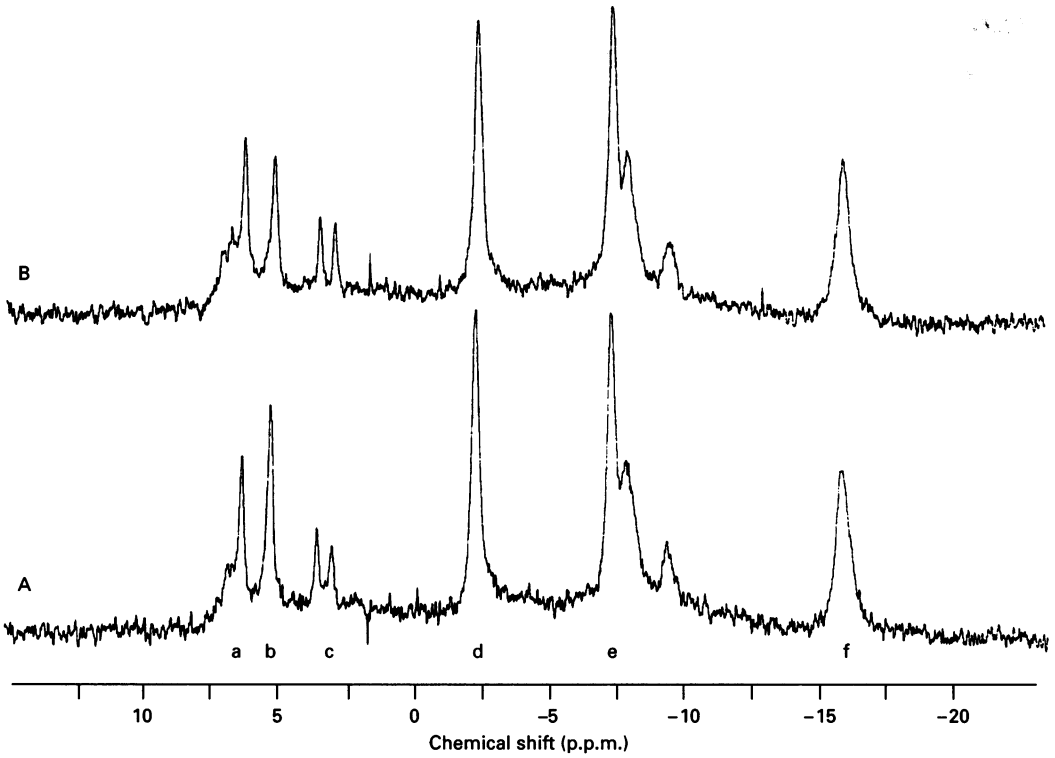

Figure 1 Complete ^{31}P-n.m.r. spectrum of a rat liver perfused with standard buffer (spectrum A) or standard buffer containing 2 μM phenylephrine, 2 mM lactate, 1 mM pyruvate, 5 mM NH_4^+ and 2 mM ornithine, omitting glucose (spectrum B)

Peak assignments: a, phosphomonoesters; b, P_i; c, phosphodiesters; d, γ-phosphate of nucleoside triphosphates and β-phosphate of nucleoside diphosphates; e, α-phosphate of nucleoside di- and tri-phosphates; f, β-phosphate of nucleoside triphosphates. Spectral parameters: repetition time, 0.5 s; number of free induction decays, 750; line-broadening, 20 Hz.

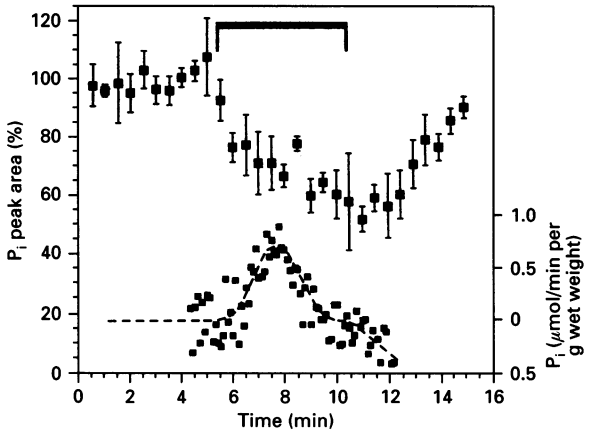

Figure 2 Level of intracellular P_i as measured by ^{31}P-n.m.r. (upper trace) and the uptake of P_i from the effluent buffer (lower trace) during stimulation with 2 μM phenylephrine, 2 mM lactate, 1 mM pyruvate, 5 mM NH_4^+ and 2 mM ornithine as indicated

The n.m.r. data are mean values $\pm$ S.E.M. for three stimulations performed on one liver. The uptake of P_i from the effluent buffer is expressed as the mean of two determinations (the S.E.M. is excluded for clarity) and was calculated from the concentration of P_i in the effluent buffer collected in 6 s aliquots and determined as described in the Experimental section.

buffer (Figure 2), and the total amount of P_i in the liver increased by 1.4 ± 0.3 μmol/g in 3 min. In addition to the decrease in n.m.r.-detectable P_i, the ATP level also decreased in the presence of phenylephrine and the metabolites (Table 1), but it is important to emphasize that this took place only after 5 min or more of stimulation, and the decrease was accompanied by the appearance of an upfield shoulder on the P_i peak (result not shown). When the liver was perfused with buffer containing glucose after the stimulation period, the P_i and ATP returned to their initial level at rates comparable with their respective decrease rates.

The mitochondrial content of Ca^{2+} was slightly elevated in livers stimulated with phenylephrine or metabolites alone [9.7 ± 1.7 and 9.2 ± 4.3 nmol/mg protein ($n = 5$ and 4) respectively], whereas when phenylephrine and the metabolites were added simultaneously a slight decrease to 5.8 ± 1.2 nmol/mg ($n = 5$) was observed in the course of 5 min.

DISCUSSION

When the liver was stimulated with phenylephrine the ATP/P_i ratio did not decrease regardless of an evident activation of the respiration. This is in accordance with published data obtained with other techniques and may be explained by the well documented activating effect of Ca^{2+} on several intramitochondrial dehydrogenases (McCormack et al., 1990; Hansford, 1991, Brown, 1992). When the metabolites were added in the absence of phenylephrine, P_i decreased and the mitochondrial P_i content was possibly slightly elevated, whereas ATP remained constant. In contrast with these data it has been reported that the P_i level increases during coupled gluconeogenesis and urea synthesis in the absence of added hormones (Tanaka et al., 1989). It is conceivable that the different results obtained by these authors

may be related to their use of a P_i-free perfusion buffer, which has been shown to induce an efflux of intracellular P_i (Vanstapel et al., 1990) and hence to decrease the steady-state level of intracellular P_i, as discussed further below.

In the presence of phenylephrine plus metabolites there was a decrease in the intracellular P_i level detected by n.m.r., whereas the total cell P_i and the mitochondrial P_i content were elevated. Phenylephrine and the metabolites induce a transition from a mainly glycolytic state to a state of activated gluconeogenesis and ureogenesis by several contributing factors. Firstly, the glucose and urea synthesis is driven by the changed Mass Action ratios of the reactions in which the metabolites take part. Secondly, phenylephrine stimulates the intramitochondrial steps of gluconeogenesis through an increased mitochondrial $NADH/NAD^+$ ratio (Sterniczuk et al., 1991), while under these conditions the cytosolic $NADH/NAD^+$ ratio was clamped at a low level, thus increasing the cytosolic concentration of oxaloacetate (Williamson et al., 1967). Carbon for gluconeogenesis may cross the mitochondrial membrane, either as aspartate in exchange for glutamate or as malate in exchange for P_i. Under these conditions, where the only exogenous nitrogen was added as ornithine and NH_4^+, the rate of aspartate/glutamate exchange was most likely limited by the relatively slow efflux of glutamate (Schoolwerth et al., 1983) formed by glutamate dehydrogenase, thus rendering the malate/P_i exchange quantitatively most important for export of carbon. It is thus likely that the accumulation of P_i into the mitochondria was driven by an increased intramitochondrial concentration of malate formed by the combined effects of the metabolites and phenylephrine. It should be noted that the same effect, i.e. a decreased intracellular P_i as measured by n.m.r., and an elevated mitochondrial P_i, could be observed upon stimulation with the metabolites alone, although the effect was quantitatively smaller. In the absence of buffer P_i the intracellular n.m.r.-detectable P_i has been shown to decrease by 40% (Vanstapel et al., 1990) and the rate of malate/P_i exchange may thus be reduced, due to the high K_m of P_i (Wohlrab, 1986). This may explain why Tanaka et al. (1989) observed an increase in the P_i under otherwise similar conditions.

It has been reported that glucagon and phenylephrine (Bygrave et al., 1990) or extracellular ATP (Zoeteweij et al., 1993) may induce the uptake of large quantities of P_i into the mitochondria by co-transport and complexation with Ca^{2+}. However, the contribution of such a mechanism was probably small, since phenylephrine did not bring about any co-accumulation of Ca^{2+} into the mitochondria under the conditions employed here. In addition to malate/P_i exchange, it is possible that a slight acidification of the cytosolic compartment also contributed to shift the distribution of P_i towards increased mitochondrial P_i by increasing the ΔpH, although, in respiring mitochondria, the ΔpH-dependence of the distribution has been shown to be much smaller than what could be expected from theoretical considerations (Greenbaum and Wilson, 1985).

When the mitochondrial P_i content increased 2-fold, the n.m.r. peak assigned to P_i at pH 7.3 decreased in intensity, and no additional peaks could be detected in the spectrum. In addition, P_i was transported from the perfusion buffer into the liver. It is thus likely that the decrease in the P_i level detected by n.m.r. reflected the uptake of P_i by mitochondria and not a loss of P_i to the buffer or the formation of any phosphorylated metabolites. This supports the view that the large n.m.r.-invisible fraction of P_i found in tissue extracts is of mitochondrial origin (Bailey et al., 1981; Adler et al., 1984). Furthermore, provided that a pH gradient of 0.3 unit or more existed across the inner membrane, i.e. the intramitochondrial pH was at least 7.6, as reported by Strzelecki et al. (1984), then the mitochondrial pool was almost completely invisible, whereas, if the pH difference was smaller, then a part of the mitochondrial P_i might be visible and contribute to the P_i peak. Such a small ΔpH is, however, not compatible with an increased P_i gradient, as discussed. It is likely that the uptake of P_i from the perfusion buffer was driven by the P_i gradient formed across the plasma membrane by the decreased cytosolic P_i level.

ATP has generally been reported to be almost completely visible during normoxic conditions (Cohen, 1983; Iles et al., 1985; Desmoulin et al., 1987), whereas during ischaemia some authors have found that the n.m.r.-visible fraction decreases (Murphy et al., 1988; Takami et al., 1988). However, in a recent report, no evidence for a decreased n.m.r. visibility of ATP could be found by using the ^1H-n.m.r. water signal as an internal standard (Masson and Quistorff, 1992). This raises the question as to why intramitochondrial ATP, but not P_i, is visible in tissues. Several possible factors such as the viscosity, the presence of paramagnetic ions and the temperature could be pointed out. Interestingly, the intramitochondrial P_i of hepatocytes (Cohen et al., 1978) and the P_i of isolated rat liver mitochondria (Ogawa et al., 1978; Hutson et al., 1992) have been shown to be visible at low temperatures and low concentrations of paramagnetic ions.

The ATP level decreased after 5 min or more of stimulation, and the decrease was accompanied by the appearance of a shoulder of the P_i resonance at pH 6.8–6.9. It is conceivable that this reflected a different steady state in the production and consumption of ATP in some population of the hepatocytes. It is unlikely that the decrease in ATP was due to anoxia, since the oxygen electrode indicated that the efflux buffer contained a surplus of oxygen and, in addition, increasing the buffer flow did not delay the decrease in ATP.

It can thus be concluded that the n.m.r. technique, in combination with other methods, may be successfully applied to study the distribution of P_i between the extracellular and intracellular spaces during stimulation with metabolites and hormones. Further experiments will be required to accurately establish the size of the n.m.r.-invisible proportion of the total mitochondrial P_i pool. Furthermore, it is conceivable that uptake of P_i into the mitochondria during gluconeogenesis and urea synthesis may contribute to regulate the total P_i level of the hepatic tissue.

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