Phosphate and calcium uptake by mitochondria and by perfused rat liver induced by the synergistic action of glucagon and vasopressin

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Co-administration of glucagon and vasopressin to rat liver perfused with buffer containing 1.3 mM- Ca^{2+} induces a 4-fold increase in P_i in the subsequently isolated mitochondria (from approx. 9 to approx. 40 nmol/mg of mitochondrial protein). This increase is not attributable to PP_i hydrolysis, and is not observed if the perfusate Ca^{2+} is lowered from 1.3 mM to 50 μM . The increase in mitochondrial P_i closely parallels that of mitochondrial Ca^{2+} ; when the increase in P_i and Ca^{2+} accumulation is maximal, the molar ratio is close to that in $\text{Ca}_3(\text{PO}_4)_2$. Measurement of changes in the perfusate P_i revealed that, whereas administration of glucagon or vasopressin alone brought about a rapid decline in perfusate P_i , the largest decrease (reflecting net retention of P_i by the liver) was observed when the hormone was co-administered in the presence of 1.3 mM- Ca^{2+} . The synergistic action of glucagon plus vasopressin was nullified by lowering the perfusate Ca^{2+} to 50 μM . The data provide evidence that, whereas glucagon may be able to alter P_i fluxes directly in intact liver, any alterations induced by vasopressin are indirect and result only from its action of mobilizing Ca^{2+} .

INTRODUCTION

Phosphate transport across cell and organellar membranes is a crucial event for maintaining an adequate cellular P_i pool(s) that is vital in cellular metabolism (see, e.g., LaNoue & Schoolwerth, 1979). In many biological systems P_i transport is often linked to cellular Ca^{2+} metabolism and transport. For example, the anion is known to have a large influence on Ca^{2+} fluxes in intact kidney cells (Borle, 1972) as well as on Ca^{2+} transport by mitochondria isolated from a range of tissues and species (Bygrave, 1978; Lehninger *et al.*, 1967). Much is known about the mechanisms of mitochondrial P_i transport (Wohlrab, 1986), but studies on P_i movement across the plasma membrane appear to have been carried out largely on cells of kidney and intestine (Bronner & Peterlik, 1984).

There is evidence that P_i exchanges only very slowly between extracellular and intracellular spaces in (unstimulated) perfused rat liver (Sestoft & Kristensen, 1979; Bracht *et al.*, 1981) and in perfused rat heart (Medina & Illingworth, 1980). On the other hand, there is evidence to suggest that glucagon, cyclic AMP and adrenaline are able to stimulate P_i exchange in perfused rat liver (De Venanzi *et al.*, 1974) and in perfused rat heart (Medina & Illingworth, 1984) and that glucagon administration to hepatocytes induces a quite rapid increase in cellular P_i content (Siess *et al.*, 1984). Moreover, administration of glucagon to the whole animal or to perfused rat liver induces an increase in the P_i content of the subsequently isolated mitochondria (Prpic *et al.*, 1978; Hughes & Barritt, 1978). Thus evidence exists that, although the movement of P_i across the liver plasma membrane is relatively slow, this may be enhanced by the action of glucagon.

Recent work in our and other laboratories has shown that influx of Ca^{2+} into hepatocytes or perfused rat liver induced by Ca^{2+} -mobilizing agents is enhanced by prior administration of glucagon (reviewed in Altin & Bygrave, 1988). This Ca^{2+} appears to accumulate in the mitochondria without inducing obvious organellar or cell damage (Altin & Bygrave, 1986; Assimacopoulos-Jeannet *et al.*, 1986). Because of the close relationship of Ca^{2+} metabolism with P_i metabolism in many biological systems, it was of interest to assess whether co-administration of

glucagon and Ca^{2+} -mobilizing hormones like vasopressin to the perfused rat liver induces a synergistic increase in mitochondrial P_i similar to that observed with Ca^{2+} . The data presented here show this to be the case.

EXPERIMENTAL

Male Wistar-strain albino rats weighing about 300 g and having free access to food were used for all experiments. They were anaesthetized with sodium pentobarbitone (50 mg/kg body wt.) administered by intraperitoneal injection. Livers were perfused with Krebs–Henseleit (1932) bicarbonate buffer equilibrated with O_2/CO_2 (19:1) as described previously (Reinhart *et al.*, 1982) at 35 °C and at a flow rate of 3.5 ml/min per g wet wt. of liver. Ca^{2+} (1.3 mM perfusate concentration unless otherwise stated) was infused by means of an infusor pump directly into the cannula feeding the portal vein to allow rapid changes to perfusate Ca^{2+} concentration to be made. All livers were pre-perfused for 15 min before hormone administration. Glucagon and vasopressin (each at 10 nM, dissolved in perfusion buffer) also were infused separately into the portal cannula by infusion syringe according to the requirements of the particular experiment. Mitochondria were prepared from livers by a rapid single-step Percoll-density-gradient procedure fully described elsewhere (Reinhart *et al.*, 1984). Immediately after isolation, the mitochondrial fraction and homogenate, when used, were extracted with ice-cold 2 M- HClO_4 /5 mM-Hepes and centrifuged for 6 min in an Eppendorf centrifuge to sediment insoluble material. Portions of the supernatant were analysed for Ca^{2+} in 0.1% (w/v) KCl by using a Varian atomic-absorption spectrophotometer. Mitochondrial P_i was measured by extracting samples (0.1 ml) with 0.4 ml of 10% (w/v) trichloroacetic acid. The extracts were left at 0 °C for 30 min, then centrifuged for 2 min in an Eppendorf centrifuge. The supernatants were removed and immediately stored overnight at –20 °C. The amount of P_i present was measured by the method of Baginski *et al.* (1967). In control experiments, we established that, under these conditions of extraction of P_i from mitochondria, the extent to which a known concentration of ATP or PP_i was hydrolysed to

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produce P_i (see below) was no greater than 5%. Similarly, and consistent with statements in the report of Baginski *et al.* (1967), the presence of neither ATP nor PP_i interfered with the P_i assay to any significant extent. To measure the perfusate P_i concentration, samples of perfusate were centrifuged for 2 min in an Eppendorf centrifuge to remove contaminating red blood cells. Appropriately diluted samples of the supernatant were then assayed for P_i (Baginski *et al.*, 1967). Protein was determined by the method of Lowry *et al.* (1951). Vasopressin and glucagon were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other reagents were of analytical-reagent grade. Where possible, results are presented as mean values $\pm$ S.E.M. Statistical significance (P) was calculated by Student's t test.

RESULTS

Changes in mitochondrial P_i content in hormone-stimulated perfused rat liver

Data in Table 1 show that infusion of vasopressin alone into the perfused rat liver induces little change in the P_i content of the subsequently isolated mitochondria, but that glucagon infusion induces an increase of approx. 35% of the control value, from 9.19 ± 0.83 to 12.40 ± 0.27 nmol of P_i /mg of mitochondrial protein ($P < 0.025$). This is in agreement with an increase in the endogenous level of P_i in mitochondria isolated from the livers of rats exposed to glucagon *in vivo* reported by us and others (Prpic *et al.*, 1978; Hughes & Barritt, 1978). When glucagon and vasopressin were co-infused, however, the P_i content, like that of Ca^{2+} , was more than additive of that achieved by treatment with either hormone alone; a significant increase over the control value (to 41.45 ± 4.07 nmol of P_i /mg of mitochondrial protein) was observed. While our work was in progress, the paper by Davidson & Halestrap (1988) appeared, in which it was shown that incubation of isolated liver cells with glucagon and vasopressin led, similarly to our data with P_i above, to increases in mitochondrial PP_i greater than those achieved with either hormone alone. In control experiments (results not shown) we established that PP_i did not interfere significantly with the assay for P_i used in our work (see the Experimental section), nor was there any significant hydrolysis of PP_i under the extraction conditions used that might account for the increase in P_i as

described in Table 1. We thus concluded that in our experiments we were indeed measuring increases in mitochondrial P_i .

Time course of Ca^{2+} and P_i accumulation by liver mitochondria during maximal stimulation by glucagon and vasopressin

In light of the observed large increase in mitochondrial P_i content induced by the co-administration of glucagon and vasopressin, a time course was made of mitochondrial Ca^{2+} and P_i contents for the period of hormone administration. The aim was to provide further information about the times at which these changes occurred, and the inter-relationship between Ca^{2+} and P_i movement into the mitochondria. The results are depicted in Table 2, with the Ca^{2+} and P_i contents at each time point representing paired results from the same animal. After 4 min of glucagon infusion, the mitochondrial Ca^{2+} and P_i contents are not significantly different from those of the control. This lack of observable change in the Ca^{2+} content is consistent with the small efflux of Ca^{2+} from the liver as monitored with the Ca^{2+} -selective electrode during the corresponding time period (Altin & Bygrave, 1986). Once vasopressin is infused, a doubling of the mitochondrial Ca^{2+} content occurs within 3 min (from 4.41 ± 0.31 to 10.17 ± 1.82 nmol/mg of protein), with no significant increase in P_i . Between 3 and 10 min of vasopressin administration, mitochondrial accumulation of both P_i and Ca^{2+} is rapid. At the end of this period (i.e. 29 min of perfusion time) the respective levels of P_i (35.73 ± 4.6 nmol/mg of protein) and Ca^{2+} (41.81 ± 1.09 nmol/mg of protein) account for approx. 85% of the maximal P_i uptake and 70% of the maximal Ca^{2+} uptake. These concentrations rise to 41.45 ± 4.07 and 56.43 ± 6.4 nmol/mg of protein respectively, when glucagon and vasopressin have been co-infused for 20 min. At this time Ca^{2+} uptake by the liver is essentially complete (Altin & Bygrave, 1986). By 30 min after removal of the hormones, mitochondrial Ca^{2+} and P_i contents have fallen to values comparable with those found to exist 5 min after the commencement of vasopressin infusion. Assimacopoulos-Jeannet *et al.* (1986) observed a similar progressive increase in mitochondrial Ca^{2+} , after the co-administration of glucagon and vasopressin to the perfused rat liver for up to 10 min, that correlated with corresponding increases in

Table 1. P_i and Ca^{2+} accumulation in mitochondria isolated from liver after the co-administration of vasopressin and glucagon

Livers were perfused with Krebs-Henseleit bicarbonate buffer with 1.3 mM- Ca^{2+} present. In one set of conditions, glucagon infusion was begun at 15 min of perfusion time and continued for a further period of 24 min. In another set vasopressin replaced glucagon, and in a third set vasopressin administration was initiated 4 min after glucagon and continued together for a further 20 min. At this time a Ca^{2+} -free Krebs-Henseleit buffer was infused containing 50 μ M-EGTA for a further 2 min to minimize access of non-mitochondrial Ca^{2+} to the mitochondria in the course of their isolation (see Reinhart *et al.*, 1984). Livers were then removed and homogenized, and mitochondria were isolated as indicated in the Experimental section. Values are means $\pm$ S.E.M. for three to six experiments for each condition.

Hormone administered	Mitochondrial content (nmol/mg of protein) of:	
	P_i	Ca^{2+}
Control	9.19 ± 0.83	4.7 ± 0.11
Glucagon (10 nM)	12.40 ± 0.27	7.53 ± 1.41
Vasopressin (10 nM)	9.31 ± 0.54	4.7 ± 0.11
Glucagon plus vasopressin	41.45 ± 4.07	56.43 ± 6.4

Table 2. Time course of Ca^{2+} and P_i accumulation in mitochondria isolated from perfused rat liver after hormone administration

Livers were perfused with Krebs-Henseleit bicarbonate buffer with 1.3 mM- Ca^{2+} present. Glucagon (10 nM) was infused at 15 min of perfusion time and vasopressin (10 nM) at 19 min. Infusion was terminated where indicated, and a Ca^{2+} -free Krebs-Henseleit buffer containing 20 μ M-EGTA was infused for a further 2 min. Livers were then removed, homogenized and fractionated, and the mitochondrial fraction was assessed for Ca^{2+} and for P_i , as indicated in the Experimental section. Means $\pm$ S.E.M. are shown for three to six separate experiments.

Time of perfusion (min)	Mitochondrial content (nmol/mg of protein) of:	
	P_i	Ca^{2+}
15	9.2 ± 0.08	4.7 ± 0.11
19	11.11 ± 0.15	4.7 ± 0.15
20	12.20 ± 0.12	9.2 ± 2.9
22	15.20 ± 1.3	11.1 ± 2.8
24	26.20 ± 1.9	22.2 ± 1.6
26	32.62 ± 3.6	27.5 ± 2.1
29	36.53 ± 4.8	42.1 ± 2.3
39	41.45 ± 4.07	56.43 ± 6.4
69	26.24 ± 2.6	19.54 ± 2.5

Fig. 1. Changes in the perfusate P_i concentration after administration of glucagon (Glu) and vasopressin (Vas) to the perfused rat liver

Livers were perfused as described in the legend to Table 1, except that the hormones were administered for the times indicated. Samples of the perfusate were collected at the times indicated and appropriate samples assayed for P_i as described in the Experimental section. Each point is the mean $\pm$ S.E.M. of three or more values.

the activities of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase.

Effects of glucagon and vasopressin on the perfusate P_i concentration

The next experiments were designed to investigate whether fluctuations in perfusate P_i concentration occur concomitant with Ca^{2+} after the administration of glucagon and vasopressin (cf. Altin & Bygrave, 1986). Results of one set of experiments carried out when vasopressin or glucagon were administered alone, or together, are shown in Fig. 1; data are expressed as the change in perfusate P_i concentration relative to the mean value for this concentration between 12 and 15 min of the pre-perfusion period, which was 1.15 ± 0.01 mM. Fig. 1(a) shows that, after vasopressin infusion, a rapid decline occurs in the perfusate P_i concentration, corresponding to a net influx of P_i into the liver, that reaches a maximum at approx. 5–6 min. The rate of P_i influx declines thereafter, to zero by about 20 min, and is followed by a slight net efflux of P_i from the liver. When glucagon instead of vasopressin is administered to the liver (Fig. 1b), P_i influx into the liver is stimulated to a greater extent; this is reflected in the greater extent to which P_i efflux subsequently occurs. Finally, Fig. 1(c) shows that, when vasopressin is co-administered 5 min after glucagon administration (i.e. conditions under which maximal Ca^{2+} influx occurs; Altin & Bygrave, 1986), the rate of P_i influx remains at a maximum for as long as both hormones are infused; only when the hormones are withdrawn does the rate of P_i influx decline in a manner similar to that with glucagon alone.

Effect of a low perfusate Ca^{2+} concentration on hormone-induced perfusate P_i concentration

Table 3 shows that, after the co-administration of glucagon and vasopressin at low perfusate Ca^{2+} concentrations, the mitochondrial P_i content (8.60 ± 0.27 nmol/mg of protein) does not change significantly from previously established values for mitochondria isolated from control (non-stimulated) liver (9.19 ± 0.83 nmol/mg of protein). This suggests that the hormones do not affect mitochondrial P_i content in the perfused liver unless extracellular Ca^{2+} is available, in which case the corresponding value for mitochondrial P_i content was 35.73 ± 4.60 nmol/mg of protein (see also Tables 1 and 2). The Ca^{2+} content of the mitochondria after perfusion at low Ca^{2+} concentrations (0.89 ± 0.04 nmol/mg of protein) is within the range found previously by us (Reinhart *et al.* 1984). Fig. 2 shows the pattern of changes in P_i fluxes induced by co-administration of glucagon and vasopressin; the experiment was carried out in a manner identical with that for Fig. 1(c), except that Ca^{2+} was absent from the perfusate buffer. It is clear that the change in

Table 3. Effect of a low perfusate Ca^{2+} concentration on mitochondrial Ca^{2+} and P_i contents after hormonal stimulation

The experiment was carried out exactly as described in Table 1, except that the Ca^{2+} concentration in the Krebs–Henseleit buffer was as indicated; glucagon and vasopressin were co-administered. Means $\pm$ S.E.M. are shown for three separate experiments.

Perfusate Ca^{2+} concn.	Mitochondrial content (nmol/mg of protein) of:	
	Ca^{2+}	P_i
1.3 mM	41.81 ± 1.09	35.73 ± 4.60
50 μ M	0.89 ± 0.04	8.60 ± 0.27

Fig. 2. Changes in the perfusate P_i concentration after administration of glucagon (Glu) and vasopressin (Vas) to the perfused rat liver in the absence of added Ca^{2+} .

Livers were perfused exactly as described in Fig. 1(c), except that the perfusion medium did not contain added Ca^{2+} . Each point is the mean $\pm$ S.E.M. of three or more values.

perfusate P_i concentration exhibits a pattern indicating significantly less uptake of the anion by the liver and a spontaneous change from influx to efflux even while the hormones are being administered. There are indications, too, that after 50 min of perfusion in these conditions a net loss of P_i has occurred from the liver.

DISCUSSION

The results presented here extend previous work on hormone-induced P_i fluxes in perfused rat liver (De Venzani *et al.*, 1974) in several fundamental respects. One of these is technical in the sense that, in this work, the more frequent sampling of the perfusate for P_i content revealed that changes in perfusate P_i occur more rapidly than was previously appreciated. Others were the synergistic action of glucagon and vasopressin on P_i fluxes, the influence of a change in perfusate Ca^{2+} concentration on P_i fluxes and the attempt to relate these fluxes both to changes in mitochondrial P_i and to changes in mitochondrial Ca^{2+} content. Finally, the data provide some insights into the relation between P_i and Ca^{2+} fluxes in the perfused rat liver induced by the actions of glucagon and vasopressin.

Experiments in the present paper demonstrated clearly that co-administration of glucagon and vasopressin to the perfused rat liver induces an increase in mitochondrial P_i content by as much as 4-fold over basal levels. Administration of glucagon alone, but not vasopressin, induced a small but significant increase in mitochondrial P_i , consistent with earlier studies carried out with intact rats (Prpic *et al.*, 1978), perfused rat livers (Hughes & Barritt, 1978) or hepatocytes (Siess *et al.*, 1984). The increase in mitochondrial P_i took place quite rapidly and in

parallel with the accumulation of Ca^{2+} by mitochondria after an initial lag period (see also Altin & Bygrave, 1986; Assimacopoulos-Jeannet *et al.*, 1986). The ratio of Ca^{2+} to P_i accumulated in the mitochondria after maximal hormonal stimulation is slightly greater than 1.5, a ratio consistent with the formation of $Ca_3(PO_4)_2$. The ability of mitochondria *in situ* to release this Ca^{2+} and P_i after cessation of hormone administration (this work, and see also Altin & Bygrave, 1986), is also consistent with this form of storage, as $Ca_3(PO_4)_2$ readily dissociates when the concentration of either ion falls below saturating levels (Blumenthal *et al.*, 1977). Some of the Ca^{2+} and P_i would be bound to membrane proteins and phospholipids, and a small amount would exist as the free ion (Lehninger, 1970).

Although we cannot entirely dismiss the possibility that some of this P_i arises from, for example, the hydrolysis of organic molecules within the mitochondria (Zahlten *et al.*, 1972), it is considered that the elevated levels of mitochondrial P_i result mainly from other sources. As pointed out above, Davidson & Halestrap (1988) have shown that another metabolic consequence of the synergistic action of glucagon and vasopressin in hepatocytes is the elevation of mitochondrial PP_i concentrations. This fact, together with their other observation that this event requires external Ca^{2+} (Davidson & Halestrap, 1987), is remarkably similar to the observations made in the present paper with P_i . As we remarked above, we do not believe, from the control experiments we performed, that the increases in P_i observed in our work result from PP_i either interfering with the assay for P_i or being hydrolysed to form P_i under the extraction conditions used here.

Direct evidence for net accumulation of P_i by the whole liver was obtained from measuring changes in the perfusate P_i concentration. Although the technique simply involves chemical measurement of the P_i concentration at different times after the administration of the hormone regimes, it is evident that, provided that considerable care is taken with this and sufficient experiments are carried out, quite reproducible results of a quantitative nature can be obtained (Figs. 1 and 2), allowing meaningful interpretations to be drawn.

Consistent with data obtained on mitochondrial P_i accumulation (Table 1), the results revealed that maximal net P_i influx occurred when both glucagon and vasopressin were infused and when the perfusate Ca^{2+} concentration was 1.3 mM (Fig. 1c). Considerably smaller extents of P_i efflux were observed under conditions in which either hormone was administered alone (Figs. 1a and 1b) or together, but with a low concentration of Ca^{2+} in the perfusate (Table 3, Fig. 2). It was of interest to observe too that the latter conditions (i.e. those described in Fig. 2) led to an eventual apparent loss of P_i from the liver. The data revealed also that maximal rates of P_i uptake continued unabated only as long as both glucagon and vasopressin were administered (Fig. 1c), and that it was not until the hormones had been withdrawn was there any indication of a diminution in the rate of P_i uptake.

In a previous report (Siess *et al.*, 1984) it was suggested that glucagon exerts its action on hepatocyte P_i fluxes, not so much by inducing P_i influx but rather by preventing P_i efflux. Although our data are consistent with this suggestion, it would appear that the actions of glucagon and vasopressin on plasma-membrane P_i fluxes are dissimilar. For instance, a comparison of the data in Fig. 1(c) with those of Fig. 2, where the only difference is the concentration of perfusate Ca^{2+} , reveals that when the perfusate Ca^{2+} is low vasopressin is ineffective in maintaining maximal rates of net P_i uptake. This suggests that, in terms of the mechanism by which the two hormones are involved in inducing P_i uptake, any action of vasopressin is indirect only and results essentially from its ability to mobilize Ca^{2+} (Altin & Bygrave,

1988). It is noteworthy too that, although vasopressin induces net loss of perfusate P_i (Fig. 1b), this action does not result in any change in mitochondrial P_i concentration (Table 1).

Finally, the molecular mechanism(s) by which the synergistic action of glucagon and the Ca^{2+} -mobilizing hormone leads to the massive accumulation of P_i in the subsequently isolated mitochondria is, like that of Ca^{2+} accumulation (see Altin & Bygrave, 1988) and PP_i accumulation (see Davidson & Halestrap, 1988), a matter of conjecture. The relevance to this of reports (see Halestrap, 1989, for a review) that glucagon stimulates energy-linked reactions in mitochondria isolated from rat livers previously treated with glucagon, for instance, remains to be determined.

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