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Inorganic pyrophosphate is located primarily in the mitochondria of the hepatocyte and increases in parallel with the decrease in light-scattering induced by gluconeogenic hormones, butyrate and ionophore A23187

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1. The effects of a variety of hormones on the PP_i content and light-scattering of isolated rat liver cells was studied. 2. The basal PP_i content was about 130 pmol/mg of cell protein, and increased after hormone addition, in parallel with a decrease in light-scattering which we have observed previously [Quinlan, Thomas, Armston & Halestrap (1983) Biochem. J. **214**, 395–404]. 3. The mean increases in PP_i content with the agonists shown (as pmol/mg of protein) were: 0.1 μM-glucagon, 25; 20 μM-phenylephrine, 30; 25 nM-vasopressin, 127; glucagon + phenylephrine, 115; glucagon + vasopressin, 382; 100 μM-ADP, 50; 15 μM-A23187, 72; 1 mM-butyrate, 80. 4. In the absence of extracellular Ca^{2+}, vasopressin had little effect on either the PP_i content or the light-scattering of hepatocytes. 5. The magnitude of the increase in PP_i content correlated with that of the decrease in light-scattering irrespective of the stimulating agent, provided that the PP_i did not exceed 300 pmol/mg of protein. Above this value little additional change in light-scattering was observed. 6. Subcellular fractionation showed that over 90% of the cellular PP_i was intramitochondrial in both control and stimulated cells. 7. The data support the conclusions of previous experiments using isolated liver mitochondria [Davidson & Halestrap (1987) Biochem. J. **246**, 715–723] that hormones increase the mitochondrial matrix volume through a Ca^{2+}-induced rise in matrix [PP_i]. 8. It is further proposed that this increase in mitochondrial [PP_i] allows entry of ADP into the mitochondria in exchange for PP_i and is therefore responsible for the increase in total mitochondrial adenine nucleotides observed after hormone treatment.

INTRODUCTION

Addition of hormones such as vasopressin, phenylephrine and glucagon to hepatocytes causes an increase in the matrix volume of their mitochondria (Quinlan *et al.*, 1983; Halestrap *et al.*, 1986). We have proposed that these volume changes are important in the hormonal stimulation of several mitochondrial processes, including respiration, pyruvate carboxylation, glutaminase and fatty acid oxidation (see Halestrap, 1986, 1988; Halestrap & Dunlop, 1986; Halestrap *et al.*, 1985). A similar increase in mitochondrial matrix volume can be induced in isolated liver mitchondria by exposing them to Ca^{2+} at concentrations similar to those found in the cytosol of hormone-stimulated hepatocytes (Halestrap *et al.*, 1986). The increase in matrix volume is associated with a Ca^{2+}-induced increase in intramitochondrial [PP_i], which is able to increase the permeability of the mitochondrial inner membrane to K^+ through an interaction with the adenine nucleotide translocase (Davidson & Halestrap, 1987). In the present paper we investigate the metabolism and compartmentation of PP_i within the intact hepatocyte. We show that PP_i is almost entirely mitochondrial (> 90%) and is increased in cells stimulated with gluconeogenic hormones in parallel with the observed decrease in light-scattering. Both processes are dependent on the presence of extracellular Ca^{2+}.

EXPERIMENTAL

Materials

Rats. Male Wistar rats (300–350 g) were starved for 24 h but allowed free access to water before preparation of hepatocytes.

Chemicals. Unless otherwise stated, the sources of all chemicals and biochemicals were the same as given by Quinlan *et al.* (1983) or Davidson & Halestrap (1987).

Methods

Preparation and incubation of isolated liver cells. Liver cells were prepared as described by Quinlan *et al.* (1983), and were at least 90% viable as judged by exclusion of Trypan Blue. Unless otherwise stated, all incubations were carried out in bicarbonate-buffered saline medium (Krebs & Henseleit, 1932) containing 20 mg of defatted dialysed bovine serum albumin/ml, supplemented with 1 mM-oleate, 10 mM-L-lactate, 1 mM-pyruvate and 2.5 mM-$CaCl_2$. Hepatocytes were preincubated under an atmosphere of O_2/CO_2 (19:1) for 30 min at 5–6.5 mg of cell protein/ml and 37 °C in an orbital shaking water bath.

Measurement of light-scattering and cell PP_i content. After preincubation, two 3.5 ml samples of cell suspension were transferred to sample and reference cuvettes of a split-beam spectrophotometer for measurement of light-scattering changes at 520 nm as described by Quinlan *et al.* (1983). After an appropriate addition to the sample cuvette, light-scattering was usually monitored until a new plateau was reached, whereupon two 1 ml samples were taken from each cuvette for cell PP_i determination. In Ca^{2+}-free experiments, Ca^{2+} was

omitted from the incubation medium and 0.1 mM-K^+EGTA was added to both cuvettes 2 min before hormone addition. Sedimentation of the cells through an oil layer (30 s at 8000 *g*), extraction in $HClO_4$, neutralization and enzymic determination of PP_i were carried out in the same manner as described for previous experiments with mitochondria (Davidson & Halestrap, 1987).

Sub-fractionation of hepatocytes. In one series of experiments, mitochondrial PP_i content was determined after rapid disruption of the hepatocytes by a combination of digitonin and shear force as described by Hoek *et al.* (1980). Digitonin (0.3 mg/mg of cell protein) was rapidly mixed with 2 ml of the cell suspension and placed into the 2.5 ml plastic syringe of a specially adapted Eppendorf 5414 centrifuge. This contained a mixing chamber revolving at 10000 rev./min, into which fitted a 25-gauge needle attached to the syringe. The cell suspension + digitonin were forced through the needle with a 2 kg lead weight, and the resulting disrupted cells were passed from the mixing chamber through a T-tube into two 1.5 ml plastic conical centrifuge tubes containing 100 μl of $HClO_4$ under a layer of silicone oil as described above. Centrifugation was continued for 1 min to ensure sedimentation of mitochondria. Correction was made for co-sedimentation of undisrupted cells and breakage of mitochondria by measuring the activity of lactate dehydrogenase (Keiding *et al.*, 1974) and citrate synthase (Srere *et al.*, 1963) respectively. These were assayed in the supernatant after cell disruption and compared with the total activities in equivalent cell suspensions incubated with 0.3 mg of digitonin/mg of cell protein or Triton X-100 (0.5%, v/v) for 30 min at 37 °C.

RESULTS

Effects of hormones and other effectors on hepatocyte PP_i content and light-scattering

In previous work we have shown that small changes in light-scattering, induced by gluconeogenic hormones, valinomycin and A23187, correlate well with changes in the mitochondrial matrix volume of hepatocytes measured *in situ* or after rapid cell disruption (Quinlan *et al.*, 1983; Halestrap *et al.*, 1986). In Fig. 1 we show typical time courses of the light-scattering changes observed after exposure of hepatocytes to 0.1 μM-glucagon, 20 μM-phenylephrine or 25 nM-vasopressin, alone or in combination, and the corresponding changes in whole-cell [PP_i]. The [PP_i] rises in parallel with the decrease in light scattering. We have not performed detailed studies on the dependence of changes in tissue PP_i content on hormone concentration. However, we have confirmed that higher concentrations of hormone than used in the present study failed to give an additional increase in PP_i, as would be expected from our previous studies on the hormone-concentration dependence of the light-scattering response (Quinlan *et al.*, 1983). The mean changes in both light-scattering and PP_i induced by hormones and other agents after equilibrium was reached are given in Table 1, where data are included from experiments on many different preparations of hepatocytes. Basal PP_i content was 130±7 pmol/mg of cell protein (mean±S.E.M. for 20 different cell preparations). Both glucagon and phenylephrine produced a small but significant increase in cell PP_i content of about 20%, accompanied by similar net changes in light-scattering. However, the temporal increase in both parameters with the various hormones was markedly different (Fig. 1).

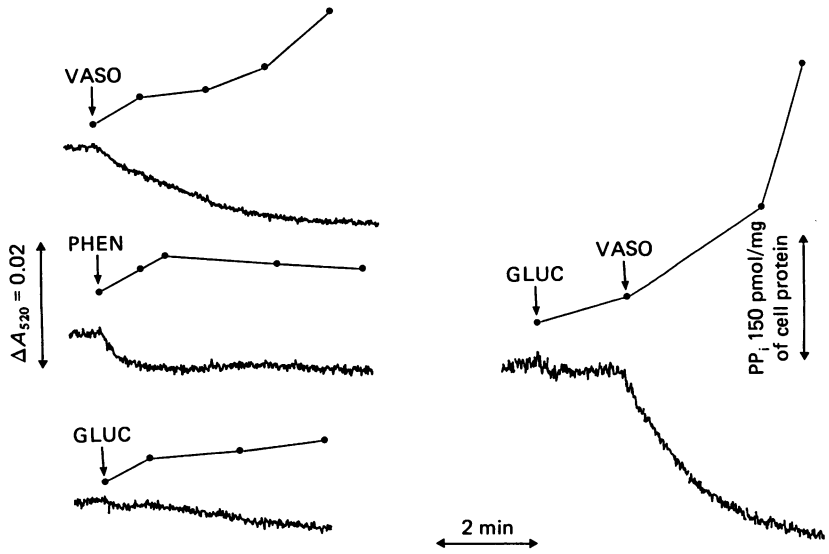

Fig. 1. Time courses of the changes in PP_i and light-scattering in response to hormones

Hepatocytes (5–6.5 mg of cell protein/ml) were preincubated for 30 min at 37 °C, under O_2/CO_2 (19:1) in an orbital shaking water bath as described in the Experimental section. After addition of hormone, duplicate 1 ml samples were withdrawn at various times for PP_i determination (●). Parallel incubations were carried out under identical conditions in the split-beam spectrophotometer, and the light-scattering changes were recorded after addition of hormone to one cuvette (lower traces). The time courses shown are representative of those measured for the effect of each hormone on at least three separate cell preparations. Concentrations of hormones were as follows: 25 nM-vasopressin (VASO), 25 μM-phenylephrine (PHEN) and 0.1 μM-glucagon (GLUC).

Table 1. Mean values of PP_i content and light-scattering changes in control and hormone-treated hepatocytes

Hepatocytes were incubated in both sample and reference cuvettes of a split-beam spectrophotometer for monitoring of light scattering. The $CaCl_2$ concentration in the incubation medium was 2.5 mM, except where otherwise stated. After addition(s) to the sample cuvette, samples of incubation medium were withdrawn from both cuvettes at the cessation of light-scattering changes for the determination of PP_i content in control and hormone-treated cells. Full details are given in the Experimental section. The final concentrations of the additions were as follows: 0.1 μM-glucagon, 20 μM-phenylephrine, 25 nM-vasopressin, 1 mM-sodium butyrate, 15 μM-A23187 and 100 μM-ADP. Where two hormones were added, the cells were incubated with glucagon for 2 min before addition of the second hormone. Values are expressed as means ± S.E.M. for the numbers of preparations shown. The statistical significance of the difference between control and hormone (or other effector)-treated cells was determined by paired Student's t test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Addition	No. of cell preparations	PP_i (pmol/mg of cell protein)			Light-scattering changes
		Control	After addition	Increment (ΔPP_i)	($10^3 \times \Delta A_{520}$)
(*a*) Hormones:					
Glucagon	9	143±9	168±13	25±7*	6.4±1.3***
Phenylephrine	5	137±14	167±18	30±7*	7.4±1.1**
Vasopressin	18	129±8	256±15	127±16***	21.9±1.8***
Vasopressin, zero Ca^{2+}	6	79±6	86±16	7±13	3.5±1.0*
Glucagon + phenylephrine	5	148±12	264±20	115±10***	24.6±1.4***
Glucagon + vasopressin	12	127±9	509±48	382±52***	30.7±1.6***
(*b*) Other effectors:					
Butyrate	5	121±10	200±27	80±22*	6.6±0.6***
A23187	4	114±10	183±17	69±12*	14.9±2.4**
ADP	3	126±11	176±10	50±4**	22.7±0.5**

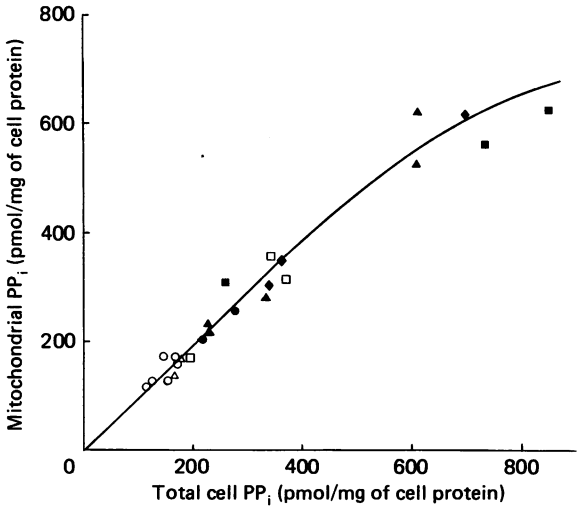

Fig. 2. Correlation between the total and mitochondrial PP_i content of intact hepatocytes incubated under various conditions

Hepatocytes were incubated in a split-beam spectrophotometer, and samples were taken for the measurement of whole-cell PP_i content and for rapid cell disruption and the measurement of mitochondrial PP_i as described in the Experimental section. The time of incubation after additions was varied to provide as wide a range of values as possible. The symbols indicate the presence or absence of additions (added at the concentrations shown in Table 1) as follows: no additions (○), butyrate (●), vasopressin (□), glucagon (△), glucagon + vasopressin (■), glucagon + phenylephrine (▲), A23187 (◆).

With glucagon there was a delay of 60–90 s after addition of hormone before initiation of the slow swelling as reported previously (Quinlan *et al.*, 1983; Halestrap *et al.*, 1986), and PP_i accumulated gradually over that period. In contrast, phenylephrine produced an initial period of rapid swelling which was complete after 1–2 min, and maximal PP_i values were attained at around 90 s. Vasopressin induced a much larger response in both light-scattering and PP_i content compared with either glucagon or phenylephrine. Pretreatment of the hepatocytes with glucagon before addition of phenylephrine or vasopressin had a synergistic effect on the increase in PP_i and the decrease in light-scattering induced by the hormones.

ADP and ATP have been reported to increase cytosolic $[Ca^{2+}]$ in hepatocytes (Bellomo *et al.*, 1984; Sistare *et al.*, 1985; Staddon & McGivan, 1985) by binding to P_2 purinergic receptors and initiating phosphatidylinositol 4,5-bisphosphate breakdown (Charest *et al.*, 1985*a*). We found that ADP at 100 μM induced light-scattering changes which were as large as those seen with vasopressin, and there was also a significant increase in PP_i (Table 1). Glucagon also potentiated the PP_i increase and light-scattering changes induced by low concentrations (10 μM) of ADP, from 11±7 to 174±8 pmol/mg of protein and ΔA_{520} from $10.4(\pm 1.1) \times 10^{-3}$ to $24.1(\pm 1.1) \times 10^{-3}$ respectively (means ± S.E.M. for three cell preparations; $P < 0.01$ by paired Student's t test).

Intracellular location of PP_i

To ascertain the intracellular distribution of the observed PP_i changes, we used a rapid fractionation technique based on disruption of the cells by digitonin

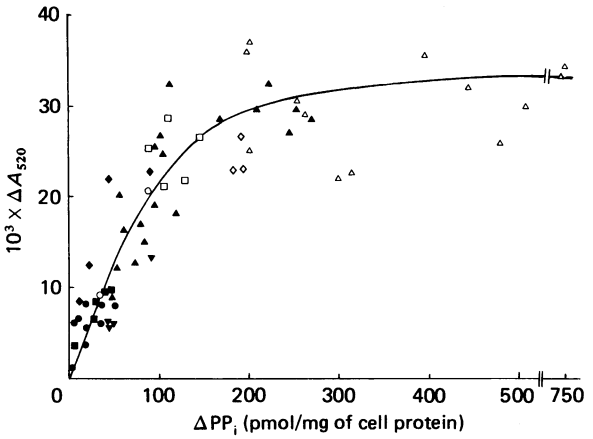

Fig. 3. Correlation between the increase in cell PP_i (ΔPP_i) and decrease in light-scattering (ΔA_{520})

Light-scattering and cell PP_i measurements were carried out after addition of hormones and/or other effectors, exactly as described in Table 1. Additions were as follows: 1 mM-butyrate (▼), 0.1 μM-glucagon (●), 20 μM-phenylephrine (■), 25 nM-vasopressin (▲), 10 μM- or 100 μM-ADP (◆), 15 μM-A23187 (○), 0.1 μM-glucagon + 20 μM-phenylephrine (□), 0.1 μM-glucagon + 25 nM-vasopressin (△), 0.1 μM-glucagon + 10 μM-ADP (◇). In the last three conditions the second effector was added 2 min after glucagon.

and shear force (see under 'Methods'). Mitochondrial PP_i was 92.1 ± 2.6% (mean ± S.E.M.) of hepatocyte PP_i in the 18 hormone-stimulated conditions and 102.3 ± 5.8% in the six controls. In Fig. 2 we show the strong correlation between measured values of whole-cell PP_i and mitochondrial PP_i under all conditions studied. Thus we may conclude that the increase in PP_i caused by hormones is almost exclusively mitochondrial. Only at very high concentrations of PP_i, such as those seen with vasopressin added in the presence of glucagon, was there evidence for a significant proportion (20%) of the PP_i being non-mitochondrial.

Dependence on extracellular Ca^{2+}

We have previously demonstrated that Ca^{2+} is important in the action of hormones on the mitochondrial volume (Quinlan *et al.*, 1983; Halestrap & Quinlan, 1986). In the present experiments (Table 1) we show that the increase in PP_i induced by vasopressin is also greatly diminished in the absence of extracellular Ca^{2+} in parallel with the diminished light-scattering response. Conversely, addition of the Ca^{2+} ionophore A23187 in the presence of 2.5 mM-$CaCl_2$ mimicked the action of vasopressin on both light-scattering and PP_i content (Table 1 and Fig. 3).

Essential role of PP_i in modulating the mitochondrial matrix volume

In isolated mitochondria, we demonstrated that Ca^{2+}-mediated changes in PP_i concentration and not Ca^{2+} itself were responsible for swelling (Davidson & Halestrap, 1987), by utilizing the facility of butyrate to undergo activation to its CoA derivative within the mitochondrial matrix, with a consequent increase in matrix PP_i (Aas & Bremer, 1968; Otto & Cook, 1982). This allows an increase in mitochondrial PP_i to be produced independently of a change in $[Ca^{2+}]$. In the current study, addition of 1 mM-butyrate increased hepatocyte PP_i content significantly and produced a net decrease in light-scattering similar to that seen with glucagon or phenylephrine (Table 1). Furthermore, the data of Fig. 3 show that, irrespective of the agonist used, there was a good and approximately linear relationship between the decrease in light-scattering of hepatocytes and the increase in their PP_i content, provided that the latter increase did not exceed about 150 pmol/mg of cell protein. However, when the change in PP_i exceeded this value, for example in the presence of glucagon and vasopressin together, little additional increase in light-scattering was observed. It is possible that, under these conditions, sufficient matrix PP_i accumulates to result in precipitation of $MgPP_i$ or $CaPP_i$, both of which have very low solubility products (Wiers, 1971; Veech *et al.*, 1980).

DISCUSSION

Subcellular location of PP_i and its hormonal regulation

In a previous paper (Davidson & Halestrap, 1987) we demonstrated that freeze-clamped livers of rats treated with glucagon or phenylephrine show a highly significant increase in tissue PP_i, from a control value of about 10 nmol/g wet wt to about 14 and 12 nmol/g wet wt. in the presence of the respective hormones. If it is assumed that all this PP_i is mitochondrial, then from the known mitochondrial content of liver it can be calculated that the mitochondrial PP_i would be about 130 pmol/mg of protein, compared with a measured value of about 110 pmol/mg of protein for isolated mitochondria (Davidson & Halestrap, 1987). These data suggest that much of the PP_i in the hepatocyte is intramitochondrial. This might be expected, since degradation of PP_i is regarded as an essential feature of those biosynthetic pathways which produce it, and the cytosol of liver contains a very active pyrophosphatase with a low K_m for PP_i (Shatton *et al.*, 1981). The cytosolic $[PP_i]$ has been calculated to be of the order of 2 nmol/g wet wt. of liver (i.e. about 5 μM) if the enzyme UTP–glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) is assumed to be in equilibrium in the cytosol (Guynn *et al.*, 1974; Veech *et al.*, 1980). This low value would also imply a mitochondrial location for most of the PP_i.

In this paper we have extended our previous studies on the PP_i content of freeze-clamped livers by showing that treatment of isolated hepatocytes with hormones, other Ca^{2+}-mobilizing agents such as A23187 and ADP, or with butyrate all caused an increase in whole-cell PP_i whose magnitude correlated with the observed decrease in light-scattering response. The time courses of both responses were also similar (Fig. 1). Furthermore, using a rapid cell-disruption technique we have confirmed that we can account for almost all of the intracellular PP_i in the mitochondrial fraction in both the control and hormone-stimulated cells (Fig. 2). These results strongly support our previous conclusion, derived from experiments with isolated mitochondria, that the swelling of mitochondria is secondary to a rise in matrix $[PP_i]$. PP_i is believed to interact with the mitochondrial adenine nucleotide carrier, causing it to act as an electrogenic K^+ channel (Davidson & Halestrap, 1987). This inter-

action with the adenine nucleotide transporter would also have the effect of allowing exchange of matrix PP_i with cytosolic ADP, leading to the net uptake of adenine nucleotides observed after hormone treatment (Bryla *et al.*, 1977; Siess *et al.*, 1977; Barritt *et al.*, 1978; Titheradge *et al.*, 1979; Titheradge & Haynes, 1980; Aprille *et al.*, 1982; Soboll & Scholz, 1986).

Role of Ca^{2+}

Vasopressin and phenylephrine increase cytosolic [Ca^{2+}] through two mechanisms (see Reinhart *et al.*, 1984; Exton, 1985). There is an initial and rapid release of Ca^{2+} from the endoplasmic reticulum as the result of an increase in inositol 1,4,5-trisphosphate derived from the hormone-induced breakdown of phosphatidylinositol 4,5-bisphosphate. This rise in [Ca^{2+}] is only transient unless extracellular Ca^{2+} is present, since Ca^{2+} will be lost from the cell (Charest *et al.*, 1985*b*; Joseph *et al.*, 1985). However, there is a second phase which involves Ca^{2+} entry from outside and may lead to a net uptake of Ca^{2+} into the cell (Reinhart *et al.*, 1984; Mauger *et al.*, 1984, 1985; Altin & Bygrave, 1985, 1986, 1987; Morgan *et al.*, 1983, 1984). The increase in cytosolic [Ca^{2+}] is translated into an increase in mitochondrial [Ca^{2+}], which can be detected by the observed activation of Ca^{2+}-sensitive enzymes within the mitochondria (McCormack, 1985*a*,*b*; Assimacopoulos-Jeannet *et al.*, 1986; Quinlan & Halestrap, 1986; Staddon & Hansford, 1986, 1987). Previously experiments on the increase in mitochondrial volume caused by vasopressin and phenylephrine showed them to be dependent on extracellular Ca^{2+} (Quinlan *et al.*, 1983) and to involve net uptake of Ca^{2+} from the medium (Halestrap *et al.*, 1986). Furthermore, we have shown that addition of Ca^{2+} at concentrations similar to those found in the cytosol of hormone-stimulated cells can induce increases in [PP_i] and matrix volume in isolated liver mitochondria similar to those observed *in situ* after hormone treatment of hepatocytes (Halestrap *et al.*, 1986; Davidson & Halestrap, 1987).

Our present results establish a similar link between increases in matrix [Ca^{2+}], matrix [PP_i] and mitochondrial swelling in the intact hepatocyte. Thus the removal of extracellular Ca^{2+} not only greatly decreased the light-scattering response induced by vasopressin but also largely abolished the increase in PP_i. The increase in cellular [Ca^{2+}] caused by phenylephrine or vasopressin is greatly enhanced when glucagon (or cyclic AMP) are added together. Under these conditions substantial net uptake of Ca^{2+} into the liver and their mitochondria can be observed (Mauger *et al.*, 1985; Poggioli *et al.*, 1986; Altin & Bygrave, 1986, 1987; Morgan *et al.*, 1983, 1984; Assimacopoulos-Jeannet *et al.*, 1986), and our present results show clearly that this is accompanied by increases in [PP_i] that are more than additive. The light-scattering response is also greatly enhanced (Table 1 and Fig. 3), but when the [PP_i] exceeded about 300 pmol/mg of cell protein no further light-scattering change was observed (Fig. 3). A similar phenomenon was observed in isolated mitochondria incubated with 0.6 mM-butyrate and then exposed to 0.9 μM-Ca^{2+}. Matrix [PP_i] rose to values as high as 3000 pmol/mg of protein, but the light-scattering response was abolished or reversed (Davidson & Halestrap, 1987). This is thought to be a consequence of the precipitation of either $MgPP_i$ or $CaPP_i$, both of which have very low solubility products (Wiers, 1971; Veech *et al.*, 1980). In similar experiments on hepatocytes we have shown that if vasopressin is added after 1 mM-butyrate the decrease in light-scattering was only 30% of that in the absence of butyrate. In contrast, the increase in PP_i was 661 pmol/mg of cell protein in the presence of butyrate, compared with 84 pmol/mg in its absence. In the presence of both butyrate and vasopressin the total PP_i content of hepatocytes in two experiments was 863 and 834 pmol/mg of cell protein. This is equivalent to about 2600 pmol/mg of mitochondrial protein, very similar to the values reached in the isolated mitochondrial experiments with Ca^{2+} and butyrate.

Taken together, the data that we have obtained strongly support a role for a Ca^{2+}-induced increase in mitochondrial [PP_i] in the action of vasopressin and phenylephrine on the mitochondrial volume. With glucagon the increase in matrix volume also appears to correlate with an increase in matrix PP_i, but there are several observations that are not compatible with Ca^{2+} inducing this change. Firstly, a significant light-scattering response is still observed with glucagon in the absence of Ca^{2+} (Quinlan *et al.*, 1983; Halestrap *et al.*, 1986). Secondly, the rise in motochondrial and cytoplasmic [Ca^{2+}] is less than with phenylephrine, and is more transient (Quinlan & Halestrap, 1986), yet changes in PP_i are similar. Thirdly, the rise in cytosolic and mitochondrial [Ca^{2+}] with glucagon can be detected within 6 s and is maximal at less than 1 min (Sistare *et al.*, 1985; Quinlan & Halestrap, 1986; Staddon & Hansford, 1986, 1987). However, the light-scattering response is not detected before 1 min and is greatest when the increase in mitochondrial NAD(P)H fluorescence has returned to basal values (Quinlan *et al.*, 1983; Quinlan & Halestrap, 1986; Halestrap *et al.*, 1986). Thus it is probable that glucagon is increasing mitochondrial PP_i by an alternative mechanism.

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