Pyrophosphate metabolism in the perfused heart and isolated heart mitochondria and its role in regulation of mitochondrial function by calcium

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1. Langendorff-perfused rat hearts were treated, either alone or in combination, with acetate, adrenaline or supraphysiological $[Ca^{2+}]$ to mimic increased workload. No significant increases in the PP_i content of the freeze-clamped tissue were observed. 2. In contrast with liver mitochondria, isolated rat heart mitochondria incubated at a free $[Mg^{2+}]$ of 1.5 mM showed little or no increase in matrix PP_i content or volume in the presence of 0.75 μM Ca^{2+} ; neither did 5 mM acetate cause heart mitochondrial PP_i to increase. 3. At 0.1 mM Mg^{2+} some increase in matrix PP_i content of heart mitochondria was observed, and increases in liver mitochondria were significantly greater. 4. Substantial increases in heart mitochondrial-matrix PP_i content were observed at 1.5 mM Mg^{2+} when $[Ca^{2+}] > 1 \mu M$, especially in the presence of acetate, or at 0.75 μM Ca^{2+} in the presence of bongkrekic acid to inhibit the adenine nucleotide translocase. 5. Total pyrophosphatase activity was similar in heart and liver mitochondrial

matrix extract and toluene-permeabilized mitochondria. 6. These data suggest that under physiological conditions Ca^{2+} does not regulate heart mitochondrial PP_i , because higher matrix $[Mg^{2+}]$ overcomes the inhibition of pyrophosphatase by Ca^{2+} , and also significant loss of PP_i from the matrix on the adenine nucleotide translocase may occur. 7. It is concluded that, in contrast with the situation in liver [Halestrap (1989) *Biochim. Biophys. Acta* **973**, 355–382], Ca^{2+} -induced PP_i -mediated increases in mitochondrial volume are unlikely to play a physiological role in the heart. 8. The substantial swelling of liver and kidney mitochondria caused by addition of supraphysiological $[Ca^{2+}]$ (20 nmol/mg of protein) was associated with significant increases in PP_i content, but was not observed in heart mitochondria. 9. This substantiates the role that we have proposed for PP_i in the opening of a non-specific pore by Ca^{2+} overload of mitochondria [Halestrap and Davidson (1990) *Biochem. J.* **268**, 153–160].

INTRODUCTION

Treatment of the perfused liver (Yamada et al., 1988; Inoue et al., 1989) and isolated hepatocytes (Davidson and Halestrap, 1988) with hormones that raise intracellular $[Ca^{2+}]$, or exposure of isolated mitochondria to physiological concentrations of Ca^{2+} (Davidson and Halestrap, 1987), leads to a substantial increase in mitochondrial-matrix $[PP_i]$. This occurs as the result of the inhibition of mitochondrial pyrophosphatase by Ca^{2+} , an effect mediated by the formation of $CaPP_i$, which is a very potent competitive inhibitor of the enzyme with respect to $MgPP_i$ (Davidson and Halestrap, 1989; Baykov et al., 1989a). The increases in matrix $[PP_i]$ are greatly potentiated by the presence of acetate or butyrate, whose intramitochondrial activation to acetyl- and butyryl-CoA produces PP_i (Aas and Bremer, 1968; Otto and Cook, 1982). We have proposed that matrix $[PP_i]$ plays an important role in the regulation of mitochondrial function in the liver under both physiological and pathological conditions (Halestrap, 1989, 1993; Halestrap et al., 1992).

In the physiological setting, hormones that increase cytosolic $[Ca^{2+}]$ also cause an increase in mitochondrial-matrix volume, which can have a profound stimulatory effect on mitochondrial metabolism (see Halestrap, 1989; Halestrap et al., 1990; McCormack et al., 1990). A rise in matrix $[PP_i]$ may be responsible, at least in part, for this increase in matrix volume (Davidson and Halestrap, 1987, 1988, 1990; Halestrap and Davidson, 1989, 1990). We have suggested that intramitochondrial PP_i binds to the adenine nucleotide translocase, displacing adenine nucleotides and facilitating the electrogenic transport of K^+ through the carrier (Davidson and Halestrap, 1987; Halestrap and Davidson, 1989, 1990). This would account for the observed swelling, and also, by PP_i exchange with extramitochondrial adenine nucleo-

tides, for the observed increase in total matrix adenine nucleotide concentration (see Halestrap, 1989). In the pathological setting, calcium overload of mitochondria leads to the formation of a non-specific pore in the mitochondrial inner membrane and a consequent loss of mitochondrial integrity (see Crompton, 1990; Gunter and Pfeiffer, 1990; Halestrap, 1991). This process is enhanced by adenine nucleotide depletion and is regulated by the conformational state of the adenine nucleotide translocase (see Halestrap and Davidson, 1990; Griffiths and Halestrap, 1991). We have proposed that the formation of mitochondrial PP_i under conditions of Ca^{2+} overload, and its subsequent binding to the translocase, may play an important role in the mechanism of pore opening (Halestrap and Davidson, 1990; Griffiths and Halestrap, 1991).

Most studies on mitochondrial PP_i metabolism have been performed on the perfused liver, isolated hepatocytes and liver mitochondria. Regulation of heart mitochondrial function by PP_i has not been investigated. However, it is known that the respiratory chain and fatty acid β -oxidation can be activated by small changes in the matrix volume of isolated heart mitochondria (Halestrap, 1987). This has the potential to play an important role in the regulation of cardiac respiration by hormones and increased workload, a role complementary to that of the activation of key mitochondrial dehydrogenases by Ca^{2+} (Halestrap, 1987; McCormack et al., 1990). In the present paper we show that Langendorff-perfused hearts treated, either alone or in combination, with acetate, adrenaline or supraphysiological $[Ca^{2+}]$ to mimic increased workload do not show significant increase in tissue PP_i . In isolated heart mitochondria an increase in matrix PP_i was only observed in the presence of both acetate and supraphysiological $[Ca^{2+}]$. These data argue against a physiological role for mitochondrial PP_i in the heart. Fur-

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thermore, we demonstrate that heart mitochondria are much more resistant to the damaging effects of loading with high concentrations of Ca^{2+} than are liver and kidney mitochondria. This correlates with the lack of an effect of the Ca^{2+} loading on heart mitochondrial PP_i content.

EXPERIMENTAL

Materials

Rat heart and liver mitochondria were prepared as described previously, by using Percoll-gradient centrifugation to remove contaminating membrane fractions (Halestrap, 1987; Whipps et al., 1987). Kidney cortex mitochondria were prepared from rat kidneys (after removal of the medulla), by a technique identical with that used for preparation of liver mitochondria. Cyclosporin A was kindly given by Sandoz, Basel, Switzerland. The sources of other biochemicals and chemicals were as cited previously (Davidson and Halestrap, 1987, 1990).

Methods

Heart perfusion

Hearts were removed from female Wistar rats (200–250 g) and immediately perfused with Krebs/Henseleit bicarbonate-buffered saline containing 11 mM glucose and 1.2 mM Ca^{2+} , by a modified Langendorff procedure (Broadley, 1979). When required, after 15 min perfusion hearts were switched to buffer containing 5 μM adrenaline, 5 mM acetate or high (4.8 mM) $[\text{Ca}^{2+}]$. At the end of the required perfusion period, ventricles were cut from the heart (while still on the cannula) and immediately freeze-clamped in tongs pre-cooled in liquid nitrogen. Hearts were then stored at -70°C overnight.

Extraction of frozen tissue

Frozen tissue was ground to a fine powder under liquid nitrogen before homogenization in 5% (w/v) HClO_4 (4 ml/g wet wt.). The extract was centrifuged at 12000 *g* for 2 min and the supernatant neutralized with 5 M KOH containing 0.5 M Mops and 20 mM EDTA. After standing on ice for 30 min, the precipitate of KClO_4 was removed by centrifugation and the supernatant used for assay of metabolites.

Incubation of mitochondria

Unless otherwise stated, mitochondria were incubated in an orbital shaking water bath at 37°C in buffer containing 125 mM KCl, 5 mM NaCl, 10 mM Mops, 7 mM Tris base, 0.5 mM EGTA, 2.5 mM potassium phosphate, 3.5 mM MgCl_2 , 1 mM potassium L-malate, 5 mM sodium L-glutamate, 1 mM ATP and 5 mg/ml dialysed defatted BSA. The buffer was equilibrated with 100% O_2 before addition of the BSA. In most experiments the necessary volume of standardized 250 mM CaCl_2 to give the required free $[\text{Ca}^{2+}]$ was added after 2 min as described previously (Davidson and Halestrap, 1987). This procedure produced a slight fall in pH (0.07 or less), which was taken into account in the calculation of free $[\text{Ca}^{2+}]$. The free $[\text{Mg}^{2+}]$ was calculated to be 1.5 mM in the standard incubation buffer. In some experiments CaEGTA/EGTA buffers (2 mM total [EGTA] present from the start of the incubation) were used to control the free $[\text{Ca}^{2+}]$ as described elsewhere (Davidson and Halestrap, 1989). At 5 min

after addition of Ca^{2+} , samples for measurement of adenine nucleotides and PP_i were removed and centrifuged through silicone oil into HClO_4 as described previously (Davidson and Halestrap, 1987). Where required, parallel measurements of mitochondrial volume were made by using $[^{14}\text{C}]\text{sucrose}$ and $^3\text{H}_2\text{O}$ as described by Halestrap et al. (1986).

Determination of metabolites

PP_i , ATP, ADP and AMP in neutralized HClO_4 extracts of hearts or mitochondria were determined enzymically by the methods described by Davidson and Halestrap (1987).

Measurement of mitochondrial swelling by using light-scattering

Mitochondria (approx. 2 mg/ml) were incubated in oxygenated buffer containing 125 mM KCl, 10 mM Mops, 7 mM Tris base, 2.5 mM succinate and 2.5 mM potassium phosphate, pH 7.2, at 37°C in a split-beam spectrophotometer as described by Armston et al. (1982) and Halestrap et al. (1986). After 2 min, CaCl_2 (final concn. 50 μM) was added to the sample cuvette and the decrease in A_{520} was monitored. Samples (1 ml) were taken from both sample and reference cuvettes and acidified with 2% (w/v) HClO_4 (final concn.) for measurement of PP_i in the incubation medium.

Statistical analysis

Values are expressed as means $\pm$ S.E.M. The statistical significance of any differences observed was calculated by paired Student's *t* test for mitochondrial experiments and unpaired *t* tests for perfused heart data. Where paired *t* tests are used, the *n* values given are for numbers of observations (each on a different mitochondrial preparation) used to calculate the mean, rather than those used in calculation of the *t* value. This was not always the same, since control conditions were available for all experiments, but not all experimental conditions were included in each individual experiment.

RESULTS AND DISCUSSION

PP_i concentrations in the perfused heart

Table 1 presents data on the effects of 5 min exposure of perfused hearts to 5 mM acetate, 5 μM adrenaline or increased extracellular $[\text{Ca}^{2+}]$, alone or in combination, on their PP_i content. Unlike the situation in liver (Davidson and Halestrap, 1988; Yamada et al., 1988; Inoue et al., 1989), no significant increases in tissue $[\text{PP}_i]$ are observed in response to any of the treatments. Longer (15 min) or shorter (2 min) exposure of the hearts to the effectors gave the same result (results not shown). Adrenaline was observed to stimulate both the rate and the force of contraction as expected (results not shown), and this is reflected in the significant increases in tissue ADP and AMP content. Acetate also caused an increase in tissue AMP, reflecting its intramitochondrial activation to acetyl-CoA and consequent formation of AMP and PP_i (Aas and Bremer, 1968). Measurement of acetyl-CoA synthetase activity showed similar activities of the enzyme to be present in liver and heart mitochondrial matrix (E. J. Griffiths, unpublished work).

Matrix volumes and PP_i content of isolated heart and liver mitochondria

In a total of 11 separate experiments in which PP_i was measured in energized isolated heart mitochondria incubated in the pres-

Table 1 PP_i and adenine nucleotide contents of rat hearts perfused under various conditions

Hearts were perfused by the Langendorff method (with 11 mM glucose and 1.2 mM Ca²⁺) for 15 min before changing to medium containing the additions indicated. Perfusion was continued for 5 min, at which point the heart was removed and immediately freeze-clamped in tongs pre-cooled in liquid nitrogen. The powdered heart was extracted in HClO₄, and metabolites were assayed after deproteinization and neutralization as described in the Experimental section. Results are expressed per g wet wt., as means $\pm$ S.E.M., and statistical significances between treated and control hearts were calculated by Student's *t* test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

	<i>n</i>	PP _i (nmol/g)	ATP (μ mol/g)	ADP (μ mol/g)	AMP (μ mol/g)	ATP + AMP + ADP (μ mol/g)	ATP/ADP
Control	4	3.62 $\pm$ 0.45	2.57 $\pm$ 0.14	0.68 $\pm$ 0.03	0.12 $\pm$ 0.01	3.37 $\pm$ 0.12	3.79 $\pm$ 0.32
5 μ M Adrenaline	3	3.86 $\pm$ 0.72	2.39 $\pm$ 0.03	1.06 $\pm$ 0.04***	0.28 $\pm$ 0.04***	3.73 $\pm$ 0.05*	2.26 $\pm$ 0.09**
5 mM Acetate	3	3.37 $\pm$ 0.10	2.25 $\pm$ 0.03	0.76 $\pm$ 0.09	0.28 $\pm$ 0.03***	3.29 $\pm$ 0.35	3.03 $\pm$ 0.39
4.8 mM Ca ²⁺	4	3.76 $\pm$ 0.58	2.41 $\pm$ 0.09	0.81 $\pm$ 0.10	0.18 $\pm$ 0.05	3.41 $\pm$ 0.19	3.07 $\pm$ 0.28
5 μ M Adrenaline + 5 mM acetate	3	3.54 $\pm$ 0.15	2.33 $\pm$ 0.12	1.15 $\pm$ 0.08***	0.37 $\pm$ 0.05**	3.85 $\pm$ 0.22	2.04 $\pm$ 0.12**
4.8 mM Ca ²⁺ + 5 mM acetate	3	3.41 $\pm$ 1.00	2.17 $\pm$ 0.15	0.94 $\pm$ 0.08**	0.46 $\pm$ 0.13**	3.57 $\pm$ 0.06	2.32 $\pm$ 0.34**

Table 2 PP_i and adenine nucleotide contents of isolated liver and heart mitochondria incubated under various conditions

Mitochondria were incubated at 37 °C in a shaking water bath under energized conditions as described in the Experimental section. After 2 min incubation, 5 mM acetate or 0.43 mM CaCl₂ (to give 0.75 μ M Ca²⁺) were added as indicated. After a further 5 min incubation, mitochondria were sedimented through silicone oil into HClO₄, and the pellet was assayed for metabolites as described in the Experimental section. Results are expressed as means $\pm$ S.E.M., and the statistical significance of control versus treated mitochondria was calculated by paired Student's *t* test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Mitochondria	Treatment	<i>n</i>	PP _i (pmol/mg)	ATP (nmol/mg)	ADP (nmol/mg)	AMP (nmol/mg)	ATP + ADP + AMP (nmol/mg)	ATP/ADP
Liver	Control	5	130 $\pm$ 10	4.17 $\pm$ 0.42	3.14 $\pm$ 0.26	1.94 $\pm$ 0.24	9.25 $\pm$ 0.87	1.33 $\pm$ 0.09
	0.75 μ M Ca ²⁺	5	260 $\pm$ 10**	4.43 $\pm$ 1.00	3.56 $\pm$ 0.10	2.17 $\pm$ 0.04	10.16 $\pm$ 1.13	1.27 $\pm$ 0.28
	5 mM acetate	5	350 $\pm$ 20*	3.59 $\pm$ 0.77	2.73 $\pm$ 0.27	3.21 $\pm$ 0.14***	9.53 $\pm$ 1.05	1.27 $\pm$ 0.29
	0.75 μ M Ca ²⁺ + 5 mM acetate	5	5518 $\pm$ 100**	4.03 $\pm$ 0.68	2.91 $\pm$ 0.21	2.51 $\pm$ 0.17	9.44 $\pm$ 0.58	1.43 $\pm$ 0.27
Heart	Control	4	180 $\pm$ 20	3.58 $\pm$ 0.06	3.30 $\pm$ 0.38	2.85 $\pm$ 0.35	9.73 $\pm$ 0.61	1.16 $\pm$ 0.17
	0.75 μ M Ca ²⁺	4	210 $\pm$ 30	4.07 $\pm$ 0.08*	3.82 $\pm$ 0.44	3.35 $\pm$ 0.11	11.23 $\pm$ 0.52**	1.10 $\pm$ 0.12
	5 mM acetate	4	170 $\pm$ 30	3.71 $\pm$ 0.12	3.18 $\pm$ 0.64	3.63 $\pm$ 0.22*	10.51 $\pm$ 0.83	1.04 $\pm$ 0.15
	0.75 μ M Ca ²⁺ + 5 mM acetate	4	170 $\pm$ 40	1.90 $\pm$ 0.15**	3.20 $\pm$ 0.53	3.68 $\pm$ 0.44	9.53 $\pm$ 0.67	0.49 $\pm$ 0.01***

Table 3 Effects of increasing concentrations of Ca²⁺ on the PP_i content of liver and heart mitochondria incubated in the presence or absence of 5 mM acetate

The experimental protocol was the same as for Table 2. However, the free [Ca²⁺] indicated was obtained by adding CaEGTA/EGTA buffers as described in the Experimental section. Results are expressed as means $\pm$ S.E.M. of the numbers of separate experiments shown in parentheses, and the statistical significance of control versus treated mitochondria was calculated by paired Student's *t* test: **P* < 0.05, ***P* < 0.02, ****P* < 0.01.

[Ca ²⁺] (μ M)	Mitochondria ...	Mitochondrial-matrix PP _i (pmol/mg of protein)			
		Liver		Heart	
		Control	5 mM Acetate	Control	5 mM Acetate
0		62 $\pm$ 8 (4)	230 $\pm$ 39 (4)*	106 $\pm$ 13 (4)	96 $\pm$ 11 (3)
0.5		121 $\pm$ 9 (3)*	422 $\pm$ 45 (3)*	137 $\pm$ 21 (3)	151 $\pm$ 11 (3)*
1.0		162 $\pm$ 19 (3)*	3243 $\pm$ 268 (3)***	140 $\pm$ 30 (4)	200 $\pm$ 4 (3)***
10.0		274 $\pm$ 48 (3)**	> 10000	266 $\pm$ 32 (3)*	> 10000

ence of 1.5 mM Mg²⁺_{free}, exposure to 0.75 μ M Ca²⁺ for 5 min gave only a very small increase in matrix PP_i from 150 $\pm$ 15 to 172 $\pm$ 14 pmol/mg of protein (*P* < 0.05). The data of Table 2 show that this effect was much smaller than that seen in liver mitochondria under the same conditions and did not reach statistical significance in the experiments reported in Table 2. Addition of 5 mM acetate to heart mitochondria also failed to give any increase in matrix PP_i, again in marked contrast with the effect seen in liver mitochondria (Table 2). However, the metabolism of acetate by heart mitochondria was confirmed by

the increase in matrix AMP content. Changing the time of exposure to Ca²⁺ or acetate from 5 min to 2 or 10 min had no effect on the matrix PP_i content (results not shown). The data of Table 2 also show a small but significant increase in total adenine nucleotide content of heart mitochondria exposed to 0.75 μ M Ca²⁺. It may be significant that the total adenine nucleotide content of the perfused heart increased by a similar magnitude in response to adrenaline (Table 1). An increase in total mitochondrial adenine nucleotides in response to Ca²⁺ has been observed previously in liver mitochondria (Davidson and Hale-

Table 4 Effect of Ca^{2+} on the matrix volume and PP_i content of liver and heart mitochondria

For measurement of mitochondrial PP_i the experimental protocol was the same as that reported in Table 2. For measurement of matrix volumes, samples were taken from parallel incubations containing $^3\text{H}_2\text{O}$ and ^{14}C sucrose as described in the Experimental section. Results are expressed as means $\pm$ S.E.M. of the numbers of separate experiments indicated in parentheses, and the statistical significance of control versus treated mitochondria was calculated by paired Student's *t* test: * $P < 0.005$.

Mitochondria ...	Liver		Heart	
	Control	0.75 μM Ca^{2+}	Control	0.75 μM Ca^{2+}
Volume ($\mu\text{l}/\text{mg}$)	1.06 $\pm$ 0.05 (6)	1.24 $\pm$ 0.04 (6)*	0.88 $\pm$ 0.08 (5)	0.93 $\pm$ 0.09 (5)
PP_i (pmol/mg)	111 $\pm$ 20 (6)	242 $\pm$ 30 (6)*	161 $\pm$ 24 (5)	184 $\pm$ 33 (5)

strap, 1987; Haynes et al., 1986), and is apparent in the experiments with liver mitochondria reported in Table 2, although not statistically significant. We have interpreted this increase as reflecting an exchange of extramitochondrial adenine nucleotides for intramitochondrial PP_i on the adenine nucleotide translocase (Davidson and Halestrap, 1987; Halestrap, 1989), although other workers have offered alternative explanations (Haynes et al., 1986; Aprile, 1988). Although ATP was routinely added to mitochondria to enhance their stability, we have found the presence of ATP to have no significant effect on the response of their matrix PP_i to 0.75 μM Ca^{2+} . Thus in the absence of added ATP and with 2.5 mM $\text{Mg}^{2+}_{\text{total}}$, matrix PP_i rose from 135 $\pm$ 39 to 198 $\pm$ 27 pmol/mg of protein (means $\pm$ S.E.M. of 5 separate experiments), whereas in the presence of 1 mM ATP and 3.5 mM $\text{Mg}^{2+}_{\text{total}}$ the equivalent values were 159 $\pm$ 40 and 205 $\pm$ 40 pmol/mg of protein.

The data of Table 3 demonstrate that significant increases in heart mitochondrial PP_i content can be induced when the mitochondria are exposed to higher concentrations of Ca^{2+} , especially when 5 mM acetate is also present. Thus increases of 100% and 5000% were observed in the PP_i content of isolated heart mitochondria incubated with 5 mM acetate in the presence of 1 or 10 μM Ca^{2+} respectively. These data were obtained by regulating the extramitochondrial $[\text{Ca}^{2+}]$ with CaEGTA/EGTA buffers, and this may account for the small differences in the absolute PP_i concentrations seen in the two experimental systems. However, considerable variability in heart mitochondrial PP_i content was observed between experiments, for which we have no explanation. Since control and experimental conditions were always paired within an experiment, this did not affect the ability to detect consistent changes in PP_i under appropriate conditions. The major conclusion that can be drawn from Table 3 is that heart mitochondria are less sensitive to effects of $[\text{Ca}^{2+}]$ on matrix PP_i than are liver mitochondria under the conditions used. Indeed, at physiological concentrations of Ca^{2+} , little or no significant change was observed in heart mitochondria, consistent with the lack of any changes in PP_i in the perfused heart (Table 1).

In liver mitochondria we have argued that changes in matrix PP_i are responsible for the changes in mitochondrial volume that are induced by Ca^{2+} (Davidson and Halestrap, 1987; Halestrap, 1989). Thus it would be predicted that under the conditions used an extramitochondrial $[\text{Ca}^{2+}]$ of 0.75 μM would not cause the matrix volume of heart mitochondria to increase. The data of Table 4 substantiate this prediction, while confirming that liver mitochondria do show a 15–20% increase in matrix volume under the same conditions (Halestrap et al., 1986; Davidson and Halestrap, 1987).

The data presented in Tables 1–4 could be explained if heart mitochondria possess a more active pyrophosphatase than do

liver mitochondria, and hence are less susceptible to inhibition of the enzyme by Ca^{2+} . However, measurements of pyrophosphatase activity in matrix extracts of liver and heart mitochondria have shown that the total enzyme activity in both cases is similar (Davidson and Halestrap, 1989). There remain three possible explanations for the tissue difference. Firstly, the relationship between intramitochondrial and extramitochondrial $[\text{Ca}^{2+}]$ might be very different in heart and liver mitochondria. Studies of the activity of the Ca^{2+} -sensitive dehydrogenases within intact energized heart and liver mitochondria incubated under conditions similar to those used here suggest that this is not the case, as does measurement of intramitochondrial $[\text{Ca}^{2+}]$ with fluorescent probes (McCormack, 1985; Denton and McCormack, 1985; Moreno-Sanchez and Hansford, 1988; McCormack et al., 1989; Wan et al., 1989; Walajtys-Rhode et al., 1992). Secondly, the liver mitochondrial matrix might contain more of an endogenous inhibitor of pyrophosphatase or less of an activator. Thirdly, heart mitochondria might transport PP_i out of the matrix more rapidly than can liver mitochondria.

Measurement of pyrophosphatase activity in permeabilized mitochondria gave similar results to those obtained with mitochondrial-matrix extracts (Dubnova and Baykov, 1992; E. J. Griffiths, unpublished work). Thus any endogenous inhibitor would have to be of low molecular mass and be lost during permeabilization. We have been unable to find evidence for such an inhibitor. However, Mg^{2+} is known both to be an activator of the enzyme and to be required for PP_i binding (Davidson and Halestrap, 1989; Ungurte et al., 1989). Indeed, the ability of Ca^{2+} to inhibit the enzyme is highly dependent on $[\text{Mg}^{2+}]$, because it is CaPP_i which competes with MgPP_i for the substrate-binding site (Davidson and Halestrap, 1989; Baykov et al., 1989a). In Table 5 we investigate the effect of varying the extramitochondrial $[\text{Mg}^{2+}]$ on the changes in matrix PP_i content induced by 0.3 μM and 0.5 μM Ca^{2+} . The data show clearly that, in liver mitochondria, basal levels and Ca^{2+} -induced changes in matrix PP_i were greater at lower extramitochondrial $[\text{Mg}^{2+}]$. This is consistent with Mg^{2+} activation of the matrix pyrophosphatase. A similar trend is apparent in heart mitochondria, but it is only at the lowest $[\text{Mg}^{2+}]$ (0.16 mM) that Ca^{2+} -induced changes in PP_i content become significant. These data are consistent with heart mitochondria maintaining a higher matrix $[\text{Mg}^{2+}]$ than liver mitochondria and hence having a more active pyrophosphatase. Little is known about the mechanism of mitochondrial Mg^{2+} transport and regulation (Brierley et al., 1987), but the use of intramitochondrial Mg^{2+} -sensitive dyes in heart mitochondria has confirmed that matrix $[\text{Mg}^{2+}]$ does respond to extramitochondrial $[\text{Mg}^{2+}]$ and that there is little or no concentration gradient across the mitochondrial inner membrane (Rutter et al., 1990; Jung et al., 1990). It has not proved possible to obtain reliable measurements of matrix $[\text{Mg}^{2+}]$ in liver mitochondria by

Table 5 Effect of increasing concentrations of Mg^{2+} on the PP_i content of liver and heart mitochondria incubated in the presence of 0, 0.3 or 0.5 μM Ca^{2+}

The experimental protocol was the same as for Table 3, except that $[Mg^{2+}]$ in the buffer was varied as indicated. The free $[Mg^{2+}]$ given represents that in the absence of Ca^{2+} ; values in the presence of Ca^{2+} were slightly greater, but always by less than 10%. Ca^{2+} was added at the concentration shown by using CaEGTA/EGTA buffers. Results are expressed as means $\pm$ S.E.M. ($n = 4$). Statistical significances were calculated by paired Student's t tests: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus 0 μM Ca^{2+} and corresponding $[Mg^{2+}]$.

Mitochondria	$[Mg^{2+}]$ (mM)		PP_i (pmol/mg of mitochondrial protein)		
	Total	Free	0 μM Ca^{2+}	0.3 μM Ca^{2+}	0.5 μM Ca^{2+}
Liver	1.00	0.16	130 $\pm$ 30	358 $\pm$ 72	542 $\pm$ 61**
	2.15	0.65	119 $\pm$ 21	291 $\pm$ 52*	478 $\pm$ 61**
	3.50	1.43	101 $\pm$ 9	286 $\pm$ 10***	292 $\pm$ 38***
	6.00	3.18	80 $\pm$ 10	131 $\pm$ 17	156 $\pm$ 17
Heart	1.00	0.16	157 $\pm$ 5	252 $\pm$ 72	414 $\pm$ 35*
	2.15	0.65	166 $\pm$ 52	219 $\pm$ 45	265 $\pm$ 43
	3.50	1.43	133 $\pm$ 18	203 $\pm$ 11	209 $\pm$ 18
	6.00	3.18	80 $\pm$ 9	155 $\pm$ 20*	190 $\pm$ 51

Table 6 Effect of bongkreikic acid on the PP_i content of liver and heart mitochondria incubated in the presence and absence of acetate and Ca^{2+}

Mitochondria were incubated at 37 °C in oxygenated buffer as described in Table 2, except that albumin was omitted from the incubation buffer. Bongkreikic acid (10 $\mu g/ml$) was present from the start of the incubation, and acetate and Ca^{2+} were added after 2 min incubation. Samples were taken after 7 min incubation for PP_i determination. Results are expressed as means $\pm$ S.E.M. of the numbers of separate experiments shown in parentheses, and the statistical significance of the effects of bongkreikic acid and Ca^{2+} was determined by paired Student's t test: * $P < 0.05$, ** $P < 0.01$.

Mitochondria ...	Mitochondrial PP_i (nmol/mg of protein)			
	Liver		Heart	
	Control	Bongkreikic acid	Control	Bongkreikic acid
Control	120 $\pm$ 15 (4)	202 $\pm$ 11 (4)*	126 $\pm$ 19 (4)	156 $\pm$ 25 (4)
0.75 μM Ca^{2+}	198 $\pm$ 42 (4)*	1180 $\pm$ 384 (4)**	171 $\pm$ 12 (4)	789 $\pm$ 108 (4)**
5 mM Acetate	—	—	106 $\pm$ 12 (10)	145 $\pm$ 28 (8)

this technique (Rutter et al., 1990), but use of 'null-point' titration suggests that matrix $[Mg^{2+}]$ may be lower in liver mitochondria than in heart mitochondria (Corkey et al., 1986). This would be consistent with our observations on the difference in sensitivity towards $[Ca^{2+}]$ of heart and liver mitochondrial PP_i content.

There is also some support for a role for PP_i transport out of mitochondria in the different response to Ca^{2+} of liver and heart mitochondrial PP_i content. It is known that the only route by which PP_i can leave the mitochondrial matrix is on the adenine nucleotide translocase, in exchange for either ADP or phosphate (Halestrap and Davidson, 1989; Halestrap et al., 1992). Heart mitochondria possess about 5 times more of this carrier than do liver mitochondria (Klingenberg, 1976), and thus might be expected to enable faster loss of PP_i from the matrix. To test this, we have performed experiments with bongkreikic acid, which, unlike carboxyatractyloside, is able to block carrier-mediated exchange of PP_i for either adenine nucleotides or phosphate (Halestrap and Davidson, 1989). These experiments were performed in the absence of albumin, which binds bongkreikic acid very tightly. Data are reported in Table 6, and it is clear that, in the presence of 10 $\mu g/ml$ bongkreikic acid, 0.75 μM Ca^{2+} caused a large increase in matrix PP_i in both liver and heart mitochondria, which was not seen in the absence of the inhibitor. In liver mitochondria bongkreikic acid caused a significant increase in matrix PP_i even in the absence of Ca^{2+} . This is consistent with a more active pyrophosphatase in heart mitochondria as a result of higher matrix $[Mg^{2+}]$ as outlined above. It could also reflect

slower endogenous rates of synthesis of PP_i in heart mitochondria than in liver mitochondria. However, even when 5 mM acetate was added to heart mitochondria the small increase in matrix PP_i caused by bongkreikic acid did not reach statistical significance, although the rise in AMP (Table 2) demonstrates that increased PP_i formation must have occurred. The pathway of PP_i synthesis in the absence of acetate is not established (Halestrap et al., 1992).

Role of PP_i in mitochondrial damage resulting from Ca^{2+} overload

We have also suggested that increases in mitochondrial PP_i may play a role in the damage to mitochondria that occurs when they become overloaded with Ca^{2+} (Crompton, 1990; Davidson and Halestrap, 1990; Griffiths and Halestrap, 1991; Halestrap, 1991). Such conditions occur during reperfusion after a period of ischaemia, and this may play a part in the tissue damage that occurs in ischaemic heart disease or transplantation of organs (see Crompton, 1990). It has been observed previously that heart mitochondria are more resistant to Ca^{2+} overload than are liver and kidney mitochondria (Halestrap, 1991; Schlegel et al., 1991). A possible explanation of this difference might be differences in PP_i metabolism in these mitochondria. The data of Table 7 and Figure 1 suggest that this is the case. Energized liver, heart and kidney-cortex mitochondria incubated in the presence of 2.5 mM phosphate were exposed to about 20 nmol of Ca^{2+}/mg of mitochondrial protein. This caused the liver and kidney mitochondria to swell, as demonstrated by the decrease in light-

Figure 1 Swelling of liver, kidney and heart mitochondria exposed to supraphysiological $[Ca^{2+}]$

Mitochondria (approx. 2 mg of protein/ml) were incubated at 37 °C in a split-beam spectrophotometer as described in the legend to Table 7, and the A_{520} was monitored continuously. After 2 min, $CaCl_2$ (50 μ M final concn.) was added to the sample cuvette only. In traces i, iv and vii, 1 μ M cyclosporin A was present in both sample and reference cuvettes, whereas in traces iii, vi and ix, 0.5 mM imidodiphosphate was present.

scattering (Figure 1), whereas heart mitochondria were largely unaffected. That the swelling occurs as a result of the opening of the non-specific pore is indicated by the inhibitory effect of 1 μ M cyclosporin A, a potent inhibitor of pore opening (Crompton et al., 1988; Broekemeier et al., 1989; Halestrap and Davidson, 1990). There is, however, a cyclosporin-insensitive component of swelling that may be due to a PP_i -mediated increase in K^+

permeability of the inner membrane (Davidson and Halestrap, 1987, 1990; Halestrap and Davidson, 1989, 1990). In conjunction with the light-scattering measurements, PP_i was measured in the incubation medium, and the data are presented in Table 7. We did not attempt to measure matrix PP_i after centrifugation of mitochondria through silicone oil, since pore opening would release PP_i from the matrix into the medium. However, in some experiments we added an inhibitor of extramitochondrial pyrophosphatase, imidodiphosphate (Smirnova et al., 1988), to ensure that little hydrolysis of released PP_i occurred. The data of Table 7 show that the Ca^{2+} -induced swelling of both liver and kidney mitochondria is accompanied by an increase in $[PP_i]$ in the incubation medium, which is substantially greater in the presence of imidodiphosphate. In contrast, the heart mitochondria showed no significant increase in PP_i content whether or not imidodiphosphate was added. Thus our results are consistent with a role of increased matrix $[PP_i]$ in pore opening, as predicted by our proposed model for the mechanisms involved.

Conclusions

The data reported in this paper show that, at physiological $[Mg^{2+}]$, PP_i metabolism by heart mitochondria is much less sensitive to Ca^{2+} than that by liver or kidney mitochondria (Tables 2, 3, 5, 7). As a result, the heart mitochondria do not show significant regulation of their matrix volume by physiological concentrations of Ca^{2+} (Table 4), nor is an effect of Ca^{2+} or acetate on mitochondrial PP_i seen in the perfused heart. Thus neither adrenaline nor high extracellular $[Ca^{2+}]$, either alone or in combination with acetate, caused an increase in tissue PP_i content (Table 1). The PP_i present within the perfused heart is likely to be mainly mitochondrial, as is the case in liver (Davidson and Halestrap, 1987, 1988). Citrate synthase measurements of freeze-clamped heart tissue and isolated heart mitochondria show that there are about 45 mg of mitochondrial protein/g wet wt. of heart. The data of Tables 2–6 show that the PP_i content of mitochondria is about 150 pmol/mg of protein, which would be equivalent to 7 nmol of PP_i /g wet wt. The measured values were about half this value (Table 1). The lack of any change in these values after treatment with adrenaline or increased extracellular $[Ca^{2+}]$ (to mimic increased workload) imply that Ca^{2+} -mediated changes in mitochondrial volume do not play an important role in the regulation of heart mitochondrial function. This contrasts with the situation in liver (McCormack et al., 1990).

The lack of an effect of sub-micromolar $[Ca^{2+}]$ on matrix $[PP_i]$ in heart mitochondria is not a consequence of greater total

Table 7 Effect of loading heart, liver and kidney mitochondria with Ca^{2+} on their total PP_i contents

Mitochondria were incubated at 37 °C in a split-beam spectrophotometer in oxygenated buffer containing 125 mM KCl, 10 mM Mops, 7 mM Tris, 2.5 mM potassium phosphate, 2.5 mM $MgCl_2$, 2.5 mM succinate and, where indicated, 0.5 mM imidodiphosphate (IMDP). After 2 min incubation 50 μ M $CaCl_2$ (final concn.) was added to the sample cuvette, and the decrease in A_{520} was monitored as shown in Fig. 1. After 6 min incubation, 0.9 ml samples were removed from the cuvettes and acidified by addition of 100 μ l of 20% (w/v) $HClO_4$. PP_i was assayed after deproteinization and neutralization as described in the Experimental section. Results are expressed as means $\pm$ S.E.M. of the numbers of separate experiments shown in parentheses. Statistical significance was calculated by paired Student's *t* test: **P* < 0.02, ***P* < 0.01, ****P* < 0.005 (versus no additions) and †*P* < 0.005 (versus control + 0.5 mM IMDP).

Mitochondria ...	Total PP_i content of incubation medium (nmol/mg of mitochondrial protein)		
	Liver	Heart	Kidney
Control	1.40 $\pm$ 0.09 (6)	1.16 $\pm$ 0.37 (5)	0.67 $\pm$ 0.07 (5)
50 μ M Ca^{2+}	1.83 $\pm$ 0.08 (6)***	1.10 $\pm$ 0.17 (5)	1.10 $\pm$ 0.13 (5)***
0.5 mM IMDP	2.75 $\pm$ 0.32 (6)*	1.88 $\pm$ 0.17 (5)**	2.39 $\pm$ 0.32 (5)***
50 μ M Ca^{2+} + IMDP	3.61 $\pm$ 0.35 (6)†	1.84 $\pm$ 0.32 (5)	3.07 $\pm$ 0.33 (5)†

activity of matrix pyrophosphatase (Davidson and Halestrap, 1989), but may reflect the higher concentration of matrix Mg^{2+} increasing the functional activity of the enzyme. In addition, the greater amount of the adenine nucleotide translocase in heart mitochondria (Klingenberg, 1976) may play a role. Thus, when this translocase was blocked with bongkrekic acid, $0.75 \mu M$ Ca^{2+} did increase matrix PP_i significantly (Table 6). The loss of PP_i from the mitochondria on the translocase in exchange for either ADP or P_i (Halestrap and Davidson, 1989) could account for the increase in heart mitochondrial adenine nucleotide content seen in response to $0.75 \mu M$ Ca^{2+} (Table 2). This may provide an important mechanism for the restoration of mitochondrial adenine nucleotides during reperfusion after a period of ischaemia or hypoxia when adenine nucleotides are lost (Neely and Feuvray, 1981). It is not possible to rule out the existence of a low-molecular-mass inhibitor of mitochondrial pyrophosphatase that is present at higher concentrations in kidney and liver mitochondria than in heart mitochondria. Indeed, it has been noted by us (Halestrap, 1993) and others (Dubnova and Baykov, 1992) that, if the activity of mitochondrial pyrophosphatase was as high as predicted from the K_m for PP_i , the matrix $[PP_i]$ and the V_{max} of the enzyme, there should be negligible amounts of PP_i within the matrix. Some of the PP_i measured may be bound tightly to either the pyrophosphatase itself (Baykov et al., 1989b) or the oligomycin-sensitive ATPase (Issartel et al., 1987). However, the increase in PP_i observed in liver mitochondria in response to acetate or sub-micromolar $[Ca^{2+}]$ cannot all be bound. Thus there must be some restraint on pyrophosphatase activity in the intact mitochondria that is lost on permeabilization and may be less in heart mitochondria.

The damaging effects of mitochondrial Ca^{2+} overload are associated with the opening of a non-specific pore (Crompton, 1990). We have proposed that this is brought about by an interaction of mitochondrial peptidyl-prolyl *cis-trans* isomerase with the adenine nucleotide translocase in the 'c' conformation and denuded of adenine nucleotides by PP_i binding (Halestrap and Davidson, 1990; Griffiths and Halestrap, 1991). It is known that adenine nucleotide depletion increases the sensitivity of mitochondria to damage by Ca^{2+} (Crompton, 1990; Gunter and Pfeiffer, 1990; Halestrap, 1991), which is consistent with our model. Our present observations that the PP_i content and swelling of heart mitochondria are less sensitive to $[Ca^{2+}]$ than is seen in liver and kidney mitochondria (Table 7 and Figure 1) provide additional support for our proposals.

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