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Inhibition of mitochondrial-matrix inorganic pyrophosphatase by physiological [Ca^{2+}], and its role in the hormonal regulation of mitochondrial matrix volume

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1. The pyrophosphatase activity in cytosolic and mitochondrial fractions of rat liver was 1.7 and 0.26 units/mg of protein respectively when assayed at 37 °C in the presence of physiological [Mg^{2+}] (0.3 mM). 2. Approx. 80% of the mitochondrial pyrophosphatase was inaccessible to extramitochondrial PP_i, of which 40% represented soluble matrix enzyme (0.38 unit/mg of matrix protein). 3. Ca^{2+} inhibited the soluble matrix enzyme; the effective $K_{0.5}$ for inhibition increased as [Mg^{2+}], an essential cofactor of the enzyme, increased. Measured values were 0.39, 1.15, 3.7, 8.3 and 12.5 μM at 0.04 mM-, 0.1 mM-, 0.3 mM-, 0.6 mM- and 1 mM-Mg^{2+} respectively. 4. The data were analysed by a kinetic model similar to that for yeast pyrophosphatase, which assumes the substrate to be $MgPP_i$ (K_m 5 μM) with Mg^{2+} also activating at an additional site ($K_{0.5}$ 23 μM). Ca^{2+} inhibits through the formation of $CaPP_i$, a strong competitive inhibitor (K_i 0.067 μM). 5. Heart mitochondria also contain a soluble matrix pyrophosphatase of similar activity to that of liver mitochondria and with the same sensitivity to [Ca^{2+}]. 6. The data provide an explanation for the increase in mitochondrial PP_i, mediated by Ca^{2+}, which is responsible for the increase in matrix volume induced by gluconeogenic hormones [Davidson & Halestrap (1988) Biochem. J. **254**, 379–384].

INTRODUCTION

Increases in cytosolic [Ca^{2+}] occur in many cells in response to physiological stimuli (Exton, 1986). The consequent rise in mitochondrial [Ca^{2+}] is accompanied by an increase in the rate of respiration and hence an increased supply of ATP to meet the cells' new requirements. This is achieved without changing the [ATP]/[ADP][P_i] ratio and involves activation of three Ca^{2+}-sensitive enzymes, pyruvate, isocitrate and 2-oxoglutarate dehydrogenases, which regulate citric acid-cycle flux and hence NADH supply for the respiratory chain (see Denton & McCormack, 1985; Quinlan & Halestrap, 1986; Denton *et al.*, 1987). However, in the liver, glucagon and Ca^{2+}-mobilizing hormones increase the NADH/NAD^+ ratio only transiently, while respiration remains stimulated (Quinlan & Halestrap, 1986). This may be a result of a Ca^{2+}-mediated increase in matrix volume (Quinlan *et al.*, 1983; Halestrap *et al.*, 1986), which leads to a stimulation of electron flow through the respiratory chain (Halestrap, 1982; Armston *et al.*, 1982). Studies *in vitro* have demonstrated that the Ca^{2+}-mediated increase in mitochondrial matrix volume correlates with an increase in matrix PP_i content with half-maximal effects on both seen at 0.3 μM-Ca^{2+} (Davidson & Halestrap, 1987). Furthermore, addition of sub-micromolar [Ca^{2+}] to energized mitochondria increases their permeability towards K^+ (Halestrap *et al.*, 1986), as does addition of PP_i to de-energized mitochondria (Davidson & Halestrap, 1987).

Subcellular fractionation of liver cells has shown that more than 95% of liver PP_i is in the mitochondrial matrix and that this pool of PP_i increases in response to Ca^{2+}-mobilizing hormones, the Ca^{2+} ionophore A23187 or butyrate, in parallel with the increase in mitochondrial volume (Davidson & Halestrap, 1988). The lack of significant concentrations of cytosolic PP_i is consistent with the widely held view that pyrophosphatase is very active in the cytosol and rapidly degrades cytosolic PP_i, so driving those synthetic reactions which produce it to completion (Shatton *et al.*, 1981). Conversely, the concentration of PP_i found in mitochondria and its increase caused by sub-micromolar [Ca^{2+}] suggest that any pyrophosphatase in the mitochondrial matrix is likely to be much less active and may be subject to inhibition by physiological concentrations of Ca^{2+}. In this paper we demonstrate that this is the case.

EXPERIMENTAL

Materials

The source of all chemicals and biochemicals was as described in Davidson & Halestrap (1987).

Methods

Preparation of cytosolic and mitochondrial-matrix fractions from liver and heart. Isolated rat liver and heart mitochondria were prepared and freed of contaminating plasma membranes and microsomes by Percoll density-gradient centrifugation as described previously (Halestrap *et al.*, 1986; Whipps *et al.*, 1987; Halestrap, 1987). After treatment of mitochondria with digitonin (0.12 mg/mg of protein) at 0 °C for 15 min to remove lysosomes and mitochondrial outer membranes (Whipps *et al.*, 1987), a matrix fraction was prepared by sonication in 50 mM-KCl/50 mM-Mops/27 mM-Tris, pH 7.2 (buffer A), and removal of membranes by centrifugation at 150000 *g* for 30 min at 4 °C. Measurement of lactate

Abbreviation used: HEDTA, *N*-(hydroxyethyl)ethylenediamine-triacetic acid.

dehydrogenase, adenylate kinase and citrate synthase activities (Vargas, 1982) demonstrated that less than 5% of the pyrophosphatase activity in the matrix fraction was due to contamination by cytosolic or inter-membrane enzymes. A cytosolic fraction was prepared by centrifuging the post-mitochondrial supernatant at 150000 *g* for 45 min. In this instance the liver was perfused with 0.9% (w/v) NaCl for 1 min to remove contaminating blood before homogenization.

Assay of pyrophosphatase activity. Pyrophosphatase was assayed at 37 °C in buffer A containing 0.5 mM-$NaPP_i$, 1 mM-EGTA, 1 mM-HEDTA, and addition of $MgCl_2$ and $CaCl_2$ to give the free concentrations of Mg^{2+} and Ca^{2+} required. Concentrations of Mg^{2+} and Ca^{2+} were calculated by a computer program from the relevant dissociation constants for the various metal ligand complexes present (Rutter & Denton, 1988). Where intact mitochondria were present in the assay (Fig. 1), [KCl] was raised to 125 mM and bongkrekic acid (10 μg/ml) was added to prevent PP_i entering the mitochondria (Halestrap & Davidson, 1988). The assay was started by addition of either mitochondria or the soluble mitochondrial matrix or cytosolic fraction. The assay was terminated at the times shown by addition of $HClO_4$ (final concn. 5%, w/v), and the PP_i remaining in the neutralized deproteinized extract was determined enzymically (Davidson & Halestrap, 1987, 1988). Enzyme activity was then calculated from the decrease in [PP_i], which was linear until most of the PP_i was hydrolysed (Figs. 1 and 2). One unit of enzyme activity is defined as catalysing the hydrolysis of 1 μmol of PP_i/min at 37 °C.

Enzyme kinetic analysis. Data were fitted to the required kinetic equation by non-linear least-squares regression analysis by using a reiterative computer program (Jones, 1970).

RESULTS

Subcellular distribution of pyrophosphatase activity

In three separate experiments the mean (±S.E.M.) pyrophosphatase activity in the cytosolic and mitochondrial fractions prepared from rat liver was 1704±54 and 261±19 munits/mg of protein respectively. These results, which have been corrected for contamination between fractions by using citrate synthase and lactate dehydrogenase (Vargas, 1982), are similar to the values reported by others (Irie *et al.*, 1970; Shatton *et al.*, 1981). To confirm that the pyrophosphatase associated with the mitochondria was not due to contaminating cytosolic enzyme, the latency of the mitochondrial activity was demonstrated as shown in Fig. 1. Intact mitochondria hydrolysed PP_i only slowly, whereas upon addition of Triton X-100 (0.2%, w/v) there was a considerable increase in activity. This latent enzyme represented 218±3 (n=3) munits/mg of total mitochondrial protein. The pyrophosphatase activity of a mitochondrial-matrix fraction free of lysosomes, cytosol, inter-membrane and inner- and outer-membrane contamination was 376±9 munits/mg of matrix protein (n = 3), which, from parallel measurements of citrate synthase activity, is equivalent to 101 munits/mg of total mitochondrial protein. The difference between the latent pyrophosphatase activity and the soluble matrix activity probably

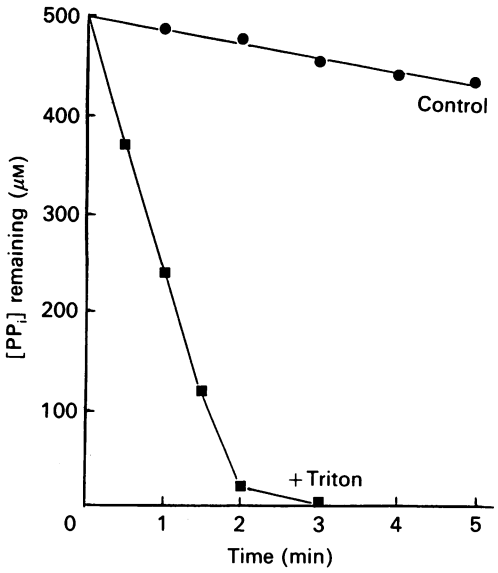

Fig. 1. Time courses of pyrophosphate hydrolysis by intact (●) and Triton-permeabilized (■) mitochondria

Pyrophosphatase was assayed at 37 °C as described in the Experimental section. The reaction was started by addition of mitochondria (1 mg of protein/ml) to assay buffer in the absence (●) or presence (■) of Triton X-100 (0.2%, w/v), and terminated at the times shown by addition of $HClO_4$ (final concn. 5%, w/v). $MgCl_2$ was present at 1.62 mM to give a free [Mg^{2+}] of 0.3 mM.

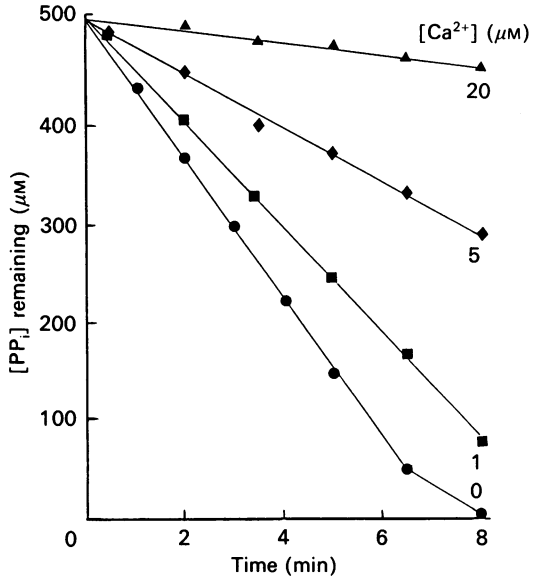

Fig. 2. Time courses of pyrophosphate hydrolysis by a liver mitochondrial-matrix preparation at various concentrations of Ca^{2+}

Pyrophosphatase was assayed at 37 °C as described in the Experimental section. The reaction was started by addition of mitochondrial-matrix fraction (0.16 mg of protein/ml) and terminated at the times shown by addition of $HClO_4$ (final concn. 5%). $MgCl_2$ was present at 1.62 mM to give a free [Mg^{2+}] of 0.3 mM, and $CaCl_2$ was added to give free concentrations (μM) of Ca^{2+} of 0 (●), 1 (■), 5 (◆) or 20 (▲).

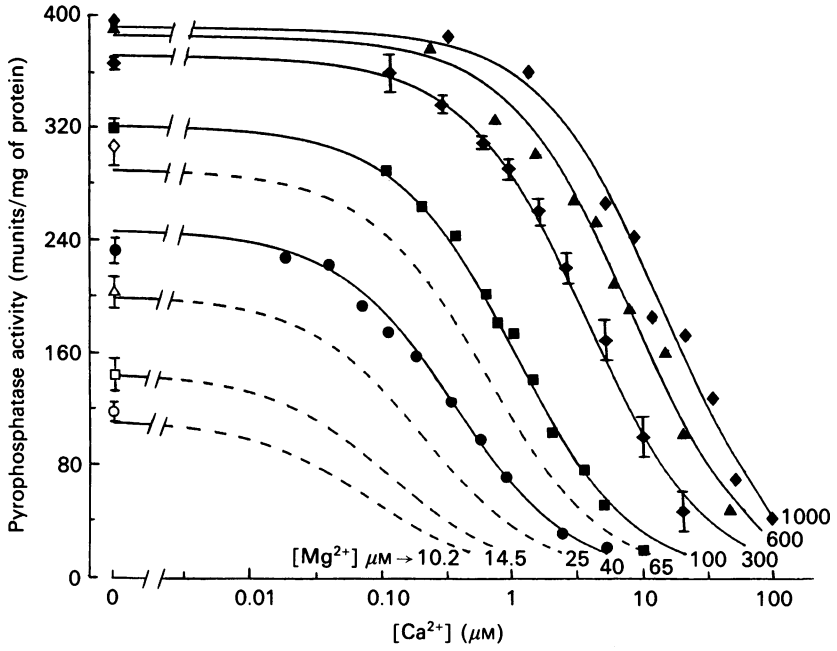

Fig. 3. Inhibition of mitochondrial-matrix pyrophosphatase by Ca^{2+} at different concentrations of Mg^{2+}

Pyrophosphatase activity was determined from time courses of PP_i hydrolysis as described in Fig. 2. The free concentration (μM) of Mg^{2+} was 10.2 (○), 14.5 (□), 25 (△), 40 (●), 65 (◇), 100 (■), 300 (◆), 600 (▲) and 1000 (♦), and [Ca^{2+}] was varied as shown. Data points are for one preparation of enzyme unless error bars are given, where values are the means ± S.E.M. of values obtained with three separate preparations. The lines drawn are calculated by least-squares regression analysis to the equation:

$$v = V_m/((1+\{(K_{m1}/[MgPP_i]) \wedge h_1\}\cdot\{1+([CaPP_i]/K_{i1}) \wedge h_1\}\cdot\{1+([PP_i]/K_{i2}) \wedge h_1\})\cdot\{1+(K_{m2}/[Mg^{2+}]) \wedge h_2\})$$

where K_{m1}, K_{m2}, K_{i1} and K_{i2} are the K_m or K_i values for $MgPP_i$, Mg^{2+}, $CaPP_i$ and PP_i respectively, h_1 and h_2 are the Hill coefficients for $MgPP_i$ and Mg^{2+} respectively, and V_m is the $V_{max.}$ of the enzyme. The total [PP_i] was 500 μM, and concentrations of $MgPP_i$, $CaPP_i$ and free PP_i were calculated as described in the Experimental section by using K_d values for $MgPP_i$ and $CaPP_i$ at pH 7.2 of 55.3 and 59.6 μM respectively. K_{m1} was set at 5 μM, and the other parameter values were calculated by reiteration and are shown in Table 1. At some [Mg^{2+}] values, inhibition by Ca^{2+} was not studied, and the theoretical curves are drawn as dashed lines.

represents membrane-bound enzyme, some of which may be a proton-translocating PP_i-synthesizing enzyme (Volk *et al.*, 1983; Baltscheffsky & Nyren, 1984).

Effects of Mg^{2+} and Ca^{2+} on the pyrophosphatase of the mitochondrial matrix

Fig. 2 shows the time course of degradation of 500 μM-PP_i by the matrix pyrophosphatase at 0.3 mM-Mg^{2+} in the presence of different Ca^{2+} concentrations. Data are given for the matrix enzyme of liver mitochondria, but the total activity of the heart enzyme was similar (341 munits/mg of matrix protein), as were its properties. The rate of PP_i degradation was constant until [PP_i] was less than 50 μM. This implies a K_m for PP_i under these conditions substantially lower than 50 μM, and too low to be determined by the present method of assay. It is also clear from Fig. 2 that Ca^{2+} is a powerful inhibitor of the enzyme. The sensitivity of the enzyme towards Ca^{2+} is decreased as the concentration of Mg^{2+}, which is essential for enzyme activity, is increased. This is shown in more detail in Figs. 3 and 4.

We have successfully analysed the data of Figs. 3 and 4 by using a model derived from that proposed for the yeast enzyme, whose kinetics have been studied extensively (Moe & Butler, 1972*a*,*b*; Ridlington & Butler, 1972; Cooperman, 1982). The substrate for the enzyme is $MgPP_i$, and Ca^{2+} inhibits primarily through formation of $CaPP_i$, which is a very strong competitive inhibitor with respect to $MgPP_i$. Free PP_i acts as a very weak competitive inhibitor for the same site. As outlined above, we were not able to calculate the K_m value for $MgPP_i$ from our data, and so have assumed a value of 5 μM, similar to that for the yeast enzyme (Cooperman, 1982). There is an additional Mg^{2+}-activating site on the enzyme, as shown clearly in the data of Fig. 4. Ca^{2+} competes very poorly for Mg^{2+} at this site, and we have ignored this in our analysis. The best-fit values (± S.E.) for all the kinetic parameters derived by least-squares regression analysis of the data are given in Table 1. Although the kinetic model allows for co-operativity at both the $MgPP_i$- and the Mg^{2+}-binding site, the best fit to the data gives values of the Hill coefficient of 0.98 and 1.01 respectively, suggesting that none occurs. The relationship between [Mg^{2+}] and the $K_{0.5}$ for Ca^{2+} was linear, as shown in Fig. 4. At 0.3 mM-Mg^{2+}, the probable matrix concentration (Corkey *et al.*, 1986), the effective $K_{0.5}$ for inhibition by Ca^{2+} was 3.7 ± 0.4 μM (mean ± S.E.M. for three separate enzyme preparations). Similar values were obtained for the enzyme from heart mitochondrial matrix (3.2 μM) and liver cytosol (2.6 μM), although studies by others suggest that the mitochondrial and cytosolic enzymes are structurally distinct (Volk *et al.*, 1983).

Table 1. Kinetic parameters derived for the mitochondrial-matrix pyrophosphatase

The data of Fig. 3 were fitted to the equation shown in the legend by least-squares regression analysis as described in the Experimental section.

Parameter	Units	Value	S.E.	95% confidence limits
$V_{max.}$	(nmol/min per mg of matrix protein)	394	6	381–408
K_m for $MgPP_i$	μM	5	–	Set value
K_i for $CaPP_i$	μM	0.067	0.004	0.059–0.074
K_i for PP_i	μM	506	159	186–825
Hill coefficient ($MgPP_i$)	–	0.98	0.03	0.92–1.04
K_m for Mg^{2+}	μM	23.1	1.5	20.1–26.1
Hill coefficient (Mg^{2+})	–	1.01	0.07	0.87–1.16

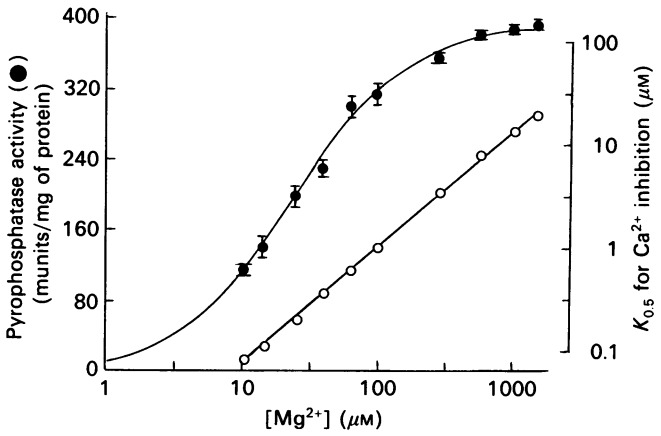

Fig. 4. Dependence on $[Mg^{2+}]$ of the rate and sensitivity to $[Ca^{2+}]$ of mitochondrial-matrix pyrophosphatase

Pyrophosphatase activities (●) at different concentrations of Mg^{2+} were taken from Fig. 3 and fitted to the same equation but assuming zero $[CaPP_i]$. The concentrations of Ca^{2+} giving half the rate found in the absence of Ca^{2+} ($K_{0.5}$; ○) were determined from the curves of Fig. 3.

DISCUSSION

Although previous studies on mitochondrial pyrophosphatases have shown inhibition by Ca^{2+}, supraphysiological concentrations of Mg^{2+} were employed in the assays, and hence effects of Ca^{2+} were only detected at concentrations of Ca^{2+} higher than those which occur physiologically (Irie *et al.*, 1970; Volk *et al.*, 1982). The significance of the present results is that they demonstrate that inhibition of the pyrophosphatase by Ca^{2+} may be of physiological importance within the mitochondria. The $K_{0.5}$ of 3.7 μM for Ca^{2+} inhibition of the pyrophosphatase is significantly higher than that for 2-oxoglutarate dehydrogenase, but of the same order as the $K_{0.5}$ for Ca^{2+} activation of purified isocitrate dehydrogenase and of pyruvate dehydrogenase phosphate phosphatase (Denton & McCormack, 1985; Denton *et al.*, 1987; Thomas *et al.*, 1986; Midgley *et al.*, 1987; Rutter & Denton, 1988). In contrast, the $K_{0.5}$ for Ca^{2+}-mediated increases in matrix volume and PP_i under physiological conditions is about 0.3 μM (Davidson & Halestrap, 1987). The order of magnitude difference between the sensitivity of the isolated pyrophosphatase and the matrix PP_i content to $[Ca^{2+}]$ resembles that between the $K_{0.5}$ for the activation of purified pyruvate dehydrogenase phosphate phosphatase by Ca^{2+} and for the activation by extramitochondrial Ca^{2+} of pyruvate dehydrogenase within intact mitochondria (Denton & McCormack, 1985; Denton *et al.*, 1987). The concentration of PP_i and the activity of pyruvate dehydrogenase within the intact mitochondria both reflect the relative activity of two enzymes, one synthetic and the other degradative and Ca^{2+}-sensitive. Unless the pyrophosphatase is inhibited by a naturally occurring inhibitor, or the PP_i present in the matrix is present as an inert metal salt rather than free PP_i, the low K_m of the pyrophosphatase suggests that it must be working under $V_{max.}$ conditions in the absence of Ca^{2+}. Possible endogenous inhibitors are ATP, ADP and P_i, but we have found that none of these has a significant effect on enzyme activity when present in the assay at 5 mM (results not shown). On addition of 0.3 μM-Ca^{2+} the resulting $CaPP_i$ would inhibit the pyrophosphatase by about 20%, but, if the enzyme is working close to $V_{max.}$ already, $[PP_i]$ could easily increase by the observed 50%. At this concentration, either PP_i synthesis must be inhibited to an equivalent extent or PP_i must be transported out of the matrix at a correspondingly faster rate. Although $CaPP_i$ is a competitive inhibitor of pyrophosphatase with respect of $MgPP_i$, an increase in $[PP_i]$ itself cannot increase the rate of PP_i hydrolysis, since the concentrations of both $CaPP_i$ and $MgPP_i$ will increase in parallel. If an additional source of PP_i is provided, such as in the presence of butyrate, whose intra-mitochondrial activation produces PP_i, the effects of inhibiting the pyrophosphatase with Ca^{2+} are even more pronounced and matrix PP_i can increase 30-fold or more in the presence of sub-micromolar Ca^{2+} (Davidson & Halestrap, 1987, 1988; Otto & Cook, 1982). The source of PP_i in the absence of butyrate is unknown, but may be a proton-translocating pyrophosphatase (Volk *et al.*, 1982; Baltscheffsky & Nyren, 1984). This would lead to a futile cycle of PP_i synthesis and breakdown, which might account for a part of the State 4 rate of respiration.

The inhibition of pyrophosphatases by Ca^{2+} appears to be a common feature of all pyrophosphatases. What makes the situation in the mitochondrial matrix of particular interest is the relatively low activity of the enzyme when compared with the activity present in the cytosol. This ensures that the matrix PP_i concentration is

sensitive to physiological changes in $[Ca^{2+}]$. We have demonstrated previously that an increase in matrix $[PP_i]$ is associated with an increase in mitochondrial matrix volume, probably as a result of PP_i interacting with the adenine nucleotide carrier and increasing the K^+ permeability of the mitochondrial inner membrane (Halestrap *et al.*, 1986; Davidson & Halestrap, 1987, 1988; Halestrap & Davidson, 1988). The present data provide the link between the increase in matrix $[Ca^{2+}]$ and the increase in matrix $[PP_i]$, which is itself responsible for causing liver mitochondria to swell in response to gluconeogenic hormones (Quinlan *et al.*, 1983). This increase in matrix volume stimulates the respiratory chain, and hence NADH utilization (Halestrap, 1982; Armston *et al.*, 1982; Quinlan & Halestrap, 1986). This is balanced by a simultaneous stimulation of NADH production through activation of the Ca^{2+}-sensitive dehydrogenases (Denton & McCormack, 1985; Denton *et al.*, 1987). In addition, such hormonally induced increases in the matrix volume of liver mitochondria are capable of stimulating glutaminase activity (McGivan *et al.*, 1985), fatty acid oxidation (Halestrap & Dunlop, 1986; Halestrap, 1987), pyruvate carboxylation (Armston *et al.*, 1982; Martin & Titheradge, 1984; Whipps & Halestrap, 1984) and citrulline synthesis (Armston *et al.*, 1982). These are all stimulated in the perfused liver and isolated hepatocytes by those hormones such as vasopressin and α-adrenergic agonists which increase cytosolic and mitochondrial $[Ca^{2+}]$ (Halestrap *et al.*, 1985).

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REFERENCES

Armston, A. E., Halestrap, A. P. & Scott, R. D. (1982) Biochim. Biophys. Acta **681**, 429–439

Baltscheffsky, M. & Nyren, P. (1984) in Bioenergetics (Ernster, L., ed.), pp. 187–206, Elsevier Science Publishers, Amsterdam

Cooperman, B. S. (1982) Methods Enzymol. **87**, 526–548

Corkey, B. E., Duszynski, J., Rich, T. L., Matschinsky, B. & Williamson, J. R. (1986) J. Biol. Chem. **261**, 2567–2574

Davidson, A. M. & Halestrap, A. P. (1987) Biochem. J. **246**, 715–723

Davidson, A. M. & Halestrap, A. P. (1988) Biochem. J. **254**, 379–384

Denton, R. M. & McCormack, J. G. (1985) Am. J. Physiol. **249**, E543–E554

Denton, R. M., McCormack, J. G., Midgley, P. J. W. & Rutter, G. A. (1987) Biochem. Soc. Symp. **54**, 127–143

Exton, J. H. (1986) Adv. Cyclic Nucleotide Protein Phosphorylation Res. **20**, 211–262

Halestrap, A. P. (1982) Biochem. J. **204**, 37–47

Halestrap, A. P. (1987) Biochem. J. **244**, 159–164

Halestrap, A. P. & Davidson, A. M. (1988) in Anion Carriers of Mitochondrial Membranes (Azzi, A., Fonyo, A., Nalecz, M. J., Vignais, P. V. & Wojtcak, L., eds.), Springer Verlag, Berlin, in the press

Halestrap, A. P. & Dunlop, J. L. (1986) Biochem. J. **239**, 559–565

Halestrap, A. P., Quinlan, P. T., Armston, A. E. & Whipps, D. E. (1985) in Achievements and Perspectives of Mitochondrial Research (Quagliariello, E., Slater, E. C., Palmieri, F., Saccone, C. & Kroon, A. M., eds.), vol. 1, pp. 469–480, Elsevier, Amsterdam

Halestrap, A. P., Quinlan, P. T., Whipps, D. E. & Armston, A. E. (1986) Biochem. J. **236**, 779–787

Irie, M., Yabuta, A., Kimura, K., Shindo, Y. & Tomito, K. (1970) J. Biochem. (Tokyo) **67**, 47–58

Jones, A. (1970) Comput. J. **13**, 301–308

Martin, A. D. & Titheradge, M. A. (1984) Biochem. J. **222**, 379–387

McGivan, J., Vadher, M., Lacey, J. & Bradford, N. (1985) Eur. J. Biochem. **148**, 323–327

Midgley, P. J. W., Rutter, G. A., Thomas, A. P. & Denton, R. M. (1987) Biochem. J. **241**, 371–377

Moe, O. A. & Butler, L. G. (1972*a*) J. Biol. Chem. **247**, 7308–7314

Moe, O. A. & Butler, L. G. (1972*b*) J. Biol. Chem. **247**, 7315–7319

Otto, D. A. & Cook, G. A. (1982) FEBS Lett. **150**, 172–176

Quinlan, P. T. & Halestrap, A. P. (1986) Biochem. J. **236**, 789–800

Quinlan, P. T., Thomas, A. P., Armston, A. E. & Halestrap, A. P. (1983) Biochem. J. **214**, 395–404

Ridlington, J. W. & Butler, L. G. (1972) J. Biol. Chem. **247**, 7303–7307

Rutter, G. A. & Denton, R. M. (1988) Biochem. J. **252**, 181–189

Shatton, J. B., Shah, H., Williams, A., Morris, H. P. & Weinhouse, S. (1981) Cancer Res. **41**, 1866–1872

Thomas, A. P., Diggle, T. A. & Denton, R. M. (1986) Biochem. J. **238**, 93–101

Vargas, A.M. (1982) J. Biochem. Biophys. Methods **7**, 1–6

Volk, S. E., Baykov, A. A., Duzhenko, V. S. & Avaeva, S. H. (1982) Eur. J. Biochem. **125**, 215–220

Volk, S. E., Baykov, A. A., Kostenko, E. B. & Avaeva, S. H. (1983) Biochim. Biophys. Acta **744**, 127–134

Whipps, D. E. & Halestrap, A. P. (1984) Biochem. J. **221**, 147–152

Whipps, D. E., Armston, A. E., Pryor, H. J. & Halestrap, A. P. (1987) Biochem. J. **241**, 835–845

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