Biochem. J. (1980) **190**, 107–117
Printed in Great Britain

Role of Calcium Ions in the Regulation of Intramitochondrial Metabolism

EFFECTS OF Na^+, Mg^{2+} AND RUTHENIUM RED ON THE Ca^{2+}-STIMULATED OXIDATION OF OXOGLUTARATE AND ON PYRUVATE DEHYDROGENASE ACTIVITY IN INTACT RAT HEART MITOCHONDRIA

Richard M. DENTON, James G. McCORMACK and Nigel J. EDGELL
Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.
Bristol BS8 1TD, U.K.

(*Received 6 December 1979*)

1. In uncoupled rat heart mitochondria, the kinetic parameters for oxoglutarate oxidation were very close to those found for oxoglutarate dehydrogenase activity in extracts of the mitochondria. In particular, Ca^{2+} greatly diminished the K_m for oxoglutarate and the $k_{0.5}$ value (concentration required for half-maximal effect) for this effect of Ca^{2+} was close to 1 μM. 2. In coupled rat heart mitochondria incubated with ADP, increases in the extramitochondrial concentration of Ca^{2+} greatly stimulated oxoglutarate oxidation at low concentrations of oxoglutarate, but not at saturating concentrations of oxoglutarate. The $k_{0.5}$ value for the activation by extramitochondrial Ca^{2+} was about 20 nM. In the presence of either Mg^{2+} or Na^+ this value was increased to about 90 nM, and in the presence of both to about 325 nM. 3. In coupled rat heart mitochondria incubated without ADP, increases in the extramitochondrial concentration of Ca^{2+} resulted in increases in the proportion of pyruvate dehydrogenase in its active non-phosphorylated form. The sensitivity to Ca^{2+} closely matched that found to affect oxoglutarate oxidation, and Mg^{2+} and Na^+ gave similar effects. 4. Studies of others have indicated that the distribution of Ca^{2+} across the inner membrane of heart mitochondria is determined by a Ca^{2+}-transporting system which is composed of a separate uptake component (inhibited by Mg^{2+} and Ruthenium Red) and an efflux component (stimulated by Na^+). The present studies are entirely consistent with this view. They also indicate that the intramitochondrial concentration of Ca^{2+} within heart cells is probably about 2–3 times that in the cytoplasm, and thus the regulation of these intramitochondrial enzymes by Ca^{2+} is of likely physiological significance. It is suggested that the Ca^{2+}-transporting system in heart mitochondria may be primarily concerned with the regulation of mitochondrial Ca^{2+} rather than cytoplasmic Ca^{2+}; the possible role of Ca^{2+} as a mediator of the effects of hormones and neurotransmitters on mammalian mitochondrial oxidative metabolism is discussed.

The transfer of Ca^{2+} across the inner membrane of rat heart and other mammalian mitochondria involves separate uptake and release processes (see Bygrave, 1978; Carafoli & Crompton, 1978; Nicholls, 1978). The uptake process is electrogenic and is inhibited rather specifically by Ruthenium Red (Rossi *et al.*, 1973) lanthanides (Mela, 1969) and also by Mg^{2+} (Crompton *et al.*, 1976*a*). Uptake probably occurs as Ca^{2+} with two net positive charges (Rottenberg & Scarpa, 1974; Heaton & Nicholls, 1976; Crompton *et al.*, 1978; Deana *et al.*, 1979), but mechanisms with partial charge compensation involving H^+, phosphate and β-hydroxybutyrate have been suggested (Reed & Bygrave, 1975; Moyle & Mitchell, 1977*a*,*b*). The release process must be able to transfer Ca^{2+} out of mitochondria against its electrochemical gradient. In heart and certain other mammalian mitochondria, this appears to be accomplished by the exchange of one Ca^{2+} ion for two or more Na^+ ions (Crompton *et al.*, 1977, 1978). In liver, kidney and smooth-muscle mitochondria the mechanism for the release process is not clearly established, but it seems likely that one Ca^{2+} ion exchanges for two or more H^+ ions (Caroni *et al.*, 1978; Fiskum & Lehninger, 1979).

The preceding paper (McCormack & Denton,

Abbreviation used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

0306-3283/80/070107-11$01.50/1

1980) showed that the activities of pyruvate dehydrogenase, NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase were all enhanced by Ca^{2+} when the enzymes were located within intact, but uncoupled, adipose-tissue mitochondria. The sensitivity to Ca^{2+} [$k_{0.5}$ (concentration required for half-maximal effect) $\simeq 1\,\mu M$ in all cases] was very close to that found previously by using mitochondrial extracts or purified enzymes (Denton *et al.*, 1972, 1978*a*; McCormack & Denton, 1979, 1980). The studies described in the present paper investigate the effects of changes in extramitochondrial Ca^{2+} on the activities of oxoglutarate dehydrogenase and pyruvate dehydrogenase in coupled rat heart mitochondria. We show that changes in extramitochondrial Ca^{2+} in the range 1 nM–1 μM alter the activities of both enzymes in parallel. Moreover, the presence of Na^+, Mg^{2+} and Ruthenium Red alters the apparent sensitivity of the enzymes to extramitochondrial Ca^{2+} as predicted from the properties of the Ca^{2+}-uptake and -release processes thought to occur in rat heart mitochondria (as outlined above). Our findings indicate that the intramitochondrial Ca^{2+} concentration in heart cells is probably only 2–3 times the cytoplasmic concentration, and moreover that it is in the range to which the Ca^{2+}-sensitive dehydrogenases within the mitochondria are sensitive. It follows that the main function of the separate Ca^{2+}-uptake and -release processes in rat heart and other mitochondria may be the regulation of intramitochondrial Ca^{2+} and thereby intramitochondrial oxidative metabolism rather than, as usually assumed, the regulation of the concentration of Ca^{2+} in the cytoplasm (see Mela, 1977; Bygrave, 1978; Carafoli & Crompton, 1978; Nicholls, 1978).

Experimental

Chemicals and biochemicals

The sources and preparations were as given in McCormack & Denton (1980). In addition, Ruthenium Red was obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Preparation of rat heart mitochondria

Albino Wistar rats (200–300 g) of either sex were used. The rats were housed at room temperature and fed *ad libitum* on a stock laboratory diet (Breeding Diet; Oxoid Ltd., Basingstoke, Hants., U.K.). For the preparation of mitochondria the rats were first anaesthetized with Sagatal (pentobarbital; 60 mg/kg) for 15 min before removal of the hearts. Each heart was immediately disrupted in 4 ml of sucrose medium [250 mM-sucrose, 20 mM-Tris/HCl, 2 mM-EGTA, 1% (w/v) albumin, pH 7.3] in a Polytron PT-20 tissue homogenizer set at one-third full speed for 3–5 s. Mitochondria were then prepared as given by Kerbey *et al.* (1976) and suspended in the same sucrose-based medium (but without albumin) to about 20 mg of protein/ml.

Incubation of mitochondria; assay of enzyme activities, protein, oxygen uptake and ATP; and handling of data

These were as described in the preceding paper (McCormack & Denton, 1980). Changes in detail are given in the appropriate legends to Figures and Tables. A unit of enzyme activity is taken as that amount of enzyme which transforms 1 μmol of substrate/min at 30°C.

Results

Kinetic parameters of oxoglutarate dehydrogenase activity in extracts of rat heart mitochondria and of oxoglutarate oxidation by uncoupled rat heart mitochondria

Table 1 compares the principal kinetic parameters for oxoglutarate dehydrogenase activity measured in extracts of rat heart mitochondria at 30°C and pH 7.3 with those found for the oxidation of oxoglutarate by rat heart mitochondria incubated with the uncoupler FCCP at the same temperature and pH. As in our previous studies with brown-adipose-tissue mitochondria (McCormack & Denton, 1980), there was a close similarity consistent with oxoglutarate dehydrogenase being rate-limiting in the oxidation of oxoglutarate by the intact uncoupled mitochondria. In particular, the kinetics with respect to oxoglutarate were close to hyperbolic, and Ca^{2+} (20 μM) greatly diminished the K_m for oxoglutarate without altering the $V_{max.}$. Values of the K_m for the oxidation of oxoglutarate by the mitochondria in both the presence and the absence of Ca^{2+} were closer to the values observed with the separated oxoglutarate dehydrogenase assayed without addition of ADP than to those found in the presence of ADP. This was also the case with the value of $k_{0.5}$ for the activation by Ca^{2+} at a low oxoglutarate concentration (0.1 mM). As expected, Sr^{2+} could replace Ca^{2+}, but the $k_{0.5}$ was some 10-fold greater. The $V_{max.}$ for oxoglutarate oxidation by the uncoupled mitochondria was equivalent to about 150 nmol of NADH (or $FADH_2$) oxidized/min per mg of mitochondrial protein. Since the maximum activity of oxoglutarate dehydrogenase measured in extracts of the mitochondria (about 120 munits/min of protein) appears to be less than this, it is possible that a proportion of oxoglutarate was oxidized beyond succinate in the intact uncoupled mitochondria.

Taken together, the results of Table 1 indicated that the properties of oxoglutarate dehydrogenase within heart mitochondria were similar to those of the separated enzyme. It should be noted that Ca^{2+}

Table 1. *Summary of kinetic parameters for: (a) oxoglutarate dehydrogenase activity in rat heart mitochondrial extracts, and (b) oxoglutarate oxidation by intact uncoupled rat heart mitochondria*

In (*a*), mitochondrial extracts were prepared and enzyme activity was assayed (as described in the Experimental section) at pH 7.3 in the presence of 2 mM-NAD^+, 1 mM-$MgCl_2$, 1 mM-thiamin pyrophosphate and 0.25 mM-CoA with additions of EGTA, $CaCl_2$, ADP and oxoglutarate as indicated. In (*b*), mitochondria (≃0.5 mg of protein) were incubated at 30°C in 1 ml of 125 mM-KCl/20 mM-Tris/HCl (pH 7.3)/5 mM-KH_2PO_4/1 mM-malate/0.5 μM-FCCP, with additions of EGTA, $CaCl_2$ and oxoglutarate as indicated. In both (*a*) and (*b*) suitable ranges of oxoglutarate concentrations and of Ca^{2+} or Sr^{2+} concentrations (obtained by using EGTA buffers) were used as appropriate. The parameters in the first four rows are derived from $v = V_{max.}/\{1 + (K_m/[S])^n\}$; however n was found not to differ significantly from 1 and is not given. The parameters in the last two rows are derived from $v = (V_{max.}/\{1 + (k_{0.5}/[M^{2+}])^n\}) + V_0$, where V_0 is the activity or oxidation rate in the presence of 0.1 mM-oxoglutarate and 5 mM-EGTA ($[Ca^{2+}] < 1$ nM). All values are expressed as means ± S.E.M. for the numbers of degrees of freedom shown in parentheses. Each value is derived from observations made on samples from at least two different preparations of mitochondria. $V_{max.}$ is given as munits/mg of protein in (*a*) and as nmol of O_2 consumed/min per mg of protein in (*b*).

		(*a*) Oxoglutarate dehydrogenase activity		(*b*) Oxoglutarate oxidation
Addition	Parameter	With no ADP	At 1 mM-ADP	
5 mM-EGTA ($[Ca^{2+}] < 1$ nM)	$V_{max.}$	121 ± 3 (16)	111 ± 8 (23)	73 ± 8 (7)
	K_m for oxoglutarate (mM)	2.30 ± 0.16 (16)	0.36 ± 0.04 (23)	3.42 ± 0.33 (7)
5 mM-EGTA plus 5 mM-$CaCl_2$ ($[Ca^{2+}] \simeq 20$ μM)	$V_{max.}$	119 ± 4 (13)	109 ± 5 (15)	76 ± 6 (7)
	K_m for oxoglutarate (mM)	0.184 ± 0.013 (13)	0.084 ± 0.005 (15)	0.230 ± 0.041 (7)
0.1 mM-Oxoglutarate	$k_{0.5}$ for Ca^{2+} (μM)	0.51 ± 0.06 (12)	0.16 ± 0.03 (15)	0.94 ± 0.15 (24)
	$k_{0.5}$ for Sr^{2+} (μM)	12.6 ± 1.1 (20)	2.8 ± 0.3 (20)	13.6 ± 2.9 (12)

(up to 20 μM) had no effect on the kinetic parameters for the oxidation of succinate in the presence of rotenone. The effects of Ruthenium Red (1 μg/ml) and NaCl (15 mM) on the activity of oxoglutarate dehydrogenase in mitochondrial extracts were investigated, but no changes in $V_{max.}$, K_m for oxoglutarate or $k_{0.5}$ for Ca^{2+} were observed. Moreover, the presence of Ruthenium Red (1 μg/ml), $MgCl_2$ (0.5 mM), NaCl (15 mM) or ionophore A23187 (0.5 μg/ml) in the mitochondrial incubation medium did not alter appreciably the corresponding parameters for oxoglutarate oxidation by uncoupled mitochondria (results not shown).

Effects of Ca^{2+} on the oxidation of oxoglutarate by coupled heart mitochondria

Typical oxygen-electrode traces obtained with rat heart mitochondria incubated in KCl-based medium (pH 7.3) at 30°C with ADP, phosphate and malate are shown in Figs. 1(*a*)–1(*c*). In the absence of any further exogenous substrate the rat heart mitochondria utilized O_2 at an appreciable rate, and this rate remained constant for at least 10 min. The addition of oxoglutarate after a preincubation period of 3 min increased the rate of O_2 consumption to a maximum of about 5-fold in the presence of either 5 mM-EGTA ($[Ca^{2+}] < 1$ nM; solid lines) or 5 mM-EGTA plus 2.5 mM-$CaCl_2$ ($[Ca^{2+}] \simeq 50$ nM; broken lines). However, the increases in O_2 uptake at low non-saturating concentrations of oxoglutarate were much greater in the presence of Ca^{2+} (50 nM). This effect of Ca^{2+} was most obvious in the absence of any further additions (Fig. 1*a*) and was diminished by 15 mM-NaCl (Fig. 1*b*) or 0.5 mM-$MgCl_2$ (Fig. 1*c*). In the experiments of Fig. 1 and all subsequent experiments, unless otherwise stated, mitochondria were added to medium containing all additions except the exogenous substrate and preincubated for 3 min before addition of oxoglutarate or other oxidizable substrate. This period of preincubation was to allow a steady-state distribution of Ca^{2+} across the mitochondrial inner membrane to be established. Longer periods of preincubation (up to 10 min) did not result in any appreciable change in the subsequent rates of oxoglutarate oxidation.

The effects of Ca^{2+} (50 nM) on the stimulation of O_2 uptake with various concentrations of oxoglutarate are shown in greater detail in Fig. 2, which summarizes results from three separate batches of rat heart mitochondria. The plots are not significantly different from hyperbolic in either the presence or the absence of extramitochondrial Ca^{2+} (50 nM). Consistent with the results of Fig. 1(*a*), Ca^{2+} lowered the K_m for oxoglutarate oxidation without altering the $V_{max.}$. Values for K_m and $V_{max.}$ were calculated by fitting all the individual observations of the three experiments to the equation $v = V_{max.}/\{1 + (K_m/[S])\}$. In the absence and presence of Ca^{2+}, the values for the K_m were 2.32 ± 0.54 and 0.54 ± 0.06 mM respectively and the values for the $V_{max.}$ were 84 ± 7 and 91 ± 4 nmol of O_2/min per mg of protein respectively. Results are given as means ± S.E.M. for 14 degrees of freedom.

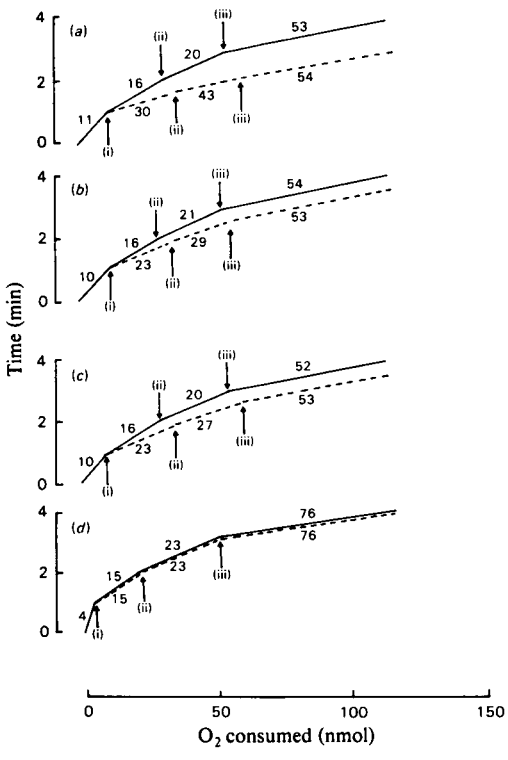

Fig. 1. *Effects of Ca^{2+}, Na^+ and Mg^{2+} on the uptake of oxygen by rat heart mitochondria with oxoglutarate (a–c) and succinate (d) as substrates*

Mitochondria (equivalent to 0.5 mg of protein) were incubated at 30°C in 1 ml of 125 mM-KCl/20 mM-Tris/HCl (pH 7.3)/5 mM-KH_2PO_4/2 mM-ADP with either 1 mM-malate (*a–c*) or 0.1 μg of rotenone/ml (*d*) and in the presence of either 5 mM-EGTA (unbroken lines) (free $[Ca^{2+}] < 1$ nM) or 5 mM-EGTA plus 2.5 mM-$CaCl_2$ (broken lines) (free $[Ca^{2+}] \simeq 50$ nM), and additionally in (*b*) 15 mM-NaCl and in (*c*) 0.5 mM-$MgCl_2$. Further additions, at the times indicated, were made as follows: in (*a–c*): (i), 0.25 mM-oxoglutarate; (ii), 0.5 mM-oxoglutarate; (iii), 20 mM-oxoglutarate; in (*d*): (i), 0.5 mM-succinate; (ii), 1 mM-succinate; (iii), 20 mM-succinate. The values given on the traces indicate nmol of O_2 taken up/min.

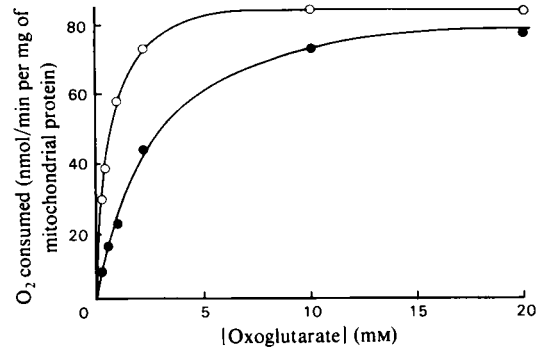

Fig. 2. *Effects of various concentrations of oxoglutarate on the oxygen uptake by rat heart mitochondria incubated in the presence or absence of Ca^{2+}*

Mitochondria (0.5 mg of protein) were incubated at 30°C in 1 ml of 125 mM-KCl/20 mM-Tris/HCl (pH 7.3)/5 mM-KH_2PO_4/1 mM-malate/2 mM-ADP and in the presence of either 5 mM-EGTA (●, free $[Ca^{2+}] < 1$ nM) or 5 mM-EGTA plus 2.5 mM-$CaCl_2$ (○, free $[Ca^{2+}] \simeq 50$ nM). Oxoglutarate was added as indicated. Experimental procedure was similar to that in Fig. 1. Each point shown represents the mean of observations made on at least three different preparations of mitochondria. See the text for calculated values.

The presence of extramitochondrial Ca^{2+} (50 nM) had no appreciable effect on the rate of O_2 uptake in the absence of added oxoglutarate (Figs. 1*a*–1*c*) or on the oxidation of non-saturating or saturating concentrations of succinate (with rotenone) (Fig. 1*d*) or glutamate (results not shown). The coupling ratios (Chance & Williams, 1956) of the mitochondria used in these studies were between 3 and 5 when oxidizing saturating concentrations of oxoglutarate or glutamate (in the presence of malate). Since Ca^{2+} at the low concentrations used in our studies did not increase the uptake of O_2 even in the absence of ADP, the coupling ratios were unaffected by Ca^{2+} (results not shown).

The maximum rates of oxoglutarate oxidation by the coupled rat heart mitochondria incubated with ADP were in the range 140–190 nmol of NADH or equivalent oxidized/min per mg of protein. This is similar to the maximum value found with the uncoupled mitochondria (Table 1) and thus slightly exceeds the maximum activity of oxoglutarate dehydrogenase found in mitochondrial extracts. It seems unlikely that the respiratory chain limits the rate of oxidation of oxoglutarate in coupled heart mitochondria incubated with ADP, as rates of O_2 consumption observed in the presence of saturating concentrations of glutamate plus malate or succinate (the latter in the presence of rotenone) were approximately double those observed at saturating concentrations of oxoglutarate. Decreasing the pH of the incubation buffer from 7.3 to 7.0 increased the rate of oxidation of 0.5 mM-oxoglutarate 3-fold without changing that of 20 mM-oxoglutarate or of non-saturating or saturating concentrations of succinate or glutamate (results not shown). Since the K_m for oxoglutarate of oxoglutarate dehydrogenase is known to be diminished with decreasing pH (McCormack & Denton, 1979, 1980), these ob-

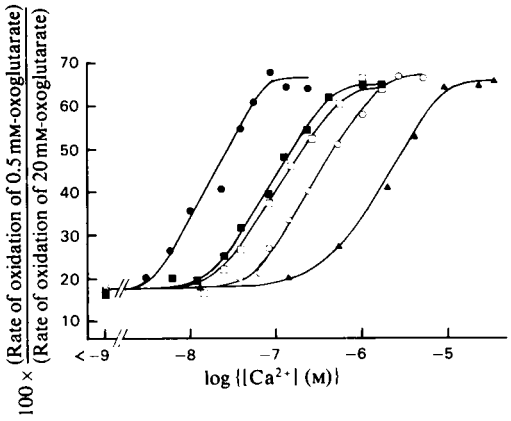

Fig. 3. *Sensitivity to Ca^{2+} of the oxidation of oxoglutarate (0.5 mM) by rat heart mitochondria in the absence and presence of Na^+, Mg^{2+} and Ruthenium Red*

Mitochondria were incubated as described in Fig. 2, in the presence of 5 mM-EGTA with additions of $CaCl_2$ to give the concentrations of free Ca^{2+} indicated, and with the additional presence of: no further additions (●); 15 mM-NaCl (■); 0.5 mM-$MgCl_2$ (□); 15 mM-NaCl plus 0.5 mM-$MgCl_2$ (○); and 1 μg of Ruthenium Red/ml (▲). Experimental procedure was similar to that in Fig. 1, and rates of oxidation at 0.5 mM-oxoglutarate are given as a percentage of the rate at a saturating oxoglutarate concentration (20 mM). The rate at 20 mM-oxoglutarate was 88 ± 7 nmol of O_2 consumed/min per mg of protein (mean ± S.E.M. for 25 observations). Each point shown represents the mean of observations made on two to six different preparations of mitochondria. See Table 4 for calculated values.

servations are also consistent with oxoglutarate dehydrogenase being rate-limiting in the oxidation of oxoglutarate by the coupled rat heart mitochondria.

We have investigated the sensitivity of oxoglutarate oxidation to activation by extramitochondrial Ca^{2+} at two different low concentrations of oxoglutarate (0.25 and 0.5 mM). Since virtually identical results were obtained at both concentrations, only results obtained by using 0.5 mM-oxoglutarate are presented. In all cases, rates of oxoglutarate oxidation are shown after subtraction of the rate of O_2 uptake observed in the absence of added oxoglutarate. In some experiments, oxoglutarate disappearance from the incubation medium was followed and found to match closely the changes in O_2 uptake. The results are expressed as a percentage of the maximum rate of oxoglutarate oxidation. This was obtained with the same sample of mitochondria by increasing the concentration of oxoglutarate to 20 mM after the rate with 0.5 mM-oxoglutarate had been established in a similar manner to that shown in Figs. 1(*a*)–1(*c*). Separate experiments showed that similar rates of oxidation were obtained by observing the increase of O_2 uptake that occurred when 20 mM-oxoglutarate was added directly after the 3 min preincubation period without oxoglutarate.

The sensitivity of the oxidation of 0.5 mM-oxoglutarate to activation by extramitochondrial Ca^{2+} is shown in Fig. 3. The overall stimulation was 3–4-fold. In the absence of added NaCl, $MgCl_2$ or Ruthenium Red (Fig. 3, solid circles) the calculated $k_{0.5}$ was about 20 nM (see Table 4).

Effects of NaCl, $MgCl_2$ and Ruthenium Red on the Ca^{2+}-activation of the oxidation of 0.5 mM-oxoglutarate by coupled rat heart mitochondria

It has been mentioned above that the addition of either NaCl (15 mM), which might be expected to stimulate Ca^{2+} efflux from mitochondria, or $MgCl_2$ (0.5 mM), which may inhibit Ca^{2+} uptake, diminished the activation of oxoglutarate oxidation by Ca^{2+} (50 nM) (Figs. 1*a*–1*c*). Results of similar experiments carried out on a number of different preparations of heart mitochondria are summarized in Table 2. Neither $MgCl_2$ nor NaCl had any effect on the oxidation of oxoglutarate (0.5 mM) in the absence of added Ca^{2+}. Consistent with the experiments shown in Figs. 1(*a*)–1(*c*), when the extramitochondrial concentration of Ca^{2+} was about 50 nM, the presence of either NaCl (15 mM) or $MgCl_2$ (0.5 mM) significantly diminished the extent of oxoglutarate oxidation. However, when the extramitochondrial concentration of Ca^{2+} was further increased to 210 nM, no statistically significant effect of either NaCl or $MgCl_2$ was apparent unless both NaCl and $MgCl_2$ were present simultaneously. These results suggested that NaCl and $MgCl_2$ may each alter the sensitivity of the oxidation of oxoglutarate to extramitochondrial Ca^{2+} without changing the maximum stimulation that can be obtained with Ca^{2+}. The results of Fig. 3 confirm this view. In the presence of either NaCl (15 mM) or $MgCl_2$ (0.5 mM) the $k_{0.5}$ for Ca^{2+} was increased by about 4-fold from 20 nM to about 80–90 nM. The effects of NaCl and $MgCl_2$ seemed to be largely independent, since in the presence of both NaCl and $MgCl_2$ the $k_{0.5}$ for Ca^{2+} was increased some 16-fold to about 320 nM (Table 4). The addition of Ruthenium Red (1 μg/ml) resulted in an even larger decrease in the sensitivity to extramitochondrial Ca^{2+}, such that very little activation was observed below 0.5 μM-Ca^{2+} and maximum stimulation required an extramitochondrial $[Ca^{2+}]$ above 20 μM.

For all the observations reported in Fig. 3, the rate of oxidation of 20 mM-oxoglutarate was essentially unaltered (values were all between 70 and 80 nmol of O_2 taken up/min per mg of protein). However, during the course of these experiments it became

Table 2. *Effects of Ca^{2+}, Sr^{2+}, Mg^{2+}, Na^+ and Ruthenium Red on the oxidation of oxoglutarate by intact rat heart mitochondria*

Mitochondria (≃0.5 mg of protein) were incubated in 1 ml of 125 mM-KCl/20 mM-Tris/HCl (pH 7.3)/5 mM-KH_2PO_4/1 mM-malate/2 mM-ADP with additions of EGTA, $CaCl_2$, $SrCl_2$, $MgCl_2$, NaCl and Ruthenium Red as indicated. The free Ca^{2+} or Sr^{2+} concentrations given refer to the extramitochondrial concentrations. Experimental procedure was as in Fig. 1, with measurement of O_2-consumption rates being made at 0.5 and 20 mM-oxoglutarate. The 'blank' rates (i.e. those before oxoglutarate addition) were subtracted from those with oxoglutarate present and the results are expressed as 100 × (rate of oxidation at 0.5 mM-oxoglutarate)/(rate of oxidation at 20 mM-oxoglutarate). The rate of oxidation at 20 mM-oxoglutarate ($V_{max.}$) was 83 ± 6 nmol of O_2 consumed/min per mg of protein (mean ± S.E.M. for 30 observations). Values are given as means ± S.E.M. for the numbers of observations in parentheses. Each value is derived from observations made on at least three different preparations of mitochondria. *Effect of other additions $P < 0.001$ when compared with appropriate value in the absence of other additions (in top row); †effect of Ca^{2+} $P < 0.001$ when compared with appropriate value with 5 mM-EGTA (in the first column); both comparisons made by Student's *t* test.

Other additions	Rate of oxidation of 0.5 mM-oxoglutarate (% of rate of oxidation of 20 mM-oxoglutarate) with: 5 mM-EGTA (control) ([Ca^{2+}] < 1 nM)	5 mM-EGTA plus 2.5 mM-$CaCl_2$ ([Ca^{2+}] ≃ 50 nM)	5 mM-EGTA plus 4 mM-$CaCl_2$ ([Ca^{2+}] ≃ 210 nM)	5 mM-EGTA plus 0.1 mM $SrCl_2$ ([Sr^{2+}] ≃ 340 nM)
None (control)	16 ± 1.0 (12)	47 ± 2.0 (20)†	47 ± 2.1 (3)†	44 ± 1.6 (6)†
15 mM-NaCl	15 ± 2.3 (3)	27 ± 2.6 (14)*	50 ± 2.5 (3)†	20 ± 3.3 (4)*
0.5 mM-$MgCl_2$	17 ± 2.6 (3)	22 ± 1.9 (5)*	51 ± 2.1 (3)†	26 ± 1.8 (3)*
15 mM-NaCl plus 0.5 mM-$MgCl_2$	17 ± 1.2 (4)	14 ± 3.3 (3)*	31 ± 2.9 (3)*†	17 ± 0.9 (3)*
Ruthenium Red (1 μg/ml)	16 ± 1.3 (3)	16 ± 1.0 (3)*	19 ± 2.1 (3)*	15 ± 1.8 (3)*

Table 3. *Effects of increasing concentrations of Ca^{2+} on the maximum rate of oxidation of oxoglutarate (20 mM) by rat heart mitochondria*

Mitochondria (≃0.5 mg of protein) were incubated in 1 ml of 125 mM-KCl/20 mM-Tris/HCl (pH 7.3)/5 mM-KH_2PO_4/1 mM-malate/2 mM-ADP with additions of EGTA and $CaCl_2$ to give the required (extramitochondrial) Ca^{2+} concentrations. Oxoglutarate (20 mM) was added as substrate and the 'blank' rate (which was similar in each case) was subtracted to give the oxoglutarate-dependent rate. The maximal rates of oxidation are given as means ± S.E.M. for the numbers of observations in parentheses. Each value is derived from observations on at least three different preparations of mitochondria. *$P < 0.05$ and **$P < 0.01$ when compared with control value (underlined) by Student's *t* test.

Approx. calculated [Ca^{2+}] (nM)	Rate of oxoglutarate oxidation (as nmol of O_2 consumed/min per mg of protein) in the presence of: No further additions	15 mM-NaCl	0.5 mM-$MgCl_2$	15 mM-NaCl plus 0.5 mM-$MgCl_2$	Ruthenium Red (1 μg/ml)
1	73.1 ± 8.0 (7)	76.4 ± 5.9 (4)	74.9 ± 3.1 (4)	69.4 ± 3.6 (4)	73.4 ± 4.9 (4)
50	72.9 ± 1.7 (5)	72.3 ± 5.6 (4)	74.0 ± 1.6 (7)	72.6 ± 4.8 (4)	76.1 ± 3.3 (3)
210	48.4 ± 3.5 (6)*	69.7 ± 4.6 (4)	71.9 ± 4.6 (3)	70.4 ± 4.7 (4)	—
470	23.0 ± 3.0 (4)**	42.9 ± 3.9 (4)*	41.2 ± 4.7 (3)*	71.8 ± 5.0 (5)	—
1050	—	33.5 ± 8.2 (3)*	26.1 ± 3.8 (3)**	36.0 ± 5.2 (4)*	70.0 ± 5.4 (5)

evident that when the concentration of extramitochondrial Ca^{2+} exceeded a certain critical value the rates of oxidation of 20 mM-oxoglutarate became progressively smaller. The critical concentration of Ca^{2+} depended on the presence of NaCl, $MgCl_2$ and Ruthenium Red (Table 3). In the absence of any of these compounds, the decrease became apparent above 100 nM-Ca^{2+}; by 200 nM-Ca^{2+} the rate was halved, and at 1 μM-Ca^{2+} little or no oxidation occurred. In the presence of either $MgCl_2$ or NaCl, the decrease was not apparent until above 210 nM-Ca^{2+}, and in the presence of both not until above 470 nM (Table 3). With Ruthenium Red, no decrease was observed even at 1 μM-Ca^{2+}. These decreases in the rate of oxidation of a saturating concentration of oxoglutarate seem to be associated with a rather general disruption of mitochondrial function, since the rates of oxidation of succinate and other substrates were also found to be diminished.

The effects of varying the concentrations of NaCl and $MgCl_2$ on the oxidation of oxoglutarate (0.5 mM)

in the presence of 120nM-Ca^{2+} were explored (results not shown). The values of K_i from these experiments calculated on the basis of all the individual observations were 1.09 ± 0.13mM (28) and 0.31 ± 0.038mM (25) for extramitochondrial NaCl and $MgCl_2$ respectively. The K_i for NaCl seemed largely unaffected by the presence of $MgCl_2$, and vice versa, since very similar K_i values were obtained in the presence of 210nM-Ca^{2+} plus either 0.5mM-$MgCl_2$ or 15mM-NaCl (as appropriate). The actual values were 1.3 ± 0.18mM (18) and 0.29 ± 0.06mM (19) for NaCl and $MgCl_2$ respectively. Results are given as means ± S.E.M. for the numbers of degrees of freedom given in parentheses. Under the conditions of these experiments appreciable binding of the extramitochondrial Mg^{2+} occurs to ADP, P_i and EGTA. When allowance is made for this binding the apparent K_i for inhibition by Mg^{2+} is about 60μM in both cases.

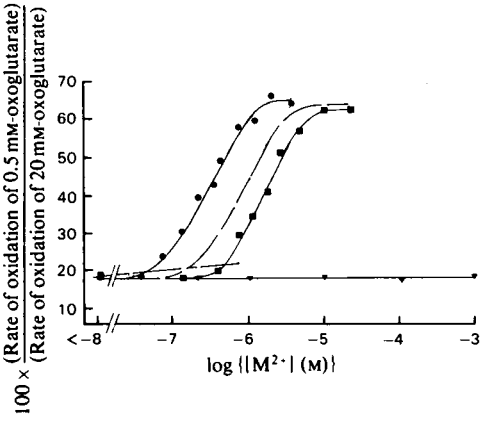

Fig. 4. *Sensitivity of the oxidation of oxoglutarate (0.5mM) by rat heart mitochondria to Mg^{2+} and Mn^{2+} and to Sr^{2+} in the presence and absence of Na^+ and Mg^{2+}*

Mitochondria were incubated as described in Fig. 2 in the presence of 5mM-EGTA with additions of $SrCl_2$, $MnCl_2$ and $MgCl_2$ to give the indicated concentrations of Sr^{2+} (●, □, ■), Mn^{2+} (△) and Mg^{2+} (▼), and with the additional presence of 15mM-NaCl (■) and 0.5mM-$MgCl_2$ (□). Experimental procedure was as in Fig. 1 and expression of the rate of 0.5mM-oxoglutarate oxidation as in Fig. 3. The rate at 20mM-oxoglutarate was 81 ± 5 nmol of O_2 consumed/min per mg of protein (mean ± S.E.M. for 20 observations). Each point shown represents the mean of observations made on two to six different preparations of mitochondria. See Table 4 for calculated values.

Effects of extramitochondrial Sr^{2+}, Mn^{2+} and Mg^{2+} on the oxidation of oxoglutarate by coupled rat heart mitochondria

Sr^{2+} was found to have very similar effects on the oxidation of oxoglutarate to those found with Ca^{2+}, but a 10–15 times higher concentration was required (Table 2 and Fig. 4). In the absence of added $MgCl_2$ or NaCl, the $k_{0.5}$ for the activation of the oxidation of 0.5mM-oxoglutarate was about 300nM (compared with about 20nM for Ca^{2+}, see Table 4). As with Ca^{2+}, the $k_{0.5}$ for Sr^{2+} was increased about 3–4-fold by either $MgCl_2$ (0.5mM) or NaCl (15mM) (Fig. 4 and Table 4).

No evidence of any activation of the oxidation of 0.5mM-oxoglutarate was observed with concentrations of Mn^{2+} up to 1μM (in MnEGTA buffers) or with concentrations of Mg^{2+} up to 1mM (Fig. 4). At concentrations of Mn^{2+} above 1μM some stimulation was observed, but this was most probably the result of the displacement of Ca^{2+} from EGTA and other extramitochondrial or intramitochondrial sites (Denton *et al.*, 1978*a*).

Table 4. *Summary of the activation constants for Ca^{2+} and Sr^{2+} on oxoglutarate oxidation by, and pyruvate dehydrogenase activity in, rat heart mitochondria in the presence and absence of Na^+, Mg^{2+} and Ruthenium Red*

Individual observations of the results shown in Figs. 3, 4 and 6 were fitted to the equation $v = (V_{max.}/\{1 + (k_{0.5}/[M^{2+}])^n\}) + V_0$, where V_0 is the enzyme activity or oxidation rate in the presence of 5mM-EGTA ($[Ca^{2+}] < 1$nM). The values given refer to the extramitochondrial concentrations of M^+ (i.e. Ca^{2+} or Sr^{2+}) required to bring about the half-maximal response. Values are expressed as means ± S.E.M. for the numbers of degrees of freedom shown in parentheses. Each $k_{0.5}$ value was computed from values obtained from observations on at least three different preparations of mitochondria.

	$k_{0.5}$ values for the activation of:			
	Oxoglutarate oxidation by:		Pyruvate dehydrogenase activity by:	
Additions	Ca^{2+} (nM)	Sr^{2+} (nM)	Ca^{2+} (nM)	Sr^{2+} (nM)
None	21 ± 3 (52)	301 ± 46 (20)	39 ± 2 (27)	328 ± 46 (20)
15mM-NaCl	82 ± 12 (33)	1410 ± 234 (18)	189 ± 25 (22)	—
0.5mM-$MgCl_2$	96 ± 14 (29)	843 ± 161 (12)	175 ± 23 (22)	—
15mM-NaCl plus 0.5mM-$MgCl_2$	328 ± 41 (28)	—	464 ± 34 (16)	—
Ruthenium Red (1μg/ml)	3290 ± 210 (19)	—	3697 ± 101 (18)	—

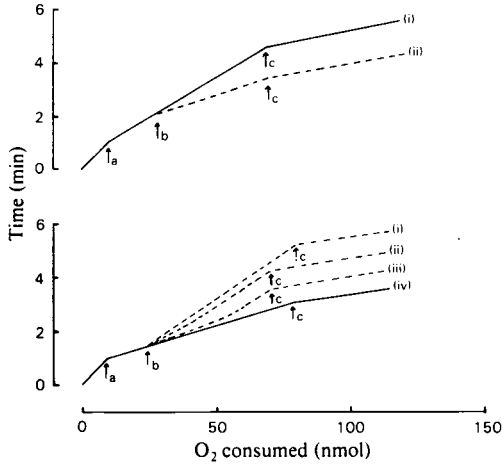

Fig. 5. *Examples of the reversibility of the activation by Ca^{2+} of oxoglutarate oxidation by rat heart mitochondria*

Mitochondria (0.5 mg of protein) were incubated at 30°C in 1 ml of 125 mM-KCl/20 mM-Tris/HCl (pH 7.3)/5 mM-KH_2PO_4/2 mM-ADP/1 mM-malate with initially, in upper panel 2.5 mM-EGTA ($[Ca^{2+}] < 1$ nM), and in lower panel 0.5 mM-EGTA plus 0.5 mM-$CaCl_2$ ($[Ca^{2+}] \simeq 50$ nM). Additions were made (at the points indicated): *a*, 0.5 mM-oxoglutarate in all cases; *c*, 20 mM-oxoglutarate in all cases; *b*, various additions as follows: top panel; trace (i), no addition (control); trace (ii), 2.5 mM-EGTA plus 2.5 mM-$CaCl_2$ ($[Ca^{2+}] \simeq 50$ nM); bottom panel; trace (i), 15 mM-NaCl; trace (ii), 4 mM-EDTA (final $[Ca^{2+}] \simeq 2$ nM); trace (iii), 4 mM-EGTA (final $[Ca^{2+}] \simeq 7$ nM); trace (iv), no addition (control).

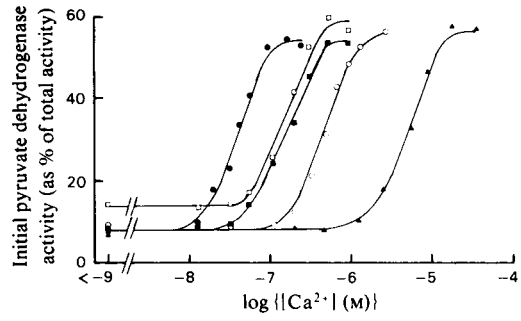

Fig. 6. *Sensitivity to Ca^{2+} of the initial activity of pyruvate dehydrogenase in rat heart mitochondria in the absence and presence of Na^+, Mg^{2+} and Ruthenium Red*

Mitochondria (0.5 mg of protein) were incubated for 4 min at 30°C in 1 ml of 125 mM-KCl/20 mM-Tris/HCl (pH 7.3)/5 mM-KH_2PO_4/1 mM-malate/15 mM-oxoglutarate in the presence of 5 mM-EGTA and additions of $CaCl_2$ to give the concentrations of free Ca^{2+} indicated, and with the additional presence of: no further additions (●); 15 mM-NaCl (■); 0.5 mM-$MgCl_2$ (□); 15 mM-NaCl plus 0.5 mM-$MgCl_2$ (○); and 1 μg of Ruthenium Red/ml (▲). Initial and total activities of pyruvate dehydrogenase were assayed as described in the Experimental section. The total activity was 91 ± 5 munits/mg of mitochondrial protein (mean ± S.E.M. for 24 observations). Similar results were obtained after incubations of 8 min duration. Each point shown represents the mean of observations made on two to six different preparations of mitochondria. See Table 4 for calculated values.

Reversibility of the effects of Ca^{2+} on the oxidation of oxoglutarate by coupled rat heart mitochondria

Fig. 5 shows some typical examples of the reversibility of the activation by Ca^{2+} of oxoglutarate oxidation by rat heart mitochondria. The upper panel shows that the addition of Ca^{2+} (about 50 nM) to the incubation medium while the mitochondria were oxidizing 0.5 mM-oxoglutarate at <1 nM-Ca^{2+} produced an apparently immediate stimulation of oxidation. Similar, apparently immediate responses were noted if Ca^{2+} was added at about 120 nM to mitochondria oxidizing 0.5 mM-oxoglutarate at <1 nM-Ca^{2+} in the presence of either 15 mM-NaCl or 0.5 mM-$MgCl_2$. On the other hand, if the mitochondria were oxidizing 0.5 mM-oxoglutarate in the presence of about 50 nM-Ca^{2+}, addition of NaCl, EDTA or EGTA resulted in a decrease in the rate of oxidation (Fig. 5, lower panel). Addition of NaCl appeared to result in an immediate decrease in the oxidation rate, whereas with the Ca^{2+} chelators a more gradual decline was observed. With excess EGTA it appeared to take at least 1 min for Ca^{2+} to be exported from the mitochondria. No effects were found under any of these conditions on the rate of oxidation of 20 mM-oxoglutarate (Fig. 5).

Effects of Ca^{2+} on the initial activity of pyruvate dehydrogenase in coupled rat heart mitochondria

In these studies, rat heart mitochondria were incubated in the presence of oxoglutarate (15 mM), malate (1 mM) and phosphate (5 mM). No extramitochondrial ADP was added to ensure high intramitochondrial concentrations of ATP, and thus of active pyruvate dehydrogenase kinase. When the extramitochondrial Ca^{2+} concentration was maintained below 1 nM with 5 mM-EGTA, the pyruvate dehydrogenase within the mitochondria was largely in its inactive phosphorylated form. In agreement with earlier studies carried out under similar conditions of incubation, the initial activity of pyruvate dehydrogenase measured in mitochondrial extracts was less than 10% of the total activity (Kerbey *et al.*, 1976; Hutson *et al.*, 1978). However, as the extramitochondrial Ca^{2+} was increased, the initial activity also increased until it reached a value of about 60% of the total activity (Fig. 6). The $k_{0.5}$ for the activation by extramitochondrial Ca^{2+} in the absence of NaCl or $MgCl_2$ was about 40 nM (Fig. 6).

Similar effects were observed with Sr^{2+}, but the $k_{0.5}$ was considerably higher (Table 4). The presence of either NaCl or $MgCl_2$ increased the $k_{0.5}$ value for extramitochondrial Ca^{2+} to about 180nM, and in the presence of both NaCl and $MgCl_2$ the $k_{0.5}$ reached 460nM (Fig. 6 and Table 4). The apparent $k_{0.5}$ in the presence of Ruthenium Red was in excess of 3.5μM. The addition of NaCl, $MgCl_2$ or Ruthenium Red had no significant effect on the initial activity of pyruvate dehydrogenase in mitochondria incubated in the absence of added Ca^{2+} or on the maximum stimulation that could be observed with Ca^{2+}. The total activity of pyruvate dehydrogenase was essentially unaltered (all values were in the range 80–100munits/mg of mitochondrial protein) for the observations given in Fig. 6. The results given in Fig. 6 were obtained after incubation of mitochondria for 4min; similar results were obtained after incubation for 8min.

Some determinations of the ATP content of the rat heart mitochondria were made to investigate the extent to which these observed changes in initial pyruvate dehydrogenase activity could have been brought about by changes in the intramitochondrial ATP/ADP concentration ratio (results not shown). However, the presence of extramitochondrial Ca^{2+} at concentrations sufficient to result in maximal increases in initial pyruvate dehydrogenase activity did not cause any significant decrease in intramitochondrial ATP content [values obtained were 7.9 ± 1.3 (4) and 7.5 ± 1.0 (3)nmol of ATP/mg of protein at <1nM- and 50nM-Ca^{2+} respectively; results given as means $\pm$ S.E.M. for numbers of observations in parentheses]. The presence of NaCl (15mM), $MgCl_2$ (0.5mM) or Ruthenium Red (1μg/ml) may all cause a small decrease in ATP content, particularly in the absence of added Ca^{2+} (to about 5.5–6.5nmol of ATP/mg of protein). However, these decreases were not associated with any consistent or marked increase in the initial activity of pyruvate dehydrogenase.

Discussion

Effects of changes in extramitochondrial Ca^{2+} on the activities of oxoglutarate dehydrogenase and pyruvate dehydrogenase within coupled rat heart mitochondria

Increasing the extramitochondrial concentration of Ca^{2+} specifically decreased the apparent K_m for the oxidation of oxoglutarate without increasing the $V_{max.}$. The most straightforward explanation is that the change in K_m results from increases in the intramitochondrial concentration of Ca^{2+} acting directly on oxoglutarate dehydrogenase, which is rate-limiting in the oxidation of oxoglutarate. It appears rather unlikely that the effects are brought about indirectly via a diminution in either the intramitochondrial ATP/ADP concentration ratio or the pH. No changes in the intramitochondrial ATP content were observed with changes in extramitochondrial Ca^{2+} in the absence of ADP. A change in ΔpH across the mitochondrial inner membrane would be expected to affect the apparent K_m for succinate oxidation (McCormack & Denton, 1980), but no such effect was found. In any case, a fall in intramitochondrial pH is most unlikely, since Ca^{2+} uptake is probably associated ultimately with the efflux of protons from mitochondria.

Except in the presence of Ruthenium Red, the $k_{0.5}$ values for the activation of the oxidation of 0.5mM-oxoglutarate by extramitochondrial Ca^{2+} in coupled heart mitochondria (Table 4) were all less than that observed with uncoupled mitochondria. This is compatible with the Ca^{2+} concentration within mitochondria being greater than that outside the mitochondria under these conditions. If calcium enters mitochondria as Ca^{2+} with a membrane potential of 180mV and no efflux pathway, then the gradient of Ca^{2+} (in:out) should approach 10^6. Clearly, even in the absence of Na^+ and Mg^{2+} ions the apparent gradient is much smaller and probably only about 45 (Table 4). The $k_{0.5}$ value for Ca^{2+} was increased by $MgCl_2$ and NaCl in an essentially independent manner, as would be expected from the properties of the Ca^{2+}-transporting system. In the presence of both $MgCl_2$ and NaCl, the $k_{0.5}$ value was 330nM, which is consistent with the intramitochondrial Ca^{2+} concentration being about 3 times the extramitochondrial concentration under these conditions.

The $k_{0.5}$ value for the activation by Ca^{2+} of pyruvate dehydrogenase phosphate phosphatase in extracts of pig and rat heart mitochondria, like the value for oxoglutarate dehydrogenase, is close to 1μM (Denton *et al.*, 1972, 1975; Randle *et al.*, 1974). Very similar values have also been obtained for the enzyme from other sources (Denton *et al.*, 1972; Severson *et al.*, 1974; McCormack & Denton, 1980) and for the increase in pyruvate dehydrogenase activity within uncoupled white-adipose-tissue and rat heart mitochondria (McCormack & Denton, 1980; J. G. McCormack & R. M. Denton, unpublished work).

It is evident from Table 4 that the sensitivity of pyruvate dehydrogenase activity within coupled heart mitochondria to changes in extramitochondrial Ca^{2+} matches closely the sensitivity of oxoglutarate oxidation. In particular, both NaCl and $MgCl_2$ increase the $k_{0.5}$ values for activation by extramitochondrial Ca^{2+} approx. 4-fold. In the presence of both NaCl and $MgCl_2$ the $k_{0.5}$ value is nearly 0.5μM, indicating that under these conditions the intramitochondrial Ca^{2+} concentration is approximately twice the extramitochondrial Ca^{2+} concentration. These effects on pyruvate dehydro-

genase activity greatly strengthen the conclusions that we have drawn from the studies on oxoglutarate oxidation, since these parameters represent two independent means of investigating the relationship between intra- and extra-mitochondrial Ca^{2+}. The activity of NAD^+–isocitrate dehydrogenase would be a third means, but unfortunately the activity of this enzyme within heart mitochondria is difficult to monitor, because of the very slow rate of transfer of citrate or *threo*-D_s-isocitrate (Chappell & Robinson, 1968).

Hansford & Cohen (1978) have also studied the activating effects of extramitochondrial Ca^{2+} on the activity of pyruvate dehydrogenase in coupled rat heart mitochondria oxidizing pyruvate and palmitoyl-L-carnitine in '50 per cent state 3'. These workers found a $k_{0.5}$ of about 300 nM in the presence of 0.9 mM-$MgCl_2$ and 1.1 mM-NaCl. They also showed that the stimulation of pyruvate dehydrogenase activity by Ca^{2+} could be reversed by addition of excess EGTA, but the reversal took some 5 min to be completed.

Since the activity of pyruvate dehydrogenase in rat heart mitochondria, and thus by inference in other mammalian mitochondria, can be altered greatly by changes in extramitochondrial Ca^{2+}, Na^+ and Mg^{2+}, it follows that the concentrations of these ions must be considered in studies on the regulation of pyruvate dehydrogenase activity in isolated mitochondria. In a number of previous studies, mitochondria have been incubated in the presence of EGTA (Martin *et al.*, 1972; Kerbey *et al.*, 1976, 1977; Hutson *et al.*, 1978; Denton *et al.*, 1978*b*). Under these conditions, the activity of the phosphatase may become much diminished owing to loss of Ca^{2+} from the mitochondria. However, at least in the absence of Na^+, the rate of loss may be rather slow and take minutes before it is completed. In this way, changes in intramitochondrial Ca^{2+} occurring in tissues may persist in isolated mitochondria (Denton *et al.*, 1978*b*).

Overall conclusions

The observations made in this study strongly support the concept of the distribution of Ca^{2+} across the inner membrane of rat heart mitochondria being determined by a cycle composed of separate uptake and efflux components (Carafoli & Crompton, 1978; Crompton & Heid, 1978; Carafoli, 1979). The only discrepancy is the apparent sensitivity of these components to Mg^{2+} and Na^+ ions. We observed half-maximal effects of these ions on oxoglutarate oxidation at about 60 μM and 1 mM respectively. These values are rather lower than those reported on studies on the individual components (Crompton *et al.*, 1976*a*,*b*). A likely explanation for this discrepancy is the much lower extramitochondrial concentrations of Ca^{2+} used in the present study. The rate of Ca^{2+} uptake into heart mitochondria can exceed the rate of efflux at high concentrations of extramitochondrial Ca^{2+} (Carafoli, 1979). Under such conditions, and especially if the extramitochondrial Ca^{2+} is buffered, as in our experiments, it is to be expected that massive loading of the mitochondria by Ca^{2+} (driven by the membrane potential) would occur eventually. We found that the capacity of the heart mitochondria to oxidize oxoglutarate and other substrates diminished above certain critical concentrations of extramitochondrial Ca^{2+}, which depended on the presence or absence of NaCl and $MgCl_2$. Presumably, this rather general loss of mitochondrial function was brought about because the rate of Ca^{2+} uptake exceeded that of efflux.

In the intact rat heart it is probable that the cytoplasmic concentration of Na^+ is in the range 5–6 mM (Lee & Fozzard, 1975), that of Mg^{2+} is above 0.25 mM (Veloso *et al.*, 1973; Hutson, 1977) and that of Ca^{2+} varies between 0.1 and 1 μM (Rasmussen & Goodman, 1977; Wollenberger & Will, 1978; Carafoli, 1979). Under these conditions, the concentration of intramitochondrial Ca^{2+} would be predicted to be in the range 0.1–2 μM on the basis of the results reported in this paper. It follows that the activities of the Ca^{2+}-sensitive dehydrogenases in heart mitochondria are likely to be enhanced when increases in cytoplasmic Ca^{2+} occur. Since such increases will be associated usually with the stimulation of muscle contraction and therefore ATP utilization, it obviously makes sense if intramitochondrial oxidative metabolism is stimulated in parallel. It has been shown in both heart and gastrocnemius muscle preparations that changes in work load are associated with parallel changes in the proportion of pyruvate dehydrogenase in its active non-phosphorylated form (Illingworth & Mullings, 1976; Hennig *et al.*, 1975). These changes in pyruvate dehydrogenase may be brought about, at least in part, by increases in intramitochondrial Ca^{2+} (Denton & Halestrap, 1979).

If, as we propose, the concentration of Ca^{2+} is an important regulator of intramitochondrial oxidative metabolism, it follows that mitochondria are unlikely to be important in the regulation of cytoplasmic Ca^{2+} under normal physiological conditions, at least in heart and other muscle. Such a role for mitochondria would necessarily mean that an inverse relationship would exist between the concentration of Ca^{2+} in the cytoplasm and in the mitochondria. It is therefore an important inference from our studies that the Ca^{2+}-transporting components in mitochondria should be considered primarily as a system for the regulation of the concentration of Ca^{2+} within mitochondria rather than being involved in the regulation of cytoplasmic Ca^{2+}.

All mammalian mitochondria so far studied

contain the three Ca^{2+}-sensitive dehydrogenases (see McCormack & Denton, 1980); thus it seems reasonable to extend the above conclusions to other tissues. For example, as we have suggested previously, the activation of pyruvate dehydrogenase in livers exposed to vasopressin may be brought about by a rise in the intramitochondrial concentration of Ca^{2+} occurring as a result of the increase in the concentration of Ca^{2+} in the cytoplasm (Hems *et al.*, 1978). There are many other examples of hormones and neurotransmitters which are thought to bring about their effects via an increase in cytoplasmic Ca^{2+} (Berridge, 1976; Rasmussen & Goodman, 1977; Michell *et al.*, 1977). Often these effects (such as increases in secretion or in transport) involve increased utilization of ATP and are thus associated with increased mitochondrial oxidative metabolism. Parallel changes in cytoplasmic and intramitochondrial Ca^{2+} may be a rather general means whereby stimulation of the supply of reducing equivalents for respiration can be achieved with minimum increases in the NAD^+/NADH and ADP/ATP ratios. Finally it should be mentioned that it is also possible that particular hormones or other extrinsic factors may alter the activity of one or both of the components of the mitochondrial transport system and thus regulate intramitochondrial Ca^{2+} without necessarily changing the concentration of Ca^{2+} in the cytoplasma.

These studies were supported by grants from the Medical Research Council. We thank Dr. Paul England for supplying computer programs.

References

Berridge, M. J. (1976) *Adv. Cyclic Nucleotide Res.* **6**, 1–98

Bygrave, F. L. (1978) *Biol. Rev. Cambridge Philos. Soc.* **53**, 43–79

Carafoli, E. (1979) *FEBS Lett.* **104**, 1–5

Carafoli, E. & Crompton, M. (1978) *Curr. Top. Membr. Transp.* **10**, 151–216

Caroni, P., Schwerzmann, K. & Carafoli, E. (1978) *FEBS Lett.* **96**, 339–342

Chance, B. & Williams, G. R. (1956) *Adv. Enzymol.* **17**, 65–134

Chappell, J. B. & Robinson, B. H. (1968) *Biochem. Soc. Symp.* **27**, 123–133

Crompton, M. & Heid, I. (1978) *Eur. J. Biochem.* **91**, 599–608

Crompton, M., Sigel, E., Salzmann, M. & Carafoli, E. (1976*a*) *Eur. J. Biochem.* **69**, 429–434

Crompton, M., Caprano, M. & Carafoli, E. (1976*b*) *Eur. J. Biochem.* **69**, 453–462

Crompton, M., Künzi, M. & Carafoli, E. (1977) *Eur. J. Biochem.* **79**, 549–558

Crompton, M., Moser, R., Lüdi, H. & Carafoli, E. (1978) *Eur. J. Biochem.* **82**, 25–31

Deana, R., Arrabaca, J. D., Mathien-Shire, Y. & Chappell, J. B. (1979) *FEBS Lett.* **106**, 231–234

Denton, R. M. & Halestrap, A. P. (1979) *Essays Biochem.* **15**, 37–77

Denton, R. M., Randle, P. J. & Martin, B. R. (1972) *Biochem. J.* **128**, 161–163

Denton, R. M., Randle, P. J., Bridges, B. J., Cooper, R. H., Kerbey, A. L., Pask, H. T., Severson, D. L., Stansbie, D. & Whitehouse, S. (1975) *Mol. Cell. Biochem.* **9**, 27–53

Denton, R. M., Richards, D. A. & Chin, J. G. (1978*a*) *Biochem. J.* **176**, 899–906

Denton, R. M., Hughes, W. A., Bridges, B. J., Brownsey, R. W., McCormack, J. G. & Stansbie, D. (1978*b*) *Horm. Cell Regul.* **2**, 191–208

Fiskum, G. & Lehninger, A. L. (1979) *J. Biol. Chem.* **254**, 6236–6239

Hansford, R. G. & Cohen, L. (1978) *Arch. Biochem. Biophys.* **191**, 65–81

Heaton, G. M. & Nicholls, D. G. (1976) *Biochem. J.* **156**, 635–646

Hems, D. A., McCormack, J. G. & Denton, R. M. (1978) *Biochem. J.* **176**, 627–629

Hennig, G., Löffler, G. & Wieland, O. H. (1975) *FEBS Lett.* **59**, 142–145

Hutson, N. J., Kerbey, A. L., Randle, P. J. & Sugden, P. H. (1978) *Biochem. J.* **173**, 669–680

Hutson, S. M. (1977) *J. Biol. Chem.* **252**, 4539–4545

Illingworth, J. A. Mullings, R. (1976) *Biochem. Soc. Trans.* **4**, 291–292

Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T. & Denton, R. M. (1976) *Biochem. J.* **154**, 327–348

Kerbey, A. L., Radcliffe, P. M. & Randle, P. J. (1977) *Biochem. J.* **164**, 509–519

Lee, C. O. & Fozzard, H. G. (1975) *J. Gen. Physiol.* **65**, 695–708

McCormack, J. G. & Denton, R. M. (1979) *Biochem. J.* **180**, 533–544

McCormack, J. G. & Denton, R. M. (1980) *Biochem. J.* **190**, 95–105

Martin, B. R., Denton, R. M., Pask, H. T. & Randle, P. J. (1972) *Biochem. J.* **129**, 763–773

Mela, L. (1969) *Biochemistry* **8**, 2481–2486

Mela, L. (1977) *Curr. Top. Membr. Transp.* **9**, 321–366

Michell, R. H., Jafferji, S. S. & Jones, L. M . (1977) *Adv. Exp. Med. Biol.* **83**, 447–465

Moyle, J. & Mitchell, P. (1977*a*) *FEBS Lett.* **73**, 131–136

Moyle, J. & Mitchell, P. (1979*b*) *FEBS Lett.* **77**, 136–145

Nicholls, D. G. (1978) *Biochem. J.* **176**, 463–474

Randle, P. J., Denton, R. M., Pask, H. T. & Severson, D. L. (1974) *Biochem. Soc. Symp.* **39**, 75–87

Rasmussen, H. & Goodman, D. B. P. (1977) *Physiol. Rev.* **57**, 421–509

Reed, K. C. & Bygrave, F. L. (1975) *Eur. J. Biochem.* **55**, 497–504

Rossi, C. S., Vasington, F. D. & Carafoli, E. (1973) *Biochem. Biophys. Res. Commun.* **50**, 846–852

Rottenberg, H. & Scarpa, A. (1974) *Biochemistry* **13**, 4811–4817

Severson, D. L., Denton, R. M., Pask, H. T. & Randle, P. J. (1974) *Biochem. J.* **140**, 225–237

Veloso, D., Guynn, R. W., Oskarsson, M. & Veech, R. L. (1973) *J. Biol. Chem.* **248**, 4811–4819

Wollenberger, A. & Will, H. (1978) *Life Sci.* **22**, 1159–1178