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Studies on mitochondrial Ca^{2+}-transport and matrix Ca^{2+} using fura-2-loaded rat heart mitochondria

James G. McCormack, Helen M. Browne and Nicola J. Dawes

Department of Biochemistry, The University of Leeds, Leeds (U.K.)

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Rat heart mitochondria were incubated for 5 min at 30 °C and at approx. 40 mg protein · ml^{-1} and in the presence of 10 μM fura-2/AM. This allowed the entrapment of free fura-2 within the mitochondrial matrix and its use as a probe for Ca^{2+}, but without affecting the apparent viability of the mitochondria. Parallel measurements of the activities of the intramitochondrial Ca^{2+}-sensitive enzymes, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, allowed an assessment of their sensitivity to measured free Ca^{2+} within intact mitochondria incubated under different conditions; the enzymes responded to matrix Ca^{2+} over the approximate range 0.02–2 μM with half-maximal effects at about 0.3–0.6 μM Ca^{2+}. Effectors of Ca^{2+}-transport across the inner membrane (e.g., Na^+, Mg^{2+}, Ruthenium red, spermine) did not appear to affect these ranges, but did bring about expected changes in Ca^{2+} distribution across this membrane. Significantly, when mitochondria were incubated in the presence of physiological concentrations of both Na^+ and Mg^{2+}, and at low extramitochondrial Ca^{2+} (< 400 nM), there was a gradient of Ca^{2+} (in:out) of less than unity; at higher extramitochondrial [Ca^{2+}] (but still within the physiological range) the gradient was greater than unity indicating a highly cooperative nature of transmission of the Ca^{2+} signal into the matrix under such conditions.

Introduction

The Ca^{2+} transport system of mammalian mitochondria comprises an electrophoretic Ca^{2+} uniporter driven by the membrane potential set up through proton extrusion by the respiratory chain, and probably two egress mechanisms, the principal of which is an electroneutral Na^+/Ca^{2+} exchange which is, ultimately, also driven by the proton gradient through subsequent Na^+/H^+ exchange, and the lesser Na^+-independent mechanism which possibly involves direct $Ca^{2+}/2H^+$ exchange (see Refs. 1 and 2). Uptake can be inhibited physiologically by Mg^{2+} and artificially by Ruthenium red [1,2] and activated by spermine [3], and Na^+/Ca^{2+} exchange can be inhibited physiologically by extramitochondrial Ca^{2+} [4] and artificially by diltiazem and other such agents more normally used as blockers of the plasma membrane Ca^{2+} channel [5]. The overall activity of uptake is about 10-fold in excess of the egress pathways [1,2].

The primary physiological function of this Ca^{2+} transport system is now widely regarded as being in the regulation of matrix Ca^{2+} in the micromolar range, and in conveying changes in cytoplasmic [Ca^{2+}] to the matrix (see Refs.. 6 and 7) rather than, as was thought earlier, that it had a key role in buffering cytoplasmic [Ca^{2+}] (see for example Ref. 2). The need to so regulate matrix Ca^{2+} ($[Ca^{2+}]_m$) resides in the fact that in mammalian tissues there are three exclusively intramitochondrial dehydrogenases that occupy key regulatory sites in oxidative metabolism which can be activated severalfold by increases in Ca^{2+} within this range [7,8]. They are the pyruvate (PDH), NAD^+-isocitrate and 2-oxoglutarate (OGDH) dehydrogenases. There is now strong evidence from studies on heart [8,9] and liver [10,11] that hormones and other agents which activate energy-requiring cytosolic events though increases in cytoplasmic [Ca^{2+}] (e.g., contraction) also, as a result, bring about increases in $[Ca^{2+}]_m$ and thus activate these

Abbreviations: PDH, the pyruvate dehydrogenase complex; PDH_a, the active, non-phosphorylated form of PDH; OGDH, the 2-oxoglutarate dehydrogenase complex; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $K_{0.5}$ value, the concentration of effector required for half-maximal response; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; $[Ca^{2+}]_m$, intramitochondrial (matrix) free [Ca^{2+}]; fura-2/AM, the acetoxymethyl ester of fura-2.

Correspondence: J.G. McCormack, Department of Biochemistry, The University of Leeds, Leeds LS2 9JT, U.K.

enzymes and, hence, oxidative metabolism and energy production [6,7,12]. The enzymes within intact mitochondria from a variety of mammalian tissues have also been shown to be activated by increases in extramitochondrial Ca^{2+} within the expected physiological range (see Ref. 13).

The above studies relied upon the development of techniques to assay for the Ca^{2+}-sensitive properties of the matrix dehydrogenases within intact mitochondria (see Refs. 6 and 7). However, their regulatory ranges for Ca^{2+} may be affected by factors such as the ATP/ADP ratio (see Ref. 14). Therefore it would be most useful to have another independent means of measuring $[Ca^{2+}]_m$. Other approaches tried include a 'null-point titration' technique (e.g., Ref. 15) which involved the application of the Ca^{2+} ionophore A23187 to mitochondria incubated with an external Ca^{2+} indicator, and also the more recent entrapment of the Ca^{2+} indicator Arsenazo III into the mitochondrial matrix by permeabilising and re-sealing the inner membrane [16]. However, these techniques have some obvious disadvantages, principally in the sensitivity of the former at low $[Ca^{2+}]_m$ [15], and the perturbation of structure in the latter [16].

Therefore, potentially a major step forward in the study of this important topic has been afforded by the recent reports that the fluorescent Ca^{2+} indicators fura-2 and quin2 can be successfully entrapped within the matrix of rat heart mitochondria by the apparently non-disruptive technique of simple pre-incubation of mitochondria with the acetoxymethyl esters of these compounds [17–19], i.e., a similar approach to that for loading into cells [20]. This has enabled continuous monitoring of $[Ca^{2+}]_m$ and Lukacs et al. [19] have made a preliminary attempt to correlate this with the activity of OGDH. In the present paper, we further refine this methodology and describe loading conditions which do not appear to affect the viability of rat heart mitochondria, and we also examine in some detail the $[Ca^{2+}]_m$ range over which both PDH and OGDH can be activated, and the gradient of Ca^{2+} ions (in : out) across the inner membrane under different conditions of incubation with various effectors of mitochondrial Ca^{2+} transport. A small part of this work has been reported briefly as a meeting abstract [21].

Materials and Methods

The acetoxymethyl esters and free acid forms of fura-2 and quin-2, and the Ca^{2+} ionophore ionomycin were purchased from Calbiochem (Cambridge, U.K.). All other chemicals and biochemicals were obtained from sources listed previously [22].

Rat heart mitochondria were prepared from male animals of 250–300 g weight using a polytron homogeniser and differential centrifugation as described previously [8]. Mitochondria were loaded with fura-2 by incubation in the final suspension medium (250 mM sucrose, 20 mM Tris, 2 mM EGTA, pH 7.4) at approx. 40 mg protein per ml for 5 min at 30 °C after the addition of 10 μM of fura-2/AM followed by a brief (1–2 s) vortexing. These conditions for loading were chosen after a preliminary series of experiments to find the best conditions (not shown), i.e., those which would allow the generation of suitable amounts of the free acid within the matrix, but which otherwise would not substantially compromise mitochondrial bioenergetic viability. The former criterion was addressed by monitoring the appearance of the Mn^{2+}-sensitive fluorescence signal in Triton-permeabilised mitochondria as described previously [17]. Under the above conditions, loading and hydrolysis of fura-2 appeared to be complete after 5 min incubation, at which time approx. 50–80 pmol of fura-2 per mg of mitochondrial protein was loaded (representing about 25–40% of the added ester); this is similar to but of a slightly shorter duration than that reported previously (approx. 8 min [17]). Lower concentrations of fura-2/AM did not yield sufficiently high signal-to-noise ratios, whilst at higher concentrations its hydrolysis in the matrix did not appear to be complete within 5 min [17], presumably because of limitations imposed by the amount of matrix esterase. With quin2 (not reported) a longer incubation period was required (20 min) and a higher concentration of ester (100 μM), as found previously [17]. The latter criterion was established by examining maximal oxygen uptake rates with various substrates, together with respiratory control and P/O ratios, and the sensitivities of matrix OGDH and PDH to extramitochondrial Ca^{2+} in the loaded mitochondria compared to the unloaded (but incubated) and also non-incubated mitochondria. These parameters were not noticeably affected by loading at up to 5 min incubation, but thereafter there was a slow deterioration in all of the parameters down to values of approx. 40–60% of control after 20 min (not shown); Lukacs et al. [19] reported 80% viability after 8 min.

Directly after loading, the mitochondria were incubated at 30 °C in a basic medium (2 ml) containing 120 mM KCl, 20 mM Tris and 5 mM KH_2PO_4 (pH 7.3 or 7.0 as indicated) and at approx. 1 mg protein per ml, in a thermostatted stirring cuvette unit housed in a Perkin-Elmer LS-5 fluorimeter; initially, some EGTA or HEDTA (0.1–2 mM as indicated) was always present; other additions were as indicated. Potassium salts were used throughout unless indicated. Ca^{2+} concentrations were varied using Ca-EGTA or Ca-HEDTA buffer systems as described previously [22]. Excitation wavelengths were 340, 380 and 365 nm, to give increases, decreases or no change in Ca^{2+}-induced fura-2 fluorescence, with emission at 500 nm [23] (10 nm slits). When NAD(P)H production was being examined exclusively, for the assay of OGDH within mitochondria, 340 (exci-

tation) and 460 (emission) nm were used [13]; in fura-2 loaded mitochondria this was assessed at 365–500 or 460 nm, i.e., using the isosbestic point (excitation) for fura-2 [23]. Amounts of PDH_a and total PDH activity were measured in samples taken from the cuvette (approx. 0.5 mg protein) after fura-2 measurements, which were then immediately sedimented ($10000 \times g$ for 20 s) and frozen prior to extraction and analysis, as previously described [22]. Total PDH in the fura-2-loaded mitochondria did not differ from that in unloaded mitochondria and was in the range of 80–100 munits per mg protein, as found previously [8], where a unit is defined as the amount required to convert 1 μmol of substrate per min at 30 °C.

Results and Discussion

Fura-2 signals were calibrated with free Ca^{2+} by first using the free acid form added to the basic incubation medium in the presence of 0.1% (v/v) Triton X-100 and mitochondria (permeabilised by the Triton) to give a protein concentration similar to that used in the incubations of intact mitochondria described below.

Corrections for intrinsic fluorescence of the mitochondria were made as described in Ref. 17; this was always small at the wavelengths used for fura-2 (less than 2% of the total signal). Calibration of the signals was therefore carried out as described by Grynkiewicz et al. [23], at various $[Ca^{2+}]$ generated by Ca-EGTA or Ca-HEDTA buffers (including at saturating Ca^{2+}, i.e., F_{max}), and also in the presence of no Ca^{2+} (i.e. F_{min}, excess EGTA), and also with excess Mn^{2+} to fully quench the signal (F_{Mn} or F_0) [23]. Any corrections for extramitochondrial free acid fura-2 were made by adding Ca^{2+} to mitochondria incubated with 1 μM Ruthenium red; this was found to be minimal in all circumstances (less than 2% of the total signal). Also Mn^{+} in the presence of Ruthenium red did not affect the signal indicating that most of the fura-2 was in the matrix. The apparent K_d values for fura-2 and Ca^{2+} using the above approach were calculated to be 149 ± 10 nM (value $\pm$ S.D. for three determinations), at pH 7.3 and 608 ± 32 at pH 7.0, using the methods described in Ref. 23.

Attempts were also made to establish the K_d actually within the matrix by selectively permeabilising the inner membrane to Ca^{2+} using the non-fluorescent Ca^{2+} ionophore ionomycin (2 μM) and thus equilibrating the matrix and extramitochondrial (buffered) Ca^{2+} pools, whilst retaining the fura-2 in the matrix. Uncoupler (FCCP, 1 μM) was also added to equilibrate protons; the further addition of valinomycin (1 μM) did not appear to affect the signals obtained (not shown). Signals appeared to be stable after this permeabilisation (as with Triton), indicating Ca^{2+} equilibration (Triton or ionomycin did not affect free acid fura-2 signals). Using this approach, the K_d values (three determinations) were 178 ± 9 nM at pH 7.3 and 571 ± 26 nM at pH 7.0. Mg^{2+} (2 mM) did not substantially affect these values [23]. Experimentation at pH 7.0 proved useful as this allowed the measurement of $[Ca^{2+}]_m$ up to higher values (up to 10 μM is feasible with accuracy [22]) owing to the higher K_d. The calibrations do, however, ultimately depend on the values of Ca-EGTA and Ca-HEDTA stability constants used [24], and it is appreciated that there is still much argument as to what these are (e.g., Ref. 25). However, the K_d values obtained agree well with results from Grynkiewicz et al. [23], given that there are also slight differences in pH, ionic strength and temperature. It was felt that the values obtained with the ionomycin/FCCP permeabilisation were the more appropriate with which to calculate $[Ca^{2+}]_m$ values, although the values calculated using Triton were not substantially different. It is also appreciated that in coupled mitochondria, the pH of the matrix is most likely to differ from the extramitochondrial pH and that the calibration will equilibrate the pH pools, and also that K_d is affected by pH. However we feel that this was the best estimation we could make at present, bearing this proviso in mind, and it is also known that the K_d is less affected by pH at values greater than 7.3 [20,23]. Perhaps a combined approach using both fura-2 and the fluorescent pH indicator BCECF (biscarboxyethyl-5(6)-carboxyfluorescein) may help to solve this problem (see Ref. 18).

The use of fura-2 to measure $[Ca^{2+}]_m$

Fig. 1 shows that the measurement of $[Ca^{2+}]_m$ using the entrapped fura-2 is entirely straightforward. Fig. 1(A) shows how the effects of Ca^{2+} on OGDH activity can be measured simultaneously; simple subtraction of a 340/500 signal obtained using unloaded mitochondria (see below) from the 340/500 signal using loaded mitochondria was most often used to calculate $[Ca^{2+}]_m$ changes under similar conditions to those of the OGDH measurement. The 380/500 signal could also be used (Fig. 1(A)). Estimations of OGDH activity in this way did not substantially differ from estimations using uninucubated and unloaded mitochondria (Fig. 1(B)) which suggests that loading did not substantially affect Ca^{2+} transport, and that the Ca^{2+} sensitivity of OGDH was not substantially affected. Fig. 1(C) shows that at saturating concentrations of 2-oxoglutarate (10 mM) (Ca^{2+} affects this K_m value) the fura-2 signal can be measured independently of changes in NAD(P)H. Such experiments could also be done in the presence of L-malate (1 mM) or succinate (5 mM) (not shown); however, a respiratory substrate had to be added for uptake. Fig. 1(C) also shows how F_{max} and F_{Mn} could be obtained to calibrate each sample. The use of the isosbestic point for fura-2 (i.e., 365(excitation)/500(emission)) allowed for the correction of any non-

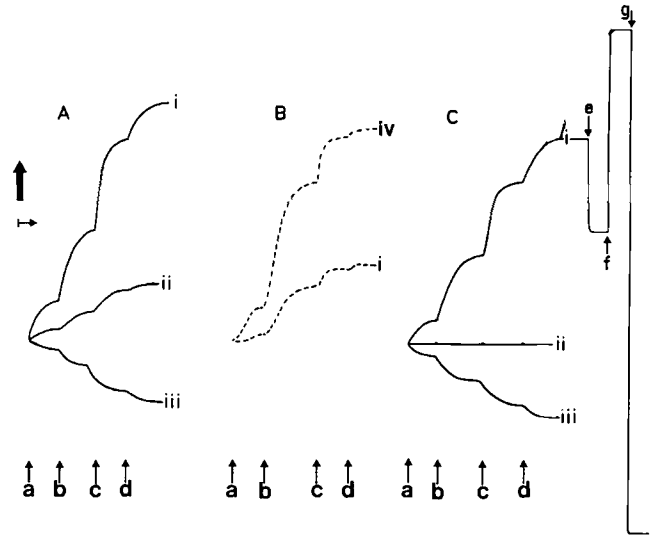

Fig. 1. Effects of increases in extramitochondrial Ca^{2+} on fluorescence signals from fura-2-loaded mitochondria. Mitochondria in A and C were loaded with fura-2 as described in Materials and Methods; in B mitochondria were incubated as for loading, but with no fura-2/AM; traces here did not differ substantially from those with mitochondria which were not incubated (as for loading) at all. Mitochondria were then transferred to the fluorimeter (at wavelength settings, i, 340 (excitation)/500 (emission); ii, 365/500; iii, 380/500; or iv, 340/460) and into buffer (at pH 7.3) which contained 0.5 mM malonate and 1 mM EGTA. Following a 2 min stabilisation period (not shown) in the presence of either 100 μM (A,B) or 10 mM (C)) 2-oxoglutarate, additions were made, sequentially at the arrows, as follows: a, 0.25 mM EGTA plus 0.25 mM $CaCl_2$ (as a buffer solution), (giving a free extramitochondrial Ca^{2+} concentration of approx. 13 nM); b, 0.25 mM EGTA plus 0.25 mM $CaCl_2$ (new free Ca^{2+} concentration of 26 nM); c, 0.5 mM EGTA plus 0.5 mM $CaCl_2$ (53 nM free Ca^{2+}); d, 1 mM EGTA plus 1 mM $CaCl_2$ (105 nM free Ca^{2+}); e, 1 μM FCCP plus 2 μM ionomycin; f, 5 mM HEDTA plus 5 mM $CaCl_2$ (6.3 μM free Ca^{2+}, to give F_{max}); g, 10 mM $MnCl_2$ (to give F_{Mn} or F_0). The large arrow indicates an increase in fluorescence (arbitrary units, but of a similar scale in each case); the barred arrow indicates 1 min and the direction of addition. Results of a typical experiment are shown; this experiment was repeated at least twice, as was the case for the traces in Figs. 2–6.

specific changes which could occur [19], as the result of for example changes in mitochondrial shape; these were found to be negligible (Fig. 1(C)), except when ADP was added (see Fig. 3), when this was corrected for. Fig. 2 shows that the Ca^{2+} effects on the enzyme appear to be saturated whilst there are still clear measurable increases in $[Ca^{2+}]_m$ occurring, and also that the increases in $[Ca^{2+}]_m$ occur more quickly than the consequent steady-state increases in matrix NAD(P)H. Samples for PDH_a and total PDH analyses were taken at the end of runs such as those shown in Fig. 2(A) by which time steady-state amounts of PDH_a will be achieved [8,13]. It should be noted that similar PDH_a responses were obtained in both loaded and unloaded mitochondria, again suggesting that loading does not substantially adversely affect the mitochondria.

Ca^{2+} distribution across the inner membrane under various conditions

Fig. 3 shows the effects of Mg^{2+}, Na^+, spermine, or Ruthenium red on Ca^{2+} uptake into the matrix, as assessed by entrapped fura-2. The expected effects are clearly evident (see Introduction). Mg^{2+} reduces the uptake rate and the final steady-state level of $[Ca^{2+}]_m$ is lowered. Ruthenium red completely blocks uptake; this again shows that the fura-2 is actually in the matrix. With Na^+, the initial uptake rate does not appear to be affected, whereas the final steady-state $[Ca^{2+}]_m$ is again diminished, reflecting the effects of Na^+ as a Ca^{2+}-egress promoter. Diltiazem (250 μM) produced the expected inhibition of these effects of Na^+ [5] (Fig. 3). Fig. 3 also shows that in the presence of Na^+ and Mg^{2+}, at the expected physiological concentrations and at low extramitochondrial $[Ca^{2+}]$, there appears in fact, to be a reverse gradient of Ca^{2+} (in : out) across the mitochondrial inner membrane; in their absence, there is a clear positive gradient (Fig. 3; see below). Spermine (0.5 mM) produced the expected promotion of Ca^{2+} uptake [4] (Fig. 3), but did not appear to affect Na^+-dependent egress (not shown), in contrast to previous reports [3]. ADP ($\pm Na^+$) did not appear to affect the distribution of Ca^{2+} substantially (Fig. 3), again perhaps in contrast to earlier expectations [26].

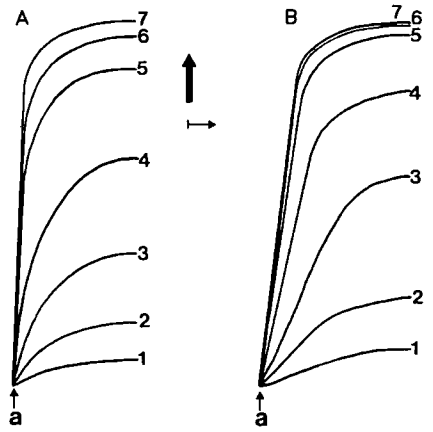

Fig. 2. Comparison of (A) $[Ca^{2+}]_m$ and (B) OGDH responses to changes in extramitochondrial Ca^{2+}. Mitochondria were loaded with fura-2 (fluorimeter settings (A) 340/500 and (B) 365/460) and incubated in media (pH 7.3) with 1 mM EGTA and 0.5 mM malonate and (A) 10 mM or (B) 100 μM 2-oxoglutarate. Following a 2 min stabilisation period, additions were made, at arrow a, for the appropriate traces as follows: 1, 0.1 mM EGTA plus 0.1 mM $CaCl_2$ (free extramitochondrial Ca^{2+} concentration approx. 5 nM); 2, 0.25 mM EGTA plus 0.25 mM $CaCl_2$ (13 nM free Ca^{2+}); 3, 0.5 mM EGTA plus 0.5 mM $CaCl_2$ (26 nM free Ca^{2+}); 4, 1 mM EGTA plus 1 mM $CaCl_2$ (53 nM free Ca^{2+}); 5, 2 mM EGTA plus 2 mM $CaCl_2$ (105 nM free Ca^{2+}); 6, 4 mM EGTA plus 4 mM $CaCl_2$ (210 nM free Ca^{2+}); 6, 0.5 mM HEDTA plus 0.5 mM $CaCl_2$ (385 nM free Ca^{2+}); 7, 1 mM HEDTA plus 1 mM $CaCl_2$ (740 nM free Ca^{2+}). Estimated $[Ca^{2+}]_m$ for the appropriate conditions in (A) were: 1, 68 nM; 2, 135 nM; 3, 290 nM; 4, 490 nM; 5, 1580 nM; 6, 3860 nM; 7, greater than 4 μM. Fluorescence and time are as indicated in Fig. 1; similar scales were drawn to aid comparison.

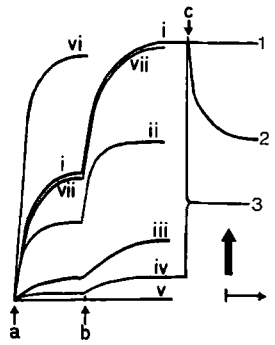

Fig. 3. Effects of Na^+, Mg^{2+}, Ruthenium red, spermine, ADP and diltiazem on $[Ca^{2+}]_m$. Mitochondria were loaded with fura-2 and initially incubated (at 340/500) with 10 mM 2-oxoglutarate, 1 mM EGTA and 0.5 mM malonate as described in Fig. 2, with the additional presence of (i) no further additions (control); (ii) 10 mM NaCl; (iii) 2 mM $MgCl_2$; (iv) 10 mM NaCl plus 2 mM $MgCl_2$; (v) 1 μM Ruthenium red; (vi) 0.5 mM spermine; or (vii) 2 mM ADP. After a 2 min stabilisation period, additions were made, sequentially at the arrows, as follows: a, 1 mM EGTA plus 1 mM $CaCl_2$ (53 nM free Ca^{2+}); b, 1 mM EGTA plus 1 mM $CaCl_2$ (105 nM free Ca^{2+}); c, 1, 10 mM NaCl plus 250 μM diltiazem, 2; 10 mM NaCl alone, 3, 0.1% Triton. The calculated $[Ca^{2+}]_m$ values directly before arrow c were: i, 1450 nM; ii, 510 nM; iii, 110 nM; iv, 35 nM; v, 0 nM; (vi, 1250 nM); vii, 1390 nM. Fluorescence and time changes are as indicated in Fig. 1.

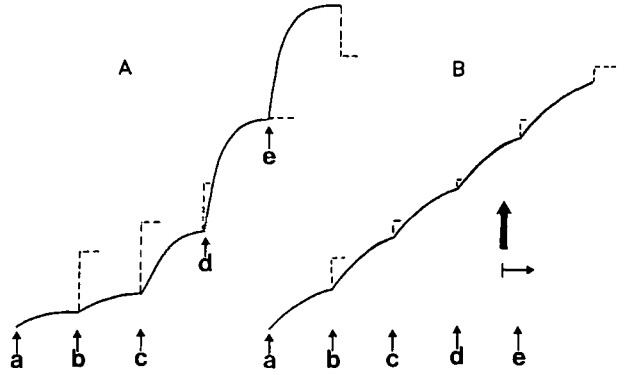

Fig. 4. Effects of increasing extramitochondrial Ca^{2+} on $[Ca^{2+}]_m$ in mitochondria incubated with either (A) Na^+ and Mg^{2+} or (B) uncoupler. Mitochondria were loaded with fura-2 and then incubated (at 340/500) at pH 7.0 and 0.5 mM malonate, 1 mM EGTA and either (A) 10 mM NaCl, 2 mM $MgCl_2$ and 10 mM 2-oxoglutarate or (B) 1 μM FCCP and 300 μM 2-oxoglutarate. After the 2 min stabilisation period, further additions were made, sequentially at the arrows, as follows: a, 0.25 mM EGTA plus 0.25 mM $CaCl_2$ (56 nM free Ca^{2+}); b, 0.25 mM EGTA plus 0.25 mM $CaCl_2$ (112 nM free Ca^{2+}); c, 0.5 mM EGTA plus 0.5 mM $CaCl_2$ (223 nM free Ca^{2+}); d, 1 mM EGTA plus 1 mM $CaCl_2$ (413 nM free Ca^{2+}); e, 2 mM EGTA plus 2 mM $CaCl_2$ (891 nM free Ca^{2+}). In B, with no $MgCl_2$, the concentrations of free Ca^{2+} were slightly lower, ranging from 52 nM to 827 nM. Essentially similar results could be obtained at pH 7.3 except that HEDTA·Ca had to be used to achieve the higher Ca^{2+} level required and thus attention had to be paid to changes in Mg^{2+} which binds to this ligand. The effects of adding Triton, at the appropriate points, are indicated by dashed lines. Fluorescence and time are as indicated in Fig. 1.

The relationships between extramitochondrial Ca^{2+} and $[Ca^{2+}]_m$, in the presence of Na^+ and Mg^{2+}, are explored more fully in Fig. 4(A), and are compared to uncoupled mitochondria (Fig. 4(B)). In the latter, there does in fact appear to be near-equilibrium of Ca^{2+} across the inner membrane (Fig. 4(B) and Fig. 7), as was thought to be the case from previous studies using the enzymes as matrix probes for Ca^{2+} [6]. Even here some substrate had to be added to achieve Ca^{2+} uptake, and uptake was sensitive to Ruthenium red, suggesting that transport is still catalysed by the proteins of the Ca^{2+}-transport system (not shown).

In contrast to the case with uncoupled mitochondria, in the coupled mitochondria incubated with Na^+ and Mg^{2+}, there appears to be a pronounced sigmoidal relationship between extramitochondrial $[Ca^{2+}]$ and $[Ca^{2+}]_m$, with a large degree of positive co-operativity in the increases in $[Ca^{2+}]_m$ with respect to those outside. Therefore, at higher extramitochondrial Ca^{2+} concentrations there is more Ca^{2+} in the matrix, in contrast to the case at lower extramitochondrial Ca^{2+} levels. Such a relationship has not been fully appreciated previously in studies using the enzymes as $[Ca^{2+}]_m$ probes [6,13,22], although Crompton [1] has pointed out that the kinetic properties of the Ca^{2+}-transport system should allow increases in cytosolic Ca^{2+} to result in amplified increases in matrix Ca^{2+}. In Table I, some attempt is made at quantification of the gradients of Ca^{2+} at different extramitochondrial $[Ca^{2+}]$ and under different conditions. One of the principle reasons underlying this positive co-operativity most likely resides

TABLE I

Estimations of the gradient of Ca^{2+} ions (in:out) across the inner membrane of fura-2-loaded mitochondria under various conditions

The gradients were calculated from estimations of $[Ca^{2+}]_m$ obtained as described in the figure legends and the known, buffered, extramitochondrial Ca^{2+} levels at three points in the Ca^{2+}-dependent activation curves of PDH and OGDH, which were: (a) early activation, i.e., values obtained between 10 and 20% of the full (100%) Ca^{2+}-dependent activations; (b) mid-point, i.e., using values obtained as in Table II and the corresponding $K_{0.5}$ values for extramitochondrial Ca^{2+}; and (c) almost saturating, i.e., between 80 and 90% of the full activation. The values given are means but can only be considered approximate and combine data obtained for both OGDH and PDH. No S.E. values are given as errors are inherent within both components; however, the ranges obtained were from approx. 25% below to 25% above the values given.

Mitochondrial incubation condition	Points at which the approximate gradient of Ca^{2+} ions (in : out) were obtained		
	(a) early enzyme activation	(b) mid-point of activation	(c) almost saturating enzyme activation
Control	19	22	25
10 mM NaCl	3	4	9
2 mM $MgCl_2$	1.5	2.0	2.3
Na^+ plus Mg^{2+}	0.3	0.8	2.0
1 μM FCCP	0.7	0.9	1.1

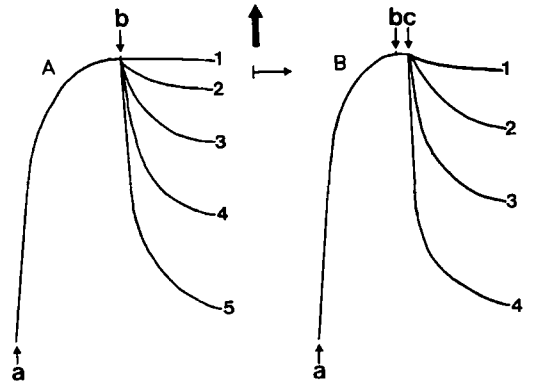

Fig. 5. (A) Effects of Na^+ on Ca^{2+} egress from mitochondria, (B) Effects of increasing extramitochondrial Ca^{2+} on Na^+-dependent egress. Mitochondria were loaded with fura-2 and incubated (pH 7.3 and at 340/500) with 10 mM 2-oxoglutarate, 0.5 mM EGTA and 0.5 mM malonate as described in Fig. 2. After a 2 min stabilisation period, 0.5 mM EGTA plus 0.5 mM $CaCl_2$ were added at arrow a to give a free Ca^{2+} concentration of 53 nM, then in A, at arrow b, additions were made as follows: 1, 10 mM KCl; 2, 1 mM NaCl; 3, 2.5 mM NaCl; 4, 10 mM NaCl; 5, 10 mM NaCl plus 5 mM EGTA (resultant free Ca^{2+} concentration of 5 nM). In B, 1 μM Ruthenium red was added at arrow b, then at arrow c, 10 mM NaCl was added together with Ca-EGTA buffers (to give the appropriate resultant free Ca^{2+} concentration), as follows: 4, 5 mM EGTA (5 nM free Ca^{2+}); 3, 0.5 mM EGTA plus 0.5 mM $CaCl_2$ (105 nM free Ca^{2+}); 2, 1.5 mM EGTA plus 1.5 mM $CaCl_2$ (211 nM free Ca^{2+}); 1, 3.5 mM EGTA plus 3.5 mM $CaCl_2$ (420 nM free Ca^{2+}).

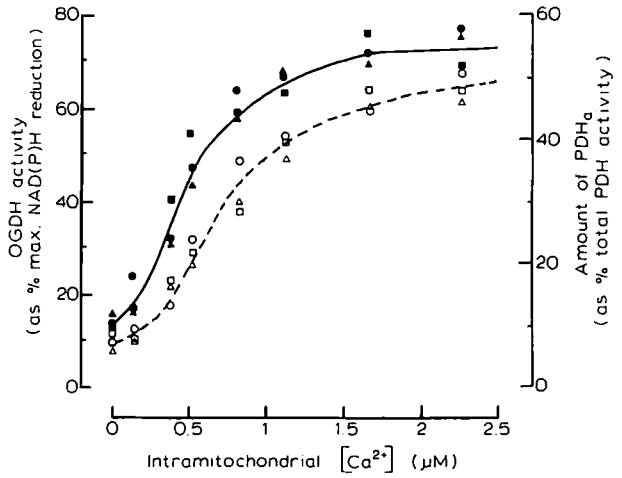

Fig. 6. The ranges of $[Ca^{2+}]_m$ over which PDH and OGDH were activated. The mean values are shown of data from 3–5 experiments of the types shown in Figs. 1–5 (for clarity S.E. values have not been shown, but were within 15% of the values shown for both parameters; see Table II) for measurements of OGDH (expressed as a percentage of the reduction at 100 μM 2-oxoglutarate compared to 5 mM (max)) (●, ■, ▲, solid line) and PDH_a (○, □, △, dashed line) for the $[Ca^{2+}]_m$ shown. For PDH_a mitochondria were incubated with 10 mM 2-oxoglutarate and 0.5 mM malonate. Incubations were at pH 7.3 and under control conditions (no further additions (●, ○)) or with 2 mM $MgCl_2$ (■, □) or 10 mM NaCl (▲, △). Mitochondria were incubated at pH 7.3 (Ca-HEDTA buffers) or at pH 7 (using Ca-EGTA buffers) and both Na^+ and Mg^{2+} gave essentially similar results.

in the fact that extramitochondrial Ca^{2+} inhibits the Na^+-dependent Ca^{2+}-egress pathway [4]. This can be demonstrated directly as shown in Fig. 5. Such inhibition is even more pronounced at higher values of extramitochondrial Ca^{2+}, such as those required for Ca^{2+} uptake in the presence of Mg^{2+}. Another reason is likely to be the sigmoidal behaviour of uptake in the presence of Mg^{2+} [1] and also some inhibitory effects of Mg^{2+} on egress (see Refs. 1 and 2).

From all the different measurements of $[Ca^{2+}]_m$ made under the various conditions (Fig. 7), and in

TABLE II

Estimations of the $K_{0.5}$ values for $[Ca^{2+}]_m$ in the Ca^{2+}-dependent activations of PDH and OGDH within fura-2-loaded rat heart mitochondria incubated under various conditions

$K_{0.5}$ values (± S.D.) were calculated as described previously [22] (with the numbers of degrees of freedom in parentheses) and were from observations made on at least three different preparations of mitochondria and from experiments of the type illustrated in Figs. 1–7. The activatory ranges in each case were from 0.02 to 2 μM. n.d. indicates not determined.

Additions to mitochondrial incubation	Calculated $K_{0.5}$ values (nM) for matrix Ca^{2+} in the activation of:	
	PDH	OGDH
None (control)	535 ± 46 (22)	376 ± 32 (38)
10 mM NaCl	398 ± 52 (16)	476 ± 60 (16)
2 mM $MgCl_2$	428 ± 37 (18)	320 ± 42 (16)
NaCl plus $MgCl_2$	602 ± 46 (28)	505 ± 35 (36)
1 μM FCCP	n.d.	586 ± 58 (20) [a]

[a] This value was determined using the oxygen electrode in parallel experiments to measure OGDH activity (see Ref. 13).

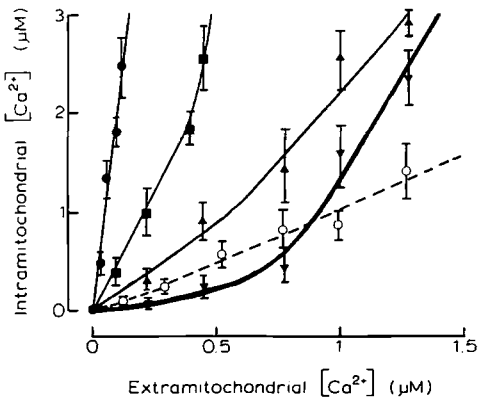

Fig. 7. Effects of Na^+ and/or Mg^{2+}, or uncoupler on Ca^{2+} distribution across the inner membrane. Data from 3–5 experiments of the types shown in Figs. 1–5 were combined as means ± S.E. for $[Ca^{2+}]_m$ at the buffer-set values of extramitochondrial Ca^{2+} shown (see Table I). Mitochondria were incubated (see legends) under control conditions (no further additions (●)) or in the presence of 10 mM NaCl (■), 2 mM $MgCl_2$ (▲) or 2 mM $MgCl_2$ plus 10 mM NaCl (▼) (thick line) or 1 μM FCCP (○, dashed line). No increases in $[Ca^{2+}]_m$ were observed in the presence of 1 μM Ruthenium red over the extramitochondrial Ca^{2+} concentrations shown. Mitochondria were incubated both at pH 7.3 and 7.0 and with both Ca-EGTA and Ca-HEDTA buffers; neither of these different conditions appeared to affect results substantially.

comparison to the measurements of the Ca^{2+}-dependent effects on PDH and OGDH (Fig. 6), some important conclusions can be made. The first is that the enzymes appear to respond to $[Ca^{2+}]_m$ over the approximate range of 0.02–2 μM Ca^{2+}; this is a similar, but slightly lower and more restricted, range when compared to the extracted enzymes [6,7]. Both PDH and OGDH respond to approximately the same range of $[Ca^{2+}]_m$, except that OGDH may respond to very slightly lower values. The $K_{0.5}$ values for Ca^{2+} activation of PDH and OGDH are again similar and within the approximate range of 0.3–0.6 μM, again slightly lower than has been estimated using the isolated enzymes [6,7], but similar to those reported for toluene-permeabilised mitochondria (see Ref. 14). So, as a first approximation, the previous uses of the enzymes as probes for $[Ca^{2+}]_m$ appears to be largely vindicated. Although the effectors of Ca^{2+} transport bring about the expected changes in Ca^{2+} distribution (Fig. 7 and Table I), they appear not to affect the $[Ca^{2+}]_m$ range over which the enzymes respond (Table II).

General discussion

The ability to load fura-2 into intact rat heart mitochondria, apparently under conditions where their functional capabilities are not noticeably impaired, offers clear advantages in the study of the regulation and role of matrix Ca^{2+} and mitochondrial Ca^{2+} transport. The loading of fura-2 achieved (see Materials and Methods), assuming a matrix volume of 1 μl/mg protein, would suggest that the internal fura-2 concentration should be of the order of 50 μM, which should be sufficient, as is evident, to allow adequate signals to be generated [20], but should not substantially buffer the changes in $[Ca^{2+}]_m$ [20]. Lukacs et al. [19], using mitochondria loaded with similar amounts of fura-2, demonstrated that the half-maximal activation of OGDH in the loaded mitochondria was achieved at a total Ca content of 4.35 nmol per mg protein; a corresponding amount of total Ca in unloaded mitochondria caused 85% activation, therefore some buffering of the matrix Ca^{2+} by fura-2 would presumably explain this. Incidentally, they reported a $K_{0.5}$ for $[Ca^{2+}]_m$ of 800 nM in the activation of OGDH, using a K_d of 135 nM, and a complete activation at 1600 nM [19]. Similarly, several studies have reported that the total Ca content over the activatory ranges of the enzymes is approximately from 0.5–5 nmol per mg protein (e.g., Refs. 11 and 15); therefore, there may be some buffering by the internal fura-2, but not much. Lukacs and Kapus [17] reported that in fura-2-loaded mitochondria (again at similar loading amounts) the $[Ca^{2+}]_m$ rose from 200 to 1800 nM as the total Ca content rose from 0.5 to 6 nmol per mg protein, whilst under similar conditions Davis et al. [18] reported increases of $[Ca^{2+}]_m$ from 0.1 to 2 μM as the total Ca content rose from about 0.5 to 2 nmol per mg protein. Both studies reported near-linear relationships between total Ca content and $[Ca^{2+}]_m$ over this range, with a tendency to larger increases in $[Ca^{2+}]_m$, with respect to total Ca, at higher values [17,18]. In the preliminary experiments noted in the Materials and Methods, no substantial differences in Ca^{2+} uptake were evident in mitochondria loaded at 5 or 15 μM fura-2/AM when compared to 10 μM, although the present studies have been carried out with a large amount of external Ca^{2+} buffer.

The present studies again substantiate the proposals that the principal function of the mitochondrial Ca^{2+}-transport system is to regulate matrix Ca^{2+}, and hence the enzymes and oxidative metabolism, and to relay changes in cytoplasmic Ca^{2+} to the matrix [6,7]. Significantly, there was even a reverse gradient of Ca^{2+} (in : out) at low concentrations of extramitochondrial Ca^{2+} in the presence of Na^+ and Mg^{2+} (Table I), arguing further against the idea that mitochondria are Ca^{2+} stores or Ca^{2+} sinks. This also suggests that $[Ca^{2+}]_m$ will be below the activatory range for the enzymes in unstimulated cells. The ability to measure matrix Ca^{2+} using fura-2 should also allow advances to be made in the study of the kinetics of the Ca^{2+}-transport system under conditions of physiological Ca^{2+} loads. The changes in matrix Ca^{2+} can be more rapidly detected using the entrapped fura-2 than by using the enzymes as matrix Ca^{2+} probes (Fig. 2). Indeed, the composite relaxation time for fura-2 around its K_d is about 5 ms [20], so changes in $[Ca^{2+}]_m$ should be reported almost instantaneously. Fig. 2 also shows that the rate of Ca^{2+} uptake at higher extramitochondrial Ca^{2+} concentrations, as might be expected during systole in the heart, is very rapid, and Fig. 5 shows that in the presence of Na^+ and low extramitochondrial Ca^{2+}, as might be expected in diastole, the rate of Ca^{2+} egress is also rather rapid. This raises the intriguing possibility that there may even be changes in $[Ca^{2+}]_m$ during the contractile cycle, and indeed attempts at computer modelling have indicated that this might be expected [1,7]. Interestingly, changes in PDH_a have been observed within the contractile cycle in in vivo dog hearts, under unstimulated conditions, from about 10% of total PDH up to about 15%, and back [27].

Another advantage with the use of fura-2 in mitochondria, compared with its use in cells, is that it is likely that in the former the indicator will only report Ca^{2+} changes from one compartment, unlike in the latter where there is evidence of the indicator locating in different cellular compartments [20]. Indeed, Davis et al. [18] have reported that in fura-2-loaded rat heart myocytes, a considerable proportion of the dye is located in the mitochondria. There are also other reports that fura-2 fluorescence is localised to mitochondria in other cell types (see Ref. 28). Therefore, in many cells in

which fura-2 is used it is likely that it will also be reporting $[Ca^{2+}]_m$; perhaps the continued development of sophisticated fluorescent-imaging technology [20] may soon allow the actual measurement of this key parameter in intact cells.

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