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Intramitochondrial and Extramitochondrial Free Calcium Ion Concentrations of Suspensions of Heart Mitochondria with Very Low, Plausibly Physiological, Contents of Total Calcium

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Abstract

The 2-oxoglutarate dehydrogenase of intact rat heart mitochondria is activated by Ca^{2+}, with 50% activation at approximately 0.5 nmol of total Ca/mg of mitochondrial protein, in the presence of P_i and Mg^{2+}. Mitochondrial Ca contents in excess of 2 nmol/mg of protein result in 100% activation of the enzyme. Investigation of Ca^{2+} release from the mitochondria using the metallochromic indicator Arsenazo III defines a $S_{0.5}$ of 5.4 ± 0.4 nmol of Ca/mg of protein, when the endogenous Ca content of the mitochondria is progressively depleted with EGTA, prior to the initiation of the release process being studied. The subsequent determination of matrix free Ca^{2+} concentration by the "null-point" technique has allowed expression of these results in terms of free concentration rather than Ca content, with an activity coefficient of approximately 0.001 for matrix Ca^{2+}. From the above, Ca^{2+} efflux from heart mitochondria is not saturated at the mitochondrial Ca contents or Ca^{2+} concentrations which give effective regulation of dehydrogenase activity. A consequence is that heart mitochondria do not buffer the pCa of the extramitochondrial medium at these Ca contents (<2 nmol/mg of protein), and this is shown in direct measurements of extramitochondrial pCa. This is taken to question the physiological significance of mitochondrial buffering of cytosolic free Ca^{2+} in normal heart.

Key Words: Mitochondrial calcium transport; calcium/sodium exchange of mitochondria; limits to buffering of extramitochondrial free calcium by mitochondria; "null-point" technique of free calcium measurement; calcium concentration of the mitochondrial matrix; control of 2-oxoglutarate dehydrogenase.

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Introduction

The view has arisen that mitochondria serve to stabilize, or buffer, the pCa of their environment (Bygrave, 1978; Nicholls, 1978; Nicholls and Crompton, 1980). They do this by virtue of having separate uptake and release pathways, which become matched in activity in the presence of a certain, characteristic concentration of extramitochondrial free Ca^{2+}, which has been referred to as the "set-point" (Becker *et al.*, 1980; Brand and de Selincourt, 1980). For this view to be valid, the release pathway, which has been defined as catalyzing a $Ca^{2+}/2\ Na^{+}$ exchange in heart mitochondria (Crompton *et al.*, 1976b; Crompton and Heid, 1978), must be saturated with its substrate, intramitochondrial Ca^{2+}. Otherwise, the accumulation of Ca^{2+} from the environment will lead to an elevated mitochondrial Ca content, an elevated rate of egress, and imperfect buffering. Notably, the Ca load has been relatively high (10–100 nmol/mg of protein), and presumably near-saturating for the release pathway, in those studies showing near-perfect buffering (Nicholls, 1978). Clearly, the normal *in vivo* mitochondrial content of Ca is of crucial importance in determining whether mitochondria do indeed serve to buffer the free Ca^{2+} concentration of the cytosol, and there is scant information on this parameter for heart muscle. However, recent studies from this laboratory on

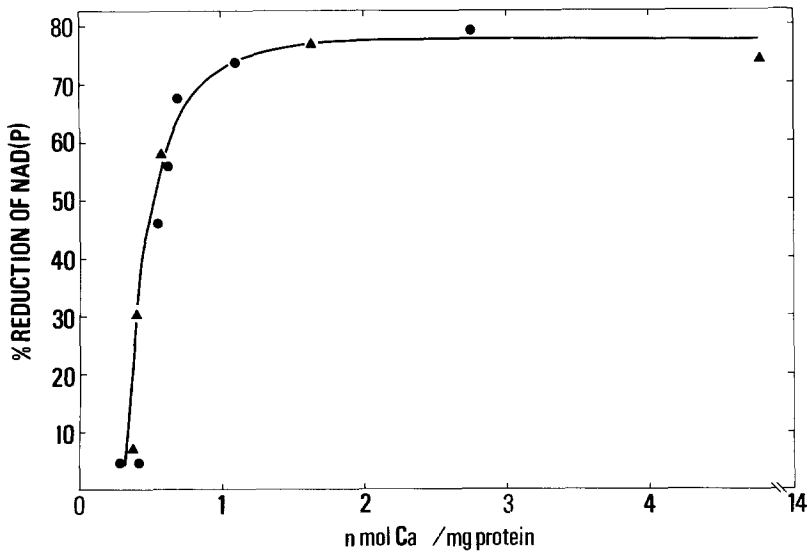

Fig. 1. Dependence of the reduction of NAD(P) by 2-oxoglutarate on the Ca content of rat heart mitochondria. Experimental details are given under Materials and Methods. Each point gives the mean result from two incubations: the different symbols denote different mitochondrial preparations.

the activation of 2-oxoglutarate dehydrogenase by Ca^{2+} in intact heart mitochondria show that control occurs at around 0.5–1 nmol of total Ca/mg of mitochondrial protein (Hansford and Castro, 1981, and this paper). The experiments reported in this paper proceed from the assumption that this control operates *in vivo,* and examine aspects of Ca^{2+} transport at these low values of Ca content. It is shown that the $Ca^{2+}/2\ Na^{+}$ exchange process is not saturated, and that heart mitochondria do not buffer extramitochondrial free Ca^{2+} concentration effectively, over the range of Ca contents allowing for dehydrogenase modulation.

Further, the "null-point" technique developed by Williamson and Murphy (1980) has been applied to heart mitochondria and shows that the sensitivity of 2-oxoglutarate dehydrogenase to Ca^{2+} is very similar when the enzyme is in its intramitochondrial locale to that shown previously for the purified enzyme by McCormack and Denton (1979). Comparison of the values for free Ca^{2+} concentration obtained by this method with values of total Ca content obtained by atomic absorption spectrophotometry further allows estimation of the activity coefficient of Ca^{2+} in the matrix of heart mitochondria.

Materials and Methods

Heart mitochondria were isolated as described by Hansford (1978), with the exception that they were given a final wash in medium omitting EGTA.

Estimation of 2-Oxoglutarate Dehydrogenase Activity

Enzyme activity was assessed in intact respiring mitochondria by measuring the degree of reduction of mitochondrial NAD during the State 4 (for terminology see Chance and Williams, 1956) oxidation of nonsaturating concentrations of 2-oxoglutarate. Mitochondrial NADH was measured fluorimetrically (Hansford, 1975), and it has been shown that changes in fluorescence can be attributed almost entirely to changes in NADH content, with a minimal contribution from changed NADPH content, provided that the mitochondria are in State 4 (Hansford and Castro, 1981). Mitochondria (4 mg of protein) were added to 2 ml of medium comprising 0.13 M KCl, 20 mM KHepes, 5 mM KP_i, 1 mM $MgCl_2$ and 0.25 mM ADP. The temperature was 25°C and the pH was 7.2. Ten minutes after the addition of mitochondria, 1 μmol of 2-oxoglutarate and 1 μmol of L-malate were added. The percentage reduction of NAD(P) presented in Fig. 1 is the reduction achieved after the phosphorylation of the added ADP, measured at 12 min, and is expressed as a percentage of the total NAD(P), given by the fluorescence in the anaerobic state minus that obtained before adding the oxoglutarate and malate. At 12.5

min, a 1.5-ml portion of the incubation was removed and centrifuged to recover the mitochondria. The mitochondrial Ca content was then determined by atomic absorption spectrophotometry, as described below. Mitochondrial Ca contents were lowered from the endogenous values characteristic of these mitochondrial preparations (2–3 nmol of Ca/mg of protein) by the inclusion of small amounts (10–200 nmol) of EGTA at the beginning of the experiments: values below 1 nmol/mg were generated by the additional inclusion of 10 mM NaCl.

Alternatively, the activity of 2-oxoglutarate dehydrogenase was inferred from the State 3 rate of O_2-uptake obtained on adding 2-oxoglutarate (to 0.5 mM) and L-malate (to 0.5 mM) to mitochondria preincubated for 5 min in the medium given above, but with ADP present at 1.5 mM. The increment in rate on adding 2-oxoglutarate was measured.

Measurement of the Kinetics of Release of Ca^{2+} from Heart Mitochondria

Ca^{2+} release to the medium was measured using the metallochromic indicator Arsenazo III (Scarpa, 1979) and the Aminco Chance DW2A dual-wavelength spectrophotometer, at the wavelength pair 675–685 nm. Mitochondria (2 mg of protein) were added to 2 ml of 0.13 M KCl, 20 mM KHepes, 5 mM KP_i, 5 mM L-malate, 5mM L-glutamate, and 30 μM Arsenazo III, containing in addition 0–20 μM $CaCl_2$ or 0–5 μM EGTA. Five minutes later, Ca^{2+} release was initiated by the addition of 40 μmol NaCl and 1.6 nmol Ruthenium Red, and the initial rate of absorbance change was recorded. The amount of intramitochondrial Ca at the moment of addition of NaCl and Ruthenium Red was estimated from the total absorbance change from that moment to the attainment of a new steady state (at 11 min), and is used as a measure of the Ca^{2+} available for translocation (the parameter S used in Fig. 2). Addition of the ionophore A23187 at this point gave rise to no further release of Ca^{2+}.

Measurement of the pCa of the Extramitochondrial Medium

The Ca^{2+} activity of the incubation was measured using an ion-selective electrode (Radiometer F2002). The voltage generated in the steady state was compared with that obtained when Ca^{2+}:EGTA buffers (Portzehl *et al.*, 1964) of known pCa (negative log of Ca^{2+} molarity) were added to the incubation at the conclusion of the experiment. Mitochondria (6.25 mg of protein) were added to 4.1 ml of medium comprising 0.13 M KCl, 20 mM KHepes, 5 mM KP_i, 10 mM NaCl, 5 mM L-glutamate, and 5 mM L-malate. The temperature was 25°C and the pH was 7.2. In addition, each incubation contained a small amount of $CaCl_2$ (0–15 nmol/mg of protein) or EGTA (0–8 nmol/mg of protein). Where indicated, $MgCl_2$ was also present at 1 mM. Ten minutes

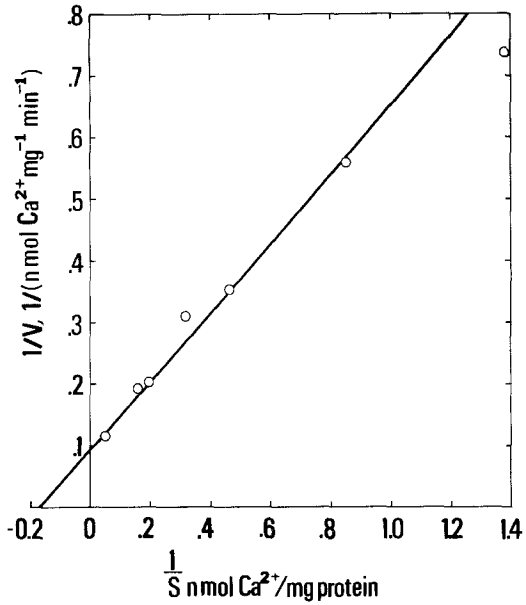

Fig. 2. The kinetics of release of Ca^{2+} from heart mitochondria at very low Ca loads. Experimental details are given under Materials and Methods. The values plotted are means from duplicate experiments.

after the addition of the mitochondria, a 1.5-ml portion was removed and centrifuged in order that the mitochondrial Ca^{2+} content could be determined by atomic absorption spectrophotometry. Different Ca^{2+}:EGTA buffers (concentration 40 mM as EGTA) were then added to the remainder of the incubation on the basis of trial and error until the pCa stabilized by the mitochondria was exactly duplicated. The final EGTA concentration was not less than 2 mM. The pCa value was then determined by reference to the final ratio of $CaCl_2$:EGTA and the stability constant. This was taken to be 1.204×10^7 at pH 7.2. Meticulous care was taken in maintaining pH 7.2 in these experiments.

In a proportion of the experiments the incubation conditions were exactly as described for the measurement of reduction of mitochondrial NAD(P) (above).

Determination of the Free Ca^{2+} Concentration of the Mitochondrial Matrix

This was approached via the "null-point" technique developed by Williamson and Murphy (1980) (see also Murphy *et al.*, 1980, for the determination of cytosolic free Ca^{2+} in hepatocytes by this method). For any

given mitochondrial load of Ca, a series of experiments was performed in which the change in the differential absorbance A675–685 nm due to Ca^{2+}-Arsenazo III binding was measured on adding the bivalent cation ionophore A23187 to a suspension of mitochondria, using a dual-wavelength spectrophotometer. Within the series, the experiments differed in the concentration of extramitochondrial $CaCl_2$ added prior to the addition of the A23187. Uptake of this added Ca^{2+} was prevented by the inhibitor Ruthenium Red (Vasington *et al.*, 1972).

Mitochondria (4 mg of protein) were suspended in a medium comprising 0.13 M KCl, 20 mM KHepes, 5 mM KP_i, 10 mM NaCl, 5 mM L-glutamate, and 5 mM L-malate, containing 30 μM Arsenazo III and 250 ng of nigericin. The pH was 7.2 and the temperature was 25°C. Five minutes later, Ruthenium Red (2 nmol) was added, followed at 5 min 10 s by an addition of $CaCl_2$. The spectrophotometer was reset and then A23187 (5 nmol) was added at 6 min 30 s. The differential absorbance change $\Delta\Delta A$ was measured, as shown in detail for Fig. 4. Finally, the ΔA 675–685 nm was returned to the value

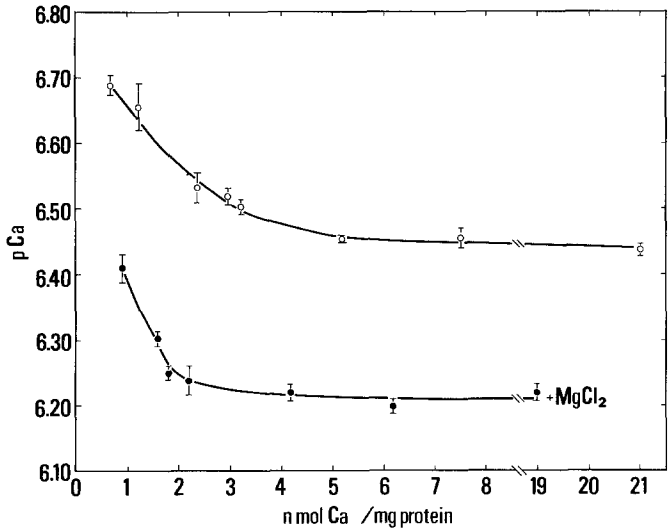

Fig. 3. Dependence of extramitochondrial pCa on the Ca content of heart mitochondria. Experimental details are given under Materials and Methods. Results are mean values ±S.E.M. from experiments with five mitochondrial preparations. Identical additions of $CaCl_2$ or EGTA resulted in similar but not identical mitochondrial Ca contents in these five preparations: thus these were 0.67 ± 0.1, 1.22 ± 0.06, 2.37 ± 0.18, 2.97 ± 0.15, 3.22 ±0.09, 5.19 ± 0.50, 7.52 ± 0.50, and 21.1 ± 1.9 nmol of Ca/mg of protein for the incubations omitting $MgCl_2$. Errors in the other experiments were comparable.

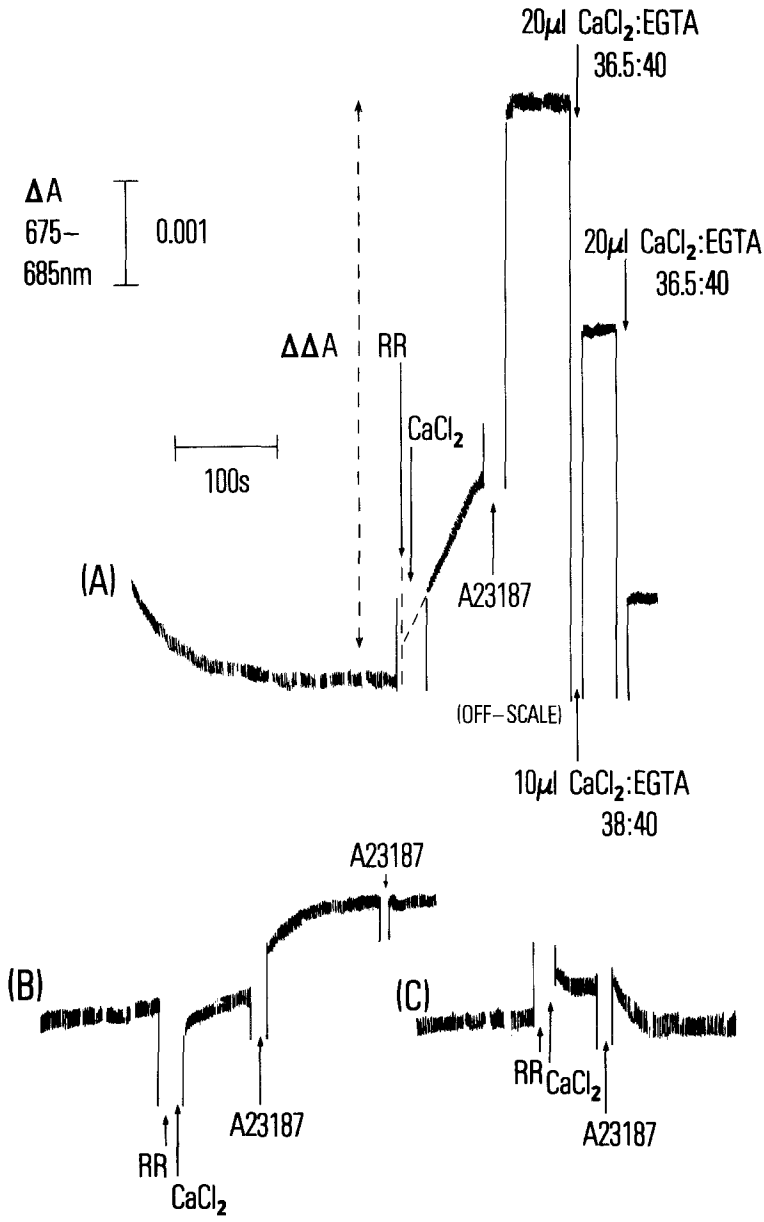

Fig. 4. Protocol for the determination of mitochondrial matrix free Ca^{2+} concentration by the "null-point" technique. The absorbance difference for the wavelength pair 675–685 nm was monitored using a dual-wavelength spectrophotometer. The change plotted in Fig. 5 ($\Delta\Delta A$) is the sum of the changes elicited by the addition of Ruthenium Red and the ionophore A23187, using the construction shown. In experiment (A), the mitochondria were not depleted of endogenous Ca, and the addition of $CaCl_2$ was 70 nmol. In (B) and (C), the mitochondria were partially depleted by the inclusion of 20 nmol of EGTA from the beginning of the experiment. In (B), the $CaCl_2$ added was 10 nmol: in (C) it was 40 nmol. The Materials and Methods section gives other experimental details, including the use of Ca^{2+}:EGTA buffers to establish the extramitochondrial free Ca^{2+} concentrations (A).

achieved directly after the addition of Ruthenium Red plus $CaCl_2$, by titration with Ca^{2+}:EGTA buffers of varying pCa value. This involved a trial-and-error procedure and is illustrated in Fig. 4A.

In some experiments, the medium contained 20 mM potassium acetate (replacing KCl) and omitted nigericin. In some experiments the protocol described above for measuring NAD(P) reduction in relation to 2-oxoglutarate dehydrogenase activity was used, with the additional inclusion of Arsenazo III and nigericin. Differential absorbance changes were measured as described in this section.

The mitochondrial pH gradient (ΔpH) was measured by acetate distribution, and the matrix volume by sucrose-inaccessible space, as described by Johnson and Hansford (1977).

Measurement of Total Mitochondrial Ca Contents

This was done by atomic absorption spectrophotometry, using a Perkin-Elmer 2380 spectrophotometer, after the mitochondria had been extracted with 1% (v/v) HCl/1% (w/v) $LaCl_3$ and the denatured protein removed by centrifugation. Inclusion of 0.1% Triton X-100 did not materially change the values.

Materials

Arsenazo III was type 1, from Sigma Chemical Co. Antipyrylazo III was from ICN Pharmaceuticals, Inc., and was recrystallized according to Scarpa (1979). Ruthenium Red was a gift from Dr. J. Wehrle, Johns Hopkins University, and had been recrystallized.

Results and Discussion

The Dependence of 2-Oxoglutarate Dehydrogenase Activity on the Mitochondrial Content of Ca

Figure 1 shows the dependence of 2-oxoglutarate dehydrogenase activity, as revealed by the reduction of NAD in State 4, on the mitochondrial content of Ca. It is seen that 50% activation is achieved at approximately 0.5 nmol/mg of protein and that activity is maximal at Ca contents of 2 nmol/mg of protein and greater. The ionic composition of the medium used, which contains 10 mM Na^+ and 1 mM Mg^{2+}, is taken to mimic the intracellular environment (Lee and Fozzard, 1975; Veloso *et al.*, 1973). Omission of Mg^{2+} results in a displacement of the curve to slightly higher values, with 50% activation at 1.0 nmol of Ca/mg of protein (Hansford and Castro, 1981). The effect of Mg^{2+}

may be to displace Ca^{2+} from intramitochondrial binding sites and thus increase the free Ca^{2+} concentration at any given total mitochondrial Ca load (but see below). The present data are preferred to those presented earlier (Hansford and Castro, 1981), because of the more nearly physiological incubation conditions: together, the two figures present the only information available on the amount of mitochondrial Ca required to activate any of the Ca^{2+}-sensitive dehydrogenases.

Although the degree of reduction of NAD in state 4 is a function of dehydrogenase activity, it may not be a linear function. For this reason, the State 3 rate of O_2-uptake with 0.5 mM 2-oxoglutarate plus 0.5 mM L-malate as substrate was also investigated as a function of Ca content. Results were substantially similar to those shown in Fig. 1, with mean values for 50% activation of 0.86 and 1.08 nmol of Ca/mg of protein, in experiments in the presence and absence of 1 mM $MgCl_2$, respectively, and using six different mitochondrial preparations (results not shown). Although easier to justify as a measure of flux through 2-oxoglutarate dehydrogenase, and hence of enzyme activity, the State 3 rate is not so precise an indicator of mitochondrial Ca content, however, as the range of activity from Ca depletion to Ca repletion is only $\times 2.2$ (cf $\times$ 15 in Fig. 1).

The Dependence of Ca^{2+} Egress from the Mitochondrion on Ca Content

The egress of Ca^{2+} from heart mitochondria shows Michaelis–Menten kinetics with respect to the intramitochondrial pool of Ca, when this is expressed in terms of nmol/mg of protein (Fig. 2). At these very low total contents of Ca, accurate allowance has to be made for endogenous Ca, and the substrate concentrations used in Fig. 2 are derived from a determination of the amount of Ca within the mitochondria at the moment that efflux is initiated by the addition of NaCl and Ruthenium Red. Five such experiments with different mitochondrial preparations gave a mean $S_{0.5}$ value of 6.05 $\pm$ 0.46 nmol/mg of protein and a V_{max} for egress of 9.95 $\pm$ 0.69 nmol/min/mg of protein. It is noted that Crompton *et al.* (1977) obtained Michaelis–Menten kinetics for the Ca^{2+}-stimulated release of $^{45}Ca^{2+}$ from heart mitochondria, and a $S_{0.5}$ of 13 μM for external Ca^{2+}. The "null-point" determinations of free Ca^{2+} concentration presented below suggest that 6.05 nmol/mg protein is equivalent to approximately 3–4 μM free Ca^{2+}. This is of the same order of magnitude as the $S_{0.5}$ derived by Crompton *et al.* (1977): the discrepancy which does exist between the results could reflect a variation of the activity coefficient of matrix Ca^{2+} at contents greater than 2.5 nmol/mg protein (which were not investigated below) or, more significantly, a lack of symmetry of the transport system, or its environment.

Repetition of these egress experiments with 1 mM $MgCl_2$ present

throughout the mitochondrial experiment gave an unchanged V_{max} of transport [12.2 ± 0.8(3) nmol/min/mg] but a significant elevation in the $S_{0.5}$ for intramitochondrial Ca [10.3 ± 1.1(3) nmol/mg of protein, $p < 0.005$]. The effect of Mg^{2+} on the affinity for (external) $^{45}Ca^{2+}$ of the $Ca^{2+}/2\ Na^{+}$ exchange process was not studied in the previous work (Crompton *et al.*, 1977).

The Partial Buffering of Extramitochondrial Ca^{2+} Concentration

From comparison of Figs. 1 and 2, the Ca^{2+} egress pathway is not saturated at mitochondrial Ca contents in the range required to show regulation of 2-oxoglutarate dehydrogenase by this metal ion. This is even more true when comparison is made with the $S_{0.5}$ value obtained for egress in the presence of 1 mM $MgCl_2$ (see text above). This suggested that mitochondria would not buffer extramitochondrial pCa under these plausibly physiological conditions, in distinction to the widely held view that this is a mitochondrial function (Bygrave, 1978; Nicholls, 1978; Nicholls and Crompton, 1980). Direct experiments employing a Ca^{2+}-selective electrode (Fig. 3) showed that this is the case. Heart mitochondria buffer the extramitochondrial free Ca^{2+} concentration very effectively when loaded with more than 5 nmol Ca/mg of protein, the range generally employed before (Nicholls, 1978; Becker, 1980; Becker *et al.*, 1980; Brand and de Selincourt, 1980), but fail to buffer at contents which we maintain are more probably physiological. The presence of 1 mM $MgCl_2$ shifts the pCa which is maintained at a given load, presumably by inhibiting the electrogenic Ca^{2+} uptake process (Crompton *et al.*, 1976a). A similar effect of Mg^{2+} was reported by Nicholls (1978) in experiments involving liver mitochondria and substantially greater Ca loads. These experiments (Fig. 3) used L-glutamate plus L-malate as substrate to allow the maintenance of a steady protonmotive force throughout the incubation. Repetition under the exact conditions of Fig. 1 gave lower pCa values during the initial 10 min substrate-depletion phase, presumably owing to a diminished membrane potential, but then a generation of pCa values very similar to those shown in Fig. 3 at the point of sampling, 2 min after the addition of 2-oxoglutarate plus L-malate. At this time, mean pCa values were 6.55 and 6.18 in the absence and presence of Mg^{2+}, respectively, with a deviation from buffering behavior below 2.4 nmol of Ca/mg of protein. Thus, the experiments of Figs. 1 and 3 can be fairly compared.

It is noted that one previous piece of work does show a variation in mitochondrial "set-point" with Ca load, using liver mitochondria (Becker *et al.*, 1980). The loads employed (10–15 nmol/mg of protein) were still an order of magnitude higher than those identified as physiologically important here: it is probably unwise to extrapolate the findings and inferences of the present paper to the liver mitochondrion.

The Null-Point Measurement of Matrix Free Ca^{2+} Concentration

Finally, an attempt was made to relate the total Ca contents discussed so far to matrix free Ca^{2+} concentration. This attempt involved the "null-point" technique of Williamson and Murphy (1980). This presupposes that the concentration of extramitochondrial free Ca^{2+} at which Ca^{2+} neither enters nor leaves the mitochondria on addition of the ionophore A23187 must be the concentration prevailing in the mitochondrial matrix. Experiments normally give rise to either uptake or release, and the null-point is obtained by plotting the extent of these changes graphically. Some examples of such experiments are illustrated in Fig. 4. When mitochondria were used without depletion of their endogenous Ca (Fig. 4A), addition of Ruthenium Red initiated a significant release of Ca^{2+}, as expected from the original work of Crompton *et al.* (1976b) and measured earlier in this study (Fig. 2). The ionophore A23187 then gave a more rapid release. However, the time of addition of the A23187 was not critical to the measurement of the extent of release; i.e., substantially the same extent of release occurred whether mediated by the A23187 or the endogenous $Ca^{2+}/2\ Na^+$ antiport system (Crompton *et al.*, 1976b). The release was extrapolated back to the point of addition of Ruthenium Red, as shown in Fig. 4A. Figures 4B and C gave examples of the smaller differential absorbance changes obtained when the mitochondria were partially Ca depleted during the prior phase of the experiment. In Fig. 4B a net release occurs on addition of the ionophore, whereas in Fig. 4C there is a net uptake, indicating that extramitochondrial free Ca^{2+} concentration exceeds intramitochondrial, after the $CaCl_2$ addition shown.

The procedure of calibrating the incubation with Ca^{2+}:EGTA buffers offers advantages over the calculation of free Ca^{2+} concentration performed by Williamson and Murphy (1980), which relies upon knowledge of all of the chelating species present. Functionally, it is limited to the buffering range of EGTA, and is not reliable above about 1.5 μM at this pH. This was sufficient for the present study, which was mainly concerned with the region 0.5–1.5 μM. Higher concentrations would be accessible using nitrilotriacetate or HEDTA, which have smaller stability constants (see Nicholls, 1978).

Results from experiments of the type shown in Fig. 4 were plotted as shown in Fig. 5, giving substantially straight lines and "null-points" corresponding to the mitochondrial matrix free Ca^{2+} concentrations. This is subject to there being no ΔpH at the moment of addition of A23187, as the ionophore catalyzes the exchange $Ca^{2+}/2\ H^+$ (Reed and Lardy, 1972). Nigericin was included in these studies to dissipate the ΔpH formed by respiration (Henderson *et al.*, 1969). Alternatively, 20 mM acetate was present, giving null-point values within the range shown in Fig. 6. Acetate distributes across the mitochondrial membrane in response to ΔpH (Chappell and Crofts, 1966) and materially neutralizes an internal alkalinity at these high concentrations.

Direct measurement of ΔpH by [^{3}H]acetate distribution gave a value indistinguishable from zero and from that achieved in experiments with FCCP and no respiratory substrate, when measurement was made at the point of addition of A23187 in the experiments of Fig. 4. Omission of Ruthenium Red in these experiments, however, resulted in a small ΔpH (0.55 ± 0.11 units), with the matrix being alkaline. Ruthenium Red was always incubated in the null-point determination experiments, and a minimal value of ΔpH is therefore presumed to apply.

Finally, null-point values are plotted against measured total Ca content in Fig. 6. Data are scattered, quite largely owing to the difficulty of atomic

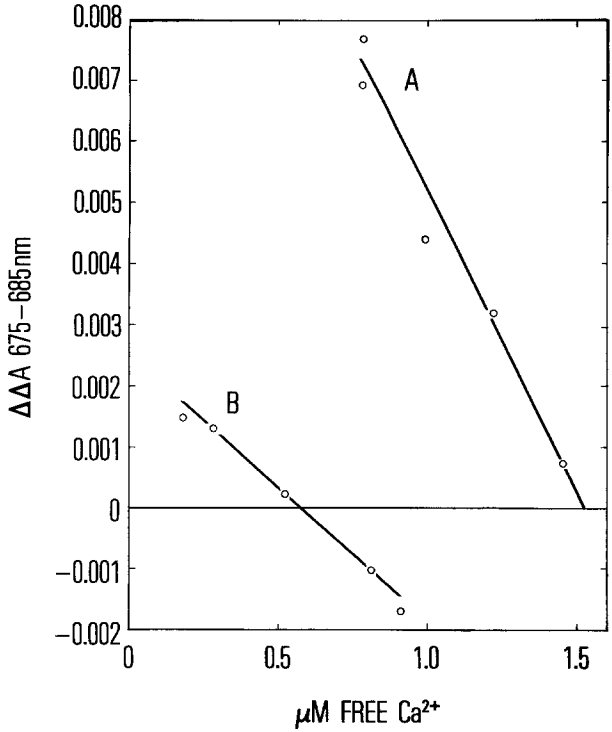

Fig. 5. The graphical determination of the matrix free Ca^{2+} concentration of undepleted and partially depleted mitochondria. The differential absorbance changes presented ($\Delta\Delta A$ 675–685 nm) were obtained in experiments of the type shown in Fig. 4. In (A) the mitochondrial preparation contained endogenous Ca, whereas in (B) this was partially depleted by the inclusion of 20 nmol of EGTA at the beginning of each experiment. The null-point determinations, corresponding to the mitochondrial free Ca^{2+} concentration, are 1.5 and 0.57 μM in (A) and (B), respectively.

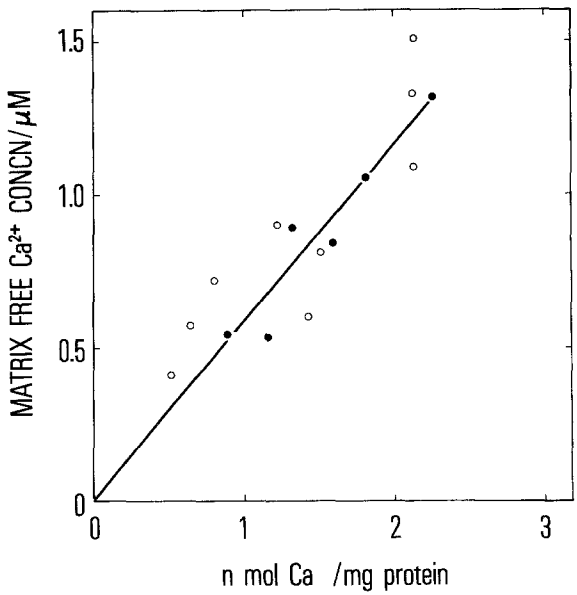

Fig. 6. Dependence of the mitochondrial matrix free Ca^{2+} concentration on the total mitochondrial Ca content. The mitochondrial Ca content was manipulated by the use of varying amounts of EGTA, included at time zero in experiments of the type of Fig. 4. It was measured by atomic absorption spectrophotometry (see Materials and Methods). The matrix free Ca^{2+} concentration was determined by the null-point method, as illustrated in Fig. 5. The solid circles represent experiments in which $MgCl_2$ was additionally present at 1 mM. Each point represents the result of one series of experiments, e.g., Fig. 5A or B: eight mitochondrial preparations were used in all.

absorption measurement of these very small amounts of Ca. However, there is a substantially linear relationship, indicating a very high degree of binding of mitochondrial Ca^{2+}, with an activity coefficient of approximately 10^{-3}. This is based upon the value for matrix space of 1 μl/mg of protein determined in this lab, which is consistent with other values obtained for heart mitochondria (LaNoue *et al.*, 1972). Although the measurements of both null-points and total Ca contents are open to error (Fig. 6), a consequence of our extension of this technique down to lower levels than those measured by Williamson and Murphy (1980), we maintain that they are quite incompatible with previous theoretical, and influential, treatments which suggested free Ca^{2+} concentrations and activity coefficients that are orders of magnitude higher (Nicholls, 1978). With reference to the enzymology, 0.5 and 1.0 nmol of Ca/mg of protein, the values identified as giving 50% activation of 2-oxoglutarate

dehydrogenase in the presence and absence of Mg^{2+} (Fig. 1 and text), correspond to 0.3 and 0.6 μM free Ca^{2+}. McCormack and Denton (1979) found a K_m for Ca^{2+} of 1 μM in studies with the purified dehydrogenase. The correspondence of these numbers is really quite close, considering the uncertainty of reproducing the matrix ionic environment in the cuvette and the impossibility of reconstructing the protein–protein interactions of the matrix in the studies of McCormack and Denton (1979).

Mg^{2+} ions have no clear effect on the relationship between free Ca^{2+} concentration and total content (Fig. 6). Thus the effect of Mg^{2+} on the studies of Fig. 1, and those described in the text, has not been shown to be due to a changed intramitochondrial buffering of Ca^{2+} and remains a puzzle. The "null-point" experiments have been repeated using the same experimental conditions as those in Fig. 1, but including nigericin and Arsenazo III, with substantially similar results [mean free Ca^2 concentration 1.68 ± 0.05(3) μM at 2.09 ± 0.08(3) nmol Ca/mg protein]. Inclusion of nigericin in the experiment of Fig. 1 caused a large diminution in the percent reduction of NAD(P) but caused no change in the Ca content required for 50% activation (0.94 nmol/mg protein, in the absence of Mg^{2+}). Thus it seems that the diminished $\Delta\psi$ presumably associated with the lower degree of NAD(P) reduction when mitochondrial Ca is depleted with nonsaturating 2-oxoglutarate as substrate is still sufficiently high for the distribution of Ca^{2+} across the mitochondrial membrane to be unchanged, relative to more active substrate (glutamate plus malate) oxidation. This may not be surprising, as the steady-state content of mitochondrial Ca has previously been shown to be insensitive to changes in $\Delta\psi$, above about 130 mV (Nicholls, 1978).

General Discussion

We have shown that the control of 2-oxoglutarate dehydrogenase in heart mitochondria by Ca^{2+} functions over the range 0–2 nmol of Ca/mg of mitochondrial protein, or approximately 0–1.2 μM free Ca^{2+} (Hansford and Castro, 1981; this paper, Figs. 1 and 6). We have also shown that this is true for pyruvate dehydrogenase interconversion (data not shown). If one accepts the purely teleological argument that this mode of control functions in the living animal, then it follows that this is also the physiological range of Ca content for heart mitochondria. Some recent, direct studies using electron probe analysis by Somlyo *et al.* (1979) have established an upper limit of approximately 3 nmol/mg of mitochondrial protein for mitochondria *in situ* in normal vascular smooth muscle, lending credence to the supposition that this is the range found in cardiac muscle too. If so, it follows from the experiments of Fig. 2 that the mitochondrial Ca^{2+}-egress pathway is not saturated, and

from this that heart mitochondria will act as only partial buffers of extramitochondrial free Ca^{2+} concentration. This is shown directly in Fig. 3.

This conclusion would lead us to support totally the proposal of Denton and McCormack (1980) that the mitochondrial transport activities serve to control the mitochondrial matrix free Ca^{2+} concentration, and not that of the cytosol. The advantage of this control is that of modulating the three enzymes which catalyze demonstrably nonequilibrium reactions in the oxidation of pyruvate and the operation of the tricarboxylate cycle, viz. pyruvate dehydrogenase (via the phosphatase), NAD-isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase (Denton *et al.*, 1972, 1978; McCormack and Denton, 1979; Hansford, 1981). Our minimizing of the mitochondrial role in cytosolic Ca^{2+} buffering is in accord with the views of Brinley *et al.* (1978) and Schweitzer and Blaustein (1980), as developed for nervous tissue and on quite different grounds.

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