Dependence of cardiac mitochondrial pyruvate dehydrogenase activity on intramitochondrial free Ca^{2+} concentration

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(1) The free Ca^{2+} concentration of the matrix of rat heart mitochondria ($[\text{Ca}^{2+}]_m$) was determined from the fluorescence of internalized indo-1. The value of the K_d of indo-1- Ca^{2+} in the mitochondrial matrix was determined to be 95 nM, on the basis of equilibration of $[\text{Ca}^{2+}]_m$ with the extramitochondrial free Ca^{2+} ($[\text{Ca}^{2+}]_o$) in the presence of rotenone, nigericin, valinomycin and Br-A23187. (2) $[\text{Ca}^{2+}]_m$ responded to energization/de-energization protocols, the inhibition of Ca^{2+} -uptake by Ruthenium Red and the potentiation of Ca^{2+} -efflux by Na^+ in a manner which was consistent with the known kinetic properties of the mitochondrial Ca^{2+} -transport processes. (3) The concentration gradient $[\text{Ca}^{2+}]_m/[\text{Ca}^{2+}]_o$ was found to be near unity (0.82 ± 0.18) when mitochondria were incubated in media containing 10 mM- Na^+ ; the additional presence of 1 mM- Mg^{2+} reduced the gradient to values below unity (0.26 ± 0.03). The polyamine spermine increased the Ca^{2+} concentration gradient in the presence of 1 mM- Mg^{2+} . (4) The fraction of pyruvate dehydrogenase in the active form (PDH_A) was found to increase with $[\text{Ca}^{2+}]_m$, with a $K_{0.5}$ for activation of approximately 300 nM- Ca^{2+} . This value of the activation constant was not affected by conditions, e.g. addition of Mg^{2+} , which changed the $[\text{Ca}^{2+}]_m/[\text{Ca}^{2+}]_o$ concentration gradient, and the presence of different oxidizable substrates, which changed the $[\text{NADH}/\text{NAD}^+]_m$ concentration ratio. Thus pyruvate dehydrogenase interconversion responds directly to changes in $[\text{Ca}^{2+}]_m$, as inferred in earlier work.

INTRODUCTION

Ca^{2+} ions serve not only to couple excitation to contraction in heart muscle (see Katz, 1970), thereby creating an energy demand, but also to activate the process of oxidative phosphorylation, thereby ensuring an enhanced energy supply. The latter process involves the activation by Ca^{2+} ions of pyruvate dehydrogenase phosphatase (Denton *et al.*, 1972; Pettit *et al.*, 1972), NAD^+ -linked isocitrate dehydrogenase (Denton *et al.*, 1978) and 2-oxoglutarate dehydrogenase (McCormack & Denton, 1979; Lawlis & Roche, 1980). Each of these dehydrogenases catalyses a non-equilibrium reaction, and activation may tend to maintain elevated mitochondrial NADH/NAD^+ ratios during periods of high work-load, thus maximizing the redox driving force for oxidative phosphorylation and flux through that process (see Koretsky & Balaban, 1987). These relations have been reviewed by Hansford (1980, 1985). Each of these dehydrogenase systems is present within the permeability barrier of the mitochondrial inner membrane, leading Denton & McCormack (1980) to suggest that the function of the mitochondrial Ca^{2+} -transport cycle was to communicate changes in cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_o$) into changes in mitochondrial matrix Ca^{2+} concentration $[\text{Ca}^{2+}]_m$. Thus, enhanced work-loads, normally associated with elevated values of $[\text{Ca}^{2+}]_o$ (Sheu & Fozzard, 1982; McCormack & England, 1983), would elicit enhanced dehydrogenase activity, and enhanced energy transduction by oxidative phosphorylation. Evidence in favour of this proposal has been recently

reviewed (Denton & McCormack, 1985; Hansford, 1985).

Although there is good agreement on the relationship between the fraction of pyruvate dehydrogenase existing in the active dephosphorylated form (PDH_A ; see reviews by Reed, 1981; Wieland, 1983) in isolated cardiac mitochondria and the concentration of free ionized Ca^{2+} ($[\text{Ca}^{2+}]_o$) in the suspending medium (Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981), much less is known of the relationship between PDH_A content and $[\text{Ca}^{2+}]_m$. This reflects the difficulty of measurement of $[\text{Ca}^{2+}]_m$. Until very recently, the only approaches consisted of the 'null-point' technique, involving the definition of a value of $[\text{Ca}^{2+}]_o$ at which there was no net flux into or out of mitochondria on rendering the mitochondrial membrane permeable to Ca^{2+} with the ionophore A23187 (Coll *et al.*, 1982; Hansford & Castro, 1982), and the insertion of the metallochromic indicator Arsenazo III into the mitochondria by a permeabilization/resealing protocol (Hayat & Crompton, 1987). The precision of the 'null-point' technique is limited by the small absorbance changes seen with the extramitochondrial metallochromic indicator when mitochondrial Ca^{2+} loads low enough to be of relevance to dehydrogenase regulation are investigated, whereas the permeability/resealing protocol may, theoretically at least, perturb the matrix composition, especially with respect to metal ions and low-molecular-mass cofactors.

Very recently, the fluorescent chelating agents developed for the measurement of cytoplasmic free Ca^{2+}

Abbreviations used: $[\text{Ca}^{2+}]_m$, mitochondrial matrix free Ca^{2+} concentration; $[\text{Ca}^{2+}]_o$, extramitochondrial free Ca^{2+} concentration; PDH_A , active form of pyruvate dehydrogenase; DTPA, diethylenetriamine- $\text{NNN}'\text{N}'\text{N}'$ -penta-acetic acid; FCCP, carbonyl cyanide p -trifluoromethoxyphenylhydrazide; HEDTA, N -(2-hydroxyethyl)ethylenedinitrilo- NNN' -tri-acetic acid; AM, acetoxymethyl ester.

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(Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1985) have been applied to the study of $[Ca^{2+}]_m$ in suspensions of isolated mitochondria (Davis *et al.*, 1987; Lukács & Kapus, 1987; Gunter *et al.*, 1988). Mitochondrial suspensions exposed to the acetoxymethyl esters of quin-2 and fura-2 generate the free acid form in the matrix, allowing the continuous recording of a fluorescence signal relating to $[Ca^{2+}]_m$. The matrix location of the chelating agent is confirmed by the dependence of the fluorescence signal upon energization/de-energization and Na^+ concentration, and the insensitivity of the chelating agent to quenching by Mn^{2+} in the presence of the Ca^{2+} -uniport inhibitor Ruthenium Red (Davis *et al.*, 1987; Lukács & Kapus, 1987).

We have used a related fluorescent chelating agent, indo-1 (Grynkiewicz *et al.*, 1985), to investigate the relationship between PDH_A content and $[Ca^{2+}]_m$ in respiring cardiac mitochondria. The sensitivity of the enzyme interconversion to Ca^{2+} is found to be slightly higher than in the previous work with mitochondrial extracts (Denton *et al.*, 1972) or with uncoupled cardiac mitochondria (Denton *et al.*, 1980). However, it is the essential similarity of these values which is emphasized. By virtue of suspending the mitochondria at known values of $[Ca^{2+}]_o$, using Ca^{2+} /EGTA buffers, we have also been able to measure the Ca^{2+} concentration gradient, $[Ca^{2+}]_m/[Ca^{2+}]_o$, and show that this is very small, varies with the presence of Mg^{2+} , polyamines and possibly ADP, and can be less than unity.

METHODS

Preparation of rat cardiac mitochondria and loading with indo-1

Mitochondria were isolated from the hearts of two male Sprague-Dawley rats, as described previously (Hansford, 1978), except that the amount of proteinase (Nagarse) used was 4 mg for two hearts and the time of digestion was 9 min.

Mitochondria (50–60 mg of protein) were loaded with indo-1 by exposure to 10–12 μM -indo-1-AM (indo-1-acetoxymethyl ester) in an incubation of 30 min at 25 °C in 2 ml of a 'loading medium' comprising 250 mM-sucrose, 10 mM- K^+ /Hepes, 1 mM- K^+ /EGTA and 0.2% (w/v) fatty-acid-free albumin (Calbiochem), of final pH 7.4. Subsequently, the mitochondria were diluted 10 times with cold loading medium and washed by sedimentation at 8000 g for 8 min, followed by resuspension to approx. 1.5 ml (25–40 mg of protein/ml). Following this procedure, mitochondria were stored on ice until they were used (up to 6 h).

Measurement of the fluorescence of indo-1

A 100 μl portion of indo-1-loaded mitochondria was added to 1.9 ml of O_2 -saturated basal medium in a plastic cuvette, equilibrated at 25 °C. This basal medium comprised 120 mM-KCl, 25 mM- K^+ /Mops, 0.5 mM- K^+ /EGTA and 5 mM-potassium phosphate, pH 7.2. Fluorescence was recorded using a Farrand A2 fluorometer, fitted with magnetic stirring of the sample and with gassing of the sample chamber with O_2 . The excitation wavelength was 333 nm and emission was collected at 400 nm. Further details are given in the appropriate Figure legend.

Measurement of PDH_A content

At the appropriate time, a 50 μl aliquot was withdrawn from the mitochondrial suspension in the fluorometer cuvette and added to 250 μl of ice-cold 'quenching' solution, which was designed to prevent further enzyme interconversion. The extract thus formed was then assayed for pyruvate dehydrogenase activity under V_{max} conditions at 25 °C. The composition of the 'quenching' solution and assay mixture have been given previously (Hansford, 1976, 1977). The reduction of NAD^+ was linear with time for at least 1 min when enzyme derived from 60–80 μg of mitochondrial protein was assayed in each cuvette. The activity thus determined is referred to as ' PDH_A content' in the text. PDH_A content is often expressed as a fraction of the total pyruvate dehydrogenase which was obtained by sampling mitochondria 2 min after the addition of 1–2 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine (FCCP) in the presence of medium containing 1 μM -free Ca^{2+} and glutamate plus malate as substrate. Values of total pyruvate dehydrogenase were 66 ± 8 nmol/min per mg of protein ($n = 17$), at 25 °C, for indo-1-loaded mitochondria. These values are comparable with those determined previously for fresh rat heart mitochondria (Hansford, 1976, 1977).

Use of Ca^{2+} /EGTA buffers

Values of free Ca^{2+} concentration stabilized using Ca^{2+} /EGTA buffers were calculated using the stability constants and the program published by Fabiato & Fabiato (1979). In all experiments using these buffers care was taken to adjust pH as precisely as possible to 7.2 at 25 °C. A Ca^{2+} -selective electrode (World Precision Instruments) was used to check values of $[Ca^{2+}]$ in the incubation solutions.

Materials

Nagarse was from Enzyme Development Corp. (New York, NY, U.S.A.). Indo-1-AM was from Calbiochem and was dissolved in dimethyl sulphoxide. Ruthenium Red, rotenone, FCCP, *N*-(2-hydroxyethyl)ethylene-dinitrilo-*NNN'*-tri-acetic acid (HEDTA), nigericin, valinomycin, diethylenetriamine-*NNN'*-*N''N''*-penta-acetic acid (DTPA) and EGTA were purchased from Sigma Chemical Co. Br-A23187 was from Calbiochem.

RESULTS AND DISCUSSION

Use of indo-1 to measure $[Ca^{2+}]_m$

Indo-1, synthesized by Grynkiewicz *et al.* (1985) as a fluorescent Ca^{2+} -chelating agent, was introduced into mitochondria in the form of the permeant acetoxymethyl (AM) ester. Fig. 1 shows fluorescence emission spectra obtained at different times of incubation of mitochondria with indo-1-AM. It is seen that there is a progressive shift to a form which emits maximally at 395 nm in the presence of Ca^{2+} . This emission is progressively more subject to quenching by the Mn^{2+} ion. Both of these properties are in accord with the published properties of the indo-1 free acid (Grynkiewicz *et al.*, 1985). Thus it is concluded from experiments of this type that there is nearly total hydrolysis of the AM ester to the free acid form in the 30 min period typically used for loading the mitochondria. The emission peak close to 450 nm would appear to represent the remaining ester form of the dye.

Fig. 1. Generation of indo-1 free acid by heart mitochondria incubated with indo-1-AM

Mitochondria (44 mg of protein) were incubated in a plastic scintillation-counter vial in 2 ml of loading medium (see the Methods section) containing $10 \mu\text{M}$ indo-1-AM at 25°C under orbital shaking (140 rev./min). After 17 min (spectra 1 and 3) or 30 min (spectra 2 and 4), aliquots of 0.1 ml were withdrawn, diluted to 1 ml with fresh loading medium and centrifuged in an Eppendorf microcentrifuge for 2 min. The supernatant was discarded, the tube was washed out with 1 ml of fresh loading medium and the pellet was resuspended in 2 ml of a medium comprising 120 mM-KCl/25 mM-Mops/0.5 mM-EGTA/0.2% (v/v) Triton X-100 (Research Products International Corp., Elk Grove Village, IL, U.S.A.) of pH 7.4. Fluorescence was excited at 333 nm and emission was studied over the range 370 to 510 nm in the presence of 1 mM- CaCl_2 (spectra 1 and 2) or after addition of 2 mM- MnCl_2 (spectra 3 and 4), using a Perkin-Elmer LS5 spectrofluorometer. The spectrum labelled 5 represents the fluorescence emission of mitochondria (3.2 mg/2 ml) not treated with indo-1-AM.

However, the fluorescence of this component was also quenched by Mn^{2+} (cf. traces 1 and 3 or 2 and 4) and the emission peak did change to the left with Ca^{2+} (results not shown), suggesting the presence of a partially hydrolysed form. To avoid any potential interference in measuring the fluorescence of the free-acid form, the emission was collected at 400 nm (Oriel interference filter, peak 400.5 nm, band width 10 nm). Fig. 1 also shows the intrinsic fluorescence of unloaded mitochondria; the contribution of this fluorescence is small at 400 nm, but was routinely monitored (see below).

Calculation of $[\text{Ca}^{2+}]_m$ from the fluorescence signal (F) requires knowledge of F_{max} and F_{min} , corresponding

to saturation of indo-1 with Ca^{2+} and complete desaturation, respectively, and of the K_d of the indo-1- Ca^{2+} chelate within the mitochondrial Ca^{2+} matrix (see Tsien *et al.*, 1982). In the present experiments, F_{min} was generated by addition of 5 mM- MnCl_2 (to a medium containing 4.25 mM-EGTA) plus 0.05% Triton X-100. Addition of a higher concentration of Mn^{2+} (up to 7.5 mM) or Triton X-100 (up to 0.1%) did not further decrease the fluorescence signal. F_{max} was achieved by the subsequent addition of a molar excess of DTPA- Ca^{2+} (10 mM) to displace Mn^{2+} from the indo-1. The contribution of extramitochondrial indo-1 to the total fluorescence signal was not routinely determined, as early experiments had indicated that it was very small (less than 5% of the total signal). These experiments relied upon the addition of Mn^{2+} in the presence of Ruthenium Red to quench fluorescence from extramitochondrial indo-1 instantaneously (see Fig. 3). Even after 6 h of storage at 0°C , mitochondrial suspensions which had been loaded with indo-1 showed no greater external signal, i.e. there was no indication of a significant leak of indo-1 from the organelles under these conditions. It is noted that, both in terms of the lack of leakage of the indo-1 and the apparent presence of only one compartment for the dye, the interpretation of these mitochondrial studies is more straightforward than corresponding studies of cardiac myocytes (Hansford, 1987; Hansford & Lakatta, 1987; Moreno-Sánchez & Hansford, 1988).

The value of K_d for indo-1- Ca^{2+} used in the calculation of $[\text{Ca}^{2+}]_m$ was determined experimentally, using suspensions of mitochondria loaded as described in the Methods section. The results of the determination are shown in Fig. 2, and the protocol is given in the legend. Briefly, the $[\text{Ca}^{2+}]_m$ was brought into equilibrium with values of $[\text{Ca}^{2+}]_o$ imposed through the use of Ca^{2+} /EGTA and Ca^{2+} /HEDTA buffers, and the fluorescence of intramitochondrial indo-1 was determined. Equilibration of Ca^{2+} across the mitochondrial membrane was effected by using the ionophore Br-A23187 (Debono *et al.*, 1981) after inhibiting respiration with rotenone. The stabilization of the fluorescence signal was taken as an index of equilibration; at this point, more Br-A23187 caused no further change. As Br-A23187 may potentially exchange Mg^{2+} for Ca^{2+} ions, though its selectivity for Ca^{2+} over Mg^{2+} is greater than for the parent compound, A23187 (Debono *et al.*, 1981), the dependence of the fluorescence signal upon extramitochondrial Mg^{2+} concentration was studied. All of the experiments shown in Fig. 2 were performed at both 0.1 mM and 1 mM free extramitochondrial Mg^{2+} concentration, and results were indistinguishable. Mitochondrial matrix free Mg^{2+} concentration is thought to be approx. 0.5 mM (Coll *et al.*, 1982), such that our studies should have involved minimal Mg^{2+} concentration gradients. The ionophore Br-A23187 may also exchange H^+ ions for Ca^{2+} ions (Reed & Lardy, 1972; Debono *et al.*, 1981), so that care was taken to dissipate any ΔpH across the mitochondrial membrane. This was achieved by adding nigericin ($2 \mu\text{M}$), with the medium used to suspend the mitochondria containing 120 mM-KCl, together with valinomycin ($3 \mu\text{M}$) or FCCP ($1 \mu\text{M}$) in the same medium. Results were the same, indicating the complete collapse of both components of the proton electrochemical gradient. Under these conditions, a K_d for intramitochondrial indo-1 of 95 nM was determined (Fig. 2). The re-

Fig. 2. Determination of K_d of intramitochondrial indo-1- Ca^{2+} chelate

(a) and (b) Protocol of the determination. Mitochondria (2.29 mg of protein/ml) which had been loaded with indo-1 as described in the Methods section were incubated in 2 ml of basal medium containing 1 mM- MgSO_4 and 5 mM-glutamate plus 5 mM-malate as respiratory substrate. Where indicated a $\text{Ca}^{2+}/\text{EGTA}$ buffer was added (to 4.25 mM-EGTA), giving 645 nM free Ca^{2+} (a) or 32 nM free Ca^{2+} (b). Subsequent additions were: rotenone (Rote) 2.5 μM ; nigericin (Nige) 2 μM ; valinomycin (Val) 3 μM ; 4-bromo A23187 (Br-A23) 4 μM . Higher concentrations of 4-bromo A23187 (up to 8 μM), nigericin (up to 4 μM) or valinomycin (up to 6 μM) did not modify the steady-state fluorescence or the kinetics of the response. Results were the same regardless of whether FCCP was used to collapse membrane potential (after appropriate correction for the quenching of the fluorescence signal) or valinomycin was used. The steady-state fluorescence achieved was recorded, and then MnCl_2 (5 mM) and Triton X-100 [0.05 % (v/v)] were added, to achieve a fluorescence minimum (F_{min}), followed by sodium DTPA/ CaCl_2 of pH 7.4 (DTPA/ Ca^{2+}) to 10 mM/10 mM, to achieve a fluorescence maximum (F_{max}). Fluorescence was excited at 333 nm and measured at 400 nm, as described in the Methods section.

determination of the K_d for indo-1 in simple solution under our present conditions (120 mM-KCl, pH 7.2, 25 °C) gave a value of 196 nM). This is slightly lower than, though similar to, the value given by Grynkiewicz *et al.* (1985); the difference may be explained on the basis of temperature (25 °C vs. 37 °C) and pH (7.2 vs. 7.05), as Gunter *et al.* (1988) have shown a decrease in K_d with increasing pH. The more substantive difference between the values of K_d determined for indo-1 free acid in simple aqueous solution and our present value for the compound within mitochondria may reflect interaction with mitochondrial protein and/or the high viscosity of the matrix.

Another control experiment deemed important to the use of indo-1 to estimate $[\text{Ca}^{2+}]_m$ involved determination of the contribution of intrinsic mitochondrial fluorescence to the total signal. When the full extent of intrinsic fluorescence was revealed by successively using ADP or FCCP and rotenone, this was found to be only 5–8 % of the total fluorescence obtained from mitochondria containing 79 ± 13 pmol of indo-1 free acid per mg of protein ($n = 10$) [see also Fig. 3(c) and (d)].

Finally, the functional consequences of loading mitochondria with indo-1 were investigated. It was found that the loading procedure, involving a lengthy incubation at 25 °C, caused only a small decrease in respiratory control ratios, when measured with pyruvate plus malate as substrates (5.1 ± 0.7 for the loaded mitochondria, vs. 7.7 ± 0.8 for the unloaded controls, $n = 8$). In addition, pyruvate dehydrogenase activity was unimpaired, as presented below. This contrasts with a loss of 2-oxoglutarate dehydrogenase activity found on loading mitochondria with fura-2 in a rather similar protocol (Lukács *et al.*, 1988).

Based on the criteria presented above, we would judge that use of indo-1-loaded mitochondria presents an attractive way of achieving a continuous estimate of mitochondrial free Ca^{2+} concentration, over the range 10 nM to 1–2 μM , with a minimum of perturbation of mitochondrial function. This sensitivity range is entirely appropriate for the study of dehydrogenase regulation by Ca^{2+} , as presented below. Other authors have recently used fura-2 in mitochondrial studies to measure $[\text{Ca}^{2+}]_m$ (Davis *et al.*, 1987; Lukács & Kapus, 1987; Gunter *et al.*, 1988). We have no direct evidence on the relative merits of the two probes; however, there is some evidence that loading with fura-2 is faster than with indo-1 (Gunter *et al.*, 1988), but that interference by intrinsic fluorescence may be more of a problem with fura-2 (T. E. Gunter, personal communication).

Studies of the fluorescence of mitochondria loaded with indo-1 could potentially be improved by the use of the ratio of the intensities of light emitted at 400 and 490 nm, these wavelengths corresponding to emission maxima of the Ca^{2+} -bound and Ca^{2+} -free forms respectively. This approach, as originally proposed by Grynkiewicz *et al.* (1985), would minimize problems due

(c) This panel presents the results of experiments of the type shown in (a) and (b) and performed at 0.1 mM ($\blacktriangle$) or 1 mM- MgSO_4 ($\circ$) with three different mitochondrial preparations (2.1–2.3 mg of protein/ml). $\blacksquare$ Represents results of experiments performed at $[\text{Ca}^{2+}] > 10 \mu\text{M}$. The K_d of the indo-1- Ca^{2+} chelate was determined to be 95 nM from this plot.

Fig. 3. Effect of Ruthenium Red on $[\text{Ca}^{2+}]_m$ and PDH_A content

Mitochondria (1.86 mg of protein/ml) were incubated in 2 ml of basal medium containing 1 mM-ATP/10 mM-NaCl and 20 mM-succinate (plus 2.5 μM -rotenone) as respiratory substrate. After 6 min, Ca^{2+} /EGTA buffer was added to give 4.25 mM-EGTA and 832 nM-free Ca^{2+} . When $[\text{Ca}^{2+}]_m$ had achieved a steady state with respect to $[\text{Ca}^{2+}]_o$, Ruthenium Red (RR) was added to give 0.5 μM . Samples (50 μl) were taken as indicated (Samp, 3 min after addition of Ca^{2+} and 0.5, 1 and 2.5 min after addition of RR) and assayed for pyruvate dehydrogenase as described in the Methods section. Calibration of the fluorescence signal was as described for Fig. 2. In trace (b), Ruthenium Red was added prior to Ca^{2+} /EGTA. In traces (c) and (d), the mitochondria (1.7 and 2.1 mg of protein/ml, respectively) contained no indo-1, and the respiratory substrates were 20 mM-succinate plus 2.5 μM -rotenone and 5 mM-glutamate plus 5 mM-malate, respectively. ADP was added to give 1 mM. Panel (e) presents results of experiments of the type shown in trace (a), conducted with three different preparations of mitochondria (1.7–1.9 mg of protein/ml). Pyruvate dehydrogenase activity is expressed as a fraction of the total enzyme, determined as described in the Methods section. Values of $[\text{Ca}^{2+}]_m$ are calculated as described in the text. Results are presented as the mean values $\pm$ S.D.

to changes of light-scattering by the mitochondrial suspension during the course of an experiment.

Relation of PDH_A content to $[\text{Ca}^{2+}]_m$

In this section we describe the results of experiments relating $[\text{Ca}^{2+}]_o$, $[\text{Ca}^{2+}]_m$ and PDH_A content of cardiac mitochondria. They derive quite largely from earlier

experiments in which the relation between $[\text{Ca}^{2+}]_o$ and PDH_A content was developed (Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981), but also exploit the indo-1 technique to measure the important parameter $[\text{Ca}^{2+}]_m$.

Fig. 3 presents a representative fluorescence recording and demonstrates the strength of this technique in allowing the kinetics of changes in $[\text{Ca}^{2+}]_m$ to be followed. Addition of Ruthenium Red, and inhibition of the Ca^{2+} uniporter (Moore, 1971), results in a rapid decrease in fluorescence, corresponding to loss of Ca^{2+} from the mitochondrial matrix. De-energization of mitochondria by FCCP also results in a similar decrease in fluorescence (results not shown). Previous experiments have documented the progressive appearance of Ca^{2+} in the suspending medium under these conditions (Crompton *et al.*, 1976b), the obverse of the present finding. By contrast, the null-point technique (Coll *et al.*, 1982; Hansford & Castro, 1982) does not allow for continuous determination of $[\text{Ca}^{2+}]_m$, although a technique recently developed by Hayat & Crompton (1987) involving the loading of resealed mitochondria with Arsenazo III does have that capability. It is noted that Na^+ was present at 10 mM in this experiment (Fig. 3a): Na^+ is necessary as a counter-ion for Ca^{2+} egress in heart mitochondria (Crompton *et al.*, 1976b), as observed in control experiments where Ruthenium Red was unable to induce Ca^{2+} release in the absence of Na^+ (results not shown).

Prior addition of Ruthenium Red completely blocks the uptake of Ca^{2+} (Fig. 3b). Note that neither the addition of Mn^{2+} after Ruthenium Red in expt. (a) nor the increase in $[\text{Ca}^{2+}]_o$ (from 5 nM to 773 nM) in the presence of Ruthenium Red in expt. (b) significantly affected the fluorescence signal, indicating a rather negligible amount of extramitochondrial dye (less than 5%). The contribution of NAD(P)H to the fluorescence signal is nil with succinate plus rotenone, but it is significant (5–8 % of total fluorescence) with glutamate plus malate as shown in experiments with unloaded mitochondria [traces (c) and (d) respectively]. Thus this contaminating fluorescence must be considered in the calculation of $[\text{Ca}^{2+}]_m$ when mitochondria are incubated with glutamate plus malate as substrates.

Fig. 3 further shows a rapid decline in PDH_A content in concert with the decline in $[\text{Ca}^{2+}]_m$ following treatment with Ruthenium Red. The values in Fig. 3 were obtained from three different mitochondrial preparations, illustrating good reproducibility. When values of PDH_A content are plotted versus $[\text{Ca}^{2+}]_m$, the relationship seen in Fig. 3(e) is obtained. It is understood that enzyme interconversion was probably not at a steady state with respect to $[\text{Ca}^{2+}]_m$ at the points of sampling, and thus quantitative conclusions concerning the affinity of the pyruvate dehydrogenase phosphatase for Ca^{2+} are better drawn from steady-state experiments presented below. A role for Ruthenium Red in antagonizing pyruvate dehydrogenase activation by Ca^{2+} in intact heart mitochondria was previously established by Denton *et al.* (1980). Similarly, the kinetics of inactivation of pyruvate dehydrogenase upon inhibition of Ca^{2+} uniporter activity, achieved with excess EGTA, were previously presented by Hansford (1981).

Fig. 4 displays the results of experiments in which pyruvate dehydrogenase interconversion was allowed to achieve a steady state at different values of $[\text{Ca}^{2+}]_o$. Proof of the steady-state relation, 9–12 min after adding

Fig. 4. Steady-state PDH_A content as a function of [Ca²⁺]_m with different respiratory substrates in state 4; also, the effect of Mg²⁺

In (a), mitochondria (1.5–2.1 mg of protein/ml) were incubated in 2 ml of basal medium containing 1 mM-ATP, 10 mM-NaCl and 20 mM-succinate as substrate, in the presence of 2.5 μM-rotenone, at a variety of different values of [Ca²⁺]_o, as described in the legend to Fig. 3. Samples (50 μl) were withdrawn in duplicate at the 9 min time-point, and assayed for pyruvate dehydrogenase activity. In (b), conditions were the same as for (a), except that the respiratory substrate was 5 mM-glutamate plus 5 mM-malate. In (c), the respiratory substrate was succinate in the presence of rotenone, and the mitochondria were preincubated with ATP as described for (a). However, unlike (a), MgSO₄ was added to 2 mM 1 min prior to the addition of the Ca²⁺/EGTA buffer. Experimental points were derived from experiments with six (panel a) or four (panels b and c) different mitochondrial preparations. Panel (d) presents the time-course of inactivation of pyruvate dehydrogenase in the presence of: ▲, glutamate (5 mM) + malate (5 mM) + ATP (1 mM); ○, succinate (20 mM) + rotenone (2.5 μM); or ●, succinate (20 mM) + rotenone (2.5 μM) + ATP (1 mM). Each data point represents the mean ± S.D. of values obtained with four different mitochondrial preparations. The effect of ATP (with succinate plus rotenone as substrate) was statistically significant: $P < 0.01$ for the 9 min time-point (Student's *t* test for paired samples). However, no significant difference was found between the 9 min and the 12 min time-points of preincubation with ATP.

mitochondria to the basal medium containing 0.5 mM-EGTA, is given in panel (d). Clearly, the PDH_A content increases with increasing values of [Ca²⁺]_m; this had been inferred from previous work (Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981), but not directly shown. Secondly, there is no unique relationship between PDH_A content and [Ca²⁺]_m. When expressed as a percentage of total pyruvate dehydrogenase, values of PDH_A are lower throughout the range of values of [Ca²⁺]_m when the substrate is succinate plus rotenone (Fig. 4a) than when it is glutamate plus malate (Fig. 4b). This is not surprising, as PDH_A content reflects the balance of kinase and phosphatase activities and the kinase is activated by NADH (Pettit *et al.*, 1975; Cooper *et al.*, 1975; Kerbey *et al.*, 1976), which is elevated in the presence of rotenone. When activation is considered, however, as opposed to absolute values of PDH_A, the $K_{0.5}$ for [Ca²⁺]_m is seen to be the same (250–320 nM) for

each substrate combination, within the accuracy of the method.

Another point to emerge from Fig. 4(d) is that added ATP tends to allow a greater degree of inactivation of pyruvate dehydrogenase. It is quite possible that the mitochondria become depleted of ATP plus ADP during the lengthy room-temperature incubation required for loading with indo-1, as a carboxyatractyloside-insensitive pathway for net flux of adenine nucleotides has been recently described (Austin & Aprille, 1984). Thus the presence of 1 mM-ATP during the 9 min equilibration protocol may serve to replete the matrix pool of adenine nucleotides.

Finally, Fig. 4(c) presents the results of experiments in which mitochondria were preincubated with succinate plus rotenone plus 1 mM-ATP (as for Fig. 4a), but in which 2 mM-MgCl₂ was added just prior to the Ca²⁺/EGTA buffer. This protocol allowed the repletion of the

Fig. 5. Steady-state values of PDH_A content as a function of $[\text{Ca}^{2+}]_m$ during state 3 respiration

Results were obtained from experiments of the type described in the legend to Fig. 4, in which 2–3 μmol of ADP was added after sampling for PDH_A content, and 40–60 s after adding ADP, more samples were taken (in duplicate) to provide the results reported here. The respiratory substrate was glutamate + malate ($\blacktriangle$) or succinate + rotenone ($\bullet$). For succinate oxidation, results are presented as means $\pm$ S.D.; five different preparations of mitochondria were used in compiling these results. For glutamate plus malate oxidation, experimental points were derived from experiments with three different mitochondrial preparations.

matrix adenine nucleotide pool, but also allowed the presence of free Mg^{2+} to modulate the activity of the Ca^{2+} -transport cycle. It is known that Mg^{2+} ions serve profoundly to inhibit the electrophoretic Ca^{2+} uniport of heart mitochondria (Sordahl, 1974; Crompton *et al.*, 1976a); Mg^{2+} also inhibits the $\text{Na}^+/\text{Ca}^{2+}$ antiport (Hansford & Castro, 1981; Crompton, 1985; Rizzuto *et al.*, 1987), although this inhibition may be relieved by ATP at constant free Mg^{2+} concentration (Hayat & Crompton, 1987). Comparison of Figs. 4(a) and (c) suggests that the $K_{0.5}$ of $[\text{Ca}^{2+}]_m$ for pyruvate dehydrogenase activation is not materially affected by Mg^{2+} . What is changed, as discussed below, is the value of $[\text{Ca}^{2+}]_o$ needed to achieve a particular value of $[\text{Ca}^{2+}]_m$.

The further addition of ADP in these studies, after state 4 PDH_A content had reached a steady value, allowed us to attempt to define the relation between PDH_A content and $[\text{Ca}^{2+}]_m$ in state 3 respiration (Chance & Williams, 1957). Although the PDH_A content is substantially higher at any given value of $[\text{Ca}^{2+}]_m$ in the presence of ADP (Fig. 5) than in its absence (Fig. 4), the sensitivity of the system to $[\text{Ca}^{2+}]_m$ is unchanged, with $K_{0.5}$ approx. 200 nM. We are aware that values of $[\text{Ca}^{2+}]_m$ calculated for mitochondria respiring in state 3 are less secure than those presented so far for state 4 respiration, as shape changes may obtrude. Thus addition of adenine nucleotides results in changes in fluorescence

emission at 400 nm that are not mirror images of those seen at 490 nm, and this is presumed to reflect changes in light scattering by the mitochondrial suspension.

The effect of ADP in increasing PDH_A content is considered to reflect a direct inhibition of the pyruvate dehydrogenase kinase, plus an indirect effect of lessening the activation of the kinase by NADH and acetyl-CoA, and has been reviewed by Hansford (1980), Reed (1981) and Wieland (1983). Both of these latter metabolites fall in concentration on transition from state 4 to state 3 in heart mitochondria (Hansford & Johnson, 1975).

Previous studies with mitochondrial extracts (Denton *et al.*, 1972) or with uncoupled cardiac mitochondria (Denton *et al.*, 1980) showed that the $K_{0.5}$ for Ca^{2+} activation of the pyruvate dehydrogenase phosphatase was approx. 1 μM . More recent studies of toluene-permeabilized mitochondria have defined a somewhat lower value for $K_{0.5}$ [0.4 μM - Ca^{2+} at a Mg^{2+} concentration of 0.18 mM (Midgley *et al.*, 1987)]. Our value of 0.2–0.3 μM - Ca^{2+} is clearly a little lower still. The slight discrepancy between our value and that of Midgley *et al.* (1987) may reflect a difference between their assumed values for matrix Mg^{2+} and that found in our indo-1-loaded mitochondria, as the sensitivity of the phosphatase to Ca^{2+} is very Mg^{2+} -dependent (Thomas *et al.*, 1986; Midgley *et al.*, 1987). Alternatively, the difference may be due to undefined effector compounds which are lost from the matrix upon toluene treatment.

When experiments of the type of Figs. 4 and 5 were repeated with mitochondria not loaded with indo-1, both the PDH_A content in the presence of extramitochondrial EGTA and that in the presence of 1.2 μM - Ca^{2+} were found to coincide with values achieved with indo-1-loaded mitochondria. Thus with 0.5 mM-EGTA and glutamate plus malate as respiratory substrates, PDH_A was 29% and 25% of total for loaded and unloaded mitochondria, respectively; at 1.2 μM - Ca^{2+} , the values were 58% and 52% for loaded and unloaded mitochondria, respectively. With 0.5 mM-EGTA and succinate plus rotenone as substrate, PDH_A was 6.6% and 2.3% of total for loaded and unloaded mitochondria, respectively; at 376 nM- Ca^{2+} , the values were 39% and 28% for loaded and unloaded mitochondria, respectively. Thus the measurement technique does not substantially perturb the quantity being measured.

The magnitude of the mitochondrial Ca^{2+} concentration gradient

Using the fluorescent probe technique it is possible to make a direct estimate of the magnitude of the Ca^{2+} concentration gradient across the mitochondrial inner membrane. In mitochondria which have been pre-treated with ATP and which are oxidizing either succinate, in the presence of rotenone, or glutamate plus malate, the gradient $[\text{Ca}^{2+}]_m/[\text{Ca}^{2+}]_o$ is approximately unity when the medium contains 10 mM- Na^+ (Fig. 6). The presence of Mg^{2+} in molar excess over ATP results in a gradient of $[\text{Ca}^{2+}]_m/[\text{Ca}^{2+}]_o$ of < 1 (Fig. 6); this is consistent with inhibition by Mg^{2+} of the electrophoretic uniporter (Crompton *et al.*, 1976a).

In general, values of $[\text{Ca}^{2+}]_o$ in excess of 1 μM lead to the saturation of the sequestered indo-1, and thus $[\text{Ca}^{2+}]_m$ cannot be studied. However, in the presence of free Mg^{2+} (and Na^+), this is not so (Fig. 6) and in this case a steep dependence of $[\text{Ca}^{2+}]_m$ is seen as a function of $[\text{Ca}^{2+}]_o$, at values in excess of 1 μM . This may be an

Fig. 6. Relation between $[Ca^{2+}]_m$ and $[Ca^{2+}]_o$.

Values of $[Ca^{2+}]_m$, determined in experiments of the type in Fig. 4, are plotted as a function of $[Ca^{2+}]_o$, as maintained with Ca^{2+} /EGTA (0–1200 nM) or Ca^{2+} /HEDTA ($Ca^{2+} > 1.2 \mu M$) buffers. Mitochondria were respiring in state 4 either with glutamate + malate or succinate + rotenone as substrate, in the presence (○) or absence (●) of Mg^{2+} . Dashed lines represent the linear regression analysis of the experimental points for each condition (correlation coefficient > 0.93). The slopes of these lines were calculated to be 0.82 ± 0.18 and 0.26 ± 0.03 (slope $\pm$ S.D.) in the absence and presence of Mg^{2+} , respectively. The slope of the relationship was significantly altered by the presence of Mg^{2+} ($P < 0.05$) as determined by a commercially available computer program (General Linear Models, SAS Institute Inc., Cary, NC, U.S.A.).

Fig. 7. Effect of spermine on $[Ca^{2+}]_m$ and PDH_A content

Indo-1-loaded mitochondria (2.1 mg of protein/ml) were incubated as described in Fig. 4(b), except that 1 mM- $MgSO_4$ was added to the basal medium and ATP was omitted. Steady-state values of $[Ca^{2+}]_m$ were achieved by addition of a Ca^{2+} /EGTA buffer to give a final $[Ca^{2+}]_o$ of $0.27 \mu M$. Where indicated, 0.5 mM-spermine (Spe) was added, or samples of 50 μl (Samp) were taken and assayed for pyruvate dehydrogenase as described in the Methods section.

example of the amplification predicted by Crompton (1985) on the grounds of the kinetic properties of the uniporter and antiporter.

The polyamine spermine results in an increase in the concentration gradient $[Ca^{2+}]_m/[Ca^{2+}]_o$ (slope = 0.39 and 0.49 for two different mitochondrial preparations and determined at 1 mM- Mg^{2+} and 10 mM- Na^+). Spermine has been shown to activate the mitochondrial Ca^{2+} uniport in the presence of Mg^{2+} (Nicchitta & Williamson, 1984; Lenzen *et al.*, 1986) and also to activate pyruvate dehydrogenase phosphatase directly (Damuni *et al.*, 1984; Thomas *et al.*, 1986). The increase in $[Ca^{2+}]_m$ seen on adding a saturating concentration of spermine (Lenzen *et al.*, 1986) to a suspension of mitochondria incubated at a $[Ca^{2+}]_o$ of 276 nM and in the presence of 1 mM- Mg^{2+} (Fig. 7) is clearly consistent with an increase in Ca^{2+} -uniport activity under these conditions. Concomitant with the increase in the concentration gradient $[Ca^{2+}]_m/[Ca^{2+}]_o$ is an increase in PDH_A content. In this case (Fig. 7), this appears as a stability of PDH_A content with time in the presence of spermine, as opposed to a progressive decline which occurs in the absence of the polyamine and ATP (see Fig. 4d). We have not obtained convincing evidence for an effect of spermine on PDH_A content when mitochondria are incubated in buffers containing EGTA and with minimal values of $[Ca^{2+}]_o$. Thus extramitochondrial polyamines do not seem to affect pyruvate dehydrogenase interconversion directly, which is consistent with an

assumption of limited permeation by a polycationic species.

Addition of ADP to respiring and indo-1-loaded heart mitochondria causes an increase of fluorescence emission measured at 400 nm (results not shown). Although this effect, which is inhibited by carboxyatractyloside, but not by oligomycin, and which thus appears to involve adenine nucleotide translocation, but not phosphorylation, would appear to involve an increase in the ratio $[Ca^{2+}]_m/[Ca^{2+}]_o$ (slope = 1.54 ± 0.56 , $P < 0.05$), there are grounds for caution. The lack of symmetrical fluorescent responses at 490 nm suggests that, instead, the adenine nucleotide may be initiating a conformational change which interferes with fluorescence emission via a change in light-scattering properties. For this reason, we have more confidence in the values of $[Ca^{2+}]_m$ determined for mitochondria respiring in state 4 (Fig. 4) and regard values derived from state 3 mitochondria (Fig. 5) as more subject to error.

Two previous approaches have indicated gradients of $[Ca^{2+}]_m/[Ca^{2+}]_o$ of slightly more than unity. The null-point technique (Hansford & Castro, 1982) was limited in precision by the small differential absorbance changes recorded and in accuracy by the potential error of gradients of pH or membrane potential persisting in the presence of nigericin (Coll *et al.*, 1982). The intention at that time was merely to establish whether $[Ca^{2+}]_m$ was in the micromolar range, allowing dehydrogenase modulation, or in the millimolar range (see, e.g. Nicholls, 1978). The other approach to this question (Denton

et al., 1980) relied upon comparison of the sensitivity to $[\text{Ca}^{2+}]_o$ of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase in respiring, coupled mitochondria and in uncoupled mitochondria. This is necessarily indirect, and could in principle be thwarted by the persistence of a small membrane potential, opposed by a ΔpH of equal magnitude, in the uncoupled state (Nicholls, 1974).

The present approach is direct, and does not seem to suffer from the problems with multiple compartments seen in studies with cells loaded with quin-2-AM or indo-1-AM (Davis *et al.*, 1987; Hansford & Lakatta, 1987; Moreno-Sánchez & Hansford, 1988). The absolute number assigned to $[\text{Ca}^{2+}]_m$ does, however, depend upon the validity of the value established for the K_d of indo-1- Ca^{2+} in the matrix. Even if this were 250 nM, as determined at pH 7.05 and 37 °C by Grynkiewicz *et al.* (1985), $[\text{Ca}^{2+}]_m$ would remain marginally lower than $[\text{Ca}^{2+}]_o$ in the presence of 1 mM free Mg^{2+} and 10 mM- Na^+ (Fig. 6). Though this result appears surprising in the light of the multitude of earlier experiments in which massive uptake of Ca^{2+} by mitochondria was studied (see e.g. Lehninger *et al.*, 1967), there is no reason why $[\text{Ca}^{2+}]_m$ has to be higher than $[\text{Ca}^{2+}]_o$ or $[\text{Ca}^{2+}]_c$. With the Ca^{2+} uniport driven by the electrochemical potential gradient of Ca^{2+} ions and the 2 Na^+ / Ca^{2+} antiport driven by ΔpH (indirectly, via Na^+/H^+ exchange; see Crompton, 1985), and with neither process achieving equilibrium, the final $[\text{Ca}^{2+}]_m/[\text{Ca}^{2+}]_o$ gradient is a function of the kinetic parameters of the two carriers and is hard to predict accurately, despite excellent progress in that direction described in an earlier review by Crompton (1985).

While this work was in preparation a paper appeared by Lukács *et al.* (1988) establishing a relation between $[\text{Ca}^{2+}]_m$ of heart mitochondria, as measured with fura-2, and 2-oxoglutarate dehydrogenase activity, as measured by the reduction of mitochondrial NAD(P)^+ . These authors determined that the $K_{0.5}$ for activation was 800 nM, making the assumption of linearity between enzyme activity and the fractional reduction of mitochondrial NAD(P)^+ , and assuming a K_d for Ca^{2+} -fura-2 of 135 nM.

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