Respiration-Dependent Calcium Ion Uptake by Two Preparations of Cardiac Mitochondria

EFFECTS OF PALMITOYL-COENZYME A AND PALMITOYLCARNITINE ON CALCIUM ION CYCLING AND NICOTINAMIDE NUCLEOTIDE REDUCTION STATE

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Ca^{2+} uptake and the effect of the uptake inhibitors palmitoyl-CoA and palmitoylcarnitine were examined in two preparations of dog cardiac mitochondria. Mitochondria prepared by using the Nagarse technique was 2.5-fold more active in respiration-dependent Ca^{2+} uptake than were mitochondria isolated by using the Polytron procedure. Palmitoyl-CoA and palmitoylcarnitine inhibited Ca^{2+} uptake in both preparations uncompetitively, with $K_{i,\text{app}}$ 0.4 and $20\text{ }\mu\text{M}$. Ca^{2+} -uptake rates were related to, or influenced by, the concentration of mitochondrial reduced nicotinamide nucleotides, with uptake slowing as this concentration decreased. When most of the nicotinamide nucleotides was oxidized, Ca^{2+} release and respiratory stimulation were observed. In the presence of Ruthenium Red and palmitoyl-CoA, oxidation of nicotinamide nucleotides was abolished and the time to Ca^{2+} release was shortened corresponding to the time of onset of nicotinamide nucleotide oxidation in the absence of Ruthenium Red. The results suggest that NAD(P)H oxidation in the presence of rotenone was a consequence of Ca^{2+} re-uptake and that net Ca^{2+} release could be observed as reduced nicotinamide nucleotide concentrations declined. Although nicotinamide nucleotide oxidation occurred in the presence of rotenone, it was not linked in an apparent manner to acyl-group metabolism (palmitoylcarnitine was less effective than palmitoyl-CoA). Therefore either a by-pass of the rotenone block or a direct interaction of NAD(P)H with the Ca^{2+} -uptake process was possible. Loss of NADH occurred before respiratory stimulation, and this loss may relate to decreased coupling efficiency at sites 2 and 3 of the respiratory chain, as suggested by others [Bhuvaneswaran & Wadkins (1978) *Biochem. Biophys. Res. Commun.* **82**, 648–654].

The ability of a variety of mitochondrial preparations to transport and retain Ca^{2+} has been intensively studied (Chance, 1965; Lehninger, 1970). Factors that modulate the rates of Ca^{2+} influx, e.g. Mg^{2+} (Scarpa & Graziotti, 1973; Hutson *et al.*, 1976), have been implicated as a physiological control over mitochondrial Ca^{2+} loading. However, information about mechanisms that operate to discharge mitochondrial Ca^{2+} has emerged more recently. A variety of naturally occurring agents such as phosphoenolpyruvate (Chudapongse & Haugaard, 1973), palmitoyl-CoA (Asimakis & Sordahl, 1977), lysophosphatidylcholine (Harris, 1977) and Na^+ (Crompton *et al.*, 1977) have been shown to effect Ca^{2+} release from mitochondria. The redox state of mitochondrial nicotinamide nucleotides has been proposed to control Ca^{2+} move-

ments, with a more oxidized state promoting Ca^{2+} efflux (Lehninger *et al.*, 1978). Cycles of Ca^{2+} uptake and release from mitochondria must occur in a readily reversible manner if the Ca^{2+} -influx and -efflux pathways are to play a role in the control of either cytosolic or matrix Ca^{2+} -affected metabolic pathways.

We have demonstrated that the non-penetrant metabolite palmitoyl-CoA causes a net oxidation of mitochondrial nicotinamide nucleotides in the presence of succinate and rotenone, with a concomitant efflux of accumulated Ca^{2+} (Wolkowicz & McMillin-Wood, 1979). The inhibitory effects of palmitoyl-CoA on mitochondrial Ca^{2+} uptake and retention are overcome with carnitine in the uptake medium and are accentuated in the presence of carnitine and malonyl-CoA. Thus an interplay of

cellular lipid metabolites at the mitochondrial membrane may have an important role in Ca^{2+} movements, apart from their non-specific surfactant properties (Harris, 1977).

It is known that different procedures for isolation of mitochondria profoundly influence the Ca^{2+} -uptake properties of these organelles. Early studies suggest that use of the proteolytic enzyme Nagarse interferes with the ability of heart mitochondria to transport Ca^{2+} (Scarpa & Graziotti, 1973). A more recent publication about the use of Nagarse describes an isolation procedure yielding mitochondria with improved respiratory activity as well as respiration-coupled Ca^{2+} accumulation (Vercesi *et al.*, 1978). This work is in agreement with a previous report where heart mitochondria isolated by using Nagarse were 1.5-fold more active than mitochondria obtained by using the Polytron procedure (Palmer *et al.*, 1977). In the present study we have examined Ca^{2+} uptake in these two different preparations of cardiac mitochondria (Palmer *et al.*, 1977) as well as the effect of the lipid metabolites palmitoyl-CoA and palmitoylcarnitine on the Ca^{2+} -influx and -efflux cycles.

Experimental

Isolation of heart mitochondria

Dog heart mitochondria were isolated from 5 g of left ventricle by the procedure of Palmer *et al.* (1977). The tissue was finely minced in cold isolation medium A [220 mM-mannitol/70 mM-sucrose/5 mM-Mops (4-morpholinepropanesulphonic acid) buffer, pH 7.4] and adjusted to a final concentration of 10 g of tissue/100 ml of medium A containing 2 mM-EGTA and 0.2% bovine serum albumin. The minced tissue was homogenized with a Polytron tissue processor (Brinkman Instruments) for 25 s at a rheostat setting of 10. The homogenate was centrifuged at 500 g in a Sorvall RC-2B centrifuge (SS-34 rotor) for 10 min. The low-speed pellet was rehomogenized after resuspension to the original volume and centrifuged at 500 g. The two supernatants were combined and the mitochondrial pellet was obtained by centrifugation at 3000 g for 10 min. The Polytron-prepared mitochondria were washed twice in medium A in the absence of EGTA and suspended in this medium at a protein concentration of 20–25 mg/ml for Ca^{2+} -uptake studies.

The washed pellet from the 500 g spin from the Polytron homogenization was resuspended in isolation medium B (100 mM-KCl/50 mM-Mops buffer, pH 7.4, containing 2 mM-EGTA and 0.2% bovine serum albumin). Nagarse was added, to a final concentration of 5 mg/g wet wt. of tissue, directly in the Potter-Elvehjem homogenizing vessel. After one pass with the pestle, the homogenate was immediately diluted 2-fold with medium B and centrifuged

at 5000 g for 5 min. The pellet was resuspended in the original volume of medium B and sedimented at 500 g for 10 min. The low-speed pellet was washed twice with medium B and the supernatants from the 500 g spins were combined and re-centrifuged at 3000 g for 10 min. The pellet was washed twice in medium A in the absence of EGTA and resuspended in this medium at a final concentration of 25 mg/ml for Ca^{2+} -uptake studies.

Protein was determined by the biuret procedure (Layne, 1957). Respiratory parameters with glutamate + malate or succinate + rotenone were determined with a Yellow Springs Instruments model 53 oxygen monitor (Yellow Springs, OH, U.S.A.) at 30°C.

Other methods

Ca^{2+} uptake and release were monitored by using an Aminco DW-2 UV/VIS dual-wavelength spectrophotometer. Mitochondria (2.2–2.8 mg) were incubated at 30°C in a medium containing 100 μM -tetramethylmurexide (tetramethylpurpurate; Calbiochem, San Diego, CA, U.S.A.), 1.67 mM-potassium phosphate buffer, pH 7.2, 1.67 mM-succinate, 10 μg of rotenone and 73 mM-KCl in 0.25 M-sucrose/10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.2, to a final volume of 3 ml. Additions to the basic uptake medium are described in the Figure legends. Ca^{2+} uptake was initiated by injection of Ca^{2+} into the cuvette. The wavelength pair 518–542 nm was employed to measure absorbance changes in the Ca^{2+} -dye complex. In one experiment where 13 μM total Ca^{2+} was injected, 20 μM purified Arsenazo III was present in the uptake medium in lieu of tetramethylmurexide. In this case the wavelength pair 685–675 nm was employed.

The oxidation state of mitochondrial nicotinamide nucleotides was followed under the same incubation conditions as described for Ca^{2+} uptake and release except that the metallochromic dye was omitted. The wavelength pair 340–370 nm was employed to follow redox changes in nicotinamide nucleotides.

Changes in pH were recorded at 30°C, in 4.5 ml of Ca^{2+} -uptake medium, containing 1.67 mM-potassium phosphate buffer, pH 7.2, 1.67 mM-succinate, 20 μg of rotenone 73 mM-KCl in 0.25 M-sucrose/1 mM-Hepes buffer, pH 7.2, at a protein concentration of 0.9 mg/ml, by using a Radiometer type PH M22s pH-meter (Radiometer, Copenhagen, Denmark) attached to a Sargent-Welch model SRLG recorder with expanded scale.

Materials

Palmitoyl-CoA and palmitoylcarnitine were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Ruthenium Red, obtained from Sigma

Chemical Co. (St. Louis, MO, U.S.A.), was recrystallized before use (Fletcher *et al.*, 1961). Arsenazo III (Sigma Chemical Co.) was purified as described by Kendrick (1976). Nagarse was purchased from the Enzyme Development Corp., New York, NY, U.S.A.

Results

Comparison of two mitochondrial fractions

Dog heart mitochondria prepared by the Polytron procedure demonstrated State-3 (Chance & Williams, 1955) respiratory rates of 265 ± 12.3 ng-atoms of oxygen/min per mg of protein with glutamate + malate and 278 ± 13 ng-atoms/min per mg in the presence of succinate + rotenone. The State-3 rates of oxygen consumption with the Nagarse-prepared mitochondria were 367 ± 15.4 and 340 ± 15.9 ng-atoms/min per mg for glutamate + malate and succinate + rotenone respectively. In the presence of $20 \mu\text{M}$ -palmitoylcarnitine State-3 rates for the Polytron- and Nagarse-prepared mitochondria were 187 and 280 ng-atoms/min per mg respectively. With all substrates there were no significant differences in State-4 rates of respiration after an ADP cycle between the Polytron- and Nagarse-prepared mitochondria, i.e. 61.3 ± 4.2 and 60.1 ± 4.6 ng-atoms/min per mg respectively. In addition, electron-microscopic examination of the mitochondrial pellets revealed little or no contamination with other membranous fragments and the mitochondria were intact. These observations are in agreement with the original report on the integrity and respiratory differences of mitochondria isolated by this procedure (Palmer *et al.*, 1977).

Initial rates of Ca^{2+} uptake were followed at various Ca^{2+} concentrations. The Polytron-prepared mitochondria demonstrated significantly lower rates of uptake, and saturation occurred at lower Ca^{2+} concentrations than with the Nagarse-prepared mitochondria (Fig. 1a). The K_m and $V_{\max}$ were about 3-fold higher for the Nagarse-prepared mitochondria, i.e. K_m $32 \mu\text{M}$ and $V_{\max}$ 410 nmol/min per mg for Polytron-prepared mitochondria, compared with K_m $91 \mu\text{M}$ and $V_{\max}$ 1176 nmol/min per mg for Nagarse-prepared mitochondria. These results support the contention of others (Vercesi *et al.*, 1978) that the Nagarse procedure yields a heart mitochondrial preparation that is superior with respect to both respiratory capabilities and the capacity for Ca^{2+} accumulation.

Effects of palmitoyl-CoA and palmitoylcarnitine on Ca^{2+} uptake and Ca^{2+} efflux

The membrane-impermeant lipid palmitoyl-CoA has been shown to cause inhibition of Ca^{2+} uptake by mitochondria and premature aerobic release of previously accumulated Ca^{2+} . The ability of carnitine to lessen the inhibitory effect of palmitoyl-CoA

Fig. 1. Respiration-supported Ca^{2+} uptake by two different preparations of dog heart mitochondria

(a) Heart mitochondria (2.5 mg) were preincubated for 3 min in the presence of 73 mM-KCl, 1.67 mM-succinate, 1.67 mM-potassium phosphate buffer, pH 7.2, 50 μg of rotenone and 100 μM -tetramethylmurexide in a final volume of 3 ml in 0.25 M-sucrose/10 mM-Hepes buffer, pH 7.2. Ca^{2+} uptake was initiated by Ca^{2+} injection and absorbance changes were measured at 518–542 nm. ●, Nagarse-prepared mitochondria, $n = 2$ –7 separate determinations (S.E.M. calculated where $n > 2$); ○, Polytron-isolated mitochondria, $n = 2$ –6 separate determinations (S.E.M. calculated where $n > 2$). (b) Double-reciprocal plot of Ca^{2+} -uptake rates from (a). ●, Nagarse-prepared mitochondria; $V_{\max}$ 1176 nmol/min per mg; K_m 91 μM ; ○, Polytron-isolated mitochondria; $V_{\max}$ 410 nmol/min per mg; K_m 32 μM .

on Ca^{2+} uptake is believed to be a consequence of palmitoylcarnitine formation and transfer to the mitochondrial matrix (Asimakis & Sordahl, 1977; Harris, 1977). On the other hand, palmitoylcarnitine, as an amphipathic metabolite, should have a significant membrane-perturbing effect (Wood *et al.*,

Fig. 2. Preincubation of dog heart mitochondria with palmitoylcarnitine: inhibition of Ca^{2+} -uptake rates

Dog heart mitochondria (2.5 mg) were preincubated in Ca^{2+} -uptake medium described in Fig. 1 legend, with or without various concentrations of palmitoylcarnitine. Ca^{2+} uptake was initiated by injection of $67 \mu\text{M}$ - Ca^{2+} . ●, Nagarse-prepared mitochondria, $n = 3-7$ separate determinations ($\pm \text{s.e.m.}$); ○, Polytron-prepared mitochondria, $n = 3-6$ separate determinations ($\pm \text{s.e.m.}$)

1977; Pitts *et al.*, 1978). Differing permeability characteristics and/or an increased fragility of the Polytron-prepared mitochondrial membranes has been proposed to explain the respiratory differences between the two mitochondrial preparations (Matlib *et al.*, 1978). Therefore it was decided to ascertain if any possible differences in the susceptibility of Ca^{2+} uptake by the two mitochondrial preparations to palmitoylcarnitine addition exist. Preincubation of mitochondria with various concentrations of palmitoylcarnitine resulted in a concentration-dependent decrease in Ca^{2+} uptake (Fig. 2). The slopes of the lines suggest that increasing concentrations of palmitoylcarnitine affect Ca^{2+} uptake by both mitochondrial preparations similarly. Further experiments were performed to determine the kinetics of inhibition of Ca^{2+} accumulation by low concentrations of palmitoyl-CoA and palmitoylcarnitine. Analysis of Ca^{2+} uptake in the presence of $2 \mu\text{M}$ -palmitoyl-CoA and/or $10 \mu\text{M}$ -palmitoylcarnitine is shown in Fig. 3. Hanes-Woolf plots (Segal, 1975) of the data demonstrate that the inhibition of Ca^{2+} uptake by either palmitoyl-CoA or palmitoylcarnitine

Fig. 3. Hanes-Woolf plots: effects of palmitoyl-CoA and/or palmitoylcarnitine on kinetics of Ca^{2+} uptake in dog heart mitochondria

Mitochondria were preincubated in the absence and in the presence of palmitoyl-CoA and/or palmitoylcarnitine. Respiration-supported Ca^{2+} uptake was initiated by addition of $67 \mu\text{M}$ - Ca^{2+} , where v is expressed as nmol/min per mg. Points represent the average uptake rates of three to eight separate experiments. The slopes of the lines were determined by regression analysis, with correlation coefficients >0.97 in all cases. (a) Polytron-prepared mitochondria: ●, control Ca^{2+} uptake; Δ, Ca^{2+} uptake in the presence of $20 \mu\text{M}$ -palmitoylcarnitine; ○, Ca^{2+} uptake in the presence of $2 \mu\text{M}$ -palmitoyl-CoA. (b) Nagarse-prepared mitochondria: ●, control Ca^{2+} uptake; Δ, Ca^{2+} uptake in the presence of $20 \mu\text{M}$ -palmitoylcarnitine; ○, Ca^{2+} uptake in the presence of $2 \mu\text{M}$ -palmitoyl-CoA; □, Ca^{2+} uptake in the presence of $10 \mu\text{M}$ -palmitoylcarnitine and $2 \mu\text{M}$ -palmitoyl-CoA.

was uncompetitive with respect to the control. The effect was similar for both mitochondrial preparations and was accentuated by preincubation with both inhibitors (Fig. 3b). The $K_{i,app}$ for palmitoyl-CoA was in the range 0.4–0.7 μM for both mito-

chondrial preparations (Table 1: see also Fig. 4). The effect of palmitoylcarnitine on Ca²⁺ uptake required higher concentrations, with $K_{i,app}$ values of 20 and 30 μM for Nagarse- and Polytron-prepared mitochondria respectively. The combination of 2 μM -palmitoyl-CoA and 10 μM -palmitoylcarnitine affected the kinetic constants for Ca²⁺ uptake in a manner predicted from the derived K_i values in Table 1. Since palmitoyl-CoA and palmitoylcarnitine affected both mitochondrial preparations similarly, the remainder of the studies were carried out by using the Nagarse isolation procedure. The latter procedure is desirable experimentally owing to higher mitochondrial yields and greater Ca²⁺-uptake capacities.

When palmitoyl-CoA concentration was varied over the range 1–4 μM , a re-plot of K_m^{-1} showed a linear response as a function of palmitoyl-CoA concentrations (Fig. 4, inset). Thus the effect of palmitoyl-CoA on mitochondrial Ca²⁺ uptake appeared to represent pure uncompetitive-inhibition kinetics, rather than a mixed hyperbolic response. In addition, the results appear to contraindicate deleterious membrane effects. The K_i for palmitoyl-CoA determined by the re-plot in this Figure is 0.4 μM .

Since palmitoylcarnitine decreases Ca²⁺ uptake in a manner kinetically similar to that of palmitoyl-CoA, albeit at higher concentrations, and since palmitoyl-CoA is known to cause Ca²⁺ efflux, the ability of palmitoylcarnitine to release Ca²⁺ was investigated. With Arsenazo III as the indicator dye, mitochondria were allowed to accumulate a small amount of Ca²⁺ (17 nmol/mg). After uptake, various concentrations of palmitoylcarnitine were injected (Fig. 5). A rapid efflux of Ca²⁺ was observed, with the rates of efflux dependent on the concentration of palmitoylcarnitine injected. However, the release of Ca²⁺ stimulated by palmitoylcarnitine differed from

Fig. 4 Hanes–Woolf plot: effect of palmitoyl-CoA concentration on the kinetics of Ca²⁺ uptake in dog heart Nagarse-prepared mitochondria

Nagarse-prepared mitochondria were preincubated for 3 min in the absence (●) and in the presence of various concentrations of palmitoyl-CoA (○, 1 μM ; □, 2 μM ; △, 3 μM ; ■, 4 μM) under Ca²⁺-uptake conditions (see Fig. 1 legend). Ca²⁺ uptake was initiated by injection of the indicated Ca²⁺ concentration into the cuvette. Inset: replot of data from Figure, where abscissa intercept = K_i and the slope = $(K_i \cdot K_m)^{-1}$.

Table 1. Effect of palmitoyl-CoA and/or palmitoylcarnitine on kinetic parameters of Ca²⁺ uptake in dog heart mitochondria

Conditions ...		Control	+ 2 μM - Palmitoyl-CoA	+ 20 μM - Palmitoylcarnitine	+ 2 μM -Palmitoyl-CoA + 10 μM -palmitoylcarnitine
Nagarse-prepared mitochondria					
K_m (μM)		114.6	30.6	54	19 (26)
V_{max} (nmol/min per mg)		1302	423	690	332 (337)
K_i (μM)		—	0.4–0.7	20	—
Polytron-prepared mitochondria					
K_m (μM)		46	6.7	29	— (6.3)
V_{max} (nmol/min per mg)		470	141	246	— (101)
K_i (μM)		—	0.6	30	—

Fig. 5. Effect of palmitoylcarnitine on Ca^{2+} release from dog heart mitochondria

Nagarse-prepared mitochondria were suspended in a medium containing 73 mM-KCl, 1.67 mM-succinate, 50 μg of rotenone, 1.67 mM-potassium phosphate buffer, pH 7.2, and 20 μM -Arsenazo III in a final total of 3 ml of 0.25 μM -sucrose/10 mM-Hepes buffer, pH 7.2. CaCl_2 (13 μM) was injected to allow Ca^{2+} uptake. Ca^{2+} movements were followed at 685–675 nm. At the completion of uptake, a base-line was established for the assay period. In other experiments, various concentrations of palmitoylcarnitine were added after completion of Ca^{2+} uptake.

palmitoyl-CoA-induced release in three ways: (1) release was immediate at all concentrations of palmitoylcarnitine used whereas with palmitoyl-CoA a concentration-dependent lag period was observed before release; (2) only a fraction of the total Ca^{2+} accumulated was released, whereas palmitoyl-CoA caused release of most of the accumulated Ca^{2+} ; (3) an occasional slow re-uptake of the released Ca^{2+} could occur in some preparations of Nagarse-prepared mitochondria, whereas addition of ATP or carnitine was necessary to reverse the palmitoyl-CoA-induced release (Harris, 1977).

To eliminate the possibility that the release and re-uptake observed were a consequence of either the low Ca^{2+} concentration used in the present study or of the high affinity of Arsenazo III for Ca^{2+} , the experiment was repeated with tetramethylmurexide (Fig. 6). Injection of 13.3 μM -palmitoylcarnitine after mitochondrial Ca^{2+} accumulation resulted in a rapid release phase with a subsequent low rate of re-uptake; when re-uptake was completed, addition of 2.6 μM -palmitoyl-CoA produced a rapid release of accumulated Ca^{2+} and no re-uptake was observed (Fig. 6a).

Since Ca^{2+} efflux from mitochondria appeared to be associated with a more oxidized state of mitochondrial nicotinamide nucleotides, with uptake dependent on the lower concentration of NADH, the effects of palmitoylcarnitine and palmitoyl-CoA on 370–340 nm absorbance changes were followed. After mitochondria had accumulated Ca^{2+} , injection of palmitoylcarnitine produced a rapid oxidation of nicotinamide nucleotides (Fig. 6b), which re-

Fig. 6. Effects of palmitoylcarnitine and palmitoyl-CoA on mitochondrial Ca^{2+} movements, nicotinamide nucleotide oxidation and proton movements

Nagarse-prepared dog heart mitochondria were suspended in Ca^{2+} -uptake medium (see Fig. 1 legend). Each trace is representative of five separate runs and measurements made as follows. (a) Mitochondria (2.8 mg) were preincubated for 2 min in Ca^{2+} -uptake medium containing 100 μM -tetramethylmurexide and 0.25 M-sucrose/10 mM-Hepes buffer, pH 7.2, final volume 3 ml. Ca^{2+} uptake was initiated by injection of CaCl_2 (final concn. 40 μM). At the completion of uptake, 13.3 μM -palmitoylcarnitine was injected and Ca^{2+} release and re-uptake were followed. At the completion of the reuptake cycle, 2.6 μM -palmitoyl-CoA was added to initiate Ca^{2+} release. Ca^{2+} movements were followed at 518–542 nm. (b) Conditions for Ca^{2+} uptake were identical with those described for (a), except that tetramethylmurexide was omitted from the uptake medium. The oxidation of mitochondrial nicotinamide nucleotides was monitored after additions of Ca^{2+} , palmitoylcarnitine and palmitoyl-CoA. Nicotinamide nucleotide oxidation was monitored at 370–340 nm. (c) Conditions for Ca^{2+} uptake were identical with those described for (b), except that the Hepes concentration was lowered to 1 mM to follow proton movements and a final volume of 4.5 ml was used. Mitochondrial protein was adjusted to a concentration of 0.93 mg/ml, as for (a) and (b). Changes in pH after addition of Ca^{2+} , palmitoylcarnitine and palmitoyl-CoA were recorded at 30°C, by using a Radiometer type PHM 22s pH-meter attached to a Sargent-Welch model SRLG recorder with expanded scale.

mained oxidized even as re-uptake of Ca^{2+} proceeded (cf. Fig. 6a). Subsequent addition of palmitoyl-CoA resulted in a further oxidation concomitant with the release of most of the Ca^{2+} from the mitochondria. Changes in pH during Ca^{2+} uptake, palmitoylcarnitine-induced release and re-up-

take and palmitoyl-CoA-mediated Ca^{2+} release are shown in Fig. 6(c). With Ca^{2+} uptake, a rapid acidification of the media occurred. Addition of palmitoylcarnitine produced re-alkalinization concomitant with Ca^{2+} release, and a subsequent slow re-acidification as Ca^{2+} re-uptake proceeded. Palmitoyl-CoA-mediated Ca^{2+} efflux was accompanied by an alkalization of the assay medium.

Fig. 7. Mitochondrial Ca^{2+} uptake and release and nicotinamide nucleotide oxidation in the presence and absence of palmitoyl-CoA

(a) Nagarse-prepared dog heart mitochondria (2.8 mg) were preincubated for 3 min in a medium containing 100 μM -tetramethylmurexide, 1.67 mM-succinate, 1.67 mM-potassium phosphate buffer, pH 7.2, 50 μg of rotenone and 73 mM-KCl in 0.25 M-sucrose/10 mM-Hepes buffer, pH 7.2, final volume 3 ml. —, Control trace; ----, trace in the presence of 4 μM -palmitoyl-CoA; in both cases 53 μM - CaCl_2 was injected and Ca^{2+} uptake and release were followed at 518–542 nm. (b) Nagarse-prepared dog heart mitochondria (2.8 mg) were preincubated for 3 min in a medium as for (a) but without tetramethylmurexide. —, Control trace; ----, trace in the presence of 4 μM -palmitoyl-CoA; in both cases 53 μM - CaCl_2 was injected and nicotinamide nucleotide oxidation was followed at 370–340 nm. Traces in (a) and (b) are representative of four separate runs each. (c) Nagarse-prepared dog heart mitochondria (2.8 mg) were preincubated for 3 min in a medium as for (b). —, Control oxygen consumption; ----, oxygen consumption in the presence of 4 μM -palmitoyl-CoA; in both cases 53 μM - CaCl_2 was injected and the rate of oxygen consumption was followed.

Since the data in Fig. 6 suggest that Ca^{2+} uptake (re-uptake, Fig. 6a) could proceed, albeit slowly, under conditions in which there is major loss in concentration of reduced nicotinamide nucleotide after palmitoylcarnitine injection, the effects of preincubation of mitochondria in the presence and absence of palmitoyl-CoA on Ca^{2+} -uptake rates and nicotinamide nucleotide oxidation were investigated. In the absence of palmitoyl-CoA, Ca^{2+} uptake by mitochondria was rapid and the Ca^{2+} accumulated was retained during the course of the assay (Fig. 7a). Preincubation with palmitoyl-CoA showed a lower rate of Ca^{2+} uptake, with subsequent Ca^{2+} efflux occurring 1 min after peak Ca^{2+} accumulation. In the control, a slight oxidation of nicotinamide nucleotides was observed on Ca^{2+} addition, with subsequent return to base-line absorbance. However, a low rate of oxidation took place towards the end of the assay period (Fig. 7b). In contrast, when mitochondria were preincubated with palmitoyl-CoA, Ca^{2+} injection was accompanied by a significant oxidation of nicotinamide nucleotides, with a further oxidation preceding and becoming maximal as Ca^{2+} release ensued. The apparent oxidation of NADH did not appear to be due to nicotinamide nucleotide efflux from the mitochondria, as no externally reactive NAD^+ could be detected in these experiments. When mitochondrial respiration was monitored under the same conditions, little or no effect on oxygen consumption could be seen under control uptake conditions (Fig. 7c). In the presence of palmitoyl-CoA the respiration rate increased markedly at a time period when peak Ca^{2+} efflux and nicotinamide nucleotide oxidation had occurred.

Effects of Ruthenium Red

The observation that NADH oxidation preceded Ca^{2+} release in the presence of palmitoyl-CoA (and that Ca^{2+} was retained in control mitochondria as slow NADH oxidation proceeded) suggested that rapid re-uptake of released Ca^{2+} resulted in the lack of observed net Ca^{2+} efflux. This process was associated with and/or dependent on nicotinamide nucleotide reduction state. To examine this possibility, the effect of Ruthenium Red, a potent inhibitor of Ca^{2+} uptake, on net Ca^{2+} release from mitochondria and on nicotinamide nucleotide oxidation was followed. Ruthenium Red was injected into the cuvette after completion of Ca^{2+} uptake and before addition of palmitoyl-CoA or palmitoylcarnitine (Fig. 8). Ruthenium Red added before Ca^{2+} completely abolished uptake, and added after Ca^{2+} had no effect on Ca^{2+} retention. Palmitoyl-CoA (2.6 μM) added alone produced rapid Ca^{2+} efflux and nicotinamide nucleotide oxidation after a concentration-dependent lag phase. When Ruthenium Red was added before palmitoyl-CoA, Ca^{2+} efflux was observed, but the release began before that seen for

Fig. 8. Effects of Ruthenium Red on Ca^{2+} efflux and nicotinamide nucleotide oxidation in the presence and in the absence of palmitoyl-CoA and palmitoylcarnitine

Nagarse-prepared dog heart mitochondria were suspended in Ca^{2+} -uptake medium. After a 2 min preincubation period, $53 \mu\text{M}$ - CaCl_2 was injected. After Ca^{2+} was accumulated, the experimental protocol was varied as follows. (a) Ca^{2+} uptake and release in the presence of $100 \mu\text{M}$ -tetramethylmurexide [$\Delta(A_{518}-A_{542})$]. —, Control $\pm 0.16 \mu\text{M}$ -Ruthenium Red (RR); ----, $2.6 \mu\text{M}$ -palmitoyl-CoA (P-CoA) added 2 min after Ca^{2+} accumulation; ····, $0.16 \mu\text{M}$ -Ruthenium Red added, followed by $2.6 \mu\text{M}$ -palmitoyl-CoA. (b) Nicotinamide nucleotide reduction state after Ca^{2+} uptake [$\Delta(A_{370}-A_{340})$]. —, Control $\pm 0.16 \mu\text{M}$ -Ruthenium Red (RR); ----, $2.6 \mu\text{M}$ -palmitoyl-CoA (PCoA) added 2 min after Ca^{2+} accumulation; ····, $0.16 \mu\text{M}$ -Ruthenium Red added, followed by $2.6 \mu\text{M}$ -palmitoyl-CoA. (c) Ca^{2+} uptake and release in the presence of $100 \mu\text{M}$ -tetramethylmurexide [$\Delta(A_{518}-A_{542})$]. Symbols as in (a) except that $13 \mu\text{M}$ -palmitoylcarnitine was added instead of $2.6 \mu\text{M}$ -palmitoyl-CoA. (d) Nicotinamide nucleotide reduction state after Ca^{2+} uptake [$\Delta(A_{370}-A_{340})$]. Symbols as in (b) except that $13 \mu\text{M}$ -palmitoylcarnitine was added instead of $2.6 \mu\text{M}$ -palmitoyl-CoA.

palmitoyl-CoA alone and the rate and extent of Ca^{2+} release appeared to be decreased (Fig. 8a). Interestingly, the oxidation of mitochondrial nicotinamide nucleotides after palmitoyl-CoA addition at this concentration was completely abolished by Ruthenium Red (Fig. 8b). The effect of Ruthenium Red on palmitoylcarnitine-induced release is shown in Figs. 8(c) and 8(d). In contrast with the decrease in efflux caused by Ruthenium Red observed in the presence of palmitoyl-CoA, an enhanced rate and extent of Ca^{2+} release were observed in the presence of the inhibitor. Again, Ruthenium Red, added before palmitoylcarnitine, abolished the slow oxidation of nicotinamide nucleotide seen in the presence of Ca^{2+} and palmitoylcarnitine alone. Polarographic traces (results not shown) demonstrated that Ruthenium Red added before palmitoyl-CoA and palmitoylcarnitine but after completion of Ca^{2+} uptake completely abolished the respiratory increases observed in the absence of Ruthenium Red.

Discussion

Two different preparations of heart mitochondria made by using the Polytron and Nagarse procedures as described by Palmer *et al.* (1977) were employed to examine respiration-supported Ca^{2+} uptake. The two mitochondrial preparations have been attributed to different subcellular localizations, with

the Polytron procedure releasing mitochondria localized at the periphery of the cell, i.e. below the sarcolemma, and the Nagarse preparation specifically isolating a mitochondrial fraction associated with the myofibrils (Palmer *et al.*, 1977). Previous reports by Hulsmann *et al.* (1968) indicated that skeletal muscle contains two types of mitochondria, and their findings were subsequently extended to the heart (Hulsmann, 1970). Our mitochondrial preparations from dog heart also demonstrate structural and functional integrity, with respiratory parameters for each preparation in the range of those reported for rat (Palmer *et al.*, 1977). In agreement with the respiratory data, the Nagarse-prepared mitochondria were almost 3-fold as active in accumulation of Ca^{2+} . Although the K_m for Ca^{2+} is from 3-fold (Polytron-prepared mitochondria) to 10-fold (Nagarse-prepared mitochondria) higher than values for heart mitochondria reported by using other methods of measurement (Crompton *et al.*, 1976; Noack & Heinen, 1977), the K_m values of the former preparation are similar to those reported by using the murexide technique (Scarpa & Graziotti, 1973). As discussed previously (Jacobus *et al.*, 1975), measurement of free Ca^{2+} in experiments utilizing metallochromic dyes is complicated by the ability of reagents to complex Ca^{2+} , and such discrepancies may be invoked to explain the K_m differences. However, since Ca^{2+} -uptake rates and nico-

tinamide nucleotide reduction states of the two preparations respond similarly to palmitoyl-CoA and palmitoylcarnitine, the use of the Nagarse procedure is desirable with respect to total protein yield and activity.

Palmitoyl-CoA has been shown previously to effect Ca^{2+} release from Ca^{2+} -loaded mitochondria (Asimakis & Sordahl, 1977) and to decrease the rate of Ca^{2+} uptake in heart mitochondria (Harris, 1977). However, the kinetic mechanisms of these effects were not investigated. The present study suggests that these two effects of palmitoyl-CoA (inhibition of Ca^{2+} uptake and Ca^{2+} release) are separate and distinct. The uncompetitive nature of the inhibition of uptake by palmitoyl-CoA implies that this compound interacts specifically with a carrier- Ca^{2+} complex, presumably at the outer aspect of the inner mitochondrial membrane. The exact nature of the kinetics indicates that the effect is a specific interaction and not a general membrane effect. An increase in affinity of the mitochondria for Ca^{2+} by 73–85% at $2\mu\text{M}$ -palmitoyl-CoA suggests that at low intracellular Ca^{2+} concentration the effect on intracellular palmitoyl-CoA on the K_m for Ca^{2+} would offset any inhibitory effect on the V_{max} of Ca^{2+} uptake. It is at least reasonable to expect that under certain pathological conditions, such as myocardial ischaemia, where both long-chain acyl-CoA and acylcarnitines accumulate (Idell-Wenger *et al.*, 1978), mitochondria may respond to Ca^{2+} in a manner described in the present and other studies (Asimakis & Sordahl, 1977; Harris, 1977). Interestingly, mitochondria isolated from ischaemic dog myocardium accumulate Ca^{2+} at lower rates, and then release the sequestered Ca^{2+} prematurely (Schwartz *et al.*, 1973).

It has been suggested that the intramitochondrial nicotinamide nucleotide reduction state is directly linked to Ca^{2+} fluxes (Lehninger *et al.*, 1978). Thus a high concentration of NADH, as in the presence of succinate + rotenone, would promote rapid Ca^{2+} entry, whereas a shift to a more oxidized ratio, after addition of acetoacetate or oxaloacetate, would favour Ca^{2+} efflux. We have previously observed (Wolkowicz & McMillin-Wood, 1979) that palmitoyl-CoA alone decreases Ca^{2+} uptake in liver mitochondria and promotes a release of accumulated Ca^{2+} that is concomitant with nicotinamide nucleotide oxidation. This effect is totally reversed by the presence of carnitine, but is enhanced by malonyl-CoA in the presence of carnitine. Since malonyl-CoA is a highly specific inhibitor of the carnitine acyltransferase located on the external aspect of the mitochondrial inner membrane (McGarry *et al.*, 1978), these results suggest that the Ca^{2+} -induced nicotinamide nucleotide oxidation is the result of palmitoyl-CoA binding to the outer surface of the mitochondrial inner membrane. Results from the present

study show that the addition of Ca^{2+} to heart mitochondria preincubated with palmitoyl-CoA produces a slight but instantaneous oxidation of nicotinamide nucleotides with a slowed Ca^{2+} uptake. Injection of palmitoylcarnitine after Ca^{2+} uptake produces oxidation of a fraction of the mitochondrial nicotinamide nucleotides that is accompanied by immediate Ca^{2+} release and slowed re-uptake of Ca^{2+} in this group of experiments.

Thus the results demonstrate that the rate of Ca^{2+} uptake is apparently related to and influenced by the reduction state of the nicotinamide nucleotides. The experiments further suggest that Ca^{2+} release may be related only indirectly to mitochondrial nicotinamide nucleotide oxidation, i.e. via stimulation of re-uptake. Evidence for this is found in Figs. 7 and 8.

Addition of palmitoyl-CoA results in a lag phase preceding release of accumulated Ca^{2+} , as previously described (Asimakis & Sordahl, 1977; Wolkowicz & McMillin-Wood, 1979). In the presence of Ruthenium Red, this lag phase is shortened and Ca^{2+} release occurs at a lower rate. The onset of Ca^{2+} release in the presence of Ruthenium Red corresponds temporally to onset of nicotinamide nucleotide oxidation after addition of palmitoyl-CoA in the absence of Ruthenium Red. In the presence of Ruthenium Red, no alteration in nicotinamide nucleotide reduction state occurs. The possibility therefore exists that Ca^{2+} release is unaffected by Ruthenium Red and the longer lag period observed in the absence of Ruthenium Red is a result of re-uptake of released Ca^{2+} . Thus the ability of palmitoyl-CoA to enhance the affinity of the uptake mechanism for Ca^{2+} could account for the lag period preceding rapid Ca^{2+} efflux and for the onset of nicotinamide nucleotide oxidation. The low initial rate of palmitoyl-CoA-induced Ca^{2+} efflux in the absence of Ruthenium Red appears to relate to the more oxidized state of the nicotinamide nucleotides, i.e. slowed re-uptake. When most of the nicotinamide nucleotides is oxidized, re-uptake is impaired and a rapid Ca^{2+} release ensues.

Rates of Ca^{2+} efflux after palmitoylcarnitine injection are higher when Ruthenium Red is present. The concentration of NADH in these mitochondria appears to be sufficient to continue active re-uptake in the absence of transport inhibitor. Thus the enhanced rates observed in the presence of both palmitoylcarnitine and Ruthenium Red are most likely to be due to inhibited re-accumulation of Ca^{2+} . A mechanism similar to that for palmitoyl-CoA can be invoked to explain the prevention by Ruthenium Red of nicotinamide nucleotide oxidation and augmented net Ca^{2+} release in response to palmitoylcarnitine.

Although the precise mechanism coupling nicotinamide nucleotide oxidation to Ca^{2+} transport is uncertain, it is curious that these effects occur in the

presence of rotenone and in a non-metabolically linked manner. Disappearance of the larger part of the reduced nicotinamide nucleotides occurs in the presence of rotenone and precedes rapid net Ca^{2+} release and respiratory stimulation. The results suggest either that these agents produce a by-pass of the rotenone block, or perhaps that the nicotinamide nucleotides respond directly to Ca^{2+} movements, specifically Ca^{2+} uptake. A previous report (Bhuvaneswaran & Wadkins, 1978) demonstrates that coupling of arsenate-uncoupled liver mitochondria in the presence of rotenone can be activated by β -hydroxybutyrate-mediated NAD^+ reduction, and suggests that coupling at sites 2 and 3 of the respiratory chain is amenable to control by the reduction state of nicotinamide nucleotides remote from these sites. In agreement with these observations, our results suggest that depletion of mitochondrial NADH leads to stimulated rates of respiration in the presence of rotenone, or possibly decreased coupling efficiency at sites 2 and 3.

Thus the oxidation of nicotinamide nucleotides preceding a net Ca^{2+} -release phase induced by acyl esters appears to be a direct consequence of Ca^{2+} cycling with a resultant dissipation of energy or coupling efficiency necessary to retain the accumulated ion. It is also proposed that acyl-CoA interacts directly and in a highly specific manner with the carrier- Ca^{2+} complex on the outer surface of the inner membrane. Questions that remain to be answered concern the apparent immediate Ca^{2+} -specific oxidation of nicotinamide nucleotides in the presence of a rotenone block as well as the mechanism by which palmitoyl-CoA induces Ca^{2+} cycling, even at low concentrations where no membrane alterations or uncoupling occur in the absence of added cations.

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