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Oxaloacetate- and acetoacetate-induced calcium efflux from mitochondria occurs by reversal of the uptake pathway

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1. Addition of oxaloacetate or acetoacetate to isolated rat liver mitochondria results in an efflux of Ca^{2+}. Concomitant with this efflux is an immediate oxidation of endogenous nicotinamide nucleotides, a fall in the mitochondrial membrane potential and an increase in the rate of respiration. The primary effect in this sequence may be either (*a*) physiologically important stimulation of a Ca^{2+}-efflux carrier, followed by Ca^{2+} re-uptake, a fall in membrane potential and increased respiration, or (*b*) physiologically unimportant damage to mitochondrial integrity, followed by a fall in membrane potential, increased respiration and Ca^{2+} efflux. 2. Ruthenium Red and EGTA will restore the increased respiratory rate to one approximating to the control rate of respiration. However, addition of lanthanide, at a concentration which inhibits the uptake but not the normal efflux of Ca^{2+}, inhibits the rate of Ca^{2+} efflux induced by oxaloacetate or acetoacetate. Therefore the observed efflux is occurring by a reversal of the uptake pathway (uniporter) and thus follows the fall in membrane potential. 3. From these results we conclude that the decrease in membrane potential and increase in the rate of respiration seen during oxaloacetate- or acetoacetate-induced Ca^{2+} efflux cannot be accounted for by rapid Ca^{2+} cycling, but are due to damage to mitochondrial integrity.

The current model for Ca^{2+} uptake and efflux from mitochondria invokes two separate pathways, an electrophoretic uniporter for uptake driven by the membrane potential, and a $Ca^{2+}/2H^+$ or Ca^{2+}/nNa^+ antiporter for efflux driven by the pH gradient (for reviews, see Nicholls & Crompton, 1980; Saris & Åkerman, 1980). The two carriers allow Ca^{2+} cycling; thus intramitochondrial free Ca^{2+} is controlled by cytosolic free Ca^{2+} at low cytosolic Ca^{2+} concentrations (Denton & McCormack, 1980), and extramitochondrial free Ca^{2+} is set at about 1 μM in the presence of larger amounts of Ca^{2+} (Nicholls, 1978).

Modulation of the activity of either carrier would consequently be of considerable interest. One putative mechanism for the control of the carriers' activity is the redox state of intramitochondrial NAD and NADP. This follows the demonstration by Lehninger *et al.* (1978) and Fiskum & Lehninger (1979) that addition of oxaloacetate or acetoacetate to rat liver mitochondria oxidized endogenous nicotinamide nucleotides and induced Ca^{2+} efflux. Lehninger *et al.* (1978) proposed an allosteric activation of the efflux pathway to explain the induced efflux. Some support for this hypothesis has come from Lötscher *et al.* (1980) and Prpić & Bygrave (1980). The suggestion that this represents a mechanism for the control of Ca^{2+} *in vivo* has been challenged by Nicholls & Brand (1980), who argued that acetoacetate sensitized mitochondria to Ca^{2+}-induced damage, since concomitant with induced efflux there was a fall in $\Delta\psi$. Both effects required critical concentrations of Ca^{2+} and P_i and could be prevented by 'protective' agents (e.g. oligomycin or ATP). Nicholls & Brand (1980) concluded that the fall in $\Delta\psi$ allowed Ca^{2+} efflux to occur via a reversal of the uniporter. Others have come to similar conclusions (Beatrice *et al.*, 1980).

Abbreviations used: pCa^{2+}, $-\log[Ca^{2+}]$ in incubation medium; $\Delta\psi$, membrane potential; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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The experiments in the present paper were designed to differentiate between two hypotheses. The first is that oxidation of endogenous nicotinamide nucleotides activates net efflux on the $Ca^{2+}/2H^+$ antiporter, resulting in Ca^{2+} efflux, faster Ca^{2+} cycling, fall in $\Delta\psi$, and increased respiration. The second is that acetoacetate addition potentiates Ca^{2+}-induced damage, resulting in a fall in $\Delta\psi$ and Ca^{2+} efflux via a reversal of the uniporter. Our results show that although elimination of Ca^{2+} cycling does protect mitochondria from the effects of acetoacetate and oxaloacetate, the Ca^{2+} efflux that is seen occurs on the uniporter. This argues against the first hypothesis and is consistent with the second.

Experimental

Materials

Oxaloacetic acid was obtained from BDH Chemicals, Poole, Dorset, U.K. Acetoacetic acid (lithium salt) was obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Bovine albumin powder (fraction V from bovine plasma) was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. CCCP was obtained from E. I. Du Pont De Nemours and Co., Central Research Department, Experimental Station, Wilmington, DE, U.S.A. All other reagents were obtained from BDH or Sigma.

Mitochondria

Rat liver mitochondria were prepared by the method of Chappell & Hansford (1972). Initial homogenization was performed in a medium containing 250mM-sucrose, 5mM-Tris/HCl, pH 7.4, and 1mM-EGTA. The mitochondrial pellet was subsequently washed in EGTA-free medium. Protein concentration was determined by the biuret method (Gornall *et al.*, 1949).

Ca^{2+} determination

The concentrations of Ca^{2+} in the incubation medium and in Ca^{2+} solutions added to the medium during the course of the experiments were determined with a Perkin–Elmer 380 atomic-absorption spectrophotometer.

Protocols

The rate of oxygen consumption and pCa^{2+} values were measured simultaneously by using a Rank oxygen electrode (4ml volume), which had been modified to incorporate a Ca^{2+}-selective electrode (Radiometer 2112, with KCl reference electrode type K801). The modification involved widening the diameter of the incubation chamber and replacing the screw-top with a cylinder of Perspex, through which two appropriately sized holes had been bored. A small needle-sized hole was cut into the side of the cylinder to allow the addition of various reagents.

The absorbance of the nicotinamide nucleotides was measured with a Perkin–Elmer 557 dual-wavelength double-beam spectrophotometer at 340–370nm.

All measurements were carried out at 30°C.

Results and discussion

Inhibition of Ca^{2+} cycling

Fig. 1 shows that prevention of Ca^{2+} cycling by Ruthenium Red or EGTA after the addition of oxaloacetate decreases the respiratory rate, as predicted by the first hypothesis.

In a medium containing 2mM-P_i, it required 12.3μM-Ca^{2+} before oxaloacetate-induced Ca^{2+} release could be observed. Addition of this amount of Ca^{2+} even without oxaloacetate increased the state-4 respiratory rate of the mitochondria from 53 to 80nmol of O/min (Fig. 1*a*). This indicates that under the conditions necessary to induce Ca^{2+} efflux, there is already some damage to mitochondrial integrity. The increased rate of respiration evoked by Ca^{2+} under these conditions was not due to Ca^{2+} cycling, since neither Ruthenium Red nor EGTA affected the rate of respiration if added after the Ca^{2+} (results not shown).

In the presence of oxaloacetate (0.5mM), the respiratory rate increased to 90nmol of O/min when Ca^{2+} was added, and after a short time Ca^{2+} efflux occurred (Fig. 1*b*). Addition of Ruthenium Red (Fig. 1*c*) or EGTA (Fig. 1*d*) decreased the accelerated rate of respiration. Similar results were obtained with acetoacetate (2mM), except that higher concentrations of P_i and/or Ca^{2+} were required before induced efflux occurred. Ruthenium Red or EGTA did not decrease the respiratory rate if added at later times (results not shown).

Thus it seems that Ca^{2+} cycling might be accelerated by oxaloacetate and acetoacetate, although this would only be true in the early stages of oxaloacetate- or acetoacetate-induced Ca^{2+} efflux. However, other interpretations of this experiment are possible; for example, it could be that the intramitochondrial Ca^{2+} concentration had not risen sufficiently to cause damage before further uptake was prevented by Ruthenium Red or EGTA. For this reason we performed the more definitive experiment using lanthanide described below.

Inhibition of oxaloacetate-induced Ca^{2+} efflux

The critical difference between the two hypotheses is that Ca^{2+} efflux is on the normal efflux pathway in the first, but on the uniporter in the second. We have taken advantage of the fact that the two carriers show very different sensitivities to inhibition by lanthanides under appropriate con-

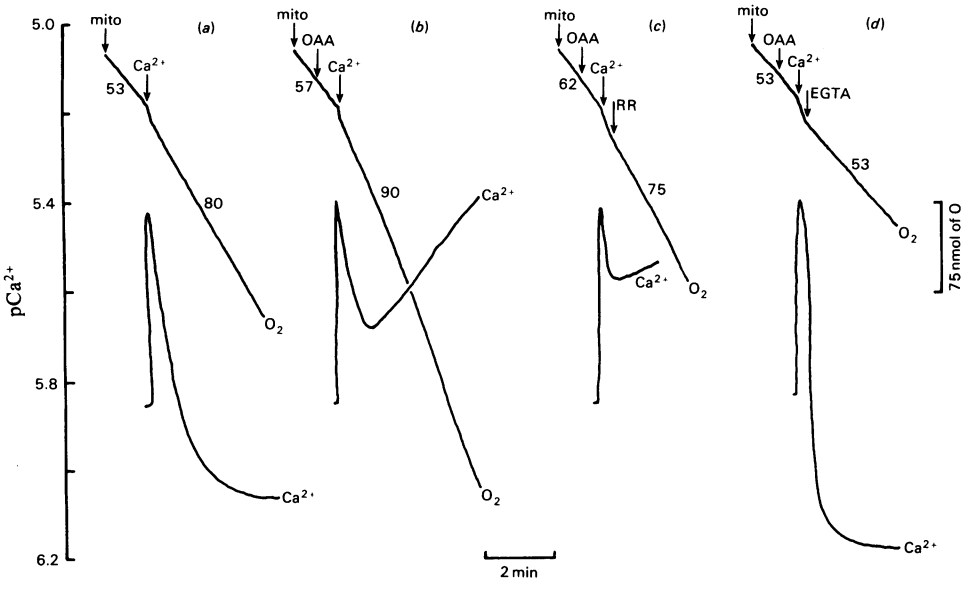

Fig. 1. *Respiratory rate in the presence of oxaloacetate: inhibition by Ruthenium Red and EGTA*
Mitochondria (mito; 5.5 mg of protein/ml) were incubated at 30°C in a medium containing 75 mM-NaCl, 10 mM-Tes, 1 μM-rotenone, 2 mM-nitrilotriacetic acid, 2 mM-phosphate, 2.2 mg of bovine serum albumin/ml, 2 mM-succinate, at a final pH of 7.0. Total $[Ca^{2+}]$ was 12.3 μM. (*a*) Control, (*b*) addition of 0.5 mM-oxaloacetate (OAA) and subsequent addition (*c*) of 1 nmol of Ruthenium Red (RR)/mg or (*d*) of 2 mM-EGTA. Numbers by the O_2 traces are respiration rates in nmol of O/min per mg of protein.

ditions (Crompton *et al.*, 1979). A lanthanide concentration was found which inhibited the uniporter substantially but had no effect on the Ruthenium Red-insensitive efflux (antiporter). This lanthanide concentration was then used to identify the carrier catalysing oxaloacetate-induced Ca^{2+} efflux.

Addition of 0.4 nmol of Nd^{3+}/mg of protein inhibited the Ca^{2+}-uptake rate by 77% (Fig. 2*a*), but did not greatly affect the rate of efflux seen in the presence of Ruthenium Red (Fig. 2*b*). At this concentration Nd^{3+} is thus a specific inhibitor of the uniporter. This concentration of Nd^{3+} inhibited oxaloacetate-induced Ca^{2+} efflux by 79% (Figs. 2*c* and 2*d*), demonstrating that the efflux was catalysed by the uniporter. This inhibition was observed even in the initial phase of induced efflux, when it can be reversed by β-hydroxybutyrate (Fig. 2*e*). Respiration was monitored to eliminate the possibility of Ca^{2+} efflux owing to the inhibition of succinate dehydrogenase. In the absence of Nd^{3+}, the mitochondria showed respiratory control and maintained a pCa^{2+} of approx 6.1 for at least 13 min. Ca^{2+} efflux induced by an uncoupler occurs via the uniporter; therefore the inhibition of Ca^{2+} efflux by Nd^{3+} on addition of CCCP (Fig. 2*f*) was measured. Ca^{2+} efflux induced by the addition of CCCP was inhibited by 45% by Nd^{3+}.

It is pertinent that Panfili *et al.* (1980) demonstrated that oxaloacetate- and acetoacetate-induced Ca^{2+} efflux was inhibited by antibody prepared against Ca^{2+}-binding glycoprotein. This antibody inhibits Ca^{2+} uptake on the uniporter, but not Ca^{2+} efflux on the Ca^{2+}/Na^{+} antiporter of heart mitochondria (Panfili *et al.*, 1981). This evidence also suggests that oxaloacetate-induced Ca^{2+} efflux occurs on the uniporter.

Nucleotide oxidation

A decrease in absorbance at 340–370 nm was seen immediately on addition of acetoacetate or oxaloacetate under conditions that did not induce Ca^{2+} efflux. Therefore oxidation of the nicotinamide nucleotides may be a necessary condition for induced efflux, but it is not sufficient. This observation is in accord with the findings of Nicholls & Brand (1980) and Wolkowicz & McMillin-Wood (1980).

Conclusions

The following observations from published data (Nicholls & Brand, 1980; Beatrice *et al.*, 1980; Wolkowicz & McMillin-Wood, 1980) and from our own work suggest strongly that oxaloacetate- and acetoacetate-induced Ca^{2+} efflux is not a physiologically important phenomenon.

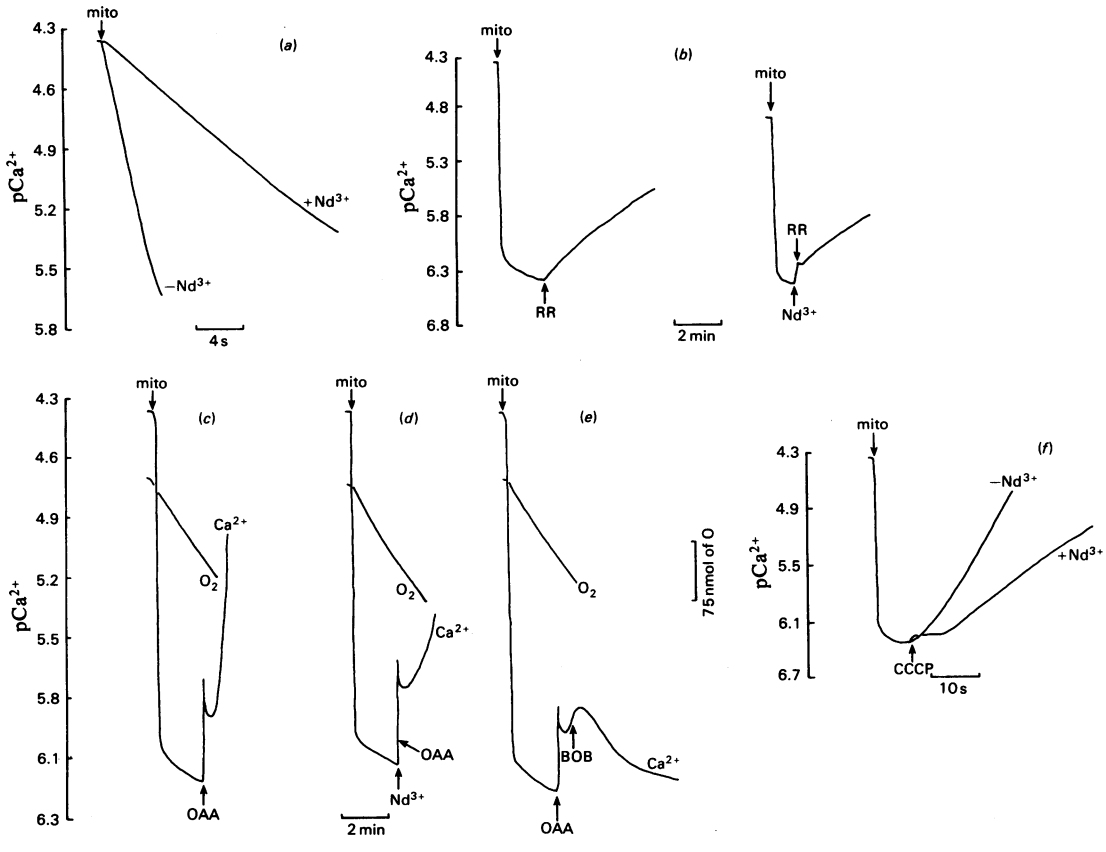

Fig. 2. *Oxaloacetate-induced Ca^{2+} release: inhibition by Nd^{3+}*

Mitochondria (mito; 1.3 mg/ml) were incubated at 30°C in a medium containing 75 mM-NaCl, 10 mM-Tris/HCl, 1 μM-rotenone, 2 mM-acetate, 2.2 mg of bovine serum albumin/ml, 2 mM-succinate, at a final pH of 7.0. Total [Ca^{2+}] was 51 μM. Where indicated Nd^{3+} (oxide) was added at 0.4 nmol/mg of protein. (*a*) Ca^{2+} uptake in the presence or absence of Nd^{3+}; (*b*) Ca^{2+} efflux after addition of 1 nmol of Ruthenium Red (RR) in the presence or absence of Nd^{3+}; (*c*) Ca^{2+} efflux induced by 0.5 mM-oxaloacetate (OAA); (*d*) inhibition of OAA-induced Ca^{2+} efflux by Nd^{3+}; (*e*) reversal of OAA-induced Ca^{2+} efflux by 5 mM-β-hydroxybutyrate (BOB); (*f*) Ca^{2+} efflux induced by 25 μM-CCCP in the presence or absence of Nd^{3+}.

(*a*) Efflux is associated with a fall in membrane potential;

(*b*) both efflux and the fall in membrane potential can be prevented by ATP or Mg^{2+} at concentrations *in vivo*, as well as by several other compounds;

(*c*) NAD(P)H oxidation is not a sufficient condition for Ca^{2+} efflux to occur;

(*d*) the concentration of Ca^{2+} required to induce efflux depends on the phosphate concentration and the acetoacetate or oxaloacetate concentration;

(*e*) induced Ca^{2+} efflux occurs on the uniporter.

We conclude that, since the Ca^{2+} efflux induced by acetoacetate and oxaloacetate occurs on the uniporter, it must therefore follow a fall in membrane potential caused by damage to the mitochondria. A stimulation of Ca^{2+} cycling caused by increased Ca^{2+} efflux and followed by a fall in membrane potential is incompatible with our results.

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