Calcium-induced damage of rat heart mitochondria

D. R. PARR, J. M. WIMHURST, and E. J. HARRIS

From the Department of Biophysics, University College London, London WC1E 6BT

AUTHORS' SYNOPSIS The damage which may be caused to heart mitochondria by the rapid uptake of calcium when oxygen is restored after a period of anoxia is monitored by the deterioration in phosphorylation performance. Methods of protecting mitochondria from this damage by preventing calcium uptake and by chelating cytoplasmic calcium are considered.

The heart has a high metabolic rate associated with its contractile function. Contraction and relaxation involve the movement of calcium within the cell, and ATP is used to re-accumulate it in specific depots. Contraction of cardiac muscle, unlike skeletal muscle, depends on the extra-cellular calcium concentration (Schaffer et al, 1972). It has been suggested (eg, Chance, 1965; Carafoli and Lehninger, 1971) that heart mitochondria may play a role in the removal of ionized calcium after each contraction, although the known rates of calcium flux in mammalian heart mitochondria may be insufficient, and the sarcoplasmic reticulum has a higher affinity for this cation (Schaffer et al, 1972). It is possible, as suggested by Spencer and Bygrave (1973), that by controlling the calcium concentration of the cytoplasm, mitochondria help to regulate such processes as glycolysis and protein and phospholipid biosynthesis, which involve calcium-activated or calcium-inhibited enzymes. The possible role of mitochondrial calcification in several pathological states has also been suggested (Cavallero et al, 1974), as has the possibility that the ability to accumulate large amounts of calcium may be connected with some cancerous states (Thorne and Bygrave, 1974).

This paper describes the calcium-mediated damage to heart mitochondria of the type which might occur on the restoration of oxygen after a period of anoxia or hypoxia. Such a situation could arise in certain clinical conditions, or after deep anaesthesia. When the mitochondrion is deprived of oxygen it first ceases to export ATP to the cytoplasm, and that which remains is used, amongst other things, to maintain the imbalance of ionized calcium. When this is exhausted, internal calcium is no longer actively retained, and leaks out into the cytoplasm (Thomas et al, 1969). In addition, calcium in the cytoplasm may become ionized as a result of the disappearance, in this situation, of physical chelating agents, especially ATP. It could be the sudden physical passage of the ionized cytoplasmic calcium back into the mitochondria when oxygen is restored which causes the membrane damage we observe; alternatively, the damage could be connected with the activation by Ca$^{2+}$ of membrane phospholipases (Scarpa and Lindsay, 1972). The damaging effect of calcium to liver mitochondria has been described by Rossi et al (1966), and many of the phenomena presented here have been described in the context of liver mitochondria.

We have used the loss of respiratory control by ADP as measured by the phosphorylation rate and the P/O ratio as an index of the damage caused to preparations of isolated mitochondria. We have investigated some ways in which this damage can be reduced, both by agents which may protect the membranes, and by those which increase the calcium chelating capacity of the mitochondrial environment.

1 This work was carried out with the aid of grants from the British Heart Foundation and the MRC. Reprint requests to D.R.P.
Methods

About 10 rat hearts were used for each preparation. All apparatus and media were precooled. Hearts were removed immediately after death and dropped into ice-cold 0.15 mol/l NaCl, in which they continued to beat briefly, expelling most of their blood. The saline was decanted off, and the hearts chopped with scissors in a medium containing 300 mmol/l sucrose and 1 mmol/l ethylene glycol bis(β-aminoethyl)-N,N'-tetracetic acid (EGTA). They were then forced through a stainless steel tissue press (with 1.32 mm diameter holes). The resulting paste was homogenized in a glass vessel with a Teflon pestle (clearance 0.22 mm) with about 50 ml of the same medium. NAGarse1 (5 mg) or a similar protease was added, and the slurry mixed and left in ice for 10 min. The homogenate was then diluted with its own volume of a similar medium containing 5 mmol/l KCl and 1 mg/ml purified bovine serum albumin (BSA), and homogenized again. The cell debris was removed by centrifugation, taking the machine up to 750 x g (10 cm rotor, MSE High Speed 18) and allowing it to come to rest without using the brake. The supernatant was poured through muslin, and centrifuged at 9900 x g for 4 min. The pellets were pooled and suspended in fresh medium, centrifuged as before, and the operation repeated. The final pellet was suspended in about 1 ml of 300 mmol/l sucrose in Tris N-2 hydroxyethylpiperazine N'-Zethanesulfonic acid (HEPES) buffer pH 7.4, and after removal of a sample for protein assay, 8 mg of BSA was added. The protein concentration was estimated by the biuret reaction, calibrated with BSA. Electron micrographs confirmed the purity of such mitochondrial preparations.

Respiratory rates and responses to ADP were measured in a Clarke type oxygen polarimeter having a special plunger with an internal well which enabled samples to be taken without disturbing the record (Harris et al, 1971). Phosphorylation rates were measured after the addition of 5 or 10 µl of 100 mmol/l ADP.

In some experiments, samples of the incubation mixture were isolated from the air by a layer of light silicone oil; mitochondria were sedimented at various times by centrifuging the samples in a Coleman microfuge, and samples of the supernatant taken immediately for assay. The method of Harris and van Dam (1968), in which mitochondria are separated from their medium and extracted by spinning through silicone oil, was also used.

ATP was assayed by measuring the change in absorbance at 351 nm caused by the reduction of NADP in a coupled system containing glucose, hexokinase and G6P dehydrogenase. Citrate was assayed by measuring the decrease in absorbance at the same wavelength caused by the oxidation of NADH in a coupled system containing citrate lyase and malate dehydrogenase. Calcium was assayed using a Techtron atomic absorption spectrophotometer.

Results

The phosphorylation performance of rat heart mitochondria, measured using an oxygen electrode, can be made worse by the addition of exogenous calcium. A typical trace is shown in Fig. 1a. The extent of the deterioration in both phosphorylation rate and P/O ratio increased with the amount of calcium added (Fig. 2). The amount of calcium required to achieve a certain deterioration varied between mitochondrial preparations, probably due to differing amounts of EGTA carried over from the preparation medium.

Periods of anoxia, as such, did not have any deleterious effect on performance, and the intervention of a period of anoxia between the addition of calcium and the second stimulation

FIG. 1 Typical oxygen electrode traces showing the effect of the addition of 345 nmol Ca2+ /mg protein on the phosphorylation performance of rat heart mitochondria with (b) and without (a) an intervening period of anoxia. In both traces mitochondria (M) were added at a concentration of 0.88 mg protein/ml, to a medium containing 150 mmol/l KCl, 10 mmol/l Tris-HEPES buffer pH 7.4 and 4.5 mmol/l each of glutamate, malate, malonate, and inorganic phosphate in a total volume of 3.3 ml at 26°C. Each ADP addition was of 300 nmol/ml.

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1 Supplied by Hughes and Hughes, Church Road, Harold Wood, Essex.
FIG. 2 The effect of adding increasing amounts of calcium on the phosphorylation rate and P/O ratio of rat heart mitochondria expressed as percentages of the control value before the addition. The incubations contained 150 mmol/l KCl, 10 mmol/l Tris-HEPES buffer pH 7.4, 4.5 mmol/l each of glutamate, malate, malonate, and inorganic phosphate, and 1.15 mg mitochondrial protein/ml in a total volume of 3.3 ml at 26°C. The rate before the addition of Ca²⁺ was between 277 and 312 nmol ATP/mg mitochondrial protein/min, and the P/O was between 2.40 and 2.80.

FIG. 3 The effect of anoxia on the calcium concentration of the medium surrounding rat heart mitochondria. Incubations contained 150 mmol/l KCl, 10 mmol/l Tris-HEPES buffer pH 7.4, 4.7 mmol/l each of glutamate, malate, and inorganic phosphate, 2.6 mmol Ca²⁺/mg protein, and 3.3 mg mitochondrial protein/ml at 21°C. All were sealed from the air at zero time, the mitochondria removed from suspension at various times by centrifugation, and the supernatant sampled. A similar suspension incubated in an oxygen electrode exhausted its oxygen in 13 min.

FIG. 4 The decrease in ATP content of a rat heart mitochondrial suspension after the addition of rotenone. Incubations contained 150 mmol/l KCl, 10 mmol/l Tris-HEPES buffer pH 7.4, 2.6 mmol/l each of glutamate, malate, and inorganic phosphate, and 3.8 mg mitochondrial protein/ml in a total volume of 3.75 ml at 21°C, and were oxygenated. Rotenone at a concentration of 1 μg/ml was added to stop the production of ATP by blocking the oxidation of endogenous NADH at zero, and samples were subsequently taken at intervals. Samples were acidified with perchloric acid, neutralized, and the ATP assayed.

cycle did not greatly exacerbate the effect of calcium alone (Fig. 1a, b). Calcium efflux occurs from mitochondria kept isolated from the air so that respiration causes exhaustion of the oxygen in the medium (Fig. 3). The mitochondria used for this experiment were supplemented with Ca²⁺; the usual amount in particles isolated with EGTA in the media was between 6 and 30 nmol/mg protein, and not all of this was necessarily released during anoxia, hence the lack of any deterioration after anoxia could be explained by a relatively small Ca²⁺ movement.

From the viewpoint of ATP content, however, the lack of appreciable deterioration after periods of anoxia was surprising, since the endogenous ATP level fell rapidly when its production was prevented (Fig. 4). A similar reduction presumably occurs in the cell during periods of anoxia, although some production may be maintained by glycolysis in the cytoplasm. Hence, it might be expected that during anoxia,
**TABLE 1**

Protective effect of ruthenium red on the phosphorylation performance of rat heart mitochondria after the addition of 209 nmol Ca\(^{2+}\)/mg protein

<table>
<thead>
<tr>
<th>Ruthenium red concentration (nmol/mg protein)</th>
<th>Phosphorylation rate (nmol ADP/mg protein min(^{-1})) Before Ca(^{2+})</th>
<th>After Ca(^{2+})</th>
<th>% protection</th>
<th>P/O ratio Before Ca(^{2+})</th>
<th>After Ca(^{2+})</th>
<th>% protection</th>
</tr>
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<tr>
<td>0.00</td>
<td>378</td>
<td>96</td>
<td>25</td>
<td>3.30</td>
<td>1.60</td>
<td>49</td>
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<tr>
<td>0.28</td>
<td>331</td>
<td>321</td>
<td>97</td>
<td>2.82</td>
<td>2.68</td>
<td>95</td>
</tr>
<tr>
<td>2.75</td>
<td>332</td>
<td>379</td>
<td>114</td>
<td>3.03</td>
<td>3.03</td>
<td>100</td>
</tr>
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Incubations contained 150 mmol/l KCl, 10 mmol/l Tris-HEPES buffer pH 7.4, 4.5 mmol/l each of glutamate, malate, malonate, and inorganic phosphate and 1.1 mg mitochondrial protein/ml in a total volume of 3.3 ml at 26°C.

**TABLE 2**

Protective effect of various respiratory substrates on the phosphorylation performance of rat heart mitochondria after the addition of 30 nmol Ca\(^{2+}\)/mg protein

<table>
<thead>
<tr>
<th>Respiratory substrates</th>
<th>Phosphorylation rate (nmol ADP/mg protein min(^{-1})) Before Ca(^{2+})</th>
<th>After Ca(^{2+})</th>
<th>% protection</th>
<th>P/O ratio Before Ca(^{2+})</th>
<th>After Ca(^{2+})</th>
<th>% protection</th>
<th>Citrate concentration in medium (umol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate + malate</td>
<td>640</td>
<td>555</td>
<td>87</td>
<td>2.77</td>
<td>2.40</td>
<td>87</td>
<td>36</td>
</tr>
<tr>
<td>Malate + glutamate + malonate</td>
<td>375</td>
<td>210</td>
<td>56</td>
<td>3.00</td>
<td>2.25</td>
<td>75</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Oxoglutarate + malonate</td>
<td>416</td>
<td>128</td>
<td>31</td>
<td>3.40</td>
<td>2.50</td>
<td>73</td>
<td>&lt;3</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>274</td>
<td>ns</td>
<td>—</td>
<td>2.52</td>
<td>ns</td>
<td>—</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Palmitoyl carnitine + malate</td>
<td>426</td>
<td>426</td>
<td>100</td>
<td>2.15</td>
<td>2.17</td>
<td>101</td>
<td>10</td>
</tr>
</tbody>
</table>

Incubations contained 150 mmol/l KCl, 10 mmol/l Tris-HEPES buffer pH 7.4, and 3 mmol/l inorganic phosphate in a total volume of 3.3 ml at 26°C. All substrates were present at a concentration of 3 mmol/l, except palmitoyl carnitine which was 30 mmol/l and β-hydroxybutyrate which was 20 mmol/l. ns = not stimulated.

**TABLE 3**

Protective effect of exogenous citrate on the phosphorylation performance of rat heart mitochondria after the addition of 345 nmol Ca\(^{2+}\)/mg protein

<table>
<thead>
<tr>
<th>Citrate concentration (mmol/l)</th>
<th>Phosphorylation rate (nmol ADP/mg protein min(^{-1})) Before Ca(^{2+})</th>
<th>After Ca(^{2+})</th>
<th>% protection</th>
<th>P/O ratio Before Ca(^{2+})</th>
<th>After Ca(^{2+})</th>
<th>% protection</th>
</tr>
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<tbody>
<tr>
<td>0.0</td>
<td>387</td>
<td>120</td>
<td>31</td>
<td>3.09</td>
<td>1.88</td>
<td>61</td>
</tr>
<tr>
<td>4.5</td>
<td>394</td>
<td>148</td>
<td>38</td>
<td>3.14</td>
<td>2.02</td>
<td>69</td>
</tr>
<tr>
<td>8.8</td>
<td>394</td>
<td>266</td>
<td>68</td>
<td>3.20</td>
<td>2.59</td>
<td>81</td>
</tr>
<tr>
<td>17.1</td>
<td>429</td>
<td>481</td>
<td>112</td>
<td>3.34</td>
<td>3.23</td>
<td>97</td>
</tr>
</tbody>
</table>

Incubations contained 150 mmol/l KCl, 10 mmol/l Tris-HEPES buffer pH 7.4, 4.5 mmol/l each of glutamate, malonate, and inorganic phosphate and 0.88 mg mitochondrial protein/ml in a total volume of 3.3 ml at 26°C.
not only is calcium released from mitochondria because of a lack of energy, but Ca\(^{2+}\) previously associated with ATP in the cytoplasm may also be released in an ionized form as the ATP is consumed. We have calculated that at pH 7.4, a cytoplasmic ATP concentration of 6.6 mmol/l would be capable of chelating all but \(2 \times 10^{-5}\) mol/l of a cytoplasmic calcium concentration of 1 mmol/l.

The calcium-induced damage was completely prevented by ruthenium red (Table I), but butacaine, dibucaine, propranolol, chlorpromazine, spermidine, and \(\alpha\)-tocopherol did not have a protective effect and the first four sometimes caused a small additional deterioration in the performance.

The extent of the calcium-induced damage varied, depending upon the respiratory substrates present (Table 2). The greatest protection was given by those substrates which were potentially most citrogenic, and the extent of the protection correlated with the amount of citrate in the medium. Similar protection could be achieved by the addition of citrate (Table 3), but 3 mmol/l phosphoenolpyruvate was ineffective.

The deterioration caused by Mn\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) was considerably less than that caused by Ca\(^{2+}\).

**Discussion**

The evidence presented here suggests that calcium-induced damage to mitochondrial membranes results from the physical effects of the active uptake of this cation subsequent to its binding at the high affinity sites which have been described by several workers (Mela, 1969; Reynafarje and Lehninger, 1969; Carafoli and Lehninger, 1971). These sites also bind Sr\(^{2+}\), Mn\(^{2+}\), and Ba\(^{2+}\) (Reynafarje and Lehninger, 1969; Vainio et al, 1970) and certain cationic dyes (Colonna et al, 1973). The uptake of the other divalent cations is slower than that of calcium but their almost complete lack of effect on phosphorylation performance might suggest the involvement of the mitochondrial phospholipase A\(_2\) which is activated by calcium (Scarpa and Lindsay, 1972); however, the time scale of the phenomenon makes this unlikely. The fact that the damage can be completely prevented using low concentrations of ruthenium red strongly indicates the involvement of high affinity sites. Ruthenium red is known to block Ca\(^{2+}\) binding at these sites at concentrations considerably below those which affect the low affinity sites and respiration (Vasington et al, 1972; Rossi et al, 1973). Our results indicate a value for the number of sites of about 2–3 mmol/mg protein, which is similar to values obtained with liver mitochondria (Reynafarje and Lehninger, 1969; Vasington et al, 1972).

The absence of protection by agents which are thought to block the low affinity sites, such as butacaine and other \(\beta\)-receptor blocking agents (Vasington et al, 1972), is understandable if these sites are concerned chiefly with calcium efflux, but might be expected to have some beneficial effect in vivo, where shedding of Ca\(^{2+}\) from the mitochondria during anoxia might be slowed down or stopped. In the present experiments, these agents caused, if anything, a deterioration, probably because blocking the low affinity sites accentuated uptake at the high affinity sites, a phenomenon noticed by Mela (1968). It is interesting that these \(\beta\)-blocking agents are also though to inhibit phospholipase activity (Seppälä et al, 1971; Scarpa and Lindsay, 1972).

It is apparent that factors which increase the amount of chelating agents, such as citrate and ATP, in the mitochondrial environment have a beneficial effect on heart mitochondria when they are subjected to the addition of calcium, and might be expected to have a similar effect during periods of anoxia when calcium is released both from the mitochondria and by dechelation in the cytoplasm. This suggests one reason for the increased vulnerability of the heart in certain conditions of disturbed metabolism, notably diabetes. The availability of both pyruvate and malate favours citrate formation, and requires the hepatic formation of malate; the generation of malate from pyruvate produced glycolytically requires energy because the carboxylation to oxalacetate and its subsequent reduction in the liver consumes ATP. No protection was afforded by the non-citrogenic substrate \(\alpha\)-hydroxybutyrate, a typical product of fat oxidation in the diabetic state. Glutamate, a product of protein breakdown, only becomes potentially citrogenic after transamination with oxalacetate produced from malate oxidation; hence, again malate is a prerequisite. Aspartate formed subsequently
in the transamination can be reconverted to oxalacetate if pyruvate is present, because activity of cytoplasmic pyruvate-oxidase transaminase leads to alanine formation (Safer and Williamson, 1973). Hence with more pyruvate than is required by the liver for malate formation, glutamate fed to the heart can yield a two carbon moiety for citrate synthesis. However, with deficient pyruvate, excess glutamate will competitively remove oxalacetate, which would otherwise have been available for condensation with acetyl groups to form citrate.

In addition to their direct effect on citrate synthesis, the availability of various anions may have a great effect on the passage of citrate out of the mitochondrion into the cytoplasm where it exerts its protective effect. England and Robinson (1969) found that with heart mitochondria, anions such as malate and aspartate caused rapid citrate efflux, while others such as malonate and glutamate did not. We thus suggest that a balanced protein/carbohydrate diet will be most likely to favour citrate formation with its resultant preservation of heart mitochondria in conditions of oxygen stress. We failed to find any protection by phosphoenolpyruvate, although this compound is able to chelate calcium; this may be explained by the finding of Chudapongse and Haugaard (1973) that this compound increases calcium efflux from heart mitochondria in the presence of certain substrates.

The presence of fluorocitrate, a highly poisonous inhibitor of citrate oxidation might have a beneficial effect on the phosphorylation performance in the presence of added calcium. Although this compound could not be used in vivo, some less drastic method of reducing citrate use may be effective, eg, the reversal of isocitrate oxidation such that oxoglutarate is reductively carboxylated. The possible use of ruthenium red in heart therapy is tenuous, not only because its lack these sites and the associated active uptake carboxylated. The possible use of ruthenium red affinity sites may itself have deleterious effects, although some species have mitochondria which may have therapeutic application in this field, and on the possible beneficial effects of other ways of controlling calcium uptake, such as reduced temperature.

The results of Hearse et al (1973) on the isolated perfused heart may involve calcium mediated damage to mitochondria. Anoxia results in enzyme leakage, which is lessened by conditions which favour glycolysis, but is accentuated by reoxygenation. Among the enzymes which leak are ones like creatine phosphokinase which are held between inner and outer mitochondrial membranes.

References


