‘Pore’ formation is not required for the hydroperoxide-induced Ca^{2+} release from rat liver mitochondria

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It has recently been suggested by several investigators that the hydroperoxide- and phosphate-induced Ca^{2+} release from mitochondria occurs through a non-specific ‘pore’ formed in the mitochondrial inner membrane. The aim of the present study was to investigate whether ‘pore’ formation actually is required for Ca^{2+} release. We find that the t-butyl hydroperoxide (tbh)-induced release is not accompanied by stimulation of sucrose entry into, K^+ release from, and swelling of mitochondria provided re-uptake of the released Ca^{2+} (‘ Ca^{2+} cycling’) is prevented. We conclude that (i) the tbh-induced Ca^{2+} release from rat liver mitochondria does not require ‘pore’ formation in the mitochondrial inner membrane, (ii) this release occurs via a specific pathway from intact mitochondria, and (iii) a non-specific permeability transition (‘pore’ formation) is likely to be secondary to Ca^{2+} cycling by mitochondria.

INTRODUCTION

Ionized calcium is an important modulator of many cellular processes. The fine tuning of Ca^{2+} requires binding to specific proteins, transport into cellular compartments and transport out of the cell. The ability of mitochondria to take up, store and release Ca^{2+} has long been recognized. Current knowledge suggests that under physiological conditions the task of intra-mitochondrial Ca^{2+} is to regulate several dehydrogenases in these organelles. When the cytosol is flooded with Ca^{2+} , e.g. in cases of emergency such as hypoxia or decreased ATP supply, mitochondria may act as safety devices against cellular Ca^{2+} poisoning, since they are able to take up transiently large quantities of Ca^{2+} with impunity [1].

Mitochondria take up and release Ca^{2+} via separate pathways. The uptake is driven by the electrochemical membrane potential ($\Delta\Psi$) built up during respiration. The mechanisms by which Ca^{2+} is released from mitochondria are not clearly understood. Release from energized mitochondria occurs by exchange of Ca^{2+} with Na^+ or H^+ [2,3] via as-yet unidentified membrane components distinct from the uptake pathway, whereas Ca^{2+} release from de-energized mitochondria may also occur by reversal of the uptake pathway [4]. Owing to the continuous operation of the mitochondrial Ca^{2+} uptake and release pathways, mitochondria ‘cycle’ Ca^{2+} across their inner membrane [5]. Under physiological conditions, when the cytosolic Ca^{2+} concentration is well below the K_m of the mitochondrial Ca^{2+} uptake system, Ca^{2+} cycling consumes little energy and is not detrimental to the organelles. Excessive cycling, as may be encountered with high Ca^{2+} loads in combination with oxidative stress, may damage mitochondria, owing to the high energy demand and disturbance of proper mitochondrial functioning [6].

The group of Crompton [7–10] and others [11–13] have proposed that Ca^{2+} may be released from mitochondria owing to a phosphate- or t-butyl hydroperoxide (tbh)-induced reversible increase of inner-membrane permeability (‘pore’ formation), which is unspecific and characterized by leakiness to small ions and proteins, swelling of mitochondria, and loss of $\Delta\Psi$. We report here that the tbh-induced Ca^{2+} efflux from intact rat liver mitochondria does not require formation of a ‘pore’ in the inner

membrane. From our results it also appears that the increase in the non-specific inner-membrane permeability is secondary to Ca^{2+} cycling and may represent the initial, reversible, phase of the Ca^{2+} -induced damage [14] to mitochondria.

MATERIALS AND METHODS

Materials

Ruthenium Red {[$\text{Ru}_3\text{O}_2(\text{NH}_3)_{14}\text{Cl}_6 \cdot 4\text{H}_2\text{O}$]; Fluka, Buchs, Switzerland} was purified as described by Luft [15]; [^{14}C]sucrose was from New England Nuclear, Boston, MA, U.S.A., and $^{45}\text{CaCl}_2$ was from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals used were of the highest purity commercially available.

Isolation of mitochondria

Liver mitochondria from female Wistar rats (180–200 g, starved overnight) were isolated by differential centrifugation [16]. Mitochondria were washed twice in a buffer containing 210 mM-mannitol, 70 mM-sucrose and 5 mM- K^+ -Hepes (Na^+ -Hepes when K^+ was measured), pH 7.4 (MSH buffer). After isolation, the mitochondria were kept on ice at a protein concentration of 80–100 mg/ml (determined by the biuret method with BSA as standard), and used within 3–5 h after preparation.

Standard incubation procedure

Mitochondria (2 mg of protein/ml) were incubated at 25 °C in 3 ml of MSH buffer containing 5 mM-rotenone and 2.5 mM-potassium succinate (sodium succinate when K^+ was measured) with continuous stirring and oxygenation. Ca^{2+} was added to give a total load of 40 nmol/mg of mitochondrial protein, and Ca^{2+} uptake was allowed to proceed for 2 min. Finally, EGTA, Ruthenium Red and tbh were added at zero time as indicated in the Figure legends.

Determination of Ca^{2+} uptake and release by mitochondria

Ca^{2+} movements across the mitochondrial inner membrane were monitored by the radioisotope technique [17]. Mitochondria were incubated by the standard procedure in the presence of

Abbreviations used: $\Delta\Psi$, electrical potential (negative inside) across the mitochondrial inner membrane; MSH buffer, 210 mM-mannitol/70 mM-sucrose/5 mM-Hepes, pH 7.4; tbh, t-butyl hydroperoxide.

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$^{45}\text{Ca}^{2+}$ (2000 d.p.m./nmol). Samples (150 μl) were withdrawn at the times indicated in the Figures, filtered through Millipore filters (0.45 μm pore size) and rinsed with $2 \times 150 \mu\text{l}$ of cold MSH buffer containing 5 mM-EGTA. The radioactivity remaining on the filters was determined in a liquid-scintillation counter.

Determination of sucrose entry into mitochondria

Sucrose entry into mitochondria was monitored by the radio-isotope technique in combination with filtration as described above. Mitochondria were incubated in accordance with the standard procedure in the presence of [^{14}C]sucrose (0.6 $\mu\text{Ci/ml}$). Samples (150 μl) were withdrawn at the times indicated in the Figures, incubated for 30 s with 5 mM-EGTA, filtered, and processed as described above.

Determination of K^+ release from mitochondria

This was done by the filtration technique. Mitochondria were incubated in accordance with the standard procedure. Samples (150 μl) were withdrawn at the times indicated in the Figures, filtered through Millipore filters (0.45 μm pore size), and the filtrate was collected. The potassium content in the filtrate was determined by flame photometry, with lithium as reference signal.

Measurement of mitochondrial swelling

Swelling of mitochondria was monitored continuously as changes in A_{540} .

RESULTS

Fig. 1 reports experiments in which tbh is added to Ca^{2+} -loaded mitochondria under conditions which allow (absence of EGTA) or prevent (presence of EGTA) re-uptake of Ca^{2+} released by mitochondria. In the absence of EGTA, tbh leads to a loss of Ca^{2+} from mitochondria which ensues after a lag time of about 8 min and soon becomes very rapid (Fig. 1a, trace c). Addition of EGTA to Ca^{2+} -loaded mitochondria (Fig. 1a, traces a and b) results in an immediate loss of Ca^{2+} , which is strongly stimulated by tbh (trace b). No Ca^{2+} is lost in the absence of both EGTA and tbh (results not shown). These results show that in intact rat liver mitochondria tbh stimulates Ca^{2+} release in the presence of EGTA. They also confirm previous reports (reviewed in [5,6,18]) that (i) mitochondria can cycle Ca^{2+} , (ii) the release phase of the cycle is stimulated by pro-oxidants, and (iii) prolonged stimulation of the release phase in Ca^{2+} -cycling mitochondria accelerates Ca^{2+} release.

In light of the recently proposed non-specific permeability changes ('pore' formation) of the mitochondrial inner membrane during Ca^{2+} release, we investigated whether or not release induced by tbh is due to non-specific membrane leakiness. The analysis of sucrose entry into mitochondria has been introduced by Crompton and co-workers [7] as the method of choice to probe for 'pore' formation. Fig. 1(b) shows that tbh added to Ca^{2+} -loaded mitochondria stimulates sucrose entry in the absence (trace c), but not in the presence (trace b), of EGTA. We obtained analogous results when we measured K^+ release from mitochondria (Fig. 1c) as an indicator of non-specific membrane permeability. Thus tbh stimulates K^+ release from Ca^{2+} -loaded mitochondria in the absence (trace c), but not in the presence (trace b), of EGTA. Finally, we monitored swelling of mitochondria (Fig. 1d) as another indicator of non-specific permeability changes of the mitochondrial inner membrane. When tbh is added to Ca^{2+} -loaded mitochondria, they swell in the absence (trace c), but not in the presence (trace b), of EGTA.

Fig. 1. tbh-induced Ca^{2+} and K^+ release from, sucrose entry into, and swelling of rat liver mitochondria in the presence or absence of EGTA

Mitochondria were incubated under standard conditions. Samples were taken for the determination of Ca^{2+} release (a), sucrose entry (b) and K^+ release (c) as described in the Materials and methods section. Swelling (d) was monitored continuously during the incubation. The reaction was started (time 0 min) by addition of 10 mM-EGTA (trace a, $\square$), 100 μM -tbh plus 10 mM-EGTA (trace b, $\circ$), or 100 μM -tbh (trace c, $\triangle$). 100 % Ca^{2+} corresponds to 40 nmol of Ca^{2+} /mg of protein. The results are from one experiment typical of three.

The results reported above show that Ca^{2+} release from mitochondria induced by tbh is not accompanied by non-specific permeability changes, provided that Ca^{2+} re-uptake is prevented

Fig. 2. tbh-induced Ca^{2+} release from, sucrose entry into, and swelling of rat liver mitochondria in the presence of Ruthenium Red

Mitochondria were incubated under standard conditions. Samples were taken for the determination of Ca^{2+} release (a) and sucrose entry (b) as described in the Materials and methods section. Swelling (c) was monitored continuously during the incubation. The reaction was started (time 0 min) by addition of 2 nmol of Ruthenium Red/mg of protein (trace a, $\square$) or 100 μM -tbh plus 2 nmol of Ruthenium Red/mg of protein (trace b, $\circ$). 100% Ca^{2+} corresponds to 40 nmol of Ca^{2+} /mg of protein. The results are from one experiment typical of three. Ca^{2+} fluxes, sucrose entry and swelling in the absence of Ruthenium Red are shown in Fig. 1, traces c.

by EGTA. Similar results were obtained with Ruthenium Red, an inhibitor of the mitochondrial Ca^{2+} -uptake route (Fig. 2). Ruthenium Red added to Ca^{2+} -loaded mitochondria causes loss of Ca^{2+} from the organelles (Fig. 2a, trace a), albeit less effectively than EGTA. Again, tbh provokes a stimulation of Ca^{2+} release (Fig. 2a, trace b). In the presence of Ruthenium Red, both sucrose entry into (Fig. 2b) and swelling of (Fig. 2c) mitochondria are induced by tbh to only a very limited extent.

Fig. 3. Ca^{2+} uptake and release by mitochondria in the presence of Ruthenium Red

Mitochondria were incubated as in Fig. 1 with non-radioactive Ca^{2+} . The reaction was started (time 0 min) by addition of 2 nmol of Ruthenium Red/mg of mitochondrial protein (trace a, $\square$), or 2 nmol of Ruthenium Red/mg of protein plus 100 μM -tbh (trace b, $\circ$), and a trace amount of ${}^{45}\text{Ca}^{2+}$ was added at time $t = 15$ s. The results are from one experiment typical of three.

The observations that (i) Ca^{2+} loss from mitochondria induced by Ruthenium Red is smaller than that induced by EGTA, and (ii) in the presence of Ruthenium Red the tbh-induced sucrose entry and swelling are slightly raised above background values, indicated that Ruthenium Red does not completely prevent re-uptake of released Ca^{2+} by mitochondria. Indeed, when trace amounts of ${}^{45}\text{Ca}^{2+}$ are added to Ca^{2+} -loaded mitochondria in the presence of Ruthenium Red, an accumulation of the radioisotope is observed (Fig. 3). As expected, Ca^{2+} resides in the organelles much longer in the absence (trace a) than in the presence (trace b) of tbh.

Besides tbh, P_i has been suggested to cause 'pore' formation in Ca^{2+} -loaded mitochondria [7–10]. We found that P_i stimulates Ca^{2+} release from, sucrose entry into and swelling of mitochondria only under conditions which allow mitochondria to cycle Ca^{2+} (Figs. 4a–c, trace c). In contrast with tbh, P_i does not stimulate Ca^{2+} release from mitochondria in the presence of EGTA (Fig. 4a, compare traces a and b). Both P_i -stimulated sucrose entry into (Fig. 4b) and P_i -stimulated swelling of mitochondria (Fig. 4c) are almost completely prevented by EGTA. Again, when Ruthenium Red replaces EGTA (Fig. 5), P_i does not evoke Ca^{2+} release, sucrose entry or swelling. Interestingly, Ca^{2+} release is even less extensive in the presence of both Ruthenium Red and P_i (Fig. 5a, trace b) than in the presence of Ruthenium Red alone (Fig. 5a, trace a), possibly owing to a diminution in the intramitochondrial free Ca^{2+} concentration caused by the formation of calcium phosphate precipitates.

DISCUSSION

The nature of the pro-oxidant-stimulated Ca^{2+} efflux route from mitochondria is still a matter of debate. Recent publications have presented evidence that Ca^{2+} can leave mitochondria via an unspecific inner-membrane 'pore', the formation of which is reversible and characterized by leakiness to small ions and proteins and swelling of the organelles [7–13].

Here we show that when mitochondria are allowed to cycle Ca^{2+} (no EGTA or Ruthenium Red present), sucrose enters, K^+ is released, and mitochondria swell. These results are fully consistent with the data of Crompton's group [7–10]. But, when Ca^{2+} cycling is prevented, either by chelating the extramito-

Fig. 4. Phosphate-induced Ca²⁺ release from, sucrose entry into, and swelling of rat liver mitochondria in the presence or absence of EGTA

Mitochondria were incubated under standard conditions. Samples were taken for the determination of Ca²⁺ release (a) and sucrose entry (b) as described in the Materials and methods section. Swelling (c) was monitored continuously during the incubation. The reaction was started (time 0 min) by addition of 10 mM-EGTA (trace a, □), 5 mM-potassium phosphate plus 10 mM-EGTA (trace b, ○), or 5 mM-potassium phosphate (trace c, △). 100% Ca²⁺ corresponds to 40 nmol of Ca²⁺/mg of protein. The results are from one experiment typical of three.

Fig. 5. Phosphate-induced Ca²⁺ release from, sucrose entry into, and swelling of rat liver mitochondria in the presence of Ruthenium Red

Mitochondria were incubated under standard conditions. Samples were taken for determination of Ca²⁺ release (a) and sucrose entry (b) as described in the Materials and methods section. Swelling (c) was monitored continuously during the incubation. The reaction was started (time 0 min) by addition of 2 nmol of Ruthenium Red/mg of protein (trace a, □) or 2 nmol of Ruthenium Red/mg of protein plus 5 mM-potassium phosphate (trace b, ○). 100% Ca²⁺ corresponds to 40 nmol of Ca²⁺/mg of protein. The results are from one experiment typical of three. Ca²⁺ fluxes, sucrose entry and swelling in the absence of Ruthenium Red are shown in Fig. 4, traces c.

chondrial ion with EGTA or by inhibiting Ca²⁺ re-uptake with Ruthenium Red, sucrose entry into, K⁺ release from and swelling of mitochondria are essentially eliminated, yet tbh-induced Ca²⁺ release occurs. The minor fluxes of sucrose and K⁺ and minor volume changes observed in the presence of Ruthenium Red are accounted for by incomplete inhibition of Ca²⁺ cycling by this compound (cf. Fig. 3).

Since addition of EGTA to tbh- or P_i-challenged mitochondria led to a restoration of ΔΨ and an entrapment of sucrose in mitochondria, it was suggested by previous investigators that EGTA induces 'pore' closure in Ca²⁺-loaded mitochondria, but release of Ca²⁺ from mitochondria in the presence of EGTA was not analysed [7-13]. Our finding that in the presence of EGTA

mitochondria do release Ca²⁺ and that the release is strongly stimulated by tbh is incompatible with the postulate that tbh-induced Ca²⁺ efflux requires a mitochondrial 'pore'.

There are two possible interpretations of our results.

(i) In the absence of EGTA or Ruthenium Red, the incubation of mitochondria with Ca²⁺ plus tbh or P_i leads to the opening of an unspecific 'pore' which can be closed by EGTA ('pore' model). Since we have shown that Ca²⁺ can leave mitochondria also in the presence of EGTA, i.e. when the putative 'pore' is closed (no sucrose entry, no K⁺ efflux, no swelling of the mitochondria), a separate release pathway for Ca²⁺ must exist, which is Ca²⁺-selective, pro-oxidant-dependent, and clearly different from the 'pore'.

(ii) Since the 'pore' model cannot explain the effect of Ruthenium Red, and since EGTA and Ruthenium Red both inhibit Ca^{2+} cycling, there is a more likely interpretation of our results: Ca^{2+} efflux from mitochondria occurs via a Ca^{2+} -selective pro-oxidant-dependent release pathway. The released Ca^{2+} can be taken up again by the mitochondria (Ca^{2+} cycling). Excessive Ca^{2+} cycling is well documented to damage mitochondria [1,14]. We therefore suggest that EGTA or Ruthenium Red prevents the non-specific permeability transition of the mitochondrial inner membrane by arresting Ca^{2+} cycling, and that the observed solute fluxes and volume changes are secondary to it. Whether non-specific permeability changes are reversible, as proposed in the 'pore' model [7–13], or reflect an irreversible breakdown of the mitochondrial inner membrane is probably determined by the duration of Ca^{2+} cycling, as shown previously by $\Delta\Psi$ measurements [19].

Interestingly, P_i induces Ca^{2+} release from, sucrose entry into and swelling of mitochondria only under cycling conditions, i.e. in the absence, but not in the presence, of EGTA or Ruthenium Red (Figs. 4 and 5). Elucidation of the mechanism by which P_i stimulates Ca^{2+} efflux requires further investigations.

We conclude from our results that (i) the tbh-induced Ca^{2+} efflux from rat liver mitochondria does not require a non-specific permeability transition of the mitochondrial inner membrane, (ii) the tbh-induced Ca^{2+} efflux occurs via a Ca^{2+} -selective pro-oxidant-dependent release pathway, and (iii) P_i acts via a pathway which is different from the tbh-stimulated Ca^{2+} release route.

Our present results accord with and extend previous findings of our own and of other groups (reviewed in [6,18]). For example, mitochondrial swelling occurs after rather than before or concomitant with Ca^{2+} efflux [20–23]. Moreover, $\Delta\Psi$, a reliable criterion for the intactness of mitochondria [24], is not lowered on addition of tbh to Ca^{2+} -loaded mitochondria when Ca^{2+} cycling is prevented [19,21,24,25]. Finally, the existence of specific inhibitors such as ATP [26], cyclosporine A [27], 4-hydroxynonenal [28], and *m*-iodobenzylguanidine [29] of the pro-oxidant-induced and 'spontaneous' Ca^{2+} release from mitochondria leaves no doubt about the intactness of mitochondria during the pro-oxidant-induced Ca^{2+} release. Therefore the eventually observed damage to mitochondria is the consequence rather than the cause of Ca^{2+} release.

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