Opening of the mitochondrial permeability transition pore by uncoupling or inorganic phosphate in the presence of Ca^{2+} is dependent on mitochondrial-generated reactive oxygen species

Alicia J. Kowaltowski, Roger F. Castilho, Anibal E. Vercesi*

Departamento de Bioquímica, I.B., UNICAMP, C.P. 6109, CEP 13084-100, Campinas, SP, Brasil

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Abstract In this study, we show that mitochondrial membrane permeability transition in Ca^{2+}-loaded mitochondria treated with carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) or inorganic phosphate (P_i) is preceded by enhanced production of H_2O_2. This production is inhibited either by ethylene glycol-bis(*b*-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) or Mg^{2+}, but not by cyclosporin A. Permeability transition is prevented either by EGTA, catalase or dithiothreitol, suggesting the involvement of Ca^{2+}, H_2O_2 and oxidation of membrane protein thiols in this mechanism. When mitochondria are incubated under anaerobiosis, no permeabilization or H_2O_2 production occurs. Based on these results we conclude that mitochondrial permeability transition induced by P_i or FCCP-uncoupling is dependent on mitochondrial-generated reactive oxygen species.

Key words: Mitochondrial permeability transition pore; Calcium; Reactive oxygen species

1. Introduction

It has long been known that mitochondrial Ca^{2+} overload leads to the state of mitochondrial membrane permeability transition [1,2], a situation associated with the opening of a mitochondrial membrane permeability transition pore (PTP) [2,3]. PTP opening can be enhanced by various agents (inducers) which include prooxidants, thiol cross-linking reagents, inorganic phosphate (P_i) and uncouplers (for reviews see refs. [2,3]).

Studies from our [4–6] and other laboratories [7–10] demonstrated that mitochondrial permeability transition induced by prooxidants such as *t*-butyl hydroperoxide and diamide was triggered by Ca^{2+}-stimulated production of reactive oxygen species (ROS) [5] which accumulated due to the exhaustion of mitochondrial antioxidants GSH and NAD(P)H [2,5,11]. The ROS attack to membrane protein thiols produces cross-linkage reactions, that may open membrane pores upon Ca^{2+} binding [4,12,13]. Mitochondrial permeabilization induced by thiol cross-linkers would be independent of ROS, due to cross-linkage promoted directly by the inducer [5,13].

*Corresponding author. Fax: (55) (192) 393124.

Abbreviations: BHT, butylhydroxytoluene; CsA, cyclosporin A; DTT, dithiothreitol; EGTA, ethyleneglycolbis(*b*-aminoethyl-ether)-*N,N,N',N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HRP, horseradish peroxidase; PTP, mitochondrial permeability transition pore; P_i, inorganic phosphate; RLM, rat liver mitochondria; ROS, reactive oxygen species; RR, ruthenium red.

The mechanisms by which P_i or uncouplers induce PTP formation remain poorly understood. The experiments presented in this manuscript were designed to ascertain whether mitochondrial-generated ROS were also involved in permeability transition induced by Ca^{2+} plus inorganic phosphate (P_i) or the uncoupler FCCP, agents frequently used as inducers of PTP opening (see refs. [2,3,14–18]).

2. Experimental procedures

2.1. Isolation of rat liver mitochondria

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar strain rats fasted overnight. These mitochondria contain 8–10 nmol/mg endogenous calcium as determined by atomic absorption spectroscopy.

2.2. Standard incubation procedure

The experiments were carried out at 30°C in a K^+-free reaction medium containing 250 mM sucrose, 10 mM Hepes-Na^+ buffer pH 7.2, 2 mM succinate, 5.0 μM rotenone and 10 μM Ca^{2+} or a standard reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes-K^+ buffer pH 7.2, 2 mM succinate, 5.0 μM rotenone and 10 μM Ca^{2+}. Other additions are indicated in the figure legends. The results shown are representative of a series of at least three experiments.

2.3. Determination of mitochondrial swelling

Mitochondrial swelling was estimated from the decrease in the absorbance at 520 nm measured in a SLM Aminco DW2000 spectrophotometer.

2.4. Oxygen uptake measurements

Oxygen concentration was measured using a Clark-type electrode (Yellow Springs Instruments Co.). The initial oxygen concentration in the reaction medium was 225 nmol/ml and was decreased by N_2 purging in anoxic incubations. The cuvette was closed immediately before starting the experiments.

2.5. Determination of H_2O_2 production

The production of H_2O_2 was determined by the horseradish peroxidase (HRP) method, as described in ref. [19]. All experiments were conducted in the presence of cyclosporin A in order to prevent artifacts secondary to mitochondrial swelling. The calibrations were performed by sequential additions of known concentrations of H_2O_2. Control experiments show that ruthenium red does not act as an electron donor to the HRP–H_2O_2 complex in our conditions.

3. Results

Bernardi and co-workers [15–17] have demonstrated that Ca^{2+}-loaded rat liver mitochondria (RLM) suspended in sucrose medium and energized by succinate oxidation undergo extensive swelling due to PTP opening when treated with FCCP after the addition of ruthenium red. Fig. 1A shows that FCCP-induced swelling of Ca^{2+}-loaded mitochondria (line f) is strongly inhibited by EGTA (line a), cyclosporin A (CsA, line

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b), dithiothreitol (DTT, line c), and catalase (line d), but not by butylhydroxytoluene (BHT, line e), an inhibitor of lipid peroxidation. Fig. 1B (lines a–f) shows that similar results were obtained when mitochondrial swelling was induced by P_i addition. The effect of CsA supports the idea that mitochondrial membrane permeabilization under these experimental conditions is associated with formation of the mitochondrial membrane permeability transition pore (PTP). The protection conferred by catalase suggests the participation of ROS in this mechanism. Catalase inactivated by boiling during 10 min, or the same protein concentration of bovine serum albumin, did not cause inhibition of mitochondrial swelling.

The mitochondrial production of H_2O_2 was then monitored under the experimental conditions of Fig. 1, in order to determine whether mitochondrial-generated ROS are, indeed, involved in the mechanism of PTP opening. Fig. 2 shows that the addition of FCCP (panel A) or P_i (panel B) to a mitochondrial suspension causes a burst of H_2O_2 production (lines a), which precedes the swelling observed in Fig. 1. This production does not occur if Ca^{2+} accumulation is prevented by the addition of ruthenium red (RR), at the beginning of the experiment (dashed lines), or if Ca^{2+} is progressively decreased by EGTA (2.5–10 μM, lines b–e), that diminishes the amount of Ca^{2+} taken up by mitochondria. Mitochondrial H_2O_2 production was also inhibited by Mg^{2+}, a known inhibitor of pore opening [2], in a dose-dependent manner (results not shown).

In order to ascertain whether opening of the FCCP or P_i-induced permeability transition pore is actually dependent on the presence of ROS, experiments were performed in the absence of molecular oxygen, a condition in which ROS cannot be formed. Previous results from our group [5] have indicated that the extent of mitochondrial swelling induced by Ca^{2+} plus *t*-butyl hydroperoxide decreases by decreasing molecular oxygen in the reaction medium. Under anaerobiosis no swelling occurs. In Fig. 3, Ca^{2+} uptake was driven by ATP-generated membrane potential ($\Delta\psi$) in both aerobic (lines b) and anaerobic (lines a) conditions. Antimycin A was present (lines a and

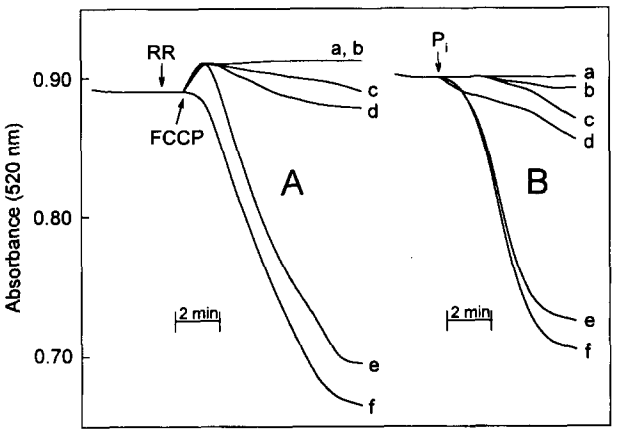

Fig. 1. Mitochondrial swelling induced by FCCP-uncoupling in the presence of RR (panel A) or P_i (panel B): effect of EGTA, Cyclosporin A (CsA), dithiothreitol (DTT), catalase and butylhydroxytoluene (BHT). Rat liver mitochondria (RLM, 0.5 mg/ml) were added to K^+-free (A) or standard reaction medium (B) in the presence of (a) 1 mM EGTA, (b) 1 μM CsA, (c) 2 mM DTT, (d) 2 μM catalase, (e) 5 μM BHT or (f) no additions. RR (1 μM), 1 μM FCCP and 1 mM P_i were added where indicated.

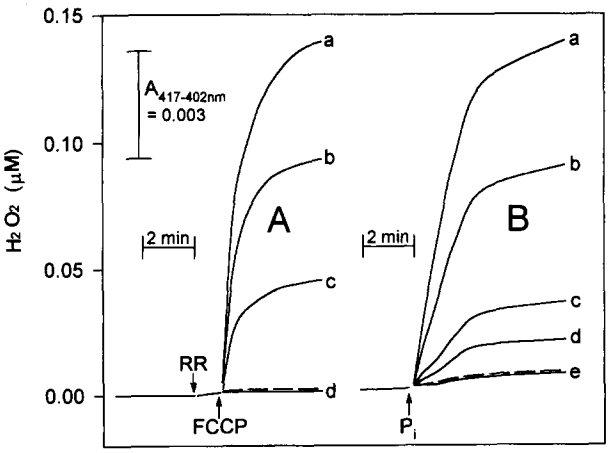

Fig. 2. Mitochondrial Ca^{2+}-dependent H_2O_2 production induced by FCCP-uncoupling in the presence of RR (panel A) or P_i (panel B): effect of EGTA and RR. RLM (0.5 mg/ml) were added to K^+-free (A) or standard reaction medium (B) in the presence of 1 μM CsA, 1 μM HRP and (a) no additions, (b) 2.5 μM EGTA, (c) 5.0 μM EGTA, (d) 7.5 μM EGTA, (e) 10 μM EGTA or (dashed lines) 1 μM RR. RR (1 μM), 1 μM FCCP and 1 mM P_i were added where indicated.

b) to prevent respiration under the conditions of lines b. $CaCl_2$ (100 μM) was used in order to give a free Ca^{2+} concentration of 25 μM in the presence of 800 μM ATP [20]. No PTP opening was observed in the absence of molecular oxygen (lines a) as evidenced by the lack of mitochondrial swelling. In the presence of molecular oxygen (lines b) extensive swelling was observed.

4. Discussion

Extensive studies have been conducted on inducers, minimal requirements for opening and reversibility characteristics of the PTP (see refs. [2,3,14–18]), but little is known about the nature and mechanism of pore formation that lead to membrane permeability transition. Previous results [4–6,12] from our laboratory suggest that mitochondrial membrane permeabilization by Ca^{2+} and *t*-butyl hydroperoxide is mediated by the oxidative attack of mitochondrial-generated ROS to membrane protein thiol groups. Other results from this laboratory indicate that intramitochondrial Ca^{2+} potentiates membrane permeabilization induced by ROS generated from 5-aminolevulinic acid oxidation [21].

In this work we have examined whether mitochondrial ROS are also involved in the mechanism of PTP opening when Ca^{2+}-loaded rat liver mitochondria were treated with FCCP or P_i, agents that do not induce situations of oxidative stress per se. The experiments shown here provided unequivocal evidence that the addition of both P_i or FCCP caused a burst of H_2O_2 production that preceded membrane permeabilization. Under the conditions in which H_2O_2 accumulation was prevented by the presence of catalase, EGTA (Fig. 1), or absence of molecular oxygen (Fig. 3), membrane permeabilization did not occur, thus supporting the notion that ROS accumulation is a key step in the mechanism of PTP formation induced by P_i or FCCP. The protection obtained in the presence of the disulfide reductant DTT indicates that both P_i and FCCP-induced PTP opening are dependent on protein thiol oxidation. It must be stressed that ROS participation in the process of permeability transition

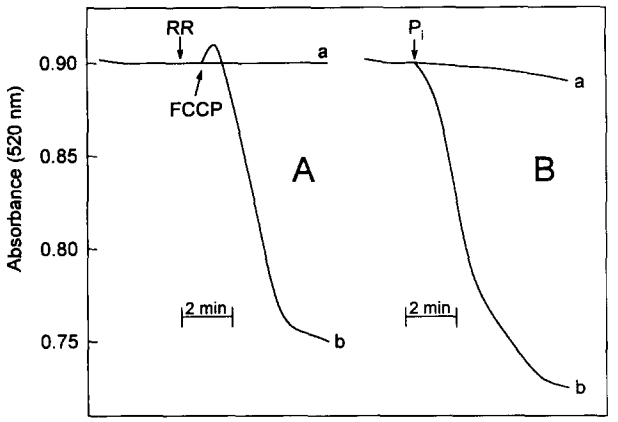

Fig. 3. Mitochondrial swelling induced by FCCP-uncoupling in the presence of RR (A) or P_i (B): role of O_2. RLM (0.5 mg/ml) were added to K^+-free (A) or standard reaction medium (B), in the absence of rotenone and succinate and in the presence of 800 μM ATP, 100 μM Ca^{2+} and 200 nM antimycin A. Lines a: incubation in the absence of molecular oxygen; lines b: incubation in the presence of molecular oxygen. RR (1 μM), 1 μM FCCP and 1 mM P_i were added where indicated. Anoxic incubations were conducted as described in section 2.

has only been demonstrated under oxidative stress conditions induced by prooxidants [4–6] or ROS generating systems [10,21].

Regarding the mechanisms underlying uncoupler-induced mitochondrial permeability transition, in 1987 Vercesi [22] demonstrated that at low membrane potentials the NAD(P)H transhydrogenase cannot sustain high levels of mitochondrial reducing power (NADPH and GSH). According to our data [5], this favors Ca^{2+}-induced ROS accumulation, thiol cross-linkage and formation of the PTP. On the other hand, Bernardi's group [15–17] has proposed that mitochondrial membrane potential drop increases the probability of PTP opening due to voltage-sensitive properties of this pore.

Recent results from our laboratory have provided evidence that Ca^{2+} stimulates electron leakage (ROS production) at the level of the reduced and semiquinone forms of coenzyme Q [6,23]. This may be caused by Ca^{2+} binding to cardiolipins with consequent disorganization of the lipid phase [24], altering interactions between electron-transferring molecules and favoring electron donation to oxygen at intermediate steps of the respiratory chain. Indeed, unpublished results from our group (Grijalba, M.T.), using electron paramagnetic resonance, demonstrated that Ca^{2+} induces lateral phase separation in heart submitochondrial particles. In this regard we have also previously shown [25] that the mitochondrial protection conferred by the local anesthetic dibucaine has a competitive character and seems to be mediated by displacement of Ca^{2+} bound to lipids at the internal surface of the inner membrane. It might be possible that alterations of membrane structure induced by Ca^{2+} are potentiated by P_i or mitochondrial uncoupling.

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