The Ca^{2+}-induced pore opening in mitochondria energized by succinate-ferricyanide electron transport

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Received 27 October 1997

Abstract The oxygen dependence of the mitochondria permeability transition pore was under study in non-respiring rat liver mitochondria. Oxygen in the medium was depleted by saturation of the incubation medium with N_2 and spontaneously by mitochondrial respiration followed by the addition of glucose/glucose oxidase. After the anaerobic state had been reached, ferricyanide has been added to support succinate-driven energization in the absence of oxygen. In the other set of the experiments KCN was added to block operation of the respiratory chain under aerobic conditions. Again, ferricyanide was added as an electron acceptor. Superoxide dismutase was added to trap superoxide anion radicals. Under either hypoxic conditions or in the presence of cyanide, calcium ions were shown to induce the permeability transition. The concentration of Ca^{2+} required was lower than under conditions of active respiration. In both cases, the transition was prevented by cyclosporine A.

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Key words: Mitochondrion; Ca^{2+}; Permeability transition; Hypoxia

1. Introduction

Obviously, aerobic life conditions are advantageous. However, in living systems molecular oxygen frequently derives ROS, whose role can be rather negative (reviewed in [1,2]). Mitochondria of eucaryotic cell are known to produce the overwhelming majority of ROS. The ROS production in mitochondria is highly regulated by mitochondrial metabolism. One of the ways to lower mitochondrial ROS production was postulated to be the mitochondrial PTP which is shown to be induced by ROS [3].

Although the mitochondrial permeability transition has been studied for decades, both its mechanism and possible physiological role is still far from being understood (reviewed in [4,5]). There is enough evidence to accept an involvement of PTP in the oxidative stress [6–8]. Recent findings of a critical role of mitochondria in apoptosis have given impetus to new studies [9–12]. The suggestion of an obligatory apoptotic step consisting in the activation of PTP assigns a critical role of mitochondria in apoptotic cascade. Therefore studies of the regulation of PTP are of great importance.

At least traces of Ca^{2+} in the incubation medium are needed for PTP activation. The oxidation of mitochondrial NAD(P)H and GSH pools and crucial SH groups in membrane proteins seem to favor permeabilization [13,14] although there are some exceptions [15,16].

Mitochondrial PTP apparently contributes to the damage induced by a reoxygenation following hypoxia since cyclosporine A diminishes the damage induced by reperfusion after a short-term occlusion of the flow through the perfused organ [17,18]. The trapping of free radicals decreases the probability of damage [19]. Free radicals are involved in PTP activation as shown by the prevention of PTP induction by the radical scavengers [20,21].

Thus, there is a number of indications in favor of studying the role of oxygen in the PTP functioning. However the problem is complicated with the overlapping effect of the change in the values of the mitochondrial membrane potential when the pO_2 around mitochondria is variable. In the living cell under hypoxic conditions, mitochondria maintain the membrane potential through the hydrolysis of ATP [22]. The attempts of studying the role of the oxygen in PTP in ATP-energized anaerobic mitochondria which have been made [23] could not give a comprehensive answer to the question since ATP itself has an inhibitory effect on PTP [24,25]. To bypass such an obstacle it is possible to energize anaerobic mitochondria utilizing ferricyanide as an electron acceptor. This approach has been used in the present study.

2. Materials and methods

2.1. Isolation of mitochondria

Rat liver mitochondria were obtained by a conventional method described elsewhere [26]. The isolation medium contained 0.3 M sucrose, 5 mM HEPES, 500 μM EDTA, pH 7.4 (adjusted by Tris). After spinning at $8000 \times g$, the mitochondrial pellet was washed in an EDTA-free medium. The incubation medium contained 0.3 M sucrose, 5 mM HEPES, 5 mM succinate, 1 μM rotenone, 2 mM K_2HPO_4, 3 μM tetraphenylphosphonium chloride, pH 7.4 (adjusted by Tris). The content of Ca^{2+} in the medium did not exceed 3 μM. Mitochondrial concentration was 1 mg protein/ml, pH 7.4 (adjusted by Tris).

2.2. Mitochondrial parameters

Four mitochondrial functions were recorded simultaneously in a special multichannel unit with a volume of 1.5 ml: oxygen consumption with a platinum Clark-type closed electrode; calcium ions with a Ca^{2+}-selective electrode; mitochondrial membrane potential with a tetraphenylphosphonium$^+$-selective electrode and mitochondrial swelling as a function of light scattering at 660 nm.

2.3. Hypoxic conditions

The incubation medium was saturated with N_2, which resulted in lowering of oxygen in a medium from 480 to 50 ng atoms/ml. Further oxygen exhaustion from the medium was spontaneous due to mitochondrial respiration and subsequent addition of glucose and glucose oxidase (5 mM and 300 units/ml respectively). For trapping of superoxide anion radicals, superoxide dismutase was added (500 units/ml). After the anaerobic state has been reached, mitochondria were energized by potassium ferricyanide (500 μM).

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Abbreviations: ROS, reactive oxygen species; PTP, permeability transition pore

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PII S0014-5793(97)01450-6

2.4. Cytochromes

Redox state of cytochromes *c* and $a+a_3$ were recorded by Aminco-DW 2000 spectrophotometer as an absorbance difference at 550/575 nm and 605/630 nm correspondingly.

2.5. Data analysis

All traces were scanned from chart papers of recorders (two double channel recorders), digitized and plotted in a single graph. The traces on the figures are representative records.

3. Results

3.1. Mitochondrial energization induced under hypoxia by the succinate and ferricyanide supplement

Under anaerobic conditions (with glucose/glucose oxidase) both cytochromes *c* and $a+a_3$ were reduced (Fig. 1A, B). However, the addition of ferricyanide resulted in the fast and complete oxidation of both cytochromes. This oxidized state lasted for a few minutes and was followed by the cytochrome reduction due to the exhaustion of ferricyanide (Fig. 1), as evidenced by the reoxidation produced by the subsequent addition of ferricyanide (not shown).

Fig. 2 represents read-outs of one of three channels (Ca^{2+} flux) as a response of isolated rat liver mitochondria to added Ca^{2+} under three different conditions, namely (A) respiration, (B) ferricyanide reduction by succinate in the presence of 1 mM KCN and (C) ferricyanide reduction by succinate under anaerobiosis. In all three cases, PTP opening was observed. Under conditions of active respiration, even 20 μM Ca^{2+} was not enough to obtain maximal PTP opening. Ferricyanide added to aerobic mitochondria didn't change the Ca^{2+} sensitivity of the PTP. On the other hand, in cyanide-inhibited or anaerobic mitochondria 5–10 μM Ca^{2+} gave the maximal response.

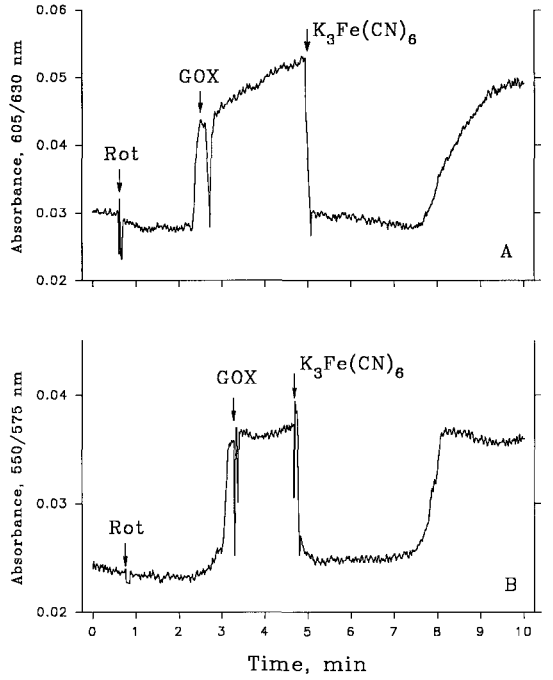


Fig. 1. The changes in the differential spectra of cytochromes $a+a_3$ (A) and *c* (B) state changes under anaerobic conditions and following energization with potassium ferricyanide. Rotenone, 1 μM (ROT), glucose oxidase 300 units/ml (GOX) and potassium ferricyanide, 500 μM were added as shown. Glucose, 5 mM and superoxide dismutase, 500 units/ml are in the incubation media.

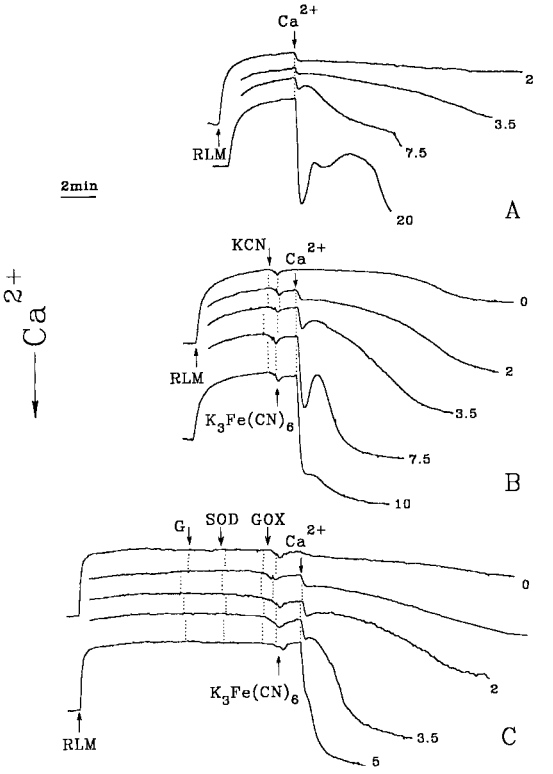


Fig. 2. Changes in Ca^{2+} distribution between rat liver mitochondria (RLM) and incubation medium recorded by the Ca^{2+}-selective electrode. A: Normoxic conditions; B: normoxic conditions with 1 mM KCN; C: anaerobic conditions (see Section 2). Numbers at the curves, Ca^{2+} concentrations (μM). Glucose, 5 mM (G), glucose oxidase, 300 units/ml (GOX), and superoxide dismutase, 500 units/ml (SOD) and 500 μM $K_3Fe(CN)_6$ were added as shown.

The observed mitochondrial changes (the collapse of the membrane potential, release of Ca^{2+} and high amplitude swelling) are more obvious in a multiplot graph of all four mitochondrial parameters at a single Ca^{2+} concentration. Fig. 3 gives such an example and summarizes the ferricyanide-induced mitochondrial sensitizing to the deleterious effect of Ca^{2+}. In this figure, the effect of added 3 μM Ca^{2+} on the measured mitochondrial parameters is shown under the three conditions mentioned above.

3.2. Effect of a radical scavenging

Ionol (buthylhydroxytoluene) commonly used as free radical scavenger (although there were data, demonstrating its side effect [27]) prevented the induction of PTP either under respiratory conditions (Fig. 4A), or hypoxic conditions reached by the removal of the oxygen by respiring mitochondria in the presence of ferricyanide without glucose-glucose oxidase system (not shown). However under deep anaerobiosis reached by the addition of glucose/glucose oxidase, Ca^{2+} induced the collapse of mitochondrial membrane potential (Fig. 4B), the Ca^{2+} release and the high amplitude swelling (not shown) could not be prevented by ionol. Since all of these Ca^{2+}-induced events are blocked by cyclosporine A, they can be ascribed to PTP (Fig. 4). Note that superoxide dismutase did not prevent the induction of PTP (Fig. 4).

4. Discussion

This study demonstrates the ability of mitochondria to undergo the permeability transitions under conditions of very low oxygen. It arises the question on the role of ROS in PTP.

One of the results of this study is the confirmation that respiratory seizure sensitizes mitochondria to the action of Ca^{2+}. Respiration impairment increases the sensitivity of the activation to Ca^{2+}, so lower concentrations are capable of activating the permeability transitions. A similar conclusion is relevant to hypoxic conditions within physiological range of oxygen, when deleterious effect of Ca^{2+} on mitochondrial metabolism is beginning to be severe. Similar data on the oxygen dependence of the PTP opening received in another model of the pore induction were presented recently [28].

Seeking an explanation for the mitochondria pore opening at low oxygen, the redox states of the respiratory electron carriers were measured. The mitochondrial energization with potassium ferricyanide resulted in the complete oxidation of cytochromes *c* and $a+a_3$. Therefore the induction of PTP under hypoxic conditions in the presence of ferricyanide may result from the highly oxidized state of the respiratory chain. This state would entail the oxidation of nicotinamide adenine dinucleotide pool, with the corresponding oxidation of vicinal thiol groups and glutathione, all known to promote permeability transitions [13,14,29]. The lower capacity to activate PTP when energized with ATP may be due to the inhibitory effect of adenine nucleotides on the permeability transitions. As for the oxygen radicals, they may represent a kind of numerous PTP inducers rather than obligatory intermediates in chain of events resulting in the PTP opening. However, if ROS are still the active members in the PTP cascade, the step(s) with ROS participation should precede the oxidation of the electron transfer carriers, since this step can be bypassed by the direct oxidation of the respiratory chain components what has been observed in this study.

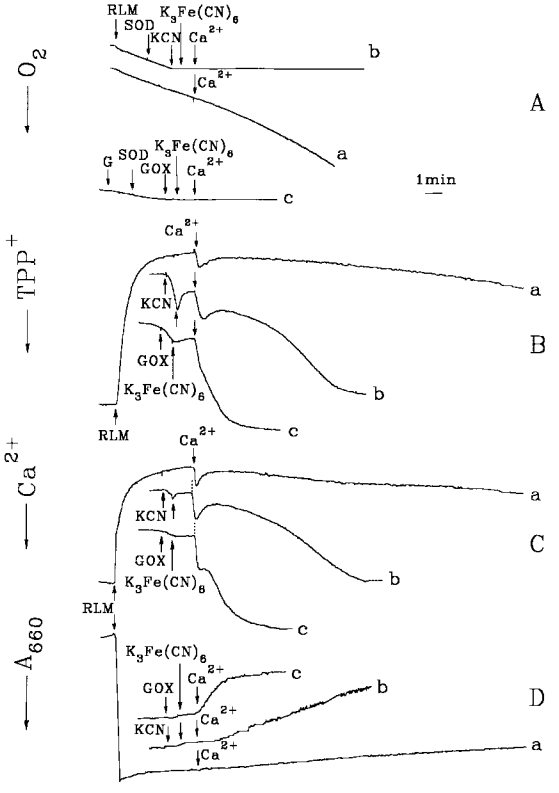


Fig. 3. The effect of 3 μM Ca^{2+} on the mitochondrial functioning under normoxic (a), normoxic, in the presence of 1 mM KCN (b) and anaerobic (c) conditions. A: Oxygen consumption; B: the membrane potential-dependent distribution of tertraphenylphosphonium (TPP^+); C: Ca^{2+} fluxes; D: light scattering (the mitochondrial volume changes). For additions, see Fig. 2. In B, C, and D, glucose and SOD were added before GOX (not shown).

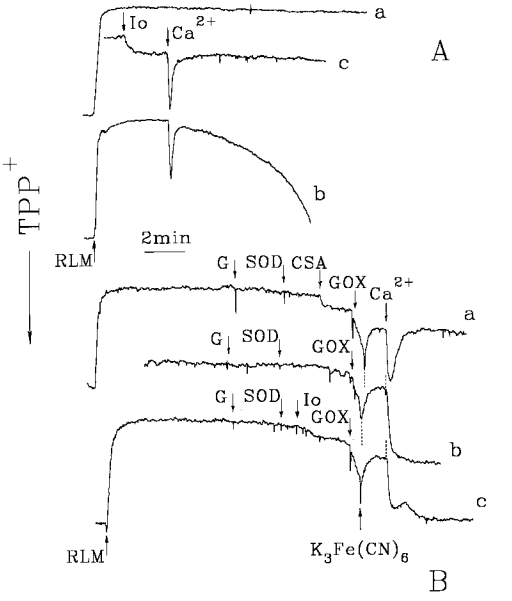


Fig. 4. The effect of ionol on the permeability transition in rat liver mitochondria under normoxic and anaerobic conditions. A: Normoxic conditions; B: anaerobic conditions. Additions, 50 μM ionol (Io), 4.5 μM Ca^{2+}, other additions as in Fig. 2. In a, the mixture was supplemented with 5×10^{-7} cyclosporine A (CSA).

Acknowledgements: Authors thank K.W. Kinnally for valid criticism and help. This work was supported by grants from Russian Foundation of Basic Research (96-04-49384 and 96-04-50940) and Volkswagen Stiftung.

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