The permeability transition pore induced under anaerobic conditions in mitochondria energized with ATP

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Abstract The role of oxygen in the induction of mitochondrial permeability transitions was studied. Oxygen consumption, swelling, membrane potential and calcium transport were recorded simultaneously in isolated rat liver mitochondria. Oxygen depletion was accomplished by saturating the medium with N_2 and allowing either mitochondrial respiration or glucose/glucose oxidase to consume the residual oxygen. Upon anaerobiosis, mitochondria were supplemented with 500 μM ATP to support succinate-driven membrane potential. Under these conditions, 100 μM Ca^{2+} induced cyclosporin A-sensitive permeability transitions. To eliminate the possible inhibition of permeability transition by high concentrations of adenine nucleotides, anaerobic mitochondria were also energized by the combination of 20 μM ADP and phosphoenolpyruvate/pyruvate kinase. These mitochondria also underwent Ca^{2+}-induced permeability transition. Under both of these conditions, namely the addition of ATP as a single or through actions of pyruvate kinase, the respiratory components were totally reduced. Thus, oxygen is not a necessary factor for mitochondria to undergo permeability transitions.

Key words: Mitochondrion; Permeability transition; Oxygen; Hypoxia

1. Introduction

The oxygen dependence of cellular functions has been studied for many years and these studies clearly indicate that aerobic life confers an evolutionary advantage for multicellular organisms. However, the advantages of aerobic metabolism are offset to some extent by the concomitant production of free radical species. The concept 'friend and foe' [1–4] reflects the dual role of oxygen. For example, leakage of electrons from the mitochondrial respiratory chain can generate a number of highly reactive oxygen species (ROS) that can oxidatively damage a variety of important intracellular targets. Cells contain extensive defensive machinery to ameliorate the harmful effects of ROS [5–7]. It has also been suggested that ROS also act as intracellular messengers [8–12], although generally as regulators of their own expression levels. Nonetheless, ROS are expected to contribute significantly to cellular oxidative stress [13–15].

Oxidative stress is thought to affect many intracellular compartments, in particular the mitochondria. Moreover, a number of studies indicate that the mitochondrial permeability transition pore (PTP) is involved in the cellular response to oxidative stress [16–19]. The PTP (also referred to as the mitochondrial megachannel) is thought to be responsible for a rapid increase in mitochondrial permeability that is sensitive to cyclosporin A (reviewed in [20,21]). Since the induction of mitochondrial PTP can be prevented by antioxidants such as catalase and free radical scavengers, it has been suggested that, under aerobic conditions, PTP depends on mitochondrially derived ROS [22–24], implying that oxygen is an obligatory requirement for PTP activation. This speculation is also consistent with the proposed involvement of both PTP and ROS in apoptosis.

Simple approaches for studying the oxygen dependence of PTP activation in isolated mitochondria do not appear to reflect the effects of hypoxia in situ. Under hypoxic conditions, mitochondria unable to maintain the membrane potential by oxidation can do so through the hydrolysis of ATP [25]. Although it is beyond the scope of this study, it should be noted that this observation illustrates the importance of the mitochondrial membrane potential in cell functioning. Furthermore, this observation supports the hypothesis that membrane potential instability may be responsible for the propagation of the apoptotic signal [26] and suggests that an examination of the oxygen dependence of PTP activation, while maintaining the membrane potential, is warranted.

Previously, we demonstrated that PTP can be induced by Ca^{2+} in anaerobic mitochondria energized by ferricyanide [27]. Since ferricyanide oxidizes mitochondrial respiratory components, two factors (lack of oxygen and respiratory chain oxidation) could contribute to the activation of PTP, complicating the interpretation of these experiments. The physiological relevance of using ferricyanide to energize mitochondria is also questionable. ATP is a more physiologically appropriate means to energize mitochondria and was used in the current studies to investigate the role of oxygen in the activation of PTP. In the present study, ATP was added either at a single concentration of 500 μM or continuously, at low concentrations, using 20 μM ADP and the regenerating system of phosphoenolpyruvate/pyruvate kinase. The steady-state level of adenine nucleotides in the latter case is sufficiently low so as not to interfere with PTP activation.

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Abbreviations: PTP, permeability transition pore; ROS, reactive oxygen species; PEP, phosphoenolpyruvate; PK, pyruvate kinase

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2. Materials and methods

2.1. Isolation of mitochondria

Rat liver mitochondria were obtained by a conventional method described elsewhere [28]. The isolation medium contained 0.3 M sucrose, 5 mM HEPES, 500 μM EDTA, pH 7.4 (adjusted with Tris). After centrifugation at 8000×*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, pH 7.4 (adjusted with 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. The time constants for Ca^{2+}-selective and tetraphenylphosphonium+-selective electrodes are approximately 1 s, obviating the detection of kinetic events in the second time scale.

2.3. Hypoxic conditions

The incubation medium was saturated with N_2, which resulted in lowering of oxygen in the medium from 480 to 50 ng atoms/ml. The remaining oxygen was eliminated either spontaneously by mitochondrial respiration or in some cases by the addition of glucose/glucose oxidase (5 mM and 300 units/ml respectively). The use of mitochondrial respiration or glucose/glucose oxidase to remove residual oxygen caused no differences between mitochondria prepared by either technique, in their basal or calcium-activated states. After the anaerobic state had been reached, mitochondria were energized either by ATP (500 μM) or by the phosphoenolpyruvate (5 mM)/pyruvate kinase (2 units)/ADP (20 μM) system.

2.4. Data analysis

All traces were scanned from chart papers of the recorders (two double channel recorders), digitized and plotted as single graphs. More than 20 experiments on the effect of hypoxic conditions on the mitochondrial functioning were carried out. The traces presented in the figures are the random choice of a single experiment where all parameters were measured. The very complicated kinetic behavior of the parameters measured made a careful statistical analysis impossible. All experiments exhibited the effects shown in the figures, however, the actual magnitude of the effects differed between experiments depending on the mitochondrial preparation.

2.5. Cytochromes

Redox state of cytochromes *c* and $a+a_3$ was recorded as the absorbance differences at 550/575 nm and 605/630 nm, respectively, using an Aminco-DW 2000 spectrophotometer.

3. Results

3.1. ATP-induced mitochondrial energization

In the presence of ATP, the Ca^{2+} activation of the mitochondrial pore was less marked than in aerobic conditions. For example, the addition of 50 μM Ca^{2+} had no effects on the membrane potential, mitochondrial calcium content and volume. The addition of a further 50 μM Ca^{2+} resulted in the induction of changes attributable to PTP activation (Fig. 1a). The same effects were observed with the single addition of 100 μM Ca^{2+} (Fig. 1b).

3.2. PEP/PK/ADP-induced mitochondrial energization

When mitochondria were supplemented with the 20 μM ADP/PEP/PK system, ATP was generated at a constant rate resulting in no more than 20 μM which was below the concentration at which ATP inhibits PTP. Mitochondria in this system were more sensitive to the effect of calcium on PTP activation, than when energized with the single addition of ATP. The addition of 100 μM Ca^{2+} resulted in very rapid changes of mitochondrial parameters, indicative of a rapid opening of the PTP (Fig. 2b, compare with Fig. 1a). Paradoxically, 50 μM Ca^{2+} produced different effects in the behavior of the calcium flux and membrane potential and mitochondrial swelling (Fig. 2a). In all cases Ca^{2+}-induced changes were prevented by 1 μM cyclosporin A (not shown).

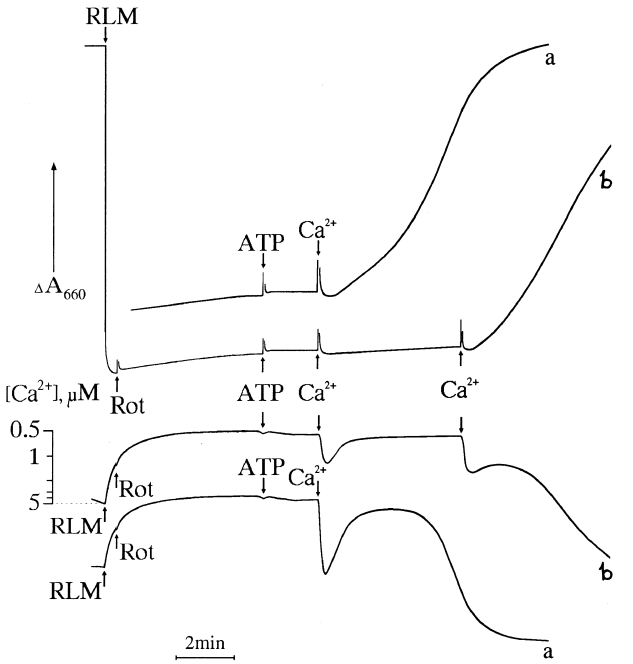

Fig. 1. The effect of energization of anaerobic rat liver mitochondria (RLM) with 500 μM ATP on the Ca^{2+}-induced changes in mitochondrial volume (upper pair of curves) and extramitochondrial Ca^{2+} (lower pair of curves). a and b: Single and succesive additions of 50 μM Ca^{2+} respectively. Additions shown by arrows. Rot, rotenone. Incubation medium as in Section 2.

3.3. Redox state of the respiratory chain

To test the redox state of the respiratory chain components, cytochrome *c* redox state was monitored as the absorbance difference at 550 and 575 nm. Fig. 3 demonstrates that energization of anaerobic mitochondria with either high ATP (Fig. 3a) or low ADP with PEP/PK maintains cytochrome *c* in a reduced state (Fig. 3b). In contrast, ferricyanide completely oxidizes mitochondrial cytochrome *c* under anaerobic conditions [27]. The redox status of cytochrome oxidase was monitored as the absorbance difference at 605 and 630 nm and indicated that this protein was completely reduced under anaerobic conditions and in the presence of adenine nucleotides, as used in the current studies (not shown).

4. Discussion

The ability of isolated mitochondria to open the non-specific megachannel or PTP in their inner membrane [29–31] has been the focus of a great number of studies (reviewed in [20]). But despite this, the functional role of PTP remains obscure. Recent data on the unique role of mitochondrial PTP in programmed cell death [32–35] have stimulated extensive studies of the mitochondrial permeability transitions. As a defense against ROS [5], PTP can regulate the extent of oxidative

damage of all intracellular targets. This takes place through the regulation of the respiration rate and thus the production of ROS. This model assigns an important role to oxygen in the induction of PTP. Although a great number of PTP inducers exist, a strict requirement of Ca^{2+} for all kinds of PTP activation is widely recognized. To study the oxygen dependence of PTP, we used calcium in the presence of inorganic phosphate, to activate PTP. The sensitivity of PTP activation to cyclosporin A was used to confirm its involvement in the observed mitochondrial permeability transitions.

Our data indicate that PTP can be induced in mitochondria under conditions of very low oxygen (lower than 10^{-6} M). Previous studies of PTP activation under low oxygen tension have produced conflicting results, possibly due to the diversity of PTP activators used in these studies [20,36,37]. We have already demonstrated PTP induction in anaerobic mitochondria energized with ferricyanide [27]. The sensitization of mitochondria to the effect of Ca^{2+} under these conditions might be due to highly oxidized respiratory chain components which are known to facilitate PTP opening [38]. In the present study, we removed the contribution of the redox state of respiratory components by energization of anaerobic mitochondria with ATP. This system is also problematic since high concentrations of adenine nucleotides can interfere with PTP activation. This potential problem was circumvented by supplying a constant and low level of nucleotides via the ADP and PEP/PK system, resulting in the maintenance of the mitochondrial membrane potential under hypoxic conditions. However, it

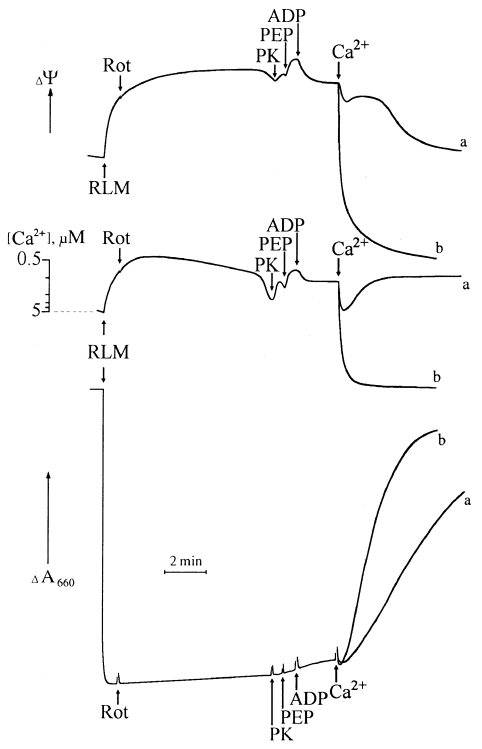

Fig. 2. The effect of energization of anaerobic rat liver mitochondria (RLM) with 20 μM ADP and the phospoenolpyruvate (PEP 5 mM)/pyruvate kinase (PK 2 units) system on the Ca^{2+}-induced changes of the membrane potential (upper pair of curves), extramitochondrial Ca^{2+} (central pair of curves) and mitochondrial volume (lower pair of curves). Ca^{2+} was added as shown in each pair for upper and lower curve, 50 or 100 μM respectively. Additions shown by arrows. Rot, rotenone. Incubation medium as in Section 2.

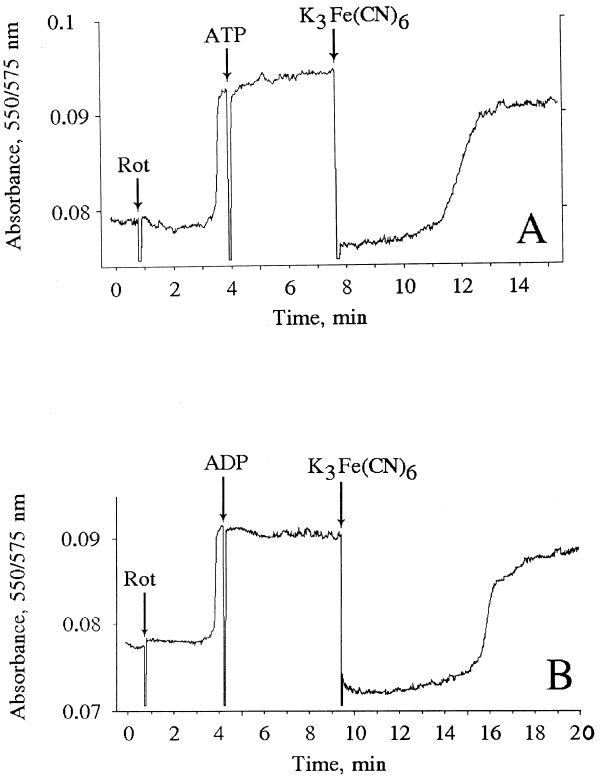

Fig. 3. Steady state of cytochrome *c*. After reaching anaerobic state resulting in a complete reduction of cytochrome *c*, ATP (500 μM) or ADP (20 μM) with PEP (5 mM)+PK (2 units) were added as shown. Ferricyanide (500 μM) was added to demonstrate cytochrome *c* oxidation.

should be noted that PEP can also affect the Ca^{2+} capacity of mitochondria, possibly through the exhaustion of the adenine nucleotide pool in the mitochondrial matrix thus releasing the PTP from the inhibition by adenine nucleotides [39,40]. The PK reaction under the conditions used results in almost complete conversion of ADP into ATP which does not interfere with PTP because of the resulting low concentrations [41]. Since the respiratory components were completely reduced under the conditions of our experiments, we can now exclude the apparent stimulation of PTP due to the oxidation of the respiratory components, as observed in our previous study [27], as contributing to the activation of PTP. Furthermore, the ability of Ca^{2+} to induce PTP activation under hypoxic conditions questions the role of ROS in this activation.

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