Mitochondrial Ca^{2+} transport and permeability transition pore opening and mitochondrial energetic status

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Abstract　The relationship between mitochondrial Ca^{2+} transport and permeability transition pore (PTP) opening as well as the effects of mitochondrial energetic status on mitochondrial Ca^{2+} transport and PTP opening were studied. The results showed that the calcium-induced calcium release from mitochondria (mCICR) induced PTP opening. Inhibitors for electron transport of respiratory chain inhibited mCICR and PTP opening. Partial recovery of electron transport in respiratory chain resulted in partial recovery of mCICR and PTP opening. mCICR and PTP opening were also inhibited by CCCP which eliminated transmembrane proton gradient. The results indicated that mitochondrial Ca^{2+} transport and PTP opening are largely dependent on electron transport and energy coupling.

Keywords: mitochondrial Ca^{2+} transport, permeability transition pore, electron transport, energy coupling.

Mitochondrial Ca^{2+} transport can regulate mitochondrial energy metabolism by affecting the activity of Ca^{2+} sensitive metabolic enzymes in mitochondrial matrix, and it can modulate [Ca^{2+}]$_c$ by Ca^{2+} uptake or release from mitochondria also[1]. It was found recently by permeability transition pore (PTP) that mitochondria carry out a special Ca^{2+} transport——Ca^{2+}-induced release of Ca^{2+} from mitochondria (mCICR). During mCICR, as a kind of organelle which can transmit or receive Ca^{2+} signal, mitochondria actively take part in cellular physiological regulation [2,3].

PTP is a proteinous pore located at the contact site between the inner and outer mitochondrial membranes. The exact composition of PTP is unknown. It is thought to involve at least hexokinase from cytosole, voltage-dependent anion channel from outer membrane, creatine kinase from intermembrane space, adenine nucleotide translocator from inner membrane, and cyclophilin D from matrix. The PTP opens by low-conductance, reversible mode and high-conductance, irreversible mode[4]. Low-conductance PTP opening is a physiological way of mitochondrial Ca^{2+} extrusion. High-conductance PTP opening, what is usually called PTP opening, is closely related to necrosis and apoptosis[3]. Therefore, mitochondrial Ca^{2+} transport and PTP opening are drawing more and more attention. Recent evidence indicates that mitochondrial Ca^{2+} transport and PTP opening and mitochondrial energetic status affect each other. It is reported that oxygen substrates can affect mitochondrial Ca^{2+} transport [5—7]. Proton selective substate of the PTP is regulated by

the redox state of the electron transport chain[8]. PTP opening is dramatically affected by the substrates used for energization[9]. With the goal of defining the effects of mitochondrial energetic status on mitochondrial Ca^{2+} transport and PTP opening, we carried out the study on the relationship between mitochondrial Ca^{2+} transport and permeability transition pore (PTP) opening as well as the effects of mitochondrial energetic status on mitochondrial Ca^{2+} transport and PTP opening. We found that mCICR induced PTP opening. Mitochondrial Ca^{2+} transport and PTP opening are largely dependent on electron transport and energy coupling.

1   Materials and methods

1.1   Materials

Rotenone, antimycine A (AA), ruthenium red (RR), rhodamine 123 (Rh123), carbonyl cyanide m-chlorophenylhydrazone (CCCP), cyclosporin A (CsA) and oligomycin were obtained from Sigma. Mannitol and bovine serum albumin (BSA) were obtained from Serva. Other reagents were obtained from local sources and were of analytical grade.

1.2   Isolation of mitochondria

Liver mitochondria were obtained from female Wistar rat (~200 g) by a standard procedure[8]. Isolation buffer contained 225 mmol/L mannitol, 75 mmol/L sucrose, 20 mmol/L Hepes, 0.5 mmol/L EGTA and 1% BSA (pH 7.2). 230 mmol/L mannitol, 70 mmol/L sucrose and 3 mmol/L Hepes were present in the PTP reaction medium (pH 7.4). The mitochondria were suspended at 12 mg protein/mL in the isolation buffer and stored on ice and diluted at 0.3 mg protein/mL in PTP reaction medium before detection.

1.3   Assay of mitochondrial Ca^{2+} transport

Mitochondrial Ca^{2+} transport was monitored at 20^oC by a dual wavelength spectrophotometer (Shimadzu 557) using arsenazo III (AIII) as Ca^{2+} indicator as described by Frei et al. [10].

1.4   Detection of mitochondrial PTP opening

（ⅰ）PTP opening detection by ultraviolet spectrophotometer. PTP opening was monitored at the decrease in OD_{540} at 20℃ by using an ultraviolet spectrophotometer (Shimadzu UV-2101PC) as described by Petronilli et al.[11].

（ⅱ）PTP opening detection by a fluorescence spectrophotometer. PTP opening was assessed by measuring the Rh123 fluorescence in the reactive system at 20℃ by using a fluorescence spectrophotometer (Hitachi F-4010) with excitation at 488 nm and emission at 525 nm after addition of 0.2 μmol/L Rh123 to mitochondria suspension as described by Fontaine et al.[9].

（ⅲ）PTP opening detection by a flow cytometer. PTP opening was assessed by measuring FSC and Rh123 fluorescence of mitochondria at 20℃ by using a flow cytometer (Becton Dickinson FACS420) with excitation at 488 nm and emission at 525 nm after addition of 0.2 μmol/L Rh123 to mitochondria suspension as described by Macouillard-Poulletier et al.[12].

2 Results

2.1 mCICR induces PTP opening

2.1.1 Low concentrations of Ca^{2+} induce mCICR, while high concentrations of Ca^{2+} induce mCICR and PTP opening.   AIII is a kind of Ca^{2+} probe characterized by Ca^{2+} sensitivity, high selectivity and membrane impermeable. The efflux and influx of mitochondrial Ca^{2+} were monitored by detecting AIII absorbance changes in the reactive system at 675—685 nm, which presents fluctuations of mitochondria Ca^{2+} [10]. Fig. 1 shows the results. Stimulated with 200 μmol/L Ca^{2+}, AIII absorbance declined rapidly. Then it rose above the baseline gradually, reflecting mitochondrial Ca^{2+} efflux evoked by rapid uptake of Ca^{2+}. This indicated that Ca^{2+} mediated mCICR, namely Ca^{2+} influx induced Ca^{2+} release from mitochondria.

PTP opening causes disappearance of mitochondrial membrane potential (Δy_m), resulting in release of Δy_m special probe Rh123 from mitochondria, and Rh123 fluorescence in the reactive system increase. PTP opening also causes permeability increase of the inner membrane, resulting in influx of medium and mitochondria swelling, then FSC, which represents the size of the mitochondria, increased and the optical density (OD), which represents the density of the mitochondria, decreased[4,12]. Treated with 200 μmol/L Ca^{2+}, the OD of the mitochondria decreased (fig. 2), the Rh123 fluorescence of the reactive system increased (fig. 3), the FSC of the mitochondria increased and the mitochondrial Rh123 fluorescence decreased. These results indicated that Ca^{2+} induced PTP opening.

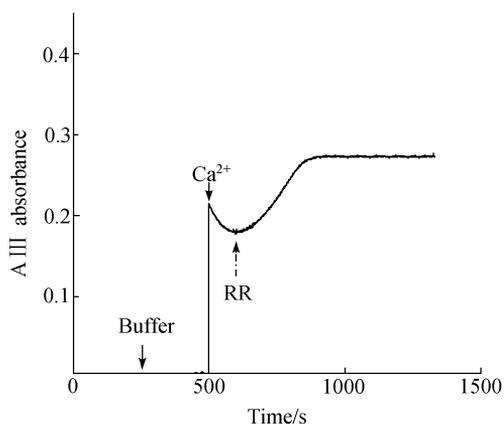Fig. 1. Ca^{2+}-induced mCICR in rat liver mitochondria. In these experiments, mitochondria were treated with 200 μmol/L Ca^{2+} (1 μmol/L RR was added 2 min after Ca^{2+}).
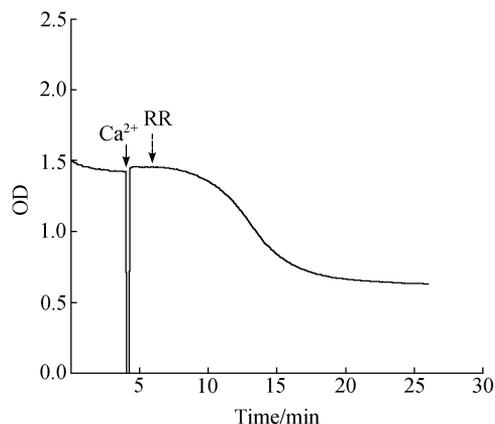
Fig. 2. Ca^{2+}-induced decrease of OD in rat liver mitochondria. In these experiments, mitochondria were treated with 200 μmol/L Ca^{2+} (1 μmol/L RR was added 2 min after Ca^{2+}).

Further experiments were performed to detect the effects of Ca^{2+} concentration on mCICR and PTP opening. Treated with less than 1 μmol/L Ca^{2+}, no changes in AIII absorbance and OD occurred. Treated with 2—20 μmol/L Ca^{2+}, OD stabilized, but there occurred AIII absorbance
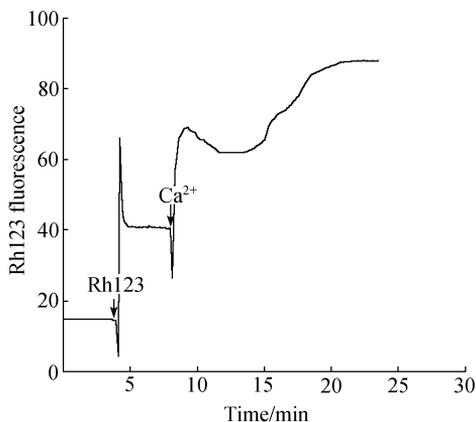
Fig. 3. Ca^{2+}-induced changes of Rhodamine123 fluorescence in the reactive system. In these experiments, mitochondria were treated with 200 µmol/L Ca^{2+}.

descent and ascent in order. Treated with more than 50 µmol/L Ca^{2+}, both AIII absorbance fluctuation and OD decrease were observed, and AIII absorbance and OD changes accelerated along with the Ca^{2+} concentration. These results indicate that mCICR and PTP opening are closely related to Ca^{2+} concentration. Low concentrations of Ca^{2+} cannot induce mCICR and PTP opening. Within a suitable concentration limit, Ca^{2+} can induce mCICR only, and high concentrations of Ca^{2+} can induce mCICR and PTP opening simultaneously. Fig. 3 shows, treated with 200 µmol/L Ca^{2+}, there occurred a transitory fluorescence wave before fluorescence of the reactive system increased at last, indicating that mitochondrial Ca^{2+} influx and efflux, namely mCICR, occurred before PTP opening. Figs. 1 and 2 show the similar results. All the above-mentioned results indicated that mCICR took place before PTP opening. Moreover, pretreated with RR, the special inhibitor of mitochondrial Ca^{2+} uniporter (the exclusive Ca^{2+} influx pathway of mitochondria), 200 µmol/L Ca^{2+} cannot cause any changes in AIII absorbance, Rh123 fluorescence of mitochondria and the reactive system, as well as FSC and OD of mitochondria, indicating that RR can inhibit mCICR and PTP opening which implied Ca^{2+}-induced mCICR and PTP opening by Ca^{2+} influx through Ca^{2+} uniporter.

2.1.2 PTP opening inhibited by mCICR inhibition. min before and 0.25 min after Ca^{2+} stimulation, respectively, no changes in AIII absorbance occurred (fig. 4). When RR was added 0.5 min after Ca^{2+}, AIII absorbance changes were observed, and the changes accelerated along with the RR added time till up to 2 min after Ca^{2+} treatment, when the changes of AIII absorbance were not affected by RR (fig. 1). Correspondingly, the OD presented identical changes (fig. 2). These results indicated that complete inhibition of mCICR results in complete inhibition of PTP opening and part inhibition of mCICR results in partial inhibition of PTP opening.

Ca^{2+}/Na$^+$ exchange is one of the mitochondrial Ca^{2+} extrusion pathways[1]. Pretreated with 1 µmol/L trifluoperazine, the inhibitor of Ca^{2+}/Na$^+$ exchanger,
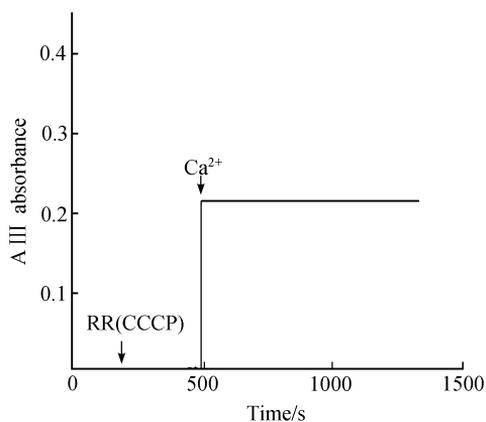
Adding RR 8, 4, 2, 1, 0.5, 0.25 and 0

Fig. 4. mCICR was inhibited by inhibition of mitochondrial Ca^{2+} influx (uncoupling). In these experiments, 1 µmol/L RR was added to inhibit Ca^{2+} influx (1 µmol/L CCCP was added to uncoupled electron transport to oxidative phosphorylation) before 200 µmol/L Ca^{2+}.

both the increase of AIII absorbance and the decrease of OD were clearly alleviated. It means also that partial inhibition of mCICR results in partial block of PTP opening.

2.1.3 mCICR not blocked by PTP inhibition.　　CsA is a special inhibitor of PTP[4]. It can inhibit PTP opening and changes of OD (fig. 5) and Rh123 fluorescence in mitochondria and reactive system. Pretreated with 1 µmol/L CsA, 200 µmol/L Ca^{2+} cannot cause increase of Rh123 fluorescence, but a transitory fluorescence wave was observed. It indicated that CsA can inhibit Ca^{2+}-induced PTP opening, but cannot inhibit mCICR. The AIII absorbance detection results showed that CsA cannot inhibit Ca^{2+}-induced AIII absorbance fluctuation (fig. 6). These results further confirmed that mCICR took place regardless of PTP opening.
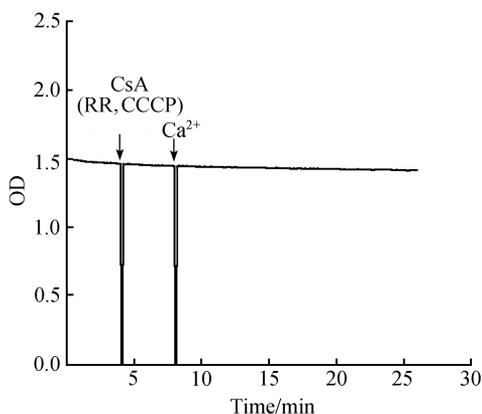

Fig. 5. OD decrease was inhibited by PTP opening inhibition (inhibition of Ca^{2+} influx or uncoupling). In these experiments, 1 µmol/L CsA was added to inhibit PTP opening (1 µmol/L RR was added to inhibit Ca^{2+} influx, 1 µmol/L CCCP was added to uncoupled electron transport to oxidative phosphorylation) before 200 µmol/L Ca^{2+}.

Fig. 6. mCICR took place when PTP opening was inhibited. In these experiments, 1 µmol/L CsA was added to inhibit PTP opening before 200 µmol/L Ca^{2+}.

2.2 mCICR and PTP opening depend on electron transport of the respiratory chain

2.2.1 mCICR and PTP opening blocked by inhibition of electron transport.　　It is known that rotenone and malonic acid are inhibitors of complexes I and II, respectively. When the oxidation of $FADH_2$ and NADH were inhibited respectively in the presence of rotenone or malonic acid, calcium still induced changes in OD (fig. 7) and AIII absorbance, though the changes weakened, implying that both mCICR and PTP opening are partly inhibited. Therefore one of the inhibitors
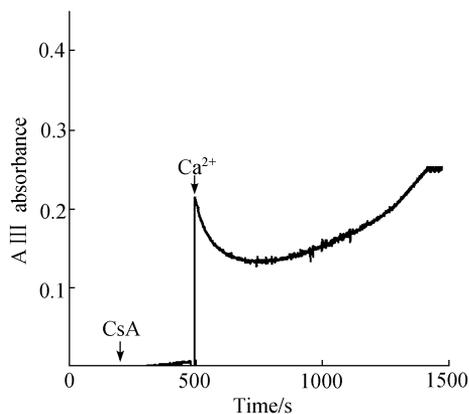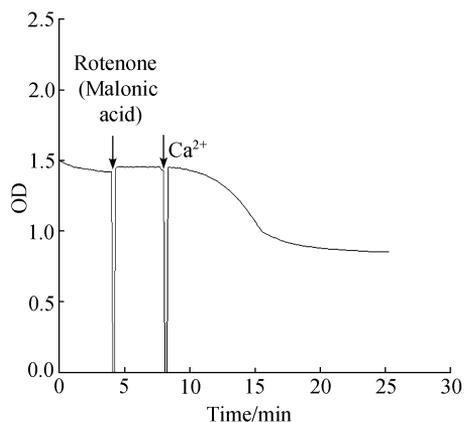

Fig.7. PTP opening was partly inhibited by part inhibition of electron transport. In these experiments, 1 µmol/L Rotenone (Melonic acid) was added to partly inhibit electron transport before 200 µmol/L Ca^{2+}.

can only partly block the electron transport in the presence of endogeneous and exogenous substrates, resulting in partial block of electron transport, and then partial inhibition of mCICR and PTP opening. When mitochondria were pretreated with rotenone and malonic acid simultaneously, or with AA, the inhibitor of complex III, and KCN, the inhibitor of complex IV, 200 μmol/L Ca^{2+} cannot induce any changes in AIII absorbance and OD, because the electron transport of the mitochondria was interrupted completely. These indicated that both mCICR and PTP opening depend on electron transport in mitochondrial respiratory chain.

2.2.2 Partial recovery of mCICR and PTP opening by partial recovery of electron transport in respiratory chain.   TMPD/ascorbate, the usually artificial electron sponsor, can provide electron to respiratory chain behind the check point of AA and partly recover the electron transport[13]. When 0.4 mmol/L TMPD/1 mmol/L ascorbate were added in the presence of rotenone and malonic acid or AA which inhibited electron transport completely, induced with 200 μmol/L Ca^{2+}, weakened changes in mitochondria OD (fig. 8) and AIII absorbance (fig. 9) were observed again. The results indicated that partial recovery of electron transport in respiratory chain can partly recover mCICR and PTP opening.
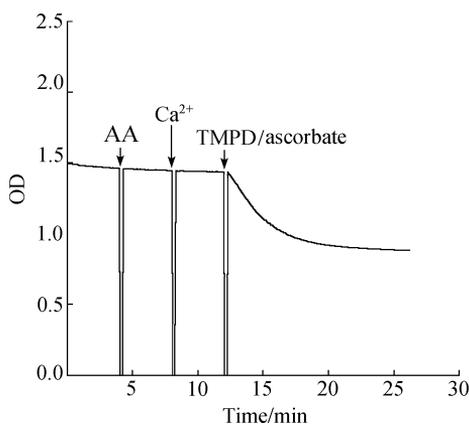

Fig. 8. PTP opening was partly recovered by partial recovery of electron transport. In these experiments, mitochondria were treated with 200 μmol/L Ca^{2+}. 1 μmol/L antimycin A was added to inhibit electron flow 4 min before and 0.4 mmol/L TMPD/1 mmol/L ascorbate were added to partly recover electron transport 4 min after 200 μmol/L Ca^{2+}.
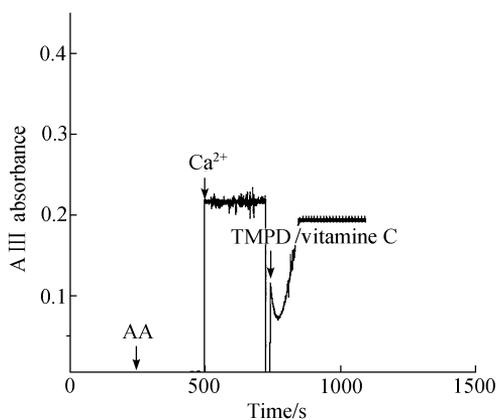
Fig. 9. mCICR was partly recovered by partial recovery of electron transport. In these experiments, mitochondria were treated with 200 μmol/L Ca^{2+}. 1 μmol/L antimycin A was added to inhibit electron flow 4 min before and 0.4 mmol/L TMPD/1 mmol/L ascorbate were added to partly recover electron transport 4 min after 200 μmol/L Ca^{2+}.

2.3   The dependence of mCICR and PTP opening on energy coupling

The protons and the electrons transport in turn in the mitochondrial respiratory chain. These results in the protons transfer from inboard to outboard of the inner membrane continuously and become transmembrane proton gradient which promotes ATP formation by ATPase. Ionophore CCCP makes energy produced by electron transport not transform into transmembrane proton potential, leading to energy uncoupling. The results showed that 200 μmol/L Ca^{2+} cannot induce

changes in OD and AIII absorbance of mitochondria pretreated with 1 μmol/L CCCP, indicating that energy coupling is necessary for mCICR and PTP opening.

3   Discussion

Ca^{2+} transport is one of the main events for mitochondria to regulate cellular physiological reaction[1]. PTP plays an important role in the mitochondrial regulation. Low-conductance PTP opening is the physiological pathway for mitochondrial Ca^{2+} release. High-conductance PTP opening, the so-called PTP opening, causes disappearance of $\Delta \psi_m$, changes of matrix osmosis and mitochondrial swelling[3,4]. Mitochondrial Ca^{2+} transport carries out its physiological or pathological responses by inducing low-conductance PTP opening or high-conductance PTP opening respectively. Ichas and co-authors suggested that rapid Ca^{2+} uptake induced low-conductance PTP opening and mCICR, slow Ca^{2+} uptake induced high-conductance PTP opening[3]. The results of this paper show that very low concentrations of Ca^{2+} cannot induce mCICR and PTP opening. Within a suitable concentration limit, Ca^{2+} can induce mCICR only, while high concentrations of Ca^{2+} can induce both mCICR and PTP opening. Furthermore, stimulated with Ca^{2+}, mCICR took place immediately and PTP opening lagged. When mCICR is completely inhibited by RR, Ca^{2+} cannot induce PTP opening. When mCICR is partly inhibited, Ca^{2+} induces PTP partial opening. Once Ca^{2+} influx occurs, mCICR and PTP opening cannot be inhibited by RR. On the contrary, when PTP is inhibited by CsA, one of the PTP inhibitors, Ca^{2+} can still induce mCICR. These results reveal that mCICR takes place before PTP opening, and PTP opening may be induced by mCICR.

Ichas et al. found that mCICR is a kind of active Ca^{2+} transport required for mitochondrial regulation of cellular physiological activity[2]. The evidence presented here that mCICR induces PTP opening suggested that mCICR may also be related to mitochondria function in cellular pathological responses. These results indicate that mitochondria carry out their physiological and pathological responses by mCICR.

Mitochondrial Ca^{2+} can promote as well as damage mitochondrial energy metabolism[1]. Meanwhile, mitochondrial energy metabolism affects mitochondrial Ca^{2+} transport[14]. Our results showed that inhibition of electron transport resulted in inhibition of mCICR, and partial recovery of electron transport in respiratory chain caused partial recovery of mCICR. These indicated that mCICR, a special kind of mitochondrial Ca^{2+} transport, depends on electron transport in mitochondrial respiratory chain.

On the one hand, PTP opening results in disappearance of $\Delta \psi_m$ and the mitochondrial energetic status [4]. On the other hand, the evidence reported by Broekemeier that one substrate of the PTP is regulated by the redox state of the electron transport chain[8], indicating that low-conductance PTP opening is affected by mitochondrial energetic status. Is PTP opening, the high-conductance PTP opening, affected by mitochondrial status? In rat skeletal muscle mitochondria, PTP opening is dramatically affected by substrates used for energization. Energized with complex

I substrate, PTP opening is clearly expedited[9]. The above-mentioned evidence suggested that mitochondrial status affected PTP opening. Our results showed simultaneous inhibition of complexes I and II or inhibition of complexes III and IV respectively, which blocks the electron transport, inhibit PTP opening completely. These results confirmed that PTP opening, as mCICR, is related to mitochondrial energetic status also.

It was reported that PTP opening of rat skeletal muscle mitochondria mainly depended on electron flow through the respiratory chain complex I[9]. However, we found that inhibition of the electron flow through complex II, III or IV all resulted in PTP opening inhibition. The further evidence showed that when providing electron to respiratory chain behind the check point of AA by the artificial electron sponsor TMPD/ascorbate, there partly recovered electron transport and the calcium can induce PTP opening again. These results indicated that once electron flow occurs in respiratory chain (even if locally), calcium can induce PTP opening.

mCICR and PTP opening depend not only on electron transport, but on energy coupling also. If treated with CCCP to make energy produced by electron transport not transformed into transmembrane proton potential, mCICR and PTP opening did not take place. However, oligomycin, the inhibitor of ATPase, had no obvious effects on mCICR and PTP opening, which means that ATP formation is not necessary for mCICR and PTP opening.

It is noticeable that during apoptosis, especially Ca^{2+}-induced apoptosis, mitochondrial Ca^{2+} transport was harmed and cellular Ca^{2+} homeostasis was perturbed, resulting in PTP opening, mitochondrial outer membrane interrupted, release of cytochrome c and apoptosis inducing factors, and apoptosis took place finally[15,16]. The fact that the mitochondrial Ca^{2+} and PTP opening can be controlled by modulation of energetic metabolism suggests that it is possible to promote or inhibit apoptosis by modulation of mitochondrial energetic status.

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