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Distinct behaviors of adenylate kinase and cytochrome c observed following induction of mitochondrial permeability transition by Ca^{2+} in the absence of respiratory substrate

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Abstract For induction of the mitochondrial permeability transition (PT) by Ca^{2+}, the addition of a respiratory substrate such as succinate is required. However, earlier studies indicated the possible induction of the mitochondrial PT by Ca^{2+} in the absence of a respiratory substrate (Hunter, D.R., and Haworth, R.A. (1979) Arch. Biochem. Biophys. 195, 453–459). In the present study, we obtained clear evidence showing that the mitochondrial PT could be induced by Ca^{2+} even in the absence of respiratory substrate. We next examined the protein release from mitochondria that accompanied the induction of PT in the absence of a respiratory substrate. Interestingly, distinct from the ordinary mitochondrial PT induced by Ca^{2+} in the presence of a respiratory substrate, which is associated with the release of mitochondrial cytochome c and adenylate kinase, the mitochondrial PT occurring in the absence of a respiratory substrate was associated with release of mitochondrial adenylate kinase but not with that of mitochondrial cytochrome c. This experimental system should be quite useful for understanding the mechanisms of protein release from mitochondria.

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Abbreviations

CsA cyclosporin A
PT permeability transition (PT)

Introduction

Mitochondria function as a major site of energy conversion in eukaryote cells. During the processes of energy conversion, the energy of nutrient molecules is first converted to the electrochemical potential difference of H^+ across the inner mitochondrial membrane generated by the respiratory chain, and this H^+ gradient across the mitochondrial inner membrane is used as a driving force for the synthesis of ATP. To enable effective energy conversion, the mitochon-

drial inner membrane is highly impermeable even to tiny solutes and ions. However, under certain conditions such as in the presence of Ca^{2+} and inorganic phosphate, the inner mitochondrial membrane becomes permeable to solutes and ions up to 1500 Da. This phenomenon is referred to as the mitochondrial permeability transition (PT), and ordinary PT is sensitive to the immunosuppressant cyclosporin A (CsA), which inhibits it (for reviews on PT, see Gunter and Pfeiffer 1990; Zoratti and Szabo 1995; Bernardi 1999).

The physiological meanings of this PT have long been uncertain. However, today, PT is well established to be involved in the early execution step of cell death. The induction of PT results in the release of proteins from the mitochondria such as cytochrome c into the cytosol, and these released mitochondrial proteins trigger the subsequent steps of cell death (for reviews, see refs. Brenner and Grimm 2006; Kroemer et al. 2007; Orrenius et al. 2007). Therefore, the question as to how mitochondrial cytochrome c is released upon induction of mitochondrial PT is very important. Probable answers to this question have been proposed; however, this topic is still controversial.

We have been studying the relationship between the changes in the status of the mitochondrial membrane and the release of mitochondrial cytochrome c (Shinohara et al. 2002; Yamamoto et al. 2004, 2005). As a result, valinomycin, which was reported to show apoptotic effects on the cultured cells, was found to cause the release of mitochondrial cytochrome c without triggering the mitochondrial PT (Shinohara et al. 2002). Essentially the same conclusion was also reported by Gogvadze *et al*. (2004). On the contrary, when mitochondria were treated with *N*, *N*'-dicyclohexylcarbodiimide (DCCD), this agent caused the mitochondrial PT; but this PT was not associated with the release of mitochondrial cytochrome c (Yamamoto et al. 2004). Furthermore, the latter study indicated the importance of 2 parameters, i.e., i) the degree of mitochondrial swelling and ii) functional electron transfer in the mitochondrial respiratory chain for the regulation of cytochrome c release.

For the induction of mitochondrial PT by externally added Ca^{2+}, a certain amount of Ca^{2+} must be accumulated in the mitochondrial matrix space; and for effective accumulation of Ca^{2+} there, an electrochemical gradient of H^{+} across the inner mitochondrial membrane is necessary. For this reason, the mitochondrial PT is generally induced by Ca^{2+} in the presence of a respiratory substrate. However, earlier studies indicated the possible induction of mitochondrial PT by Ca^{2+} in the absence of a respiratory substrate as well (Hunter and Haworth 1979). If this change caused by Ca^{2+} in the absence of a respiratory substrate actually reflects the induction of mitochondrial PT, this experimental system would allow us to examine whether functional electron transfer in the mitochondrial respiratory chain is required for the release of cytochrome c from mitochondria. Thus, in the present study, we first characterized the membrane status of mitochondria treated with Ca^{2+} in the absence of a respiratory substrate. Furthermore, its association with the release of cytochrome c was also investigated.

Materials and methods

Materials

Cyclosporin A (CsA) was kindly provided by Novartis Pharma Inc. (Tokyo). ECL kit (code RPN2106) and anti-rabbit IgG conjugated to peroxidase (code NA934V) were obtained from Amersham Biosciences (Bucks, UK).

Preparation of mitochondria

Mitochondria were isolated from the livers of normal male Wistar rats according to the method described previously (Shinohara et al. 2002; Yamamoto et al. 2004, 2005). For determination of the protein concentration of mitochondrial suspensions, mitochondrial proteins were first solubilized with 1% SDS, and then subjected to Biuret analysis using bovine serum albumin as a standard.

Measurement of absorbance change of mitochondrial suspensions

The change in the absorbance of mitochondrial suspensions was examined essentially as described previously at 25°C (Shinohara et al. 2002; Yamamoto et al. 2004, 2005). Briefly, mitochondria were suspended in + Pi medium (200 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4) to a final protein concentration of 0.7 mg protein/ml. Time-dependent changes in the absorbance of mitochondrial suspensions was monitored at 540 nm with a Shimadzu spectrophotometer, model UV-3000. Ca^{2+} was added to the mitochondrial suspension to a final concentration of 100 μM. When a respiratory substrate was used, 5 mM succinate (plus 0.5 μg rotenone/mg mitochondrial protein) was added to the incubation medium. To test the sensitivity of the PT to cyclosporin A (CsA), we added the drug to make a final concentration of 2.5 μM.

Transmission electron microscopic analysis of mitochondrial configuration

The mitochondrial configuration was analyzed by transmission electron microscopy according to the method published earlier (Shinohara et al. 2002; Yamamoto et al. 2004, 2005).

Antibody preparation and measurement of protein release from mitochondria

Antibody against cytochrome c was raised by using a synthetic peptide with the amino acid sequence of HTVEKGGKHKTGPNLHGLFC as immunogen, as described previously (Shinohara et al. 2002). Similarly, antibody against adenylate kinase 2 was prepared by using a synthetic peptide with the amino acid sequence of TVKQAEMLDDLMDKR KEKLDC, corresponding to amino acid residues 104 – 123 of rat adenylate kinase 2 (accession number D13061) plus a C-terminal artificially introduced cysteine residue.

To examine the protein release from mitochondria after treatment under certain conditions, we promptly centrifuged an aliquot (500 µl) of the mitochondrial suspension. The pelleted mitochondria were resuspended in 500 µl of the incubation medium, and 9 µl of this suspension and 12 µl of the supernatant were individually subjected to SDS-PAGE. Western blotting was carried out essentially as described earlier (Shinohara et al. 2002; Yamamoto et al. 2004, 2005).

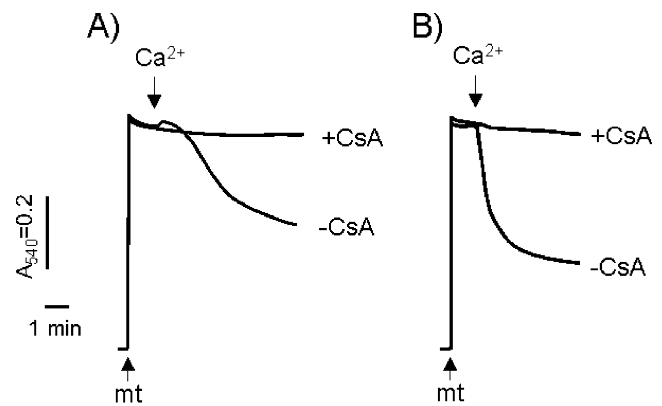

Fig. 1 Turbidity change in mitochondrial suspension induced by Ca^{2+}. Panels **A** and **B** represent the changes in the turbidities of mitochondrial suspensions induced by Ca^{2+} in the presence and absence, respectively, of the respiratory substrate succinate. In the experiment in panel A, 5 mM succinate (plus 0.5 µg rotenone/mg mitochondrial protein) was included in the incubation medium as a respiratory substrate. Ca^{2+} and cyclosporin A (*CsA*) were added to final concentrations of 100 µM and 2.5 µM, respectively

Results and discussion

Hunter and Haworth reported the possible induction of mitochondrial PT by Ca^{2+} in the absence of a respiratory substrate (Hunter and Haworth 1979). However, it was uncertain whether these changes in the membrane status caused by Ca^{2+} in this case were the same as those observed in the presence of a respiratory substrate. To answer this question, we compared changes in the status of the mitochondrial membrane caused by Ca^{2+} in the presence and absence of a respiratory substrate.

As shown in Fig. 1a, when Ca^{2+} was added to the mitochondrial suspension in the presence of the respiratory substrate succinate, a gradual decrease in the absorbance of the mitochondrial suspension was observed; and this change was completely suppressed by the addition of cyclosporin A (CsA). This absorbance decrease is well regarded as one of the typical features of the ordinary mitochondrial PT. When Ca^{2+} was added to the mitochondrial suspension in the absence of the respiratory substrate, a much quicker and deeper decrease in the absorbance of the mitochondrial suspension was observed (Fig. 1b). This change was also completely prevented by the addition of CsA, suggesting that the mitochondrial PT could be induced even in the absence of respiratory substrate in a CsA-sensitive manner.

To confirm whether the changes in the absorbance of the mitochondrial suspension caused by Ca^{2+} reflected the induction of the mitochondrial PT, we examined the status of the mitochondrial membrane by transmission electron microscopy. As shown in Fig. 2a, in the presence of the respiratory substrate, Ca^{2+} caused a loss of the normal structure of the cristae of the inner mitochondrial membrane, and this change was completely suppressed by the addition of CsA. These changes are regarded to reflect the increase in the permeability of the inner mitochondrial membrane. Similar changes in the status of the mitochondrial membrane were also induced by Ca^{2+} in the absence of succinate and in a CsA-sensitive fashion (Fig. 2b). These results clearly indicate that the mitochondrial PT could be induced by Ca^{2+} even in the absence of respiratory substrate. The increase in the permeability of the mitochondrial membrane induced by Ca^{2+} in the absence of the respiratory substrate was also confirmed by measuring the shrinkage of mitochondria upon the addition of polyethylene glycol, as described previously (Yamamoto et al. 2005; Pfeiffer et al. 1995; Sultan and Sokolove 2001) (data not shown).

We next assessed whether the PT induced by Ca^{2+} in the absence of succinate was associated with the release of mitochondrial cytochrome c. As shown in Fig. 3, in the presence of the respiratory substrate (+ succi), Ca^{2+} caused release of mitochondrial cytochrome c; and this release was completely abolished by treatment with CsA, as consistently observed in past studies. On the contrary, when the mitochondrial PT was induced by Ca^{2+} in the absence of the respiratory substrate (- succi), the release of mitochondrial cytochrome c was not observed. To examine whether this lack of release of cytochrome c upon induction of the mitochondrial PT in the absence of the respiratory substrate is a common property of mitochondrial intermembrane proteins, we also examined the distribution of adenylate

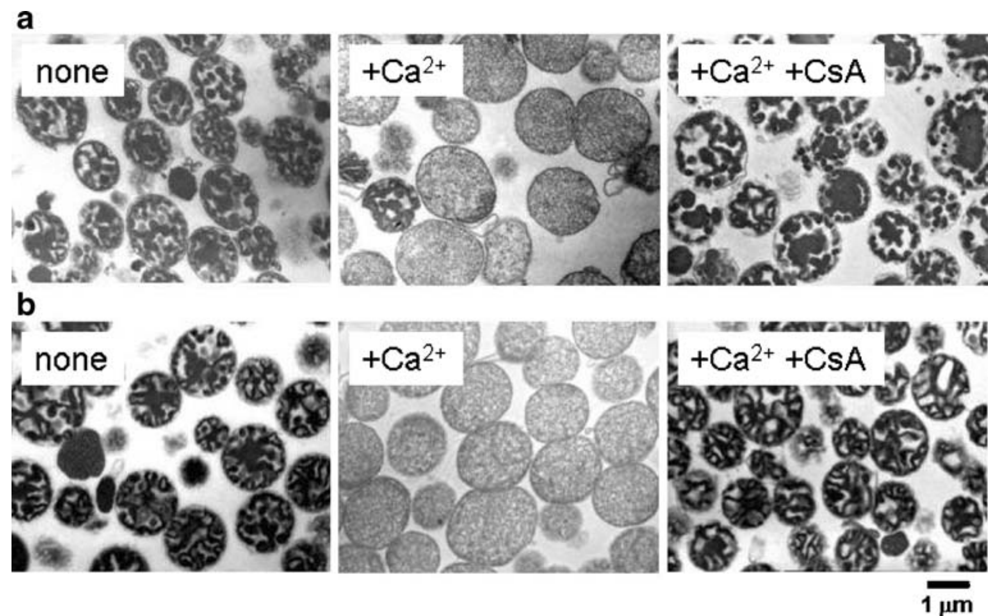

Fig. 2 Morphological changes in mitochondria examined by transmission electron microscopy. Photographs in panels **a** and **b** represent the morphologies of mitochondria observed in the presence and absence, respectively, of the respiratory substrate succinate. When used, 5 mM succinate (plus 0.5 µg rotenone/ mg mitochondrial protein) was included in the incubation medium. Photos labeled "none" show the morphology of non-treated mitochondria; and those labeled "+Ca^{2+}" and "+Ca^{2+} +CsA," the morphology of mitochondria treated with 100 µM Ca^{2+} and with 100 µM Ca^{2+} plus 2.5 µM CsA, respectively. Bar indicates 1 µm for all photos.

kinase, a marker protein located in the intermembrane space and released from mitochondria upon induction of the PT by Ca^{2+} in the presence of a respiratory substrate (Scarlett and Murphy 1997; Crouser et al. 2003). The release of adenylate kinase has been mainly evaluated by measuring the activity of the enzyme released into the cytosol. At least 3 genes encoding its isozymes have been identified, and type 1 – 3 isozymes are mainly detected in the cytosol, mitochondrial intermembrane space, and matrix, respectively (Köhler et al. 1999). Thus, immunodetection of the type-2 isozyme, adenylate kinase 2, in the pellet and supernatant obtained from a mitochondrial suspension is more informative for a distribution analysis of proteins located in the mitochondrial intermembrane space. As shown in Fig. 3, the immuno-signal of adenylate kinase 2 (AK2) was observed in the mitochondrial pellet (P) from the mitochondrial samples not treated with Ca^{2+} (control). However, almost al immuno-signals were observed in the supernatant (S) of the mitochondria treated with Ca^{2+}, regardless of the presence or absence of the respiratory substrate. These results clearly indicate that the induction of the PT by Ca^{2+} in the absence of a respiratory substrate is sufficient to cause the release of proteins from the intermembrane space but not sufficient for the release of cytochrome c. It should be also noted that the degree of the release of cytochrome c observed in the mitochondria treated with Ca^{2+} in the presence of the respiratory substrate (+ succi, +Ca^{2+}) was much more moderate than that of adenylate kinase 2 in the same mitochondrial sample. However, this difference accords well with the results reported previously (Crouser et al. 2003).

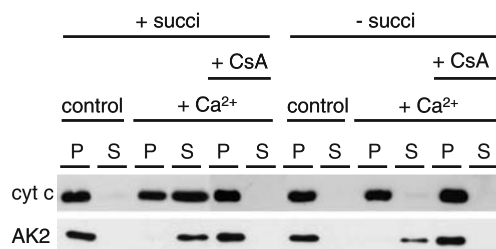

Fig. 3 Release analysis of cytochrome c and adenylate kinase 2 from mitochondria treated with Ca^{2+} in the presence and absence of respiratory substrate. For examination of the effects of Ca^{2+} treatment of mitochondria in the presence or absence of succinate on the release of cytochrome c and adenylate kinase 2, mitochondria incubated under either condition were promptly centrifuged. The pellet (*P*) and supernatant (*S*) thus obtained from individual mitochondrial suspensions were subjected to SDS-PAGE followed by immuno-detection of cytochrome c and adenylate kinase 2 (*AK2*). Ca^{2+} and cyclosporin A (*CsA*) were added to final concentrations of 100 µM and 2.5 µM, respectively

Cytochrome c is well known as a member of the respiratory chain, and to exist in a membrane-bound form at the outer surface of the inner mitochondrial membrane. Therefore, under normal conditions, most of the cytochrome c is observed in the intermembrane space of mitochondria. However, under certain conditions, cytochrome c may be released from the mitochondria into the cytosol. Once released into the cytosol, it triggers the subsequent execution steps towards cell death. Nowadays, 2 processes for the release of cytochrome c from mitochondria are thought to exist, i.e., PT-dependent and Bax/ Bak-dependent permeabilization of the outer mitochondrial membrane. In the former process, opening of nonspecific pores in the inner mitochondrial membrane, i.e., induction of the mitochondrial PT, causes osmotic swelling of

mitochondrial matrix and rupture of the outer mitochondrial membrane. For the latter process, several mechanisms have been proposed, but the exact mechanism is still not yet fully established.

In general, PT-dependent permeabilization of the outer mitochondrial membrane is caused by the addition of Ca^{2+} to the mitochondria energized by a respiratory substrate. Possible induction of the PT by addition of Ca^{2+} to the mitochondria in the absence of a respiratory substrate was also reported (Hunter and Haworth 1979), but its features have not been well characterized. In the present study, we first examined the status of the inner membrane of the mitochondria treated with Ca^{2+} in the absence of a respiratory substrate, and succeeded in obtaining clear evidence of the occurrence of the PT. When we assessed the release of cytochrome c and adenylate kinase 2 from mitochondria treated with Ca^{2+} in the absence of succinate, adenylate kinase 2 was released but cytochrome c was not.

Regarding the behavior of proteins present in the intermembrane space of mitochondria, previous studies revealed their protein species-dependent distinct behaviors, i.e., not all proteins in the intermembrane space are released from mitochondria simultaneously. For instance, the release of cytochrome c can occur before or after the release of apotosis inducible factor, AIF (Modjtahedi et al. 2006). Similarly, Smac/DIABLO was reported to be released from mitochondria before cytochrome c and AIF (Arnoult et al. 2003). These distinct behaviors of proteins in the intermembrane space are thought to be due to differential release mechanisms or to distinct mechanisms of mitochondrial retention. However, interpretation of the observed results is difficult, because these results would be dependent on the apoptotic models used in these studies. On the contrary, interpretation of the results obtained in the present study is much easier; i.e., rupture of the outer mitochondrial membrane, as judged by the non-specific release of the intermembrane isozyme adenylate kinase 2 caused by induction of the mitochondrial PT in the absence of a respiratory substrate, was not sufficient to cause the release of cytochrome c. This conclusion accords well with our previous conclusion stating the importance of functionally active mitochondrial respiration for the release of cytochrome c (Yamamoto et al. 2005), and also supports a previous paper describing the involvement of a detachment process in the release of cytochrome c from the inner mitochondrial membrane (Ott et al. 2002). Thus, the experimental system described presently would be very effective for understanding the release mechanisms of mitochondrial proteins.

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