Journal of Bioenergetics and Biomembranes, Vol. 37, No. 5, October 2005 (© 2005)
DOI: 10.1007/s10863-005-8641-6

Two Critical Factors Affecting the Release of Mitochondrial Cytochrome *c* as Revealed by Studies Using *N,N′*-Dicyclohexylcarbodiimide as an Atypical Inducer of Permeability Transition

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Received August 2, 2005; accepted August 18, 2005

N,N′-dicyclohexylcarbodiimide (DCCD) was earlier reported to have stimulatory effects on mitochondrial respiration and to induce mitochondrial swelling, when it was added to mitochondrial suspensions. These data seem to imply that DCCD caused the mitochondrial permeability transition (PT), but this possibility had never been investigated. In the present study, effects of DCCD on the mitochondrial structure and function were studied in detail. DCCD was found to induce mitochondrial PT in a cyclosporine A-insensitive manner. Electron microscopic analysis also supported the induction of the mitochondrial PT by DCCD. However, different from many other PT inducers, DCCD failed to cause massive release of mitochondrial cytochrome *c*. To understand the relationship between the induction of mitochondrial PT and the release of mitochondrial cytochrome *c*, we compared the actions of DCCD on mitochondrial structure and function with those of Ca^{2+}, known as an ordinary PT inducer. As a result, two parameters considered to be critical for controlling the release of mitochondrial cytochrome *c* on the induction of PT were mitochondrial volume and the velocity of mitochondrial oxygen consumption.

KEY WORDS: *N,N′*-Dicyclohexylcarbodiimide (DCCD); mitochondria; permeability transition; apoptosis; cytochrome *c* release.

INTRODUCTION

The inner mitochondrial membrane is highly resistant for permeation of solutes and ions, because the electrochemical gradient of H^+ across the inner membrane is utilized as the driving force for ATP synthesis. However, under certain conditions, such as in the presence of Ca^{2+} and inorganic phosphate (Pi), the permeability of inner mitochondrial membrane is known to be markedly increased. Today, this transition is referred to as the permeability transition (PT), and is believed to result from the formation of a proteinous pore, PT pore, which makes the mitochondrial membrane permeable to various solutes and ions smaller than 1500 Da (for recent reviews, see Gunter and Pfeiffer, 1990; Zoratti and Szabo, 1995; Bernardi, 1999). The physiological importance of

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Key to abbreviations: CsA, cyclosporine A; DCCD, *N,N′*-dicyclohexylcarbodiimide; PEG, polyethylene glycol; Pi, inorganic phosphate; PT, permeability transition; SF6847, 3,5-di-*tert*-butyl-4-hydroxy-benzylidene malononitrile; TEM, transmission electron microscopy; VDAC, voltage-dependent anion channel.

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the PT has long been uncertain; however, recent studies have revealed that cytochrome *c* is released from mitochondria on the induction of the PT and that this release then triggers subsequent steps of programmed cell death, i.e., apoptosis (for reviews, see refs. Vander Heiden and Thompson, 1999; Kroemer and Reed, 2000; Bernardi *et al*., 2001). Thus, the PT is considered to be one of the major regulatory steps of apoptosis. However, the molecular features of the PT pore are still uncertain. Furthermore, the question as to how the release of cytochrome *c* is effected by the induction of the PT is also still obscure. Thus, for an understanding of how the release of mitochondrial cytochrome *c* is regulated, measurement of such release from isolated mitochondria under various experimental conditions would seem to be very important.

N,N′-dicyclohexylcarbodiimide (DCCD), a chemical generally used for the formation of peptide bonds, is known to be reactive with various side chains of amino acids such as carboxyl groups and sulfhydryl groups, when it is added to an aqueous solution of proteins (for review, see Azzi *et al*., 1984). Thus, when DCCD is added to a mitochondrial suspension, chemical modification of several proteins would be expected to occur, resulting in changes in the functions of particular ones (Azzi *et al*., 1984). Of these effects, the most well studied is the effect of DCCD on FoF_1-ATPase. DCCD is known to inhibit FoF_1-ATPase by covalent modification of subunit c in Fo. Chemical modifications of other mitochondrial proteins such as porin, which exists in the outer mitochondrial membrane (De Pinto *et al*., 1985, 1993; Nakashima *et al*., 1986; Nakashima, 1989), and those of the respiratory complex (Azzi *et al*., 1984), have also been reported. In addition to these effects, DCCD was also shown to accelerate mitochondrial respiration and mitochondrial swelling (Chavez *et al*., 1990). The effects of DCCD on mitochondrial structure and function were similar to those of Ca^{2+}; however, the mechanisms by which DCCD causes these effects are still uncertain.

In this study, we characterized the detailed effects of DCCD on mitochondrial structure and function. As a result, DCCD was found to cause a PT that could be distinguished from the ordinary PT induced by Ca^{2+} by a difference in either cyclosporine A (CsA) sensitivity or the size of the PT pore formed. Thus DCCD was concluded to be an atypical PT inducer. Interestingly, the PT induced by DCCD was not accompanied by any significant change in the release of mitochondrial cytochrome *c*. Based on these results, critical parameters controlling cytochrome *c* release are discussed.

MATERIALS AND METHODS

Materials

DCCD and polyethylene glycol (PEG) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka) and Nacarai Tesque (Kyoto), respectively. Cyclosporin A (CsA) was kindly provided by Novartis Pharma Inc. (Tokyo). SF6847 (malonoben, code 0479) was obtained from Tocris Cookson Ltd. (Bristol).

Preparation of Mitochondria

Liver mitochondria were isolated from normal male Wistar rats according to the method described earlier (Shinohara *et al*., 1998, 2002; Yamamoto *et al*., 2004).

Measurements of Mitochondrial Oxygen Consumption and Swelling

Mitochondrial function and absorbance change were examined essentially as described earlier (Shinohara *et al*., 1998, 2002; Yamamoto *et al*., 2004). Briefly, mitochondria (0.7 mg protein/ml) suspended in − Pi medium (200 mM sucrose, 10 mM KCl, 10 mM Tris-Cl buffer; pH 7.4) were energized with 10 mM succinate in the presence of rotenone (0.5 μg rotenone/mg protein), and their respiration was measured at 25°C with a Clark oxygen-electrode (Yellow Springs, code 5331) in a total volume of 2.2 ml. Mitochondrial swelling in the same medium was monitored by measuring the absorbance at 540 nm with a Shimadzu spectrophotometer, model UV-3000. When actions of Ca^{2+} were measured, + Pi medium (250 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4) was used instead of − Pi medium.

Measurement of Permeability of Mitochondrial Membrane to PEG

To examine the permeability of the mitochondrial membrane, we measured the effects of PEGs of various molecular sizes on the turbidity of mitochondrial suspensions, as described (Pfeiffer *et al*., 1995; Sultan and Sokolove, 2001). Briefly, mitochondria were first treated with a certain reagent; and then, after complete induction of swelling, 1.1 ml of 300 mOsM PEG solution was added. Changes in the turbidity of the reaction mixture were monitored at 540 nm.

Transmission Electron Microscopic Analysis of Mitochondrial Configuration

Mitochondrial configuration was analyzed by transmission electron microscopy according to the method published earlier (Shinohara *et al.*, 1998, 2002; Yamamoto *et al.*, 2004).

Detection by Western Blotting of Cytochrome *c* Released From Mitochondria

Antiserum against cytochrome *c* was prepared as described previously (Shinohara *et al.*, 2002). To determine the amount of cytochrome *c* released from the mitochondria, after treatment under certain conditions, we placed an aliquot (500 μl) of mitochondrial suspension in an Eppendorf centrifuge tube, promptly centrifuged it, and separated the supernatant from the pellet. The pelleted mitochondria were resuspended in 500 μl of the incubation medium, and 9 μl of this suspension and 15 μl of the supernatant were individually subjected to SDS-PAGE. Western blotting was carried out essentially as described earlier (Shinohara *et al.*, 2002; Hashimoto *et al.*, 1999).

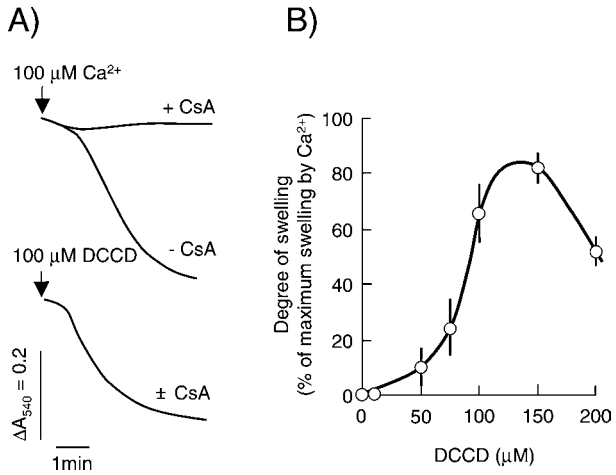

Fig. 1. Turbidity change in mitochondrial suspension induced by DCCD. In panel A, typical traces of the turbidity change in a mitochondrial suspension induced by 100 μM Ca^{2+} (upper trace) or by 100 μM DCCD (lower trace) are shown. It should be noted that the effects of Ca^{2+} and DCCD were measured in the presence and absence of Pi, respectively. Effects of CsA (1 μM) on Ca^{2+}- and DCCD-induced mitochondrial swelling are also shown. In panel B, the degree of mitochondrial swelling observed with DCCD at various concentrations is shown as relative to the degree of maximum swelling observed with 100 μM Ca^{2+} in the presence of Pi ($\Delta A_{540} = 0.29$) and as mean values ± SD of three independent runs.

RESULTS

Previously, Chávez *et al.* reported that addition of DCCD to mitochondria accelerated mitochondrial oxygen consumption and swelling of the mitochondria (Chavez *et al.*, 1990). Although these results would seem to indicate that DCCD induced mitochondrial PT, this possibility has never been investigated. Thus, we examined whether the actions of DCCD on the mitochondria reflect the induction of mitochondrial PT.

Induction of mitochondrial swelling is one of the common features of mitochondria PT. Further addition of a PEG solution to preswollen mitochondria causes shrinkage of the mitochondria, reflecting the increased permeability of the inner mitochondrial membrane (Pfeiffer *et al.*, 1995; Sultan and Sokolove, 2001). When mitochondrial PT is induced by Ca^{2+}, both of these reactions, i.e., mitochondrial swelling and shrinkage of preswollen mitochondria by PEG, are observed; and they can be completely prevented by the addition of CsA, known as a specific inhibitor of the ordinary PT. This experimental system is one of the most reliable procedures to judge whether mitochondrial PT is induced. Thus, we first compared the actions of DCCD with those of Ca^{2+} in this experimental system. Different from the actions of Ca^{2+}, which exclusively require inorganic phosphate (Pi) in the incubation medium for induction of the mitochondrial PT, acceleration of mitochondrial oxygen consumption and induction of mitochondrial swelling caused by DCCD were much stronger in the absence of Pi than in its presence (Chavez *et al.*, 1990). Thus, in this study, we compared the actions of DCCD in the absence of Pi with features of the ordinary PT induced by Ca^{2+} in the presence of Pi. The actions of DCCD on mitochondrial structure and function in the presence of Pi were more moderate than those observed in the absence of Pi, but their characteristics were essentially the same as those in the presence of Pi.

As shown in Fig. 1(A), DCCD induced remarkable swelling of the mitochondria, as well as Ca^{2+}. Effects of DCCD were dose-dependent up to 150 μM; and above this concentration, the degree of mitochondrial swelling was rather decreased (Fig. 1(B)). However, the effects of DCCD were different from those of Ca^{2+} in the following two points: First, the magnitude of the maximum absorbance change induced in the mitochondrial suspension by DCCD was slightly smaller than that induced by Ca^{2+} (about 80% of Ca^{2+}-induced swelling). Second, different from the action of Ca^{2+}, that of DCCD was insensitive to CsA, known as a specific inhibitor of the ordinary PT (Fig. 1(A)). We next compared the effects of DCCD and Ca^{2+} on the permeability of mitochondrial membrane by measuring the changes in the turbidity of the

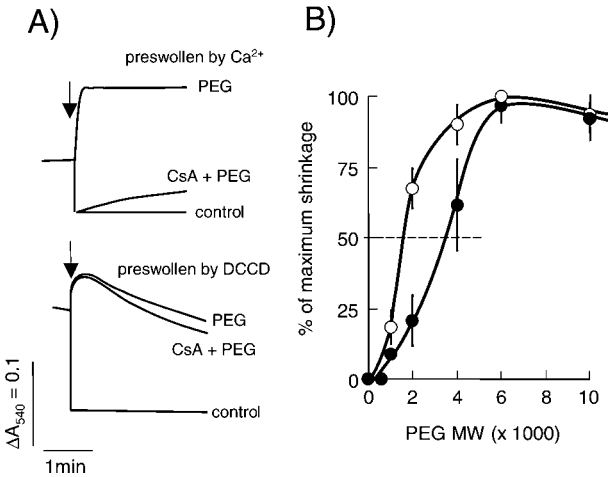

Fig. 2. Effects of PEG on the turbidity of preswollen mitochondrial suspensions. In panel A, typical traces of the turbidity change following the addition of a PEG6000 solution to a suspension of mitochondria preswollen by 100 μM Ca^{2+} (upper traces) and 150 μM DCCD (lower traces) are shown. In panel B, the degree of shrinkage of preswollen mitochondria induced by the addition of PEGs having various sizes is shown as relative to the maximum increase in mitochondria turbidity induced by the addition of PEG6000 to the Ca^{2+}-treated mitochondria ($\Delta A_{540} = 0.135$). Open and closed symbols represent the results of mitochondria preswollen by 100 μM Ca^{2+} and 150 μM DCCD, respectively. Results are given as mean values ± SD of three independent runs.

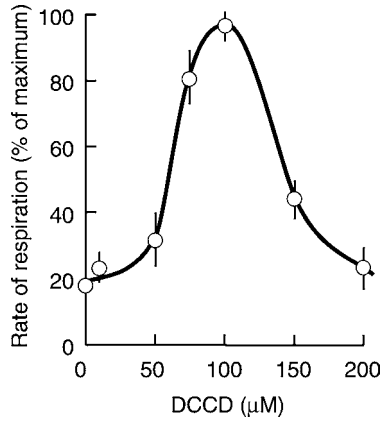

Fig. 3. Effects of DCCD on the rate of mitochondrial oxygen consumption. The rate of mitochondrial oxygen consumption in the absence or presence of DCCD at various concentrations was measured in the − Pi medium (200 mM sucrose, 10 mM KCl, 10 mM Tris-Cl buffer; pH 7.4) at 25°C, with 10 mM succinate (plus 0.5 μg rotenone/mg protein) used as a respiratory substrate. Results are shown as relative to the maximum rate of oxygen consumption induced by DCCD (112 natomsO/mg/min) and as mean values ± SD of three independent runs.

reaction mixture on the addition of PEG to mitochondria preswollen by these reagents. As shown in Fig. 2(A), the addition of PEG to mitochondria preswollen by DCCD caused an increase in mitochondrial turbidity, reflecting mitochondrial shrinkage, as in the case of mitochondria preswollen by Ca^{2+}. Thus, DCCD was concluded to induce mitochondrial PT as well as Ca^{2+}. The PEG-induced shrinkage of mitochondria preswollen by Ca^{2+} was CsA sensitive, whereas the PEG effect on the mitochondria preswollen by DCCD was almost completely insensitive to CsA, consistent with the fact that mitochondrial swelling induced by DCCD was insensitive to CsA. Furthermore, when the effects of PEG on mitochondria were titrated with PEGs having various molecular weights, the half exclusion size of the PT pore induced by Ca^{2+} was about 1500 Da; in contrast, that induced by DCCD was estimated to be approximately 3500 Da, thus indicating that the size of the PT pore induced by DCCD was larger than that induced by Ca^{2+} (Fig. 2(B)). These results clearly indicate that DCCD opened a PT pore that was distinct from that induced by Ca^{2+} with respect to sensitivity to CsA and exclusion size.

In the case of Ca^{2+}, respiration was accelerated on the induction of the PT. Thus, we next measured the effects of DCCD on the velocity of mitochondrial oxygen consumption. As shown in Fig. 3, DCCD caused acceleration of oxygen consumption in a dose-dependent manner up to 100 μM; but above this concentration, the rate of oxygen consumption was significantly decreased. This inhibitory effect of the higher concentrations of DCCD on the mitochondrial oxygen consumption was attributable to the inhibition of the respiratory chain by DCCD, because the slow oxygen consumption observed in the presence of higher concentrations of DCCD was not accelerated even by the addition of a protonophoric uncoupler, SF6847 (data not shown; see also Azzi *et al.*, 1984). It is noteworthy that even under the condition at which mitochondrial respiration was significantly inhibited (150–200 μM DCCD), significant swelling of mitochondria was still observed (see Fig. 1(B)).

When the ordinary PT is induced by Ca^{2+}, disappearance of the inner mitochondrial membrane, reflecting the increased permeability, is observed (Shinohara *et al.*, 1998, 2002; Yamamoto *et al.*, 2004; Beatrice *et al.*, 1982; Petronilli *et al.*, 1993; Jung *et al.*, 1997). Thus, we next examined the effects of DCCD on the TEM appearance of mitochondria. As shown in Fig. 4, Ca^{2+} caused remarkable swelling of mitochondria and disappearance of inner membrane structure, as reported previously (Shinohara *et al.*, 1998, 2002; Yamamoto *et al.*, 2004; Beatrice *et al.*, 1982; Petronilli *et al.*, 1993; Jung *et al.*, 1997). Likewise, DCCD also showed similar effects on the mitochondrial morphology and membrane structure; however, its effect seemed to be much more moderate than that of Ca^{2+}. First,

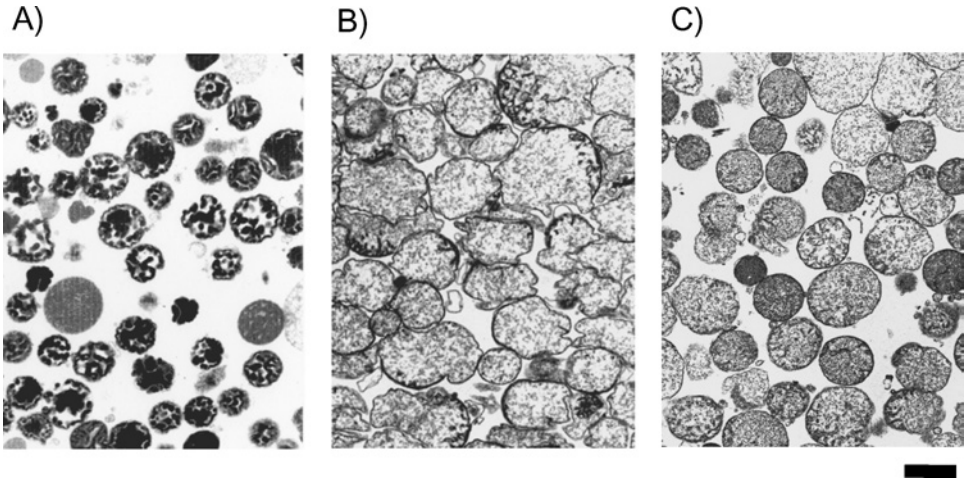

Fig. 4. Effects of DCCD on mitochondrial configuration. Configurations of control mitochondria (A), Ca^{2+}-treated (B), and DCCD-treated mitochondria (C) were examined by transmission electron microscopy. These mitochondrial samples were prepared as follows: For control mitochondria, mitochondria were brought to the energized state for 5 min in the + Pi medium (250 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4) at 25°C by using 10 mM succinate (plus 0.5 μg rotenone/mg protein) as a respiratory substrate. For Ca^{2+}-treated mitochondria, mitochondria were incubated under the same conditions as used for the control mitochondria except for the further supplementation with 100 μM Ca^{2+}. For DCCD-treated mitochondria, they were brought to the energized state for 5 min in − Pi medium containing 100 μM DCCD. After these treatments, mitochondria were promptly harvested and subjected to the fixation processes. Bar under "C" indicates 1 μm for all panels.

in comparison to the Ca^{2+}-treated mitochondria, DCCD-treated ones showed an inner membrane that was not totally damaged; and some of their crista structure remained. Second, sizes of mitochondria treated with DCCD were slightly smaller than those of Ca^{2+}-treated mitochondria. Namely, in comparison to the diameter of control mitochondria, 0.87 ± 0.21 μm ($n = 50$), those of Ca^{2+}- and DCCD-treated mitochondria were determined to be 2.01 ± 0.55 μm ($n = 35$) and 1.65 ± 0.36 μm ($n = 40$), respectively. These differences in TEM appearances between Ca^{2+}-treated and DCCD-treated mitochondria do not reflect the differences in the degree of progression of PT-induction, but reflect the different status of the PT-induced mitochondria, because similar results were obtained even when incubation time was prolonged (data not shown). Furthermore, the observed difference in size

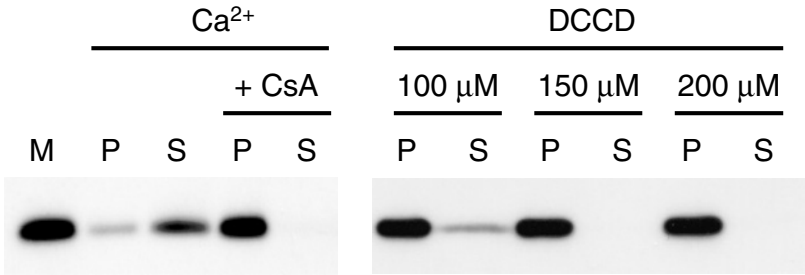

Fig. 5. Effects of DCCD on the location of cytochrome *c*. To examine the effects of DCCD further, mitochondria were first incubated in − Pi medium with DCCD at the indicated concentration, and then they were separated from the medium by prompt centrifugation. Both the pelleted mitochondria and supernatant were subjected to the SDS-PAGE and subsequent Western analysis using specific antibody against cytochrome *c*. "M" indicates the results for non-treated mitochondria (6.3 μg). The typical effect of 100 μM Ca^{2+} on the release of mitochondrial cytochrome *c* observed in the +Pi medium, and the sensitivity of the release to CsA, are also shown as a control.

between Ca^{2+}-treated and DCCD-treated mitochondria is in agreement with the fact that magnitude of mitochondrial swelling induced by DCCD was smaller than that induced by Ca^{2+} (Fig. 1(B)).

Upon induction of the ordinary PT, the release of mitochondrial cytochrome *c* is often observed (Scarlett and Murphy, 1997). Since cytochrome *c* released from mitochondria to the cytosol is known to trigger subsequent steps of apoptosis, it was very interesting to us to examine whether cytochrome *c* could be released from mitochondria when they were treated with DCCD. As shown in Fig. 5, Ca^{2+} caused a significant release of cytochrome *c* from mitochondria, and this release was completely abolished by cyclosporine A. In contrast, DCCD failed to induce massive release of cytochrome *c*. However, it should be noted that a small amount of cytochrome *c* was detected in the supernatant of mitochondrial suspension treated with 100 μM DCCD but not in those treated with DCCD at higher concentrations.

DISCUSSION

DCCD was previously reported to stimulate mitochondrial oxygen consumption, but its action mechanism was uncertain (Chavez *et al*., 1990). In the present study, we further characterized its effects on mitochondria by examining whether it could induce the mitochondrial PT. As a result, DCCD was found to induce mitochondrial PT in a CsA-insensitive manner. As described above, one of the major reaction sites of DCCD in mitochondria is the Fo, and covalent modification of Fo by DCCD causes inhibition of FoF_1-ATPase. It is noteworthy that oligomycin, a well-known specific inhibitor of FoF_1-ATPase, is known to inhibit the ordinary PT induced by Ca^{2+} (Novgorodov *et al*., 1994). Thus, with respect to the PT, these two inhibitors of Fo show opposite effects of induction and inhibition of the mitochondrial PT.

We also examined whether release of cytochrome *c* could be observed when the mitochondria PT was induced by DCCD. As a result, different from the case of the ordinary PT induced by Ca^{2+}, when mitochondria were treated with DCCD, no massive release of mitochondrial cytochrome *c* was observed. Since DCCD may cause intermolecular cross-linking of proteins, possible immobilization of cytochrome *c* by formation of cross-linking between cytochrome *c* and the other mitochondrial proteins, resulting the inhibition of cytochrome *c* release from mitochondria, should be considered. However, in such a case, changes in the mobility of cytochrome *c* in an acrylamide gel containing SDS would be expected. However, this was not the case, for the mobility of cytochrome *c* from DCCD-treated mitochondria was completely identical to that in those from control mitochondria. Thus, we simply concluded that DCCD did not induce cytochrome *c* release even under the condition in which permeability of the inner mitochondrial membrane was elevated.

The molecular mechanism causing cytochrome *c* release from mitochondria is still in controversy and uncertain (Vander Heiden and Thompson, 1999; Kroemer and Reed, 2000; Bernardi *et al*., 2001). Until now, release of mitochondrial cytochrome *c* is believed to occur when the mitochondrial PT is induced. In contrast, we recently reported that cytochrome *c* could be released from mitochondria by valinomycin, even though this reagent does not induce mitochondrial PT (Shinohara *et al*., 2002). Conversely, the present results clearly showed that the mitochondrial PT could be induced without inducing the release of mitochondrial cytochrome *c*. Therefore, we expect these findings to give us clues regarding the process involved in the release of mitochondrial cytochrome *c*. Presently, we would like to further discuss probable factors controlling the release of mitochondrial cytochrome *c*.

Shimizu *et al*. (1999) reported that cytochrome *c* is released via the voltage-dependent anion channel (VDAC) present in the outer mitochondrial membrane. This observation is very interesting, for VDAC is also known to be modified by DCCD (De Pinto *et al*., 1985, 1993; Nakashima *et al*., 1986; Nakashima, 1989), and inhibition of channel activity of VDAC by DCCD could be considered as a probable answer to the question as to why DCCD failed to induce the release of cytochrome *c*, even though it caused the mitochondrial PT. In this context, the question as to whether the transport function of VDAC is inhibited by DCCD or not, is very important. Although this function of VDAC was reported to be insensitive to DCCD treatment (De Pinto *et al*., 1985; Nakashima *et al*., 1986), Shafir *et al*. (1998) showed that the channel activity of VDAC in the sarcoplasmic reticulum could be inhibited by DCCD when VDACs were purified, reconstituted into a planar lipid membrane, and exposed to the high electric potential, thus indicating that the effects of DCCD on channel activity of VDAC may be dependent on the experimental conditions. However, under the present experimental conditions, possible inhibition of VDAC by DCCD could be excluded, since externally added PEGs could still penetrate the outer membrane of DCCD-treated mitochondria to stimulate mitochondrial shrinkage (Fig. 2).

For understanding the key factors controlling the release of mitochondrial cytochrome *c*, comparison of the actions of Ca^{2+} and DCCD should be effective, because these two reagents showed remarkable differences in the aspect of cytochrome *c* release, although both of them

could induce mitochondrial PT, as was shown in Figs. 1, 2, and 4. The actions of DCCD were different from those of Ca^{2+} in terms of the following points: First, different from the PT induced by Ca^{2+}, that induced by DCCD was CsA insensitive. Second, Ca^{2+} requires Pi for induction of mitochondrial PT, but DCCD did not. Third, the degree of mitochondrial swelling induced by DCCD was smaller than that induced by Ca^{2+}. Results of TEM analysis also supported this difference. Finally, the effect on the rate of mitochondrial oxygen consumption was also different between Ca^{2+} and DCCD. Namely, Ca^{2+} stimulated mitochondrial respiration in a dose-dependent manner up to approximately 100 μM; and above this concentration, the rate of respiration was constant at almost maximum (data not shown). In contrast, DCCD accelerated mitochondrial oxygen consumption up to 100 μM; but above this concentration, acceleration of mitochondrial oxygen consumption was significantly inhibited in a dose-dependent manner (Fig. 3), due to its inhibitory effects on the respiratory complex.

In addition to the ordinary PT sensitive to CsA, PT insensitive to it was reported to be induced by atypical PT inducers such as cyanine dyes (Yamamoto *et al*., 2004; Yamashita *et al*., 2003) or metal ions (Jiang *et al*., 2001; Almofti *et al*., 2003; Li *et al*., 2003). Even in the case of the PT insensitive to CsA, when it was induced, the release of cytochrome *c* could be observed, indicating CsA sensitivity of induced PT not to be correlated with the release of cytochrome *c*. The presence or absence of Pi was also not well correlated with the release of cytochrome *c* (data not shown). Therefore, the remaining two parameters of (i) degree of mitochondrial swelling and (ii) status of the mitochondrial respiratory system would seem to be important for the regulation of cytochrome *c* release. As stated above, when mitochondria were treated with 100 μM DCCD, both of massive mitochondrial swelling and acceleration of mitochondrial oxygen consumption were observed. Under this condition, slight release of mitochondrial cytochrome *c* was observed (Fig. 5). When mitochondria were treated with DCCD at concentrations higher than 100 μM, the amplitude of the mitochondrial swelling was not markedly different from that induced by 100 μM DCCD. However, under these conditions, mitochondrial oxygen consumption was significantly inhibited; and the release of mitochondrial cytochrome *c* was not observed. Based on these results, not only induction of mitochondrial swelling but also functionally active mitochondrial respiration would seem to be required for the release of cytochrome *c*. Furthermore, differences in mitochondrial size between Ca^{2+}-treated and DCCD-treated mitochondria are noteworthy. As stated above, the size of mitochondria treated with DCCD was slightly smaller than that of those treated with Ca^{2+}. Therefore, extensive swelling would appear to be required to induce massive release of mitochondrial cytochrome *c*.

Recently, extensive studies have been performed on the functional properties of mitochondria prepared from genetically modified animals or cells (Bauer *et al*., 1999; Li *et al*., 2004; Zamora *et al*., 2004; Kokoszka *et al*., 2004; Baines *et al*., 2005; Nakagawa *et al*., 2005; Basso *et al*., 2005). For better understanding of the relationship between the release of mitochondrial cytochrome *c* and the induction of the mitochondrial permeability transition, in addition to such studies using genetically modified experimental animals or cells, the characterization of the PT induced by various chemicals is also important.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid for scientific research (No. 14370746 [to Y.S.]) from the Ministry of Education, Science, and Culture of Japan, and by a fellowship from Katayama Chemical Industries, Co., Ltd. (Osaka) to T.Y.

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