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Ca^{2+}-induced Reactive Oxygen Species Production Promotes Cytochrome *c* Release from Rat Liver Mitochondria via Mitochondrial Permeability Transition (MPT)-dependent and MPT-independent Mechanisms

ROLE OF CARDIOLIPIN*

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Release of cytochrome *c* from mitochondria is considered a critical, early event in the induction of an apoptosis cascade that ultimately leads to programmed cell death. Mitochondrial Ca^{2+} loading is a trigger for the release of cytochrome *c*, although the molecular mechanism underlying this effect is not fully clarified. This study tested the hypothesis that distinct Ca^{2+} thresholds may induce cytochrome *c* release from rat liver mitochondria by membrane permeability transition (MPT)-dependent and independent mechanisms. The involvement of reactive oxygen species (ROS) and cardiolipin in the Ca^{2+}-induced cytochrome *c* release was also investigated. Cytochrome *c* was quantitated by a new, very sensitive, and rapid reverse-phase high performance liquid chromatography method with a detection limit of 0.1 pmol/sample. We found that a low extramitochondrial Ca^{2+} level (2 μM) promoted the release of ~13% of the total alamethicin releasable pool of cytochrome *c* from mitochondria. This release was not depending of MPT; it was mediated by Ca^{2+}-induced ROS production and cardiolipin peroxidation and appears to involve the voltage-dependent anion channel. High extramitochondrial Ca^{2+} level (20 μM) promoted ~45% of the total releasable pool of cytochrome *c*. This process was MPT-dependent and was also mediated by ROS and cardiolipin. It is suggested that distinct Ca^{2+} levels may determine the mode and the amount of cytochrome *c* release from rat liver mitochondria. The data may help to clarify the molecular mechanism underlying the Ca^{2+}-induced release of cytochrome *c* from rat liver mitochondria and the role played by ROS and cardiolipin in this process.

Mitochondria play a central role in apoptosis induction. Release of cytochrome *c* from mitochondria is considered a critical, early event in the induction of apoptosis cascade that ultimately leads to programmed cell death (1–3). The mechanisms regulating cytochrome *c* release are still not fully understood. Known molecular triggers for the release of cytochrome *c* include pro-apoptotic members such as Bax and Bac and BH3-only proteins such as Bid and Bim (for review see Refs. 4–6). Calcium is also considered an important trigger for the release of cytochrome *c* from mitochondria (7–9). The currently recognized mechanism for Ca^{2+}-induced cytochrome *c* release involves opening of the mitochondrial permeability transition (MPT)[1] (10–12). Pore transition results in a dramatic drop in the mitochondrial transmembrane electrical potential ($\Delta\Psi$), osmotic swelling of the mitochondrial matrix, rupture of the outer mitochondrial membrane, and the release of cytochrome *c*. Relatively high levels of extramitochondrial added Ca^{2+} are required to achieve this effect, and different mechanisms selective for brain *versus* liver have been suggested (13).

Although MPT induction was originally considered the basic mechanism responsible for cytochrome *c* release from mitochondria in response to Ca^{2+} accumulation, more recent studies suggest that Ca^{2+}-induced cytochrome *c* release may occur in an MPT-independent manner (14, 15). Low extramitochondrial Ca^{2+} level can induce cytochrome *c* release from brain mitochondria without apparent MPT opening, mitochondrial swelling, and rupture of the outer membrane (14). Liver mitochondria appear to be less resistant than brain mitochondria to Ca^{2+}-induced MPT opening and subsequent cytochrome *c* release (13). However, a relatively low level of added Ca^{2+} to rat liver mitochondria is sufficient to induce cytochrome *c* release without any observable large amplitude swelling or drop in membrane potential (15).

A growing number of data show that reactive oxygen species (ROS), mainly produced by mitochondria, can be involved in cell death by promoting cytochrome *c* release from mitochondria (16–18). ROS can produce an oxidative stress leading to cell destruction as observed during necrosis. In several instances, ROS derived from mitochondria are also involved in the initiation phase of apoptosis contributing to cell death signaling. The involvement of ROS in cytochrome *c* release from mitochondria is well established. However, the exact mechanism by which ROS promote cytochrome *c* release from mitochondria and subsequent apoptosis process remains unclear.

Cardiolipin, a phospholipid almost exclusively located in the

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[1] The abbreviations used are: MPT, mitochondrial permeability transition; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel; HPLC, high pressure liquid chromatography; RR, ruthenium red.

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inner mitochondrial membrane, has been emerging as an important player in the control of the mitochondrial phase of the apoptotic process (19–22). Cardiolipin at the contact sites has been claimed to provide specificity for targeting of tBid to the mitochondria (23, 24). In addition to its role in controlling many mitochondrial processes such as the activity of respiratory chain complexes and anion carriers (for review see Ref. 25), cardiolipin molecules bind cytochrome *c* to the outer surface of the inner mitochondrial membrane (26, 27). Thus, conditions leading to alteration of this binding, such as changes in the cardiolipin content and/or oxidative damage to its unsaturated acyl chains (28–31) should promote cytochrome *c* detachment from the mitochondrial membrane, which can be considered an initial step in the release of cytochrome *c* from mitochondria. Accordingly, we have proposed that mitochondrial-mediated ROS production promotes cytochrome *c* release from mitochondria by a two-step process consisting of the dissociation of this protein from cardiolipin, caused by alteration of the interaction between this phospholipid and cytochrome *c*, followed by permeabilization of the outer mitochondrial membrane probably by interaction with VDAC (32).

Recent studies reported that Ca^{2+} accumulation by mitochondria is associated to stimulation of mitochondrial ROS production (33–36) by a mechanism not well understood. On the basis of the observations reported above, it is conceivable that Ca^{2+}, ROS, and cardiolipin might play a coordinated role in the mechanism of cytochrome *c* release from mitochondria and subsequent apoptosis. These aspects were explored in the present investigation. This work describes the effects of relatively low and relatively high extramitochondrial Ca^{2+} levels added to rat liver mitochondria on various bioenergetic parameters such as cytochrome *c* release, ROS production, membrane potential, and mitochondrial swelling as well as on the cardiolipin level and its degree of peroxidation. We found that low (2 μM) and high (20 μM) Ca^{2+} levels promote cytochrome *c* release from rat liver mitochondria by a mechanism that is MPT-independent and MPT-dependent, respectively. These effects of Ca^{2+} are mediated by ROS and cardiolipin.

EXPERIMENTAL PROCEDURES

Reagents—Safranin O, scopoletin, and horseradish peroxidase were from Sigma. Dichlorofluorescein was from Fluka. All other reagents were of analytical grade. Anti-VDAC antibody was from Calbiochem.

Preparation of Mitochondria—Rat liver mitochondria were isolated in ice-cold medium containing 250 mM sucrose, 10 mM Tris-HCl, 1 mM EGTA, pH 7.4, by differential centrifugation of liver homogenates essentially as described previously (32). Mitochondrial pellet was resuspended in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4 and stored in ice. Mitochondrial protein concentration was measured by the biuret method using serum albumin as standard.

Standard Incubation Procedure—Mitochondria (0.5 mg protein/3 ml) were incubated at 25 °C in a medium containing 150 mM sucrose, 50 mM KCl, 5 mM Tris, pH 7.4, 0.25 mM Pi, 10 μM EGTA, and 1 μM rotenone. Extramitochondrial Ca^{2+} was adjusted by using Ca^{2+}/EGTA buffer. For calculation the concentration of free Ca^{2+} we used the complexing constants according to Ref. 37.

Fluorometric Determination of Mitochondrial H_2O_2 Production—The rate of mitochondrial hydrogen peroxide production was estimated by measuring the linear fluorescence increase induced by H_2O_2 oxidation of dichlorofluorescein to the fluorescent dichlorofluorescein in the presence of horseradish peroxidase (38). Fluorescence was determined at 488 nm for excitation and 525 nm for emission. Rat liver mitochondria (0.5 mg of protein) were suspended in 3 ml of the standard incubation medium supplemented with 1.5 μM rotenone, 5 mM succinate, 7.5 μg of horseradish peroxidase, and 1 μM dichlorofluorescein. The production of hydrogen peroxide was induced by the addition of Ca^{2+}. The amount of H_2O_2 produced was calculated by measuring the fluorescence changes induced by the addition of known amounts of H_2O_2. H_2O_2 production was also measured by the scopoletin method. The results were practically similar to those obtained with the dichlorofluorescein method.

Measurement of Mitochondrial Membrane Potential—The membrane potential of intact liver mitochondria was measured following the safranin O fluorescence quenching at 525 nm (excitation), 575 nm (emission) with a Jasco FP-750 spectrofluorometer (39). Freshly isolated mitochondria (0.5 mg of protein) were suspended in 3 ml of the standard incubation medium supplemented with 1.5 μM rotenone and 8 μM safranin O. The generation of the transmembrane potential was induced by the addition of 5 mM succinate as substrate.

Detection of Cytochrome c Release—Cytochrome *c* content, in the supernatant, was determined by using a 5-mm C4 reverse-phase column (150 × 4.6 mm) on a Hewlett Packard series 1100 high performance liquid chromatography (HPLC) chromatograph. A gradient of 20% acetonitrile in water with trifluoroacetic acid (0.1% v/v) to 60% acetonitrile in water with trifluoroacetic acid (0.1% v/v) over 12 min with a flow rate of 1 ml/min was used. Absorption at 393 nm was used (40). To improve the sensitivity of the method, the supernatants were supplemented with bovine serum albumin to a final concentration of 25 μM (41).

Analysis of Cardiolipin in Mitochondrial Membranes—Cardiolipin was analyzed by HPLC using a Hewlett Packard series 1100 gradient liquid chromatograph. Lipids from heart mitochondria were extracted with chloroform/methanol by the procedure of Bligh and Dyer (42). Lipid extraction was carried out on ice immediate after the preparation of mitochondria in the presence of butylated hydroxytoluene and under a nitrogen atmosphere. Phospholipids were separated by the HPLC method previously described (43) with a Lichrosorb Si60 column (4.6 × 250 mm). The chromatographic system was programmed for gradient elution using two mobile phases: solvent A, hexane/2-propanol (6:8, v/v) and solvent B, hexane/2-propanol/water (6:8:1.4, v/v/v). The percentage of solvent B in solvent A was increased in 15 min from 0–100%. Flow rate was 1 ml/min and detection was determined at 206 nm. The peak of cardiolipin was identified by comparison with the retention time of standard cardiolipin and rechromatographed by TLC.

Analysis of Peroxidized Cardiolipin—Peroxidized cardiolipin was identified by normal-phase HPLC as described above with UV detection at 235 nm, indicative of conjugated dienes (44–46). The resulting peak was rechromatographed by TLC and used as standard.

Mitochondrial Swelling—To measure mitochondrial swelling that accompanies the opening of the permeability transition pore mitochondria (0.5 mg of protein/3 ml) were suspended in the standard incubation medium supplemented with 1.5 μM rotenone and 5 mM succinate. The mitochondrial swelling was monitored continuously at 540 nm.

RESULTS

Most of the published data concerning cytochrome *c* release from mitochondria have been obtained from Western blotting or absorbance spectroscopy. In particular, Western blotting is a semiquantitative method that cannot give precise information about the amount of cytochrome *c* released. For direct evaluation of Ca^{2+}-induced cytochrome *c* release from isolated liver mitochondria, extramitochondrial cytochrome *c* was analyzed by a new, very rapid, and sensitive method based on reverse-phase HPLC. This method first introduced by Piklo *et al.* (40) and improved by Crouser *et al.* (41) allowed us to quantitate cytochrome *c* with a detection limit around 0.1 pmol/sample.

The release of cytochrome *c* was measured in mitochondria supplemented with the oxidizable substrate succinate in the presence of rotenone after the addition of relatively low and relatively high concentrations of added Ca^{2+}. The mitochondrial cytochrome *c* content as quantified by this technique was 160 ± 14 pmol/mg protein, in line with the results reported by others (41). The addition to mitochondria of alamethicin, an artificial channel former, induced the release of 121 ± 13 pmol cytochrome *c*/mg protein, which corresponds to 70% of the total amount of mitochondrial cytochrome *c*. The amount of cytochrome *c* released upon treatment with alamethicin was taken as the maximal amount of this hemoprotein that could be released by mitochondria under our experimental conditions. As shown in Fig. 1, in the absence of added Ca^{2+} no detectable amount of cytochrome *c* was liberated from control mitochondria during 10 min of incubation, which indicates the integrity of our mitochondrial preparation. However, the addition of 2 μM Ca^{2+} (12 nmol/mg protein) or 20 μM (120 nmol/mg protein)

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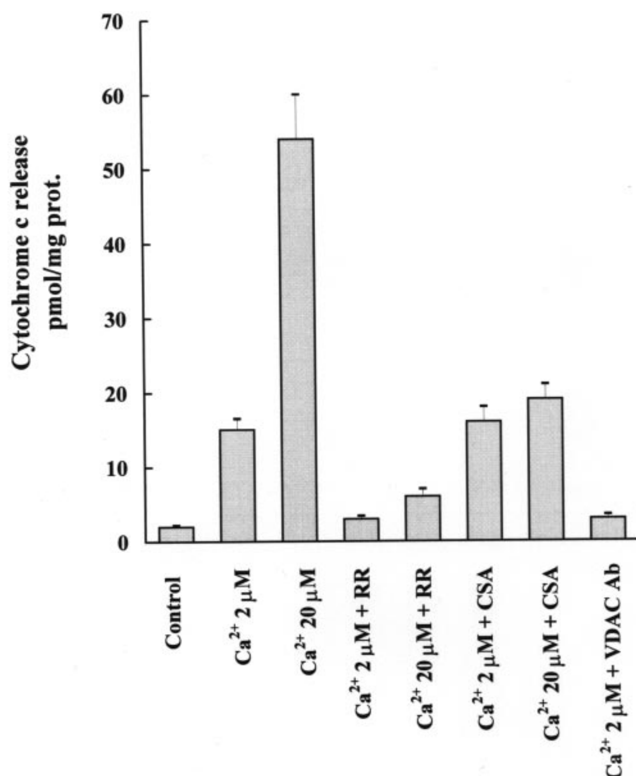

FIG. 1. **Ca^{2+}-induced release of cytochrome *c* from rat liver mitochondria and the effect of ruthenium red, cyclosporin A, and anti-VDAC antibody.** Mitochondria were incubated in the standard medium at 25 °C in the presence of 5 mM succinate and where indicated 2.5 μM RR, 1 μM cyclosporin A (*CSA*). Where present, anti-VDAC antibody (*VDAC Ab*) (0.3 μg/ml) was preincubated for 5 min with mitochondria. After 1 min the incubation was started by the addition of 2 or 20 μM free Ca^{2+}. 10 min later, mitochondria were centrifuged and the supernatant was withdrawn, filtered, and then injected into the HPLC to determine cytochrome *c* content. All values are expressed as mean ± S.E. of four separate experiments.

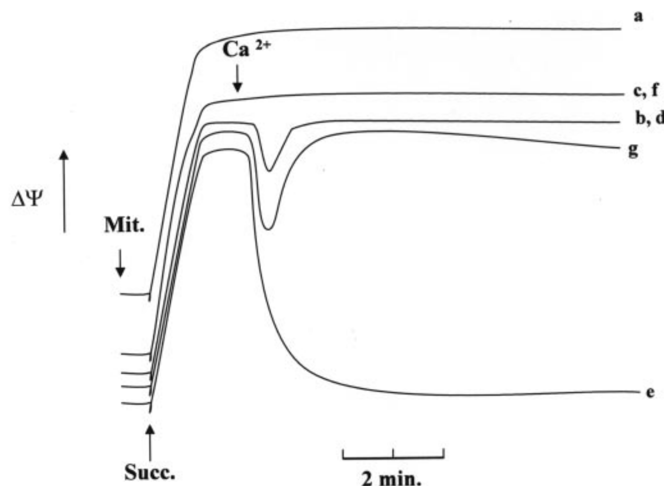

FIG. 2. **Changes in mitochondrial membrane potential induced by Ca^{2+} addition and the effect of ruthenium red, cyclosporin A, and anti-VDAC antibody.** Mitochondria were incubated in the standard medium. The membrane potential was induced by the addition of 5 mM succinate and monitored for at least 10 min (*trace a*). Further additions were as follows: 2 μM free Ca^{2+} (*trace b*); 2 μM free Ca^{2+}, 2.5 μM RR (*trace c*); 2 μM free Ca^{2+}, VDAC Ab (*trace d*); 20 μM free Ca^{2+} (*trace e*); 20 μM free Ca^{2+}, 2.5 μM RR (*trace f*); 20 μM free Ca^{2+}, 1 μM cyclosporin A (*trace g*). Where present cyclosporin A or ruthenium red was added 1 min before Ca^{2+} addition. Results shown are typical of five different experiments.

induced the release of 15 ± 1.5 or 54 ± 6 pmol of cytochrome *c*/mg protein, respectively. Complete prevention of Ca^{2+} influx into the mitochondrial matrix by inhibition of Ca^{2+} uniporter by ruthenium red totally abolished the Ca^{2+}-induced cytochrome *c* release, indicating that Ca^{2+} accumulation inside mitochondria is needed for this effect. The effect of cyclosporin A, an inhibitor of mitochondrial permeability transition, was tested on the release of cytochrome *c* induced by these two Ca^{2+} levels. Cyclosporin A did not prevent the release of cytochrome *c* induced by low [Ca^{2+}], while it inhibited the extraquote of cytochrome *c* release induced by high [Ca^{2+}]. Pretreatment of the isolated mitochondria with an anti-VDAC antibody (47) prevented the release of cytochrome *c* induced by a low Ca^{2+} level.

To ascertain the possible involvement of MPT in the release of cytochrome *c* from mitochondria in the presence of added Ca^{2+}, experiments were carried out recording the changes in the mitochondrial membrane potential (Fig. 2). Mitochondrial membrane potential ($\Delta\Psi$) was generated by the addition of succinate as substrate in the presence of rotenone (*trace a*). The addition of low [Ca^{2+}] to mitochondria resulted in a transient reduction in $\Delta\Psi$ (*trace b*) as Ca^{2+} uptake occurs via an electrophoretic uniporter that draws upon the $\Delta\Psi$ for active Ca^{2+} accumulation. Approximately 1 min after the addition of Ca^{2+}, $\Delta\Psi$ recovered back to its control level and remained stable for at least 10 min. The addition of ruthenium red (RR) totally abolished the Ca^{2+}-induced transient reduction of mitochondrial transmembrane $\Delta\Psi$ (*trace c*). By contrast, the anti-VDAC antibody had no effect on this $\Delta\Psi$ decrease (*trace d*) indicating that this compound did not affect Ca^{2+} accumulation by mitochondria. As expected, the succinate-induced polarization was rapidly and totally abolished by the addition of high [Ca^{2+}] (*trace e*). This effect was abolished by the Ca^{2+} uniporter inhibitor RR (*trace f*) or by cyclosporin A, a known inhibitor of the Ca^{2+}-induced pore opening (*trace g*). These results indicate that two distinct Ca^{2+} thresholds can determine the closed or unclosed state of the permeability transition pore in rat liver mitochondria. This conclusion is further supported by experiments in which mitochondrial volume changes were measured.

As reported in Fig. 3 the addition of low Ca^{2+} to rat liver mitochondria did not cause any appreciable decrease of light scattering during the first 10 min of incubation, indicating the absence of pore opening. By contrast, the addition of high Ca^{2+} resulted in a rapid and full activation of MPT, which was abolished by cyclosporin A.

It has been reported that Ca^{2+} loading by mitochondria results in a stimulation of H_2O_2 production (33–36), although the mechanism responsible for this stimulation remains unclear. Fig. 4 shows the production of H_2O_2 in mitochondria supplemented with the oxidizable substrate succinate in the presence of rotenone after the addition of low or high levels of Ca^{2+}. The addition of free Ca^{2+} at 2 μM resulted in a stimulation of H_2O_2 generation (*trace b*). This H_2O_2 production was more pronounced when 20 μM free Ca^{2+} was added (*trace c*). Complete prevention of Ca^{2+} influx into the mitochondrial matrix by inhibition of uniporter by ruthenium red almost completely abolished the Ca^{2+}-induced H_2O_2 production (*trace d*), confirming the requirement of Ca^{2+} entry into mitochondria for this effect. The stimulation of H_2O_2 production induced by low [Ca^{2+}] was not inhibited by cyclosporin A (*trace e*), indicating that MPT is not involved in this process nor by anti-VDAC antibody (*trace f*). The stimulation of H_2O_2 production in the presence of high [Ca^{2+}] was largely reduced by cyclosporin A (*trace g*) suggesting the possible involvement of MPT in this process.

Cytochrome *c* is bound to cardiolipin at the level of the inner mitochondrial membrane. The molecular interaction between cytochrome *c* and cardiolipin has been extensively studied (26,

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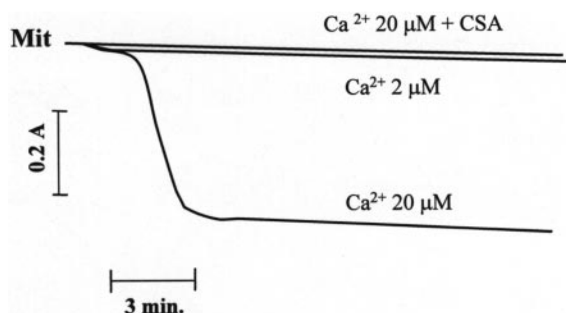

FIG. 3. **Effect of different Ca^{2+} concentrations on mitochondrial swelling.** Rat liver mitochondria (0.5 mg protein/ml) were incubated in the standard medium supplemented with 5 mM succinate. After 1 min 2 μM or 20 μM free Ca^{2+} was added. Where present, 1 μM cyclosporin A was added 1 min before Ca^{2+} addition. Changes in mitochondrial volume were monitored spectrophotometrically at 540 nm for at least 10 min. Results shown are typical of five different experiments.

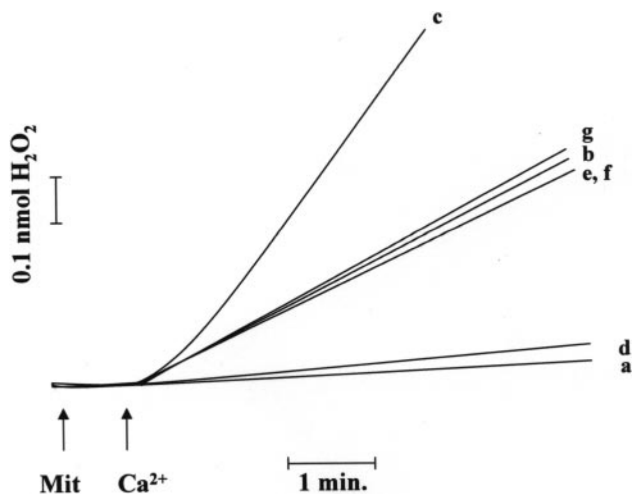

FIG. 4. **Ca^{2+}-induced H_2O_2 production in respiring rat liver mitochondria and the effect of ruthenium red, cyclosporin A, and anti-VDAC antibody.** The mitochondrial production of H_2O_2 was measured as described under "Experimental Procedures." Rat liver mitochondria were incubated in the standard incubation medium supplemented with 5 mM succinate. The following additions were made: control in the absence of Ca^{2+} (*trace a*); 2 μM free Ca^{2+} (*trace b*); 20 μM free Ca^{2+} (*trace c*); 20 μM free Ca^{2+}, 2.5 μM RR (*trace d*); 2 μM free Ca^{2+}, 1 μM cyclosporin A, (*trace e*); 2 μM free Ca^{2+}, VDAC Ab (*trace f*); 20 μM free Ca^{2+}, 1 μM cyclosporin A (*trace g*). Cyclosporin A and ruthenium red were added 1 min before Ca^{2+} addition. Results shown are typical of five different experiments.

27). We have recently reported that mitochondrial ROS production is associated to cardiolipin oxidative damage and cytochrome *c* release from mitochondria (30, 32). The content of cardiolipin was measured in mitochondria in the presence of low and high Ca^{2+} concentrations by a very sensitive HPLC technique set up in our laboratory with a detection limit of 0.5 nmol (43). As shown in Fig. 5, addition of low $[Ca^{2+}]$ to mitochondria resulted in a loss of cardiolipin content compared with control mitochondria. This loss of cardiolipin was more pronounced in the presence of high $[Ca^{2+}]$. The addition of RR prevented the loss of cardiolipin induced by low and high Ca^{2+} concentrations. Pretreatment of the isolated mitochondria with an anti-VDAC antibody had no effect on the cardiolipin loss induced by low $[Ca^{2+}]$ (results not shown).

The loss in the cardiolipin content observed in mitochondria following Ca^{2+} accumulation could be caused by cardiolipin peroxidation because of ROS attack to its unsaturated acyl chains. To asses this, the content of peroxidized cardiolipin was measured in mitochondria by an HPLC method based on the absorbance at 235 nm, indicative of the formation of conjugated

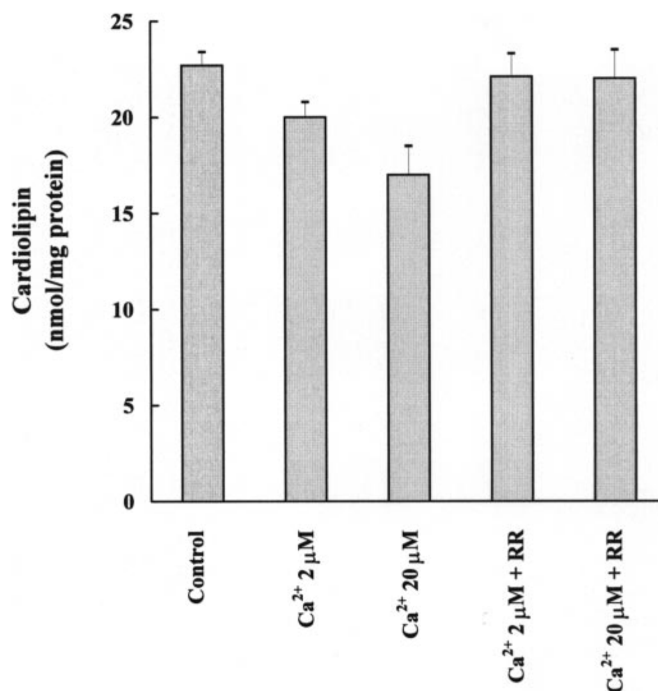

FIG. 5. **Ca^{2+}-induced loss in the mitochondrial cardiolipin content and prevention by ruthenium red.** Rat liver mitochondria were incubated in the standard medium at 25 °C supplemented with 5 mM succinate. After 1 min of incubation 2 or 20 μM free Ca^{2+} was added. 10 min later, mitochondria were centrifuged and the cardiolipin content was measured on the lipid extract of the mitochondrial pellet as described under "Experimental Procedures." Where indicated, 2.5 μM ruthenium red was added 1 min before Ca^{2+} addition. All values are expressed as mean ± S.E. of four separate experiments.

dienes (45, 46). As shown in Fig. 6, an increase in the level of peroxidized cardiolipin was observed in mitochondria in the presence of low $[Ca^{2+}]$ compared with control mitochondria. A more pronounced level of peroxidized cardiolipin was detected when a high level of $[Ca^{2+}]$ was added to mitochondria. Ruthenium red prevented the Ca^{2+}-induced increase in cardiolipin peroxidation.

DISCUSSION

A number of studies have demonstrated that mitochondrial Ca^{2+} loading contributes to the induction of molecular cell death cascades in several different paradigms (7–9, 48). Ca^{2+}-induced release of mitochondrial cytochrome *c* into the cytosol is an early and key step linking the triggering phase of apoptosis to the executioning phase. Two main hypotheses concerning the mechanism of Ca^{2+}-induced cytochrome *c* release from mitochondria have been suggested. One involves the activation of MPT by Ca^{2+} and mitochondrial depolarization and swelling followed by the rupture of the outer mitochondrial membrane causing cytochrome *c* release (10–12, 49, 50). The second hypothesis, obtained mainly with brain mitochondria, suggests that a distinct threshold of extramitochondrial Ca^{2+} in low micromolar range is sufficient to mediate the release of cytochrome *c* independent of MPT without mitochondrial swelling or outer membrane rupture (14). Evidence for cytochrome *c* release at low Ca^{2+} levels in rat liver mitochondria in the absence of observable manifestation of MPT was already presented. This effect was explained on the basis that low Ca^{2+} loading is sufficient to induce MPT in a subpopulation of mitochondria (15).

We have investigated the effect of low Ca^{2+} on cytochrome *c* release from rat liver mitochondria using a new, very sensitive, and rapid reverse HPLC method with a detection limit of 0.1 pmol. We found that extramitochondrial Ca^{2+} level as low as 2

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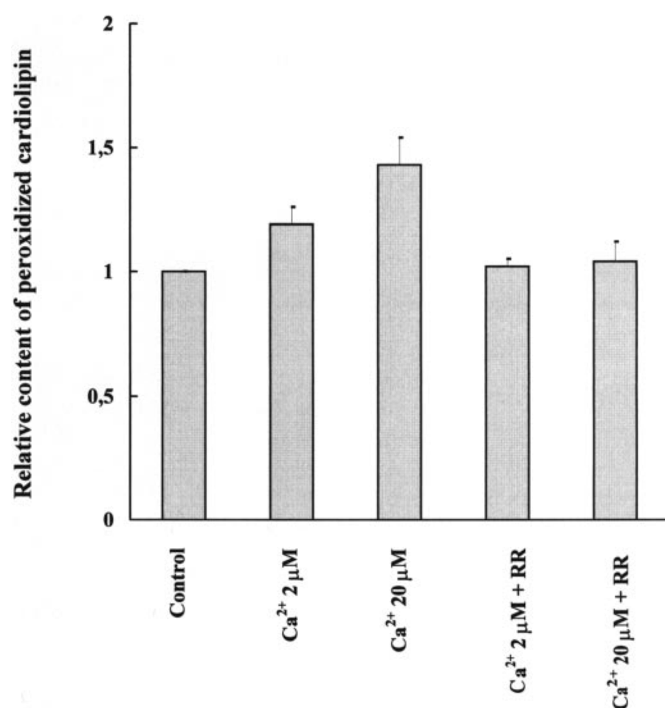

FIG. 6. **The effect of Ca^{2+} on the cardiolipin peroxidation in rat liver mitochondria.** Experimental conditions are similar to those described in the legend to Fig. 1. Mitochondrial content of peroxidized cardiolipin was determined by the HPLC technique described under "Experimental Procedures." The content of peroxidized cardiolipin is expressed as peak area (at 235 nm) per mg of phospholipids, and peak area of the control is assumed as one unit. All values are expressed as mean ± S.E. of four separate experiments.

μM (12 nmol/mg protein) promoted the release of ~13% of the total alamethicin-releasable pool of cytochrome *c* from mitochondria. This release was not inhibited by cyclosporin A. In addition, no change in the membrane potential nor in the mitochondrial swelling was observed following incubation of mitochondria for 10 min in the presence of low $[Ca^{2+}]$ (a condition under which cytochrome *c* was observed), thus excluding the involvement of the MPT in this process. These results indicate that the integrity of the mitochondrial inner membrane and matrix space was preserved following low $[Ca^{2+}]$ addition to mitochondria. By contrast, when a high Ca^{2+} level (20 μM) was added to mitochondria, a substantial amount of cytochrome *c* (~45% of the maximal releasable amount) was released. This release was inhibited by cyclosporin A indicating activation of MPT in this process as further documented by classical membrane depolarization and swelling experiments (Figs. 3 and 4).

Release of cytochrome *c* from mitochondria appears to be largely mediated by ROS. However the exact mechanism by which ROS induce cytochrome *c* release from mitochondria and subsequent apoptosis is still not well understood. Recent findings have shown that Ca^{2+} overload is accompanied by ROS production (33–36). We have confirmed these results showing that addition of micromolar Ca^{2+} level to rat liver mitochondria in the presence of rotenone and oxidizable substrate succinate induces a stimulation of ROS production (Fig. 4). This stimulation was dose-dependent and was completely abolished by ruthenium red, a known inhibitor of the Ca^{2+} influx into the mitochondria. This indicates that Ca^{2+}-induced ROS production depends on Ca^{2+} accumulation into the mitochondrial matrix. The mechanism by which Ca^{2+} accumulation promotes ROS generation is unclear. One possible explanation is that by binding to cardiolipin molecules Ca^{2+} may impair the flow of electrons within the electron transport chain at the site, most likely within complex III, which is proximal to the site of ROS generation (51). In fact, it has been reported that complex III contains tightly bound cardiolipin molecules that appear to be essential for the catalytic function (52, 53). The involvement of cardiolipin in the function of complex III is also supported by the results of our recent study showing inactivation of this enzyme complex by nonyl-L-acridine, a compound that interacts specifically with cardiolipin (54).

Because Ca^{2+} accumulation by mitochondria stimulates ROS generation and given that these species are highly reactive and short lived, mitochondrial constituents might be exposed to oxidative stress. Among mitochondrial constituents, cardiolipin appears particularly susceptible to ROS attack either because of its high content of unsaturated fatty acids (90% represented by linoleic acid) or because of its location in the inner mitochondrial membrane, near to the site of ROS production. We found that the addition of low $[Ca^{2+}]$ to mitochondria is associated with a decrease in the mitochondrial cardiolipin content (Fig. 5), caused by ROS-induced cardiolipin peroxidation and shown by the increased level of conjugated dienes (Fig. 6). These effects were totally abolished by ruthenium red. The fact that ruthenium red prevents Ca^{2+}-induced cytochrome *c* release, ROS production, and cardiolipin peroxidation indicates that all these processes are caused by Ca^{2+} accumulation inside mitochondria. Furthermore, the existence of a good quantitative correlation among these processes suggests a possible causal link among them.

Taken together, our results indicate that addition of low $[Ca^{2+}]$ to rat liver mitochondria promotes a cascade of events consisting of ROS production, leading to oxidative damage to cardiolipin with subsequent detachment of cytochrome *c* from the inner mitochondrial membrane, and then releasing cytochrome *c* through the outer mitochondrial membrane. As discussed above, this Ca^{2+}-induced cytochrome *c* release is independent of MPT. How might Ca^{2+} induce cytochrome *c* release in the absence of MPT? Recently, we reported (confirming previous results in the literature (17)) that reactive oxygen species produced by the mitochondrial respiratory chain induce MPT-independent cytochrome *c* release by selective permeabilization of outer mitochondrial membrane through interaction with VDAC (32, 55). The fact that cytochrome *c* release induced by low $[Ca^{2+}]$ is inhibited by an anti-VDAC antibody supports the possibility that a similar mechanism might be involved in the cytochrome *c* release induced by low $[Ca^{2+}]$.

At a relatively high Ca^{2+} level, the mechanism of cytochrome *c* release would involve ROS production leading to cardiolipin peroxidation with subsequent detachment of cytochrome *c* from inner mitochondrial membrane followed by ROS-dependent MPT opening. In fact, it has been reported that ROS and high intramitochondrial Ca^{2+} may act together to trigger MPT opening, probably by oxidizing critical thiol groups of adenine nucleotide translocase, a known reported constituent of the pore (36). The possibility that ROS and Ca^{2+} could interact with cardiolipin molecules tightly bound to adenine nucleotide translocase inducing conformational change to this protein, thus causing MPT opening, should also be taken into consideration. Permanent MPT opening causes cytochrome *c* release via matrix swelling and rupture of the mitochondrial membrane.

In summary, our data indicate that an increase in mitochondrial $[Ca^{2+}]$ loading into micromolar range is sufficient to promote cytochrome *c* release from rat liver mitochondria without opening of MPT. This effect of Ca^{2+} is mediated by ROS inducing cardiolipin peroxidation, cytochrome *c* detachment from inner mitochondrial membrane and outer mitochondrial membrane permeabilization through VDAC. Relatively high micromolar Ca^{2+} level is required to open MPT to release a substan-

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tial amount of cytochrome *c*. This effect is also mediated by ROS generation and cardiolipin peroxidation.

Ca^{2+} overload has been suggested to be the final common pathway of all types of cell death. The low and high [Ca^{2+}]-dependent mechanisms of cytochrome *c* release from rat liver mitochondria mediated by ROS and cardiolipin might reflect two distinct physiopathological situations. Under a pathological condition, such as during ischemia-reperfusion injury, high mitochondrial Ca^{2+} loading could promote cell death through necrosis, whereas lower Ca^{2+} loading induced by milder insults could promote cell death through apoptosis.

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