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Flow Cytometric Analysis of Ca^{2+}-Induced Membrane Permeability Transition of Isolated Rat Liver Mitochondria

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Summary **The membrane permeability transition (MPT) of mitochondria plays an important role in the mechanism of apoptotic cell death in various cells. Classic type MPT is induced by Ca^{2+} in the presence of inorganic phosphate and respiratory substrate, and is characterized by various events including generation of reactive oxygen species (ROS), membrane depolarization, swelling, release of Ca^{2+} and high sensitivity to cyclosporine A. However, the sequence of these events and the effect of antioxidants on their events remain obscure. Flow cytometry is a convenient method to investigate the order of events among various functions occurring in MPT using a limited amount of mitochondria (200 μl of 0.02 mg protein/ml) without contamination by other organelles. Flow cytometric analysis revealed that Ca^{2+} sequentially induced ROS generation, depolarization, swelling and Ca^{2+} release in mitochondria by a cyclosporine A-inhibitable mechanism. These results were supported by the finding that Ca^{2+}-induced MPT was inhibited by antioxidants, such as glutathione and N-acetylcysteine. It was also revealed that various inhibitors of Ca^{2+}-induced phospholipase A_2 suppressed all of the events associated with Ca^{2+}-induced MPT. These results suggested that ROS generation and phospholipase A_2 activation by Ca^{2+} underlie the mechanism of the initiation of MPT.**

Key Words: **antioxidant, membrane permeability transition, flow cytometric analysis, mitochondria, phospholipase A_2**

Introduction

Apoptosis plays an important role in various physiological processes including embryonic development, maintenance of tissue and cell homeostasis, and in the pathogenesis of various diseases [*1–3*]. Among various organelles [*4–7*] mitochondria play the most important roles in the process of apoptosis by inducing membrane permeability transition (MPT). Opening of MPT pores releases apoptosis-related proteins including cytochrome c from mitochondria to cytosol thereby activating the caspase cascade [*4*, *8*]. Mitochondria thus play pivotal roles in determining cell survival and death through energy transduction and release of apoptosis-related proteins, respectively.

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In the presence of inorganic phosphate (Pi) and respiratory substrates, Ca^{2+} induces typical classic type MPT characterized by its dependency on Ca^{2+} and energy metabolism, mitochondrial depolarization, swelling, release of Ca^{2+}, and high sensitivity to cyclosporine A, a specific inhibitor of MPT [*4*, *9*, *10*]. Although Ca^{2+} loading into mitochondria induces cytochrome c release, the molecular mechanism and sequence of events leading to cell death remain unclear.

Reactive oxygen species (ROS) produced by a variety of physiological and pathological metabolisms [*11–13*] function as critical second messenger in a variety of intracellular signaling pathways [*14*, *15*]. We previously reported that mitochondria generated ROS followed by the induction of MPT [*10*]. Although the generation of ROS has been postulated to be one of the early events that induce MPT [*15*], the effects of antioxidants on Ca^{2+}-induced mitochondrial swelling and other events leading to MPT remain obscure. Since flow cytometric analysis is an excellent method for the analysis of mitochondrial swelling, depolarization, Ca^{2+} release and ROS generation [*16–18*], we analyzed a sequence of events occurring in small amount of mitochondria using a FACScan analyzer.

Materials and Methods

Chemicals

Bromophenacyl bromide (BPB), chlorpromazine (CP), fatty acid free bovine serum albumin (BSA), N-acetylcysteine (NAC), quinacrine (QC), ruthenium red (RR), cyclosporine A (CsA) and trifluoperazine (TFP) were obtained from Sigma Co. Ltd. (Saint Louis, MO). Ca^{2+}-dependent secretary phospholipase A2 (cPLA2) α inhibitor was obtained from Calbiochem (Darmstadt, Germany). 2',7'-Dichlorodihydrofluorescein diacetate (H2DCF-DA), hydroethidine (HE), tetramethylrhodamine-ethyl-ester (TMRE) and 10-nonyl acridine orange (NAO) were obtained from Molecular Probes (Eugene, OR). 1-[2-Amino-5-(-dimethylamino-6-dimethylammonio-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid (Rhod 2)-tetraacetoxymetyl (AM) was obtained from Dojindo Co. Ltd. (Kumamoto, Japan). Cyanine dye, 3,3'-dipropyl-2,2'-thiodicarbocyanine iodide (diS-C3-(5)), a cyanine dye, was obtained from the Hayashibara Biochemical Laboratories (Okayama, Japan). All other chemicals were of analytical grade and obtained from Nacalai Tesque (Kyoto, Japan). NAO, TMRE, hydroethidine and CsA were dissolved in DMSO and stored at 4°C until use.

Isolation of rat liver mitochondria

After fasting Wistar rats overnight, excised rat livers were homogenized in 0.25 M sucrose containing 10 mM Tris-HCl buffer (pH 7.4) and 1 mM EDTA at 4°C. Mitochondria were isolated from the homogenates by the method of Hogeboom as described previously [*19*].

Assay for mitochondrial functions

Oxygen consumption and oxidative phoshorylation of mitochondria were measured by an oxygen electrode [*10*]. Mitochondria (0.25 mg protein/ml) were incubated in a medium consisting of 250 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl and 10 mM Tris-HCl buffer (pH 7.4) at 25°C. Mitochondria used for the experiments maintained a high respiratory control ratio (RCR of 5.0) and ADP/O ratio (1.7) in the presence of Pi and succinate.

Mitochondrial swelling was monitored by the change in light scattering at 540 nm and recorded by a Hitachi fluorescence spectrophotometer (650-10LC) equipped with a thermostatically controlled cuvette holder and a magnetic stirrer [*10*]. Mitochondrial membrane potential was measured by the fluorescence intensity of diS-C3-(5) (0.2 μg/ml) at 670 nm during excitation at 622 nm by a Hitachi 650-10LC [*10*].

Flow cytometry

Flow cytometric analysis was carried out using a FACScan equipped with a 488-nm Argon laser (Becton Dickinson, San Jose, CA). Data from the experiments were analyzed using the CELLQuest software (Becton Dickinson) as described previously [*16–18*]. To exclude debris in the side scatter (SSC) and forward scatter (FSC) modes, 50,000 events per sample within this gate (R1) were collected using the "low" setting for sample flow rate. Mitochondria were selectively stained with NAO (100 nM, excitation at 488 nm and emission at 525 nm) that binds to cardiolipin in the inner mitochondrial membrane [*17*, *20*]. TMRE (100 nM, excitation at 488 nm and emission at 590 nm), HE (10 μM, excitation at 495 and emission at 580 nm) and Rhod 2 (2.5 μM, excitation at 488 nm and emission at 576 nm) were used to measure membrane potential, ROS and release of Ca^{2+}, respectively [*16–18*, *21–24*]. TMRE accumulated in mitochondria in membrane potential dependent manner. Binding of Ca^{2+} to Rhod 2 increases its fluorescence. Thus, it has been used to monitor changes in $[Ca^{2+}]$ within the mitochondrial matrix [*24*]. HE also used to detect ROS, especially superoxide [*22*].

Mitochondria (0.1 mg protein/ml) were stained under dark conditions with either NAO, HE, TMRE or Rhod 2-AM in 1 ml of standard medium (3 mM HEPES buffer, pH 7.4, containing 70 mM sucrose, 230 mM mannitol, 1 μM EDTA and ~10 μM contaminating Ca^{2+}) in the presence of 0.5 mM Pi and 2.5 mM succinate under dark conditions at 25°C for 3 min. Then, adding various concentration of Ca^{2+} in the presence or absence of various reagents induced MPT. After 20 seconds~5 min, membrane depolarization, Ca^{2+}-release and ROS generation of NAO-positive mitochondria were analyzed by FACScan for changes in FL2-H of TMRE,

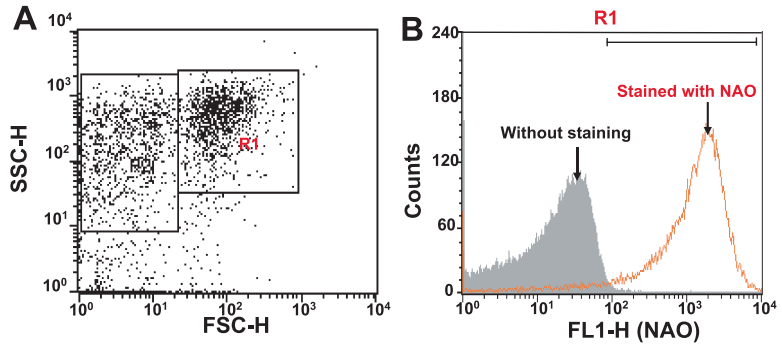

Fig. 1. Selection of mitochondria by the light scattering profile and NAO staining. A) On the FSC/SSC plot of the isolated mitochondria, we gated the largest population with reasonable FSC values as R1, assuming that they are mitochondrial fraction. B) When the gated R1 fraction was analyzed for NAO fluorescence, almost all events in the gate R1 were positive for NAO (orange line) when compared to samples without NAO staining (gray area), which confirmed that they were mitochondrial fraction.

Rhod 2 and HE. ROS generation and mitochondrial swelling were also analyzed by FACScan based on the changes in FL1-H of H_2DCF-DA and in SSC and FSC of NAO-positive particles before and after adding Ca^{2+}. Protein concentrations were determined by the method of Bradford using BSA as a standard [*25*].

Results

Analysis of light scattering properties of mitochondria

To analyze the relationship among Ca^{2+}-induced membrane depolarization, swelling, Ca^{2+} release and ROS generation, mitochondria were selected from rat liver based on their light-scattering properties using a FACScan analyzer. The purity of mitochondrial preparations was determined by staining with NAO [*18*]. Fig. 1A shows the FSC/SSC plot of the isolated rat liver mitochondria. We gated the largest population with reasonable FSC values as R1, assuming that they are mitochondrial fraction. When the gated R1 population was analyzed for NAO fluorescence, almost all events were positive for NAO (Fig. 1B), which confirmed that they were mitochondrial fraction. Thus, the gated R1 events were analyzed in the following experiments.

Effect of succinate and Ca^{2+} on the membrane potential of isolated mitochondria

It is well-known that mitochondrial membrane depolarization occurs prior to the occurrence of MPT [*4*]. We tested whether the flow cytometric technique was useful for the analysis of the mitochondrial membrane depolarization, and the effect of Ca^{2+} on TMRE fluorescence intensity. When the mitochondria were added with only TMRE, FL2-H intensity increased (Fig. 2), probably due to the endogenous membrane potential. Adding Pi and succinate, a respiratory substrate, further increased the intensity of fluorescence. In contrast, exogenously-added Ca^{2+} decreased the FL2-H fluorescence and the peak shifted to the left, which indicated depolarization (Fig. 2). These results indicate that flow cytometry is useful for the analysis of the sequence of MPT in isolated mitochondria.

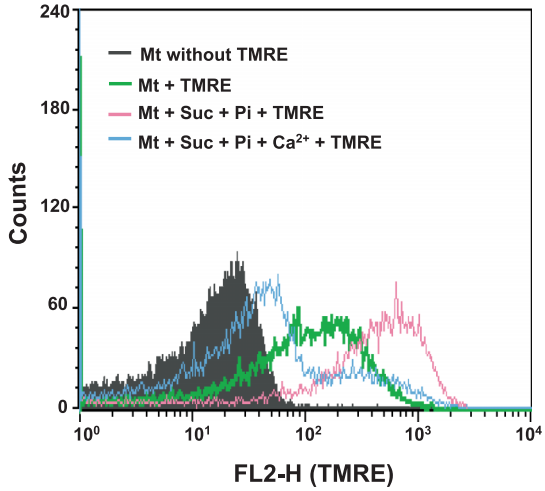

Fig. 2. Effect of succinate and Ca^{2+} on TMRE fluorescence intensity. Membrane depolarization was analyzed by flow cytometry for changes in FL2-H in the gated R1 events. Membrane potential was detected by TMRE. Mitochondrial background fluorescence (without TMRE staining) (black area). Membrane potential in the absence of Pi and succinate (Suc) (green line) and in the presence of Pi and Suc (pink line). Induction of membrane depolarization by 40 μM Ca^{2+} for 5 min incubation in the presence of respiratory substrate (blue line). Similar results were obtained in 3 separate experiments.

Effect of succinate and cyclosporine A on Ca^{2+}-induced mitochondrial events

Since Ca^{2+}-induced MPT occurred by some energy-dependent and CsA-inhibitable mechanism, we analyzed the

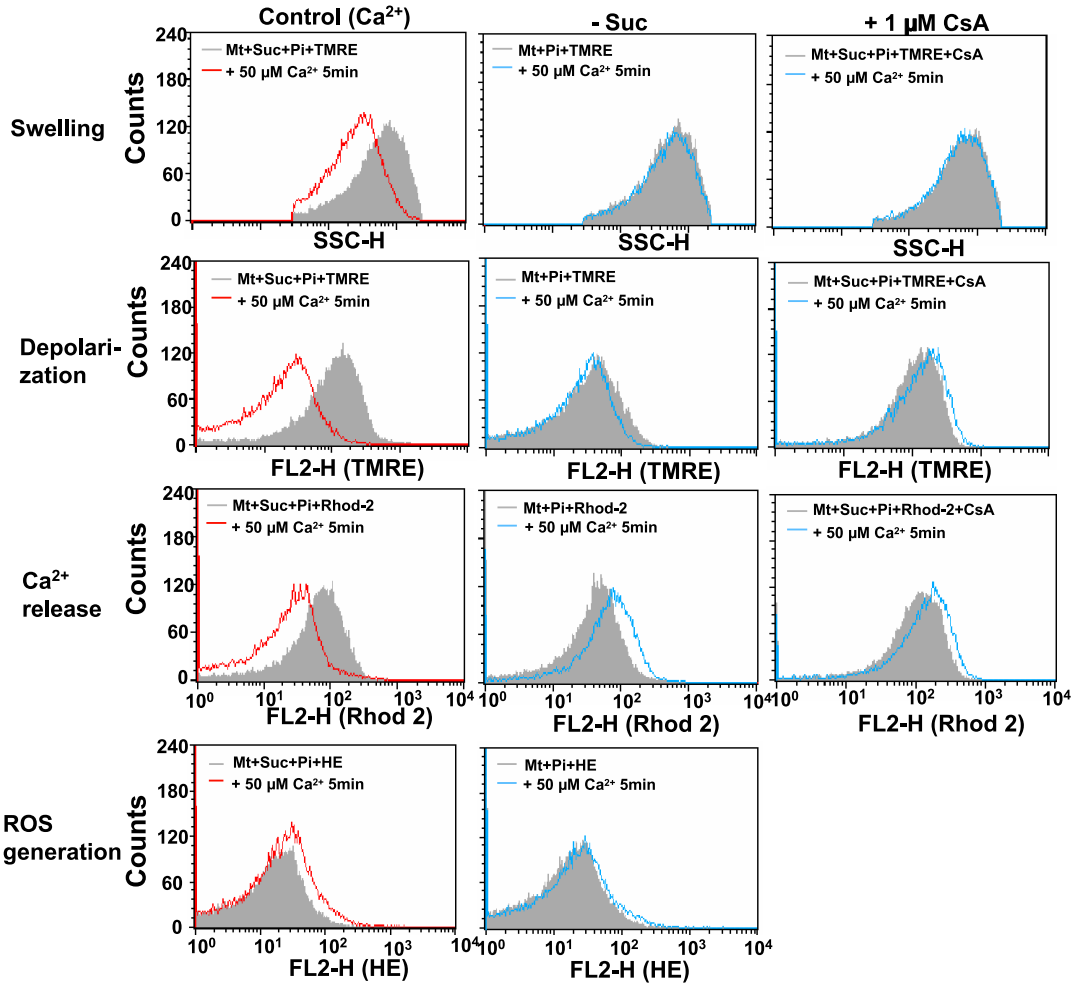

Fig. 3. Effect of respiratory substrate and cyclosporine A on the Ca^{2+}-induced swelling, depolarization, Ca^{2+} release and ROS generation of isolated mitochondria. Experimental conditions were the same as described in Figure 2. Membrane depolarization, Ca^{2+}-release and ROS generation were analyzed with TMRE, Rhod 2 and hydroethidine (HE), respectively, and detected with flow cytometry for changes in FL2-H in the gated R1. Mitochondrial swelling was also analyzed simultaneously with these events by SSC. (Left, Control) MPT was induced by addition of 50 μM Ca^{2+} for 5 min at 25°C. Mitochondria were incubated in the standard medium containing 2.5 mM succinate and 0.5 mM Pi before addition of Ca^{2+}. (Center, -Succ) The effect of the absence of respiratory substrate on the Ca^{2+}-induced MPT. Mitochondria were incubated in standard medium containing 0.5 mM Pi without succinate (Suc). (Right, +CsA) The effect of cyclosporine A on the Ca^{2+}-induced MPT. Mitochondria were incubated in the presence of 1 μM CsA in the standard medium containing 2.5 mM succinate and 0.5 mM Pi before addition of Ca^{2+}. Similar results were obtained in 3 separate experiments.

effects of succinate and CsA on various events observed with MPT. In the presence but not in the absence of succinate, ROS generation, depolarization, swelling and Ca^{2+} release were observed with Ca^{2+}-treated mitochondria (Fig. 3). All of these Ca^{2+}-induced events associated with MPT of Ca^{2+}-treated mitochondria were suppressed by CsA (Fig. 3). Since CsA affected the fluorescence intensity of HE, ROS generation by mitochondria could not be measured.

Although most experiments were carried out using 0.1~1 mg mitochondrial protein/ml, the extent of the Ca^{2+}-induced events in mitochondria occurred independently from these concentrations. At protein concentrations between 0.02 to 0.1 mg/ml, mitochondria showed typical patterns of depolarization, Ca^{2+} release and swelling 5 min after the treatment with 5~15 μM Ca^{2+} (Fig. 4). These results indicate that various functions can be analyzed with a relatively small amount of mitochondria.

Sequence of events occurring with Ca^{2+}-induced membrane permeability transition

To determine the sequence of events occurring with Ca^{2+}-induced mitochondrial MPT, flow cytometric analysis of swelling, depolarization, Ca^{2+} release, and ROS generation was carried out with isolated mitochondria which were

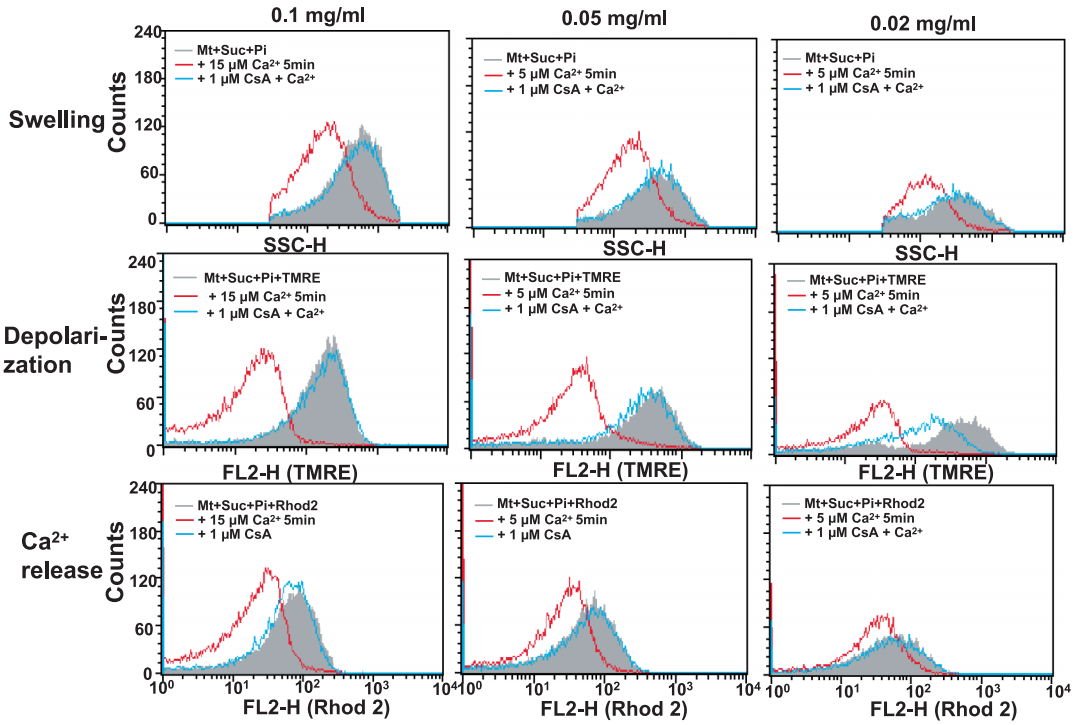

Fig. 4. Effect of mitochondrial concentration on the flow cytometric analysis of mitochondrial functions. Experimental conditions were the same as described in Fig. 3. Used mitochondrial concentrations were 0.02 to 0.1 mg/ml. Used Ca^{2+} concentrations were 5~15 µM. Similar results were obtained in 3 separate experiments.

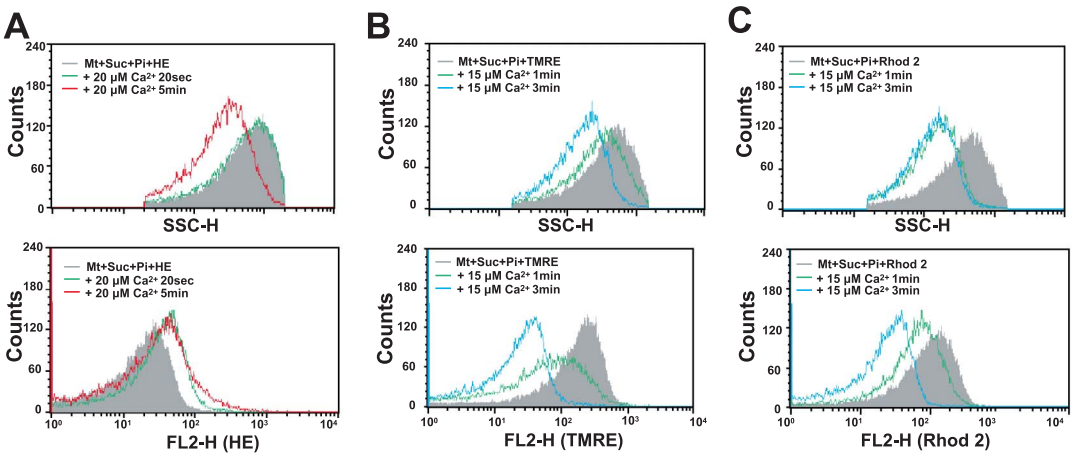

Fig. 5. Causal sequence of various events occurring in Ca^{2+}-induced MPT. Experimental conditions were the same as described in Fig. 3. A) Effect of Ca^{2+} on the ROS generation of mitochondria. B) Effect of Ca^{2+} on the depolarization of mitochondrial membrane. C) Effect of Ca^{2+} on the release of loaded Ca^{2+} from mitochondria. The experiments B and C were performed using different mitochondrial preparations. Thus, the time course of the mitochondrial swelling was slightly different. Similar results were obtained in 3 separate experiments.

stained with either TMRE, Rhod 2, or HE for analysis. The mitochondrial swelling decrease in SSC of Argon laser at 488 nm was comparable to the decrease in light scattering at 540 nm in a fluorescence spectrophotometer (data not described) [*16*]. As shown in Fig. 5, Ca^{2+}-induced ROS generation was detectable from the increase in FL2-H fluorescence (green line) of HE. The increased HE fluorescence (green line) was detectable before the onset of mitochondrial swelling detected in SSC-H (Fig. 5A). The decrease of TMRE fluorescence in FL2-H (green line) reflecting Ca^{2+}-induced depolarization occurred more rapidly than mitochondrial swelling (Fig. 5B). In contrast, mitochondrial

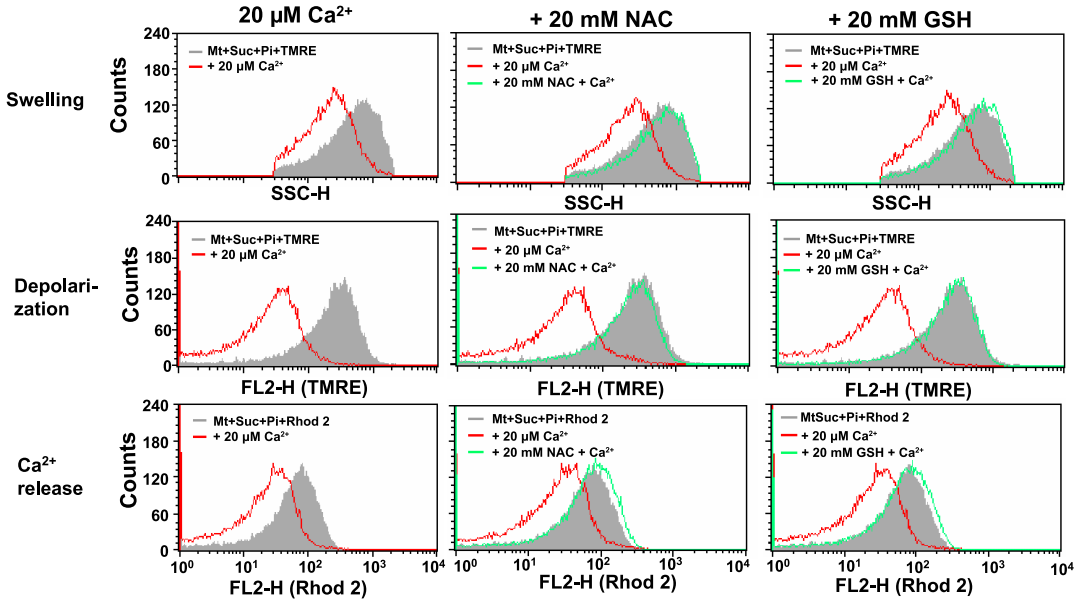

Fig. 6. Effects of NAC and GSH on the Ca^{2+}-induced MPT in isolated mitochondria analyzed by flow cytometry. Experimental conditions were the same as described for Fig. 3. 20 mM NAC or GSH were added in the standard medium containing 2.5 mM succinate and 0.5 mM Pi before addition of Ca^{2+}.

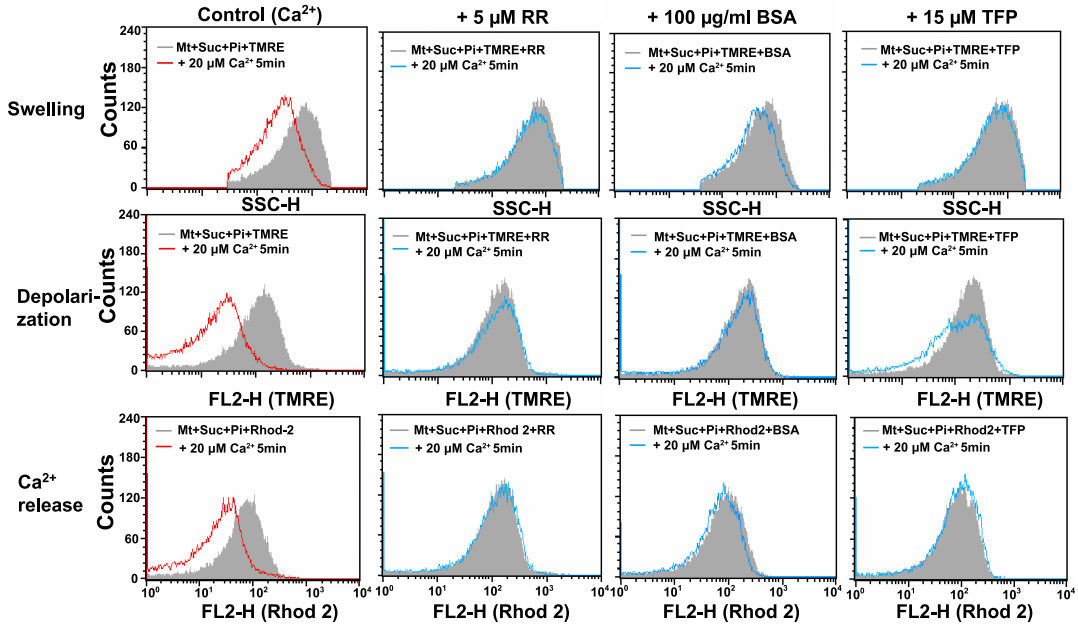

Fig. 7. Effect of RR, BSA and TFP on the various Ca^{2+}-induced events in isolated mitochondria. Experimental conditions were the same as described for Fig. 3. Various reagents were added in the standard medium before addition of Ca^{2+}. The concentrations of RR, BSA and TFP were 5 μM, 100 μg/ml and 15 μM, respectively.

swelling occurred more rapidly than the release of Ca^{2+} detected by the decrease of Rhod 2 fluorescence in FL2-H (green line in Fig. 5C). ROS generation was also detectable from the increase in the fluorescence of H_2DCF-DA (data not shown).

Effect of antioxidants on the Ca^{2+}-induced changes in mitochondrial functions

Since ROS generation is one of the early events in Ca^{2+}-induced MPT, we tested the effect of antioxidants on the Ca^{2+}-induced depolarization, swelling and Ca^{2+} release of mitochondria. Reduced GSH, a typical hydrophilic antioxi-

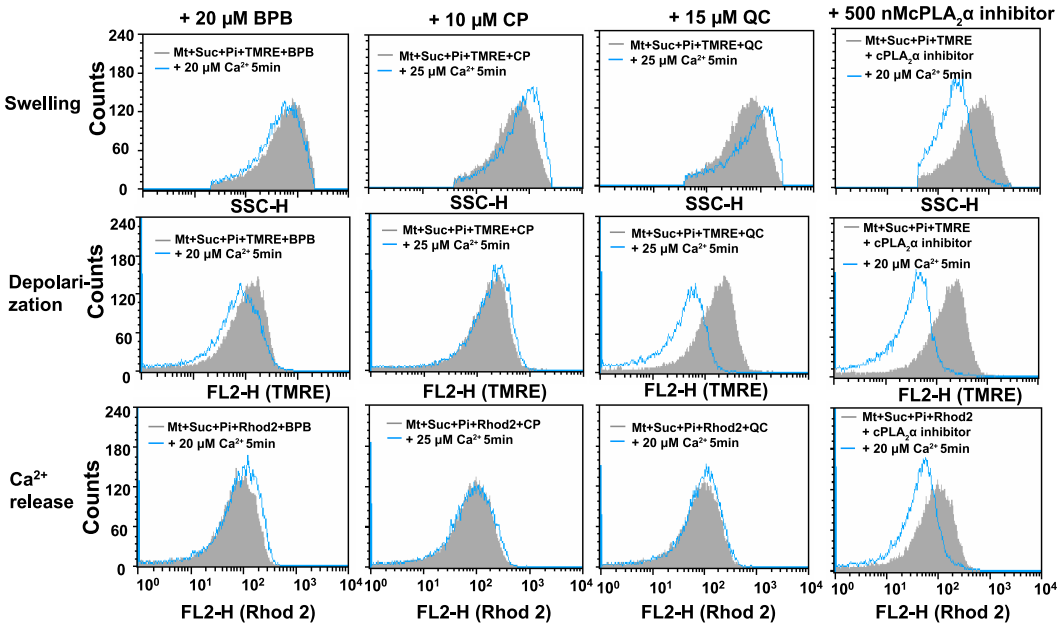

Fig. 8. Effect of PLA_2 inhibitors on the Ca^{2+}-induced depolarization, swelling and Ca^{2+}-release of isolated mitochondria. Experimental conditions were the same as described for Fig. 7. The concentration used of BPB, CP, QC and $cPLA_2\alpha$ inhibitor were 20 µM, 10 µM, 15 µM and 0.5 µM, respectively. Similar results were obtained in 3 separate experiments.

dant, suppressed the Ca^{2+}-induced swelling, depolarization and release of Ca^{2+} in isolated mitochondria in a concentration dependent manner (Fig. 6). Similar effects were also observed with NAC, a hydrophilic antioxidant. We also studied ROS production by HE and, contrary to our expectation, found that HE fluorescence increased in the presence of GSH and NAC (data not shown). Additional experiment showed that even without mitochondria, GSH increased the HE fluorescence in xanthine-xanthine oxidase system. Thus, HE may not be a suitable ROS detector when GSH and NAC were present in the system.

Effect of RR and inhibitors of phospholipase A_2 on Ca^{2+}-induced swelling, depolarization, and Ca^{2+}-release of mitochondria

To elucidate the mechanism of Ca^{2+}-induced MPT, we tested the effect of RR, an inhibitor of Ca^{2+} uniporter [*26*] that inhibits Ca^{2+} influx into mitochondrial matrix, on mitochondrial swelling, depolarization and release of loaded Ca^{2+}. Analysis using SSC-H, FL2-H (TMRE), and FL2-H (Rhod 2) revealed that Ca^{2+}-induced mitochondrial swelling, depolarization and Ca^{2+} release were suppressed by RR (Fig. 7). Similar inhibition was observed with BSA that binds free fatty acid. Furthermore, various inhibitors of phospholipase A_2 (PLA_2), such as TFP, BPB, CP and QC suppressed the Ca^{2+}-induced mitochondrial swelling, depolarization and Ca^{2+} release [*27–32*] (Fig. 8). In contrast, inhibitor of cytosolic $cPLA_2$ [*32*] failed to suppress these changes induced by Ca^{2+}. These results suggested that both Ca^{2+} uniporter and PLA_2 play important role in the mechanism of Ca^{2+}-induced MPT. Furthermore, PLA_2 inhibitors did not affect significantly the ROS generation demonstrated by HE fluorescence (data not shown).

Discussion

The present work describes the sequence of events that elicited Ca^{2+}-induced MPT in isolated mitochondria without being affected by cytosol and other organelles. Kinetic analysis using FACScan equipment revealed that the sequence of events occurring during the process of Ca^{2+}-induced MPT were Ca^{2+}-uptake into mitochondria, which was followed by ROS generation and activation PLA_2, depolarization, swelling, and then efflux of the loaded Ca^{2+}.

In this experiment we measured mitochondrial swelling by SSC, a parameter for the complexity of the target object, and not by FSC, a parameter for the object size. This was because the actual measurement showed that SSC was more sensitive than FSC in the detection of mitochondrial swelling. Probably the mitochondrial swelling result in the simplification of inner membrane structure and SSC decreases more sensitively than the increase in FSC [*33*, *34*].

Recent studies using newly developed multi channel analyzers have revealed that selective ion leaks occur prior to the onset of permeability transition [*35*]. Although Ca^{2+} plays important roles in cell signaling for cell survival, it accumulates in mitochondria by an energy-dependent mechanism and triggers the reaction causing MPT, a prerequisite to

cell death. We previously described that CsA inhibited the ROS generation from mitochondria [*10*]. The present work demonstrates that ROS generation is an initial step of the sequence of events triggering Ca^{2+}-induced MPT of mitochondria.

In the present experiments, it was found that inhibitors of Ca^{2+}-induced PLA_2, RR and BSA suppressed the various events in MPT. These results suggested that PLA_2 might affect the early events in MPT. Several investigators have reported that Ca^{2+} accumulated in mitochondria stimulated PLA_2 and released free fatty acid and lysophosphatides, which activate the caspase cascade [*36–39*]. RR suppressed the uniport channel found in the inner mitochondrial membrane [*26*, *40*]. It is known that free fatty acids elicit CsA-sensitive MPT and induce mitochondrial swelling [*41–43*] by a mechanism that is suppressed by fatty acid binding BSA [*27*, *28*]. In this context, ischemia/reperfusion increased free fatty acids in rat brain, and CsA and TFP effectively suppressed the reperfusion-induced release of fatty acids [*37*]. These results indicate that MPT seems to involve the uncoupling effect of fatty acids generated by activated PLA2 [*43*]. However, it was reported that mitochondrial Ca^{2+} could be released spontaneously by MPT without generating free fatty acid although long-term incubation of mitochondria significantly increased the products of PLA_2 [*32*]. This result suggests that the accumulation of fatty acids in mitochondria might be the consequence rather than the cause of MPT, and that free fatty acid generated in mitochondria might sustain the permeable state.

It is well known that PLA_2 consists of a wide variety of enzymes [*44*]. Thus, it is very important to identify the isoform involving in MPT mechanism. The PLA_2 isoform that is localized within mitochondria is different from the cytosolic isoform of PLA_2 ($cPLA_2$), and is most likely to be a low-molecular-mass PLA_2 [*45*], which belong to group IIA PLA_2s [*46*]. It is interesting to note that TFP, BPB, CP and QC, but not $cPLA_2\alpha$ inhibitor, are potent inhibitors for group IIA PLA_2 [*45*, *47–49*]. This finding indicates that the group IIA PLA_2s are involved in the Ca^{2+}-induced mitochondrial swelling. In addition, recent report showed that Ca^{2+}-independent $PLA_2\gamma$ ($iPLA_2\gamma$) is also localized in mitochondria, and is involved in Ca^{2+}-induced mitochondrial MPT [*50*]. Thus, the possible involvement of this isoform should be studied further.

Since Ca^{2+}-induced MPT is associated with ROS generation, some antioxidants were expected to inhibit the occurrence of mitochondrial swelling and depolarization [*12*, *51–53*]. However, only limited information is available for the inhibitory effect of antioxidant on mitochondrial swelling [*12*, *54*]. Recent studies showed that some peptide having strong antioxidant activity accumulated in mitochondria and inhibited the Ca^{2+}-induced swelling [*55*, *56*]. Since thiol-specific antioxidants suppressed the Ca^{2+}-induced swelling of mitochondria, Ca^{2+}-induced reactions might involve the oxidation of the critical thiol groups such as those in adenine nucleotide translocator (ANT) [*10*, *52*, *53*]. In this context, Ca^{2+} has been postulated to modify the reactivity of mitochondrial membrane protein thiols with N-ethylmaleimide and mersalyl [*57*]. The present work showed that antioxidant NAC and GSH inhibited the occurrence of Ca^{2+}-induced mitochondrial swelling and depolarization. These observations are consistent with the hypothesis that oxidation of critical thiol underlies the mechanisms for the induction of Ca^{2+}-induced MPT and mitochondrial dysfunction [*10*].

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Abbreviations

ANT, adenine nucleotide translocator; BPB, bromophenacyl bromide; BSA, bovine serum albumin; diS-C3-(5), 3,3'-dipropyl-2,2'-thiodicarbocyanine iodide; CP, chlorpromazine; HE, hydroethidine; H2DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; CsA, cyclosporine A; MPT, membrane permeability transition; NAC, N-acetylcysteine; NAO, 10-nonyl acridine orange; PLA2, phospholipase A2; QC, quinacrine; Rhod 2; 1-[2-Amino-5-(-dimethylamino-6-dimethylammonio-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid; RR, ruthenium red; ROS, reactive oxygen species; SSC, side scatter; FSC, forward scatter; TFP, trifluoperazine; TMRE, tetramethylrhodamine-ethyl-ester.

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