Mitochondrial membrane potential modulates regulation of mitochondrial Ca\(^{2+}\) in rat ventricular myocytes

Masao Saotome, Hideki Katoh, Hiroshi Satoh, Shiro Nagasaka, Shu Yoshihara, Hajime Terada, and Hideharu Hayashi

Third Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan

Submitted 15 June 2004; accepted in final form 18 November 2004

Mitochondrial membrane potential modulates regulation of mitochondrial Ca\(^{2+}\) in rat ventricular myocytes. Am J Physiol Heart Circ Physiol 288: H1820–H1828, 2005. First published December 2, 2004; doi:10.1152/ajpheart.00589.2004.—Although recent studies focused on the contribution of mitochondrial Ca\(^{2+}\) to the mechanisms of ischemia-reperfusion injury, the regulation of mitochondrial Ca\(^{2+}\) under pathophysiological conditions remains largely unclear. By using saponin-permeabilized rat myocytes, we measured mitochondrial membrane potential (\(\Delta \Psi_m\)) and mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{m}}\)) at the physiological range of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{c}}\); 300 nM) and investigated the regulation of [Ca\(^{2+}\)]\(_{\text{m}}\) during both normal and dissipated \(\Delta \Psi_m\). When complete \(\Delta \Psi_m\) dissipation was achieved by FCCP (0.3–1 \(\mu\)M), [Ca\(^{2+}\)]\(_{\text{m}}\) remained at one-half of the control level despite no Ca\(^{2+}\) influx via the Ca\(^{2+}\) uniporter. The \(\Delta \Psi_m\) dissipation by FCCP accelerated calcein leakage from mitochondria in a cyclosporin A (CsA)-sensitive manner, which indicates that \(\Delta \Psi_m\) dissipation opened the mitochondrial permeability transition pore (mPTP). After FCCP addition, inhibition of the mPTP by CsA caused further [Ca\(^{2+}\)]\(_{\text{m}}\) reduction; however, inhibition of mitochondrial Na\(^{+}/\text{Ca}^{2+}\) exchange (mitoNCX) by a Na\(^{+}\)-free solution abolished this [Ca\(^{2+}\)]\(_{\text{m}}\) reduction. Cytosolic Na\(^{+}\) concentrations that yielded one-half maximal activity levels for mitoNCX were 3.6 mM at normal \(\Delta \Psi_m\) and 7.6 mM at \(\Delta \Psi_m\) dissipation. We conclude that the mitochondrial Ca\(^{2+}\) uniporter accumulates Ca\(^{2+}\) in a manner that is dependent on \(\Delta \Psi_m\) at the physiological range of [Ca\(^{2+}\)]\(_{\text{c}}\). 2) \(\Delta \Psi_m\) dissipation opens the mPTP and results in Ca\(^{2+}\) influx to mitochondria; and 3) although mitoNCX activity is impaired, mitoNCX extrudes Ca\(^{2+}\) from the matrix even after \(\Delta \Psi_m\) dissipation.

permeability transition pore; Na\(^{+}/\text{Ca}^{2+}\) exchange; depolarization; ischemia-reperfusion injury

ACCUMULATING EVIDENCE REVEALS that mitochondria play primary roles in fatal cell damage during ischemia-reperfusion (33). Key events that occur during ischemia include cytosolic Ca\(^{2+}\) elevation, ATP depletion, high P\(_i\) concentration, depolarized membrane potential, and acidic pH. On reperfusion and recovery of normal pH, a burst of reactive oxygen species occurs, mitochondrial Ca\(^{2+}\) overload ensues, and these lead to opening of the mitochondrial permeability transition pore (mPTP; Refs. 8, 12, 34). Opening of the mPTP allows water and solutes \(\leq 1,500 \text{ Da}\) in size to enter the matrix and cause mitochondrial swelling, rupture of the outer mitochondrial membrane, and release of cytochrome c or apoptosis-inducing factor, which initiates apoptotic programmed cell death (12, 24, 34). Because previous studies (8, 12, 13) have shown that the opening of the mPTP is largely dependent on mitochondrial Ca\(^{2+}\), it is important to understand the properties of the regulatory systems for mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{m}}\)) during pathophysiological conditions such as ischemia and reperfusion.

Under normal conditions, the mitochondrial inner membrane remains impermeable to ions and possesses several Ca\(^{2+}\) transport systems for the regulation of [Ca\(^{2+}\)]\(_{\text{m}}\) (4, 6, 10, 11). Ca\(^{2+}\) accumulation into mitochondria occurs via the Ca\(^{2+}\) uniporter, which is driven by the negative charge of the mitochondrial membrane potential (\(\Delta \Psi_m\)). The Ca\(^{2+}\) uniporter is activated by extra-mitochondrial Ca\(^{2+}\), ADP, and spermine and is inhibited by Mg\(^{2+}\) and ruthenium red (RuR; Refs. 10, 11). Extrusion of mitochondrial Ca\(^{2+}\) is mediated primarily via mitochondrial Na\(^{+}/\text{Ca}^{2+}\) exchange (mitoNCX) in cardiac myocytes. The mitoNCX is activated by nigericin and the pH or Na\(^{+}\) gradients between the matrix and the cytosol and is inhibited by diltiazem, clonazepam, CGP-37157, and Mg\(^{2+}\) (6, 10, 11). For another Ca\(^{2+}\) efflux pathway, mitochondria have the slow, relatively small Ca\(^{2+}/\text{H}^{+}\) exchange, which is not dominant in cardiac tissues (10, 11). Furthermore, recent studies (4, 8, 10, 11, 18) also suggest a possible contribution by the mPTP to Ca\(^{2+}\) homeostasis in both the cytosol and mitochondria.

Despite the considerable attention given to the pathophysiological significance of mitochondrial Ca\(^{2+}\), the regulation and/or modulation of mitochondrial Ca\(^{2+}\) during pathophysiological conditions such as ischemia-reperfusion injury are unclear. In previous studies, information about mitochondrial Ca\(^{2+}\) was obtained using isolated mitochondria, whereby the structural and functional properties of organelles were seriously affected, and other cellular architectures were separated from the mitochondria. In this study, we measured [Ca\(^{2+}\)]\(_{\text{m}}\) in saponin-permeabilized rat ventricular myocytes and investigated how \(\Delta \Psi_m\) depolarization affects [Ca\(^{2+}\)]\(_{\text{m}}\) and mitochondrial Ca\(^{2+}\) transport systems such as the Ca\(^{2+}\) uniporter, the mPTP, and mitoNCX.

MATERIALS AND METHODS

Cell isolation and solutions. This investigation is in conformance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Isolated myocytes were obtained from male Sprague-Dawley rats (body wt, 250–300 g) by enzymatic dissociation, and the

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cells were kept in a modified Kraftbrühe solution (15, 29) that contained (in mM) 70 KOH, 40 KCl, 20 KH2PO4, 3 MgCl2, 50 glutamic acid, 10 glucose, 10 HEPES, and 0.5 EGTA (pH 7.4 with KOH). Just before the experiment, cells were placed in a chamber and perfused with a normal Tyrode solution composed of (in mM) 143 NaCl, 5.4 KCl, 0.5 MgCl2, 0.25 NaH2PO4, 1 CaCl2, 5.6 glucose, and 5 HEPES (pH 7.4 with NaOH). All experiments were conducted at room temperature (22°C) within 6 h of cell isolation.

Measurement of \([Ca^{2+}]_{\text{m}}\) in skinned myocytes. Isolated rat ventricular myocytes were loaded with 20 \(\mu\)M rhod-2-AM at room temperature for 60 min. To remove cytosolic rhod-2, the sarcolemmal membrane was permeabilized by perfusion of saponin (0.05 mg/ml) in a Ca2+-free internal solution that contained (in mM) 50 KCl, 80 potassium aspartate, 4 sodium pyruvate, 20 HEPES, 3MgCl2·6H2O, 3 Na2ATP, 5.8 glucose, and 3 EGTA (pH 7.3 with KOH). After the sarcolemmal membrane was permeabilized, the free Ca2+ concentration in the internal solution (\([Ca^{2+}]_{\text{c}}\)) was increased according to the experimental protocol. The \([Ca^{2+}]_{\text{c}}\) was obtained by mixture of EGTA and CaCl2 and was calculated using the WIN MAXC 2.1 computer program (provided by Stanford University).

Experiments were performed using a laser-scanning confocal microscope (LSM 510; Zeiss) coupled to an inverted microscope (Axiovert S100; Zeiss) with a 63 water-immersion objective lens (numerical aperture, 1.3). Cells were excited with the 514-nm-wavelength argon laser, and images were acquired through a >560-nm long-pass filter. For quantitative analysis of the changes in rhod-2 signals, fluorescence intensities at identical regions of interest (20 × 20 pixels) were monitored every 30 or 60 s.

To localize mitochondria, some myocytes were coloaded with rhod-2-AM and Mito Tracker Green (excitation wavelength, 488 nm; emission wavelength, through a 505–530-nm band-pass filter). RuR (1 \(\mu\)M), which is an inhibitor of the mitochondrial Ca2+ uniporter, or FCCP (0.0–1 \(\mu\)M), which is an uncoupler of the mitochondrial respiratory chain, was used to modify \([Ca^{2+}]_{\text{m}}\).

Measurement of \(\Delta V_m\). For \(\Delta V_m\) measurement, cells were initially permeabilized and loaded using a continuous perfusion of the fluorescent indicator tetramethylrhodamine ethyl ester (TMRE; 20 nM). TMRE was excited at a 543-nm wavelength with a helium-neon laser, and the emission signals were collected through a 580-nm long-pass filter. For the steady measurements of time-dependent fluorescence changes, images were recorded after 20 min of TMRE perfusion.

Imaging of mPTP opening. To evaluate mPTP opening, myocytes were loaded with calcein-AM (1 \(\mu\)M) for 15 min, and the sarcolemmal membrane was permeabilized to remove cytoplasmic dyes. This method allows for selective loading of calcein in mitochondria. On the opening of the mPTP, entrapped calcein is released from the mito-
chondrial matrix (20, 30). Calcein was excited at a 488-nm wavelength, and emission was collected through a 505- to 550-nm band-pass filter.

Chemicals and data analysis. All chemicals were obtained from Sigma (St. Louis, MO), and fluorescent dyes were purchased from Molecular Probes (Eugene, OR). Data are presented as means ± SE; n describes the number of cells or experiments. Statistical analyses were performed using t-test or ANOVA. A level of P < 0.05 was accepted as statistically significant.

RESULTS

Determination of [Ca2+]m. We first compared the rhod-2 signals before and after permeabilization of the sarcolemmal membrane in the rhod-2-AM-loaded myocytes to determine the fraction of fluorescence signals that originated from the mitochondria. Figure 1A demonstrates the dual-stained images of rhod-2 and Mito Tracker Green (which was used to identify the localization of mitochondria). As shown in Fig. 1A (left), the distributions of rhod-2 (top) and Mito Tracker Green (middle) were not identical before the permeabilization, and there were red areas that were loaded only by rhod-2 in the superimposed image (bottom). However, after the sarcolemmal membrane was permeabilized in the same cell (Fig. 1A, right), both fluorescent indicators overlapped almost completely (yellow in the superimposed image). These results indicate that when cells are loaded with rhod-2-AM, some amount of dye remains in the cytosolic space, and this dye was released after the permeabilization. As shown in Fig. 1B, the rhod-2 signal decreased rapidly after membrane permeabilization and increased in response to incremental [Ca2+]c changes to 177, 350, and 600 nM. Summarized data in Fig. 1C demonstrate that after membrane permeabilization, the rhod-2 signal decreased to 15 ± 1% of its intensity before permeabilization (P < 0.01; n = 4), and the incremental [Ca2+]c changes increased the rhod-2 signal to 60 ± 5% ([Ca2+]c = 177 nM; P < 0.01 vs. 0 Ca2+ after saponin) and 159 ± 7% ([Ca2+]c = 350 nM; P < 0.01 vs. [Ca2+]c = 177 nM) of the control value (before saponin), respectively.

To confirm that the changes of rhod-2 signals in permeabilized myocytes indeed reflect [Ca2+]m, we examined the effects of mitochondrial Ca2+ uniporter inhibition by RuR, which is an inhibitor of the Ca2+ uniporter. As shown in Fig. 2A, repeated exposure to the 300 nM [Ca2+]c (without drugs) was permeabilized in the same cell (Fig. 1A, right), both fluorescent indicators overlapped almost completely (yellow in the superimposed image). These results indicate that when cells are loaded with rhod-2-AM, some amount of dye remains in the cytosolic space, and this dye was released after the permeabilization. As shown in Fig. 1B, the rhod-2 signal decreased rapidly after membrane permeabilization and increased in response to incremental [Ca2+]c changes to 177, 350, and 600 nM. Summarized data in Fig. 1C demonstrate that after membrane permeabilization, the rhod-2 signal decreased to 15 ± 1% of its intensity before permeabilization (P < 0.01; n = 4), and the incremental [Ca2+]c changes increased the rhod-2 signal to 60 ± 5% ([Ca2+]c = 177 nM; P < 0.01 vs. 0 Ca2+ after saponin) and 159 ± 7% ([Ca2+]c = 350 nM; P < 0.01 vs. [Ca2+]c = 177 nM) of the control value (before saponin), respectively.

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caused rapid increases in the rhod-2 signal, whereas in the cells pretreated with 1 μM RuR, the rhod-2 signal increase was completely inhibited even in 300 nM [Ca\textsuperscript{2+}]\textsubscript{i}. (Fig. 2B). The inhibitory effects of the Ca\textsuperscript{2+} uniporter on the rhod-2 signal were further verified by treating cells with 0.3 μM FCCP, which is a respiratory chain uncoupler that abolishes the driving force for the Ca\textsuperscript{2+} uniporter via ΔΨ\textsubscript{m} depolarization. FCCP was applied to the Ca\textsuperscript{2+}-free solution, and [Ca\textsuperscript{2+}]\textsubscript{i} was increased to 300 nM in the presence of FCCP. FCCP pretreatment also inhibited the rhod-2 signal increase that was caused by addition of 300 nM Ca\textsuperscript{2+} (Fig. 2C). Summarized data in Fig. 2D show that rhod-2 intensities in the permeabilized myocytes increased by the second addition of 300 nM Ca\textsuperscript{2+} (control, 88 ± 2% of the first 300 nM Ca\textsuperscript{2+}; n = 4), and that pretreatment with RuR (2 ± 9%; P < 0.01 vs. control, n = 5) or FCCP (4 ± 5%; P < 0.01 vs. control; n = 5) abolished the increase in the rhod-2 signal. These results indicate that 1) Ca\textsuperscript{2+} influx into mitochondria is mainly mediated via the Ca\textsuperscript{2+} uniporter, 2) the driving force for the Ca\textsuperscript{2+} uniporter largely depends on ΔΨ\textsubscript{m}, and 3) there is no net mitochondrial Ca\textsuperscript{2+} uptake via the Ca\textsuperscript{2+} uniporter during conditions of ΔΨ\textsubscript{m} dissolution.

Because the rhod-2 signal in permeabilized myocytes increased in response to the change in [Ca\textsuperscript{2+}]\textsubscript{i}, and this rhod-2 signal increase was abolished by inhibition of the mitochondrial Ca\textsuperscript{2+} uniporter (by RuR or FCCP), we confirmed that rhod-2 signals in the permeabilized myocytes represent [Ca\textsuperscript{2+}]\textsubscript{i}.

Effects of ΔΨ\textsubscript{m} on [Ca\textsuperscript{2+}]\textsubscript{i}. In this series of experiments, we investigated the effects of ΔΨ\textsubscript{m} depolarization on [Ca\textsuperscript{2+}]\textsubscript{i} in permeabilized myocytes. Figure 3A shows a representative recording of TMRE intensity (which represents ΔΨ\textsubscript{m}) after application of FCCP to the internal solution ([Ca\textsuperscript{2+}]\textsubscript{i} = 300 nM). Lower concentrations of FCCP (0.01–0.1 μM) depolarized ΔΨ\textsubscript{m} in a dose-dependent manner, whereas 1 μM FCCP dissipated ΔΨ\textsubscript{m} completely. Figure 3B shows a typical recording of rhod-2 intensity after FCCP application to 300 nM [Ca\textsuperscript{2+}]\textsubscript{i}. Lower concentrations of FCCP (0.01–0.1 μM) dose dependently decreased [Ca\textsuperscript{2+}]\textsubscript{i}, whereas [Ca\textsuperscript{2+}]\textsubscript{i} remained at almost one-half of the control level even after application of 1 μM FCCP. Figure 3C shows that when ΔΨ\textsubscript{m} was partially depolarized by lower concentrations of FCCP [0.01 μM: 83 ± 2% of value before FCCP administration (control); P < 0.01 vs. control; n = 4; and 0.1 μM: 37 ± 4%; P < 0.01 vs. 0.01 μM FCCP; n = 13], there were corresponding reductions in [Ca\textsuperscript{2+}]\textsubscript{i} [0.01 μM: 85 ± 5% of value before FCCP administration (control); P < 0.05 vs. control; n = 4; and 0.1 μM: 67 ± 6%; P < 0.01 vs. 0.01 μM FCCP; n = 7]. However, under conditions of complete ΔΨ\textsubscript{m} dissipation by 0.3 or 1 μM FCCP (which means no driving force for the Ca\textsuperscript{2+} uniporter), the reductions in [Ca\textsuperscript{2+}]\textsubscript{i} were incomplete, and [Ca\textsuperscript{2+}]\textsubscript{i} remained at 53 ± 1% (0.3 μM; n = 4) and 55 ± 4% (1 μM; n = 6) of the control value, respectively. Because FCCP might alter the properties of mitochondrial Ca\textsuperscript{2+} transport systems through changes in matrix pH, we also tested the effects of the K\textsuperscript{+} ionophore valinomycin on ΔΨ\textsubscript{m} and [Ca\textsuperscript{2+}]\textsubscript{i}. When the application of 10 nM valinomycin dissipated ΔΨ\textsubscript{m}, the decrease of [Ca\textsuperscript{2+}]\textsubscript{i} also remained at one-half of the control level (data not shown). These results suggest that when FCCP is applied, changes in matrix pH might not be responsible for the changes in the mitochondrial Ca\textsuperscript{2+} transport systems. Because the mitochondrial Ca\textsuperscript{2+} influx is mainly mediated via the Ca\textsuperscript{2+} uniporter, and the driving force for the Ca\textsuperscript{2+} uniporter depends on ΔΨ\textsubscript{m}, these results suggest that 1) mitochondrial Ca\textsuperscript{2+} influx via the Ca\textsuperscript{2+} uniporter decreased when ΔΨ\textsubscript{m} was partially depolarized, and 2) Ca\textsuperscript{2+}-regulating pathways other than the Ca\textsuperscript{2+} uniporter were involved when ΔΨ\textsubscript{m} was completely dissipated.

Imaging of mPTP opening by calcein. Because it has been reported that the mPTP serves as a mitochondrial Ca\textsuperscript{2+} flux pathway, and depolarization of ΔΨ\textsubscript{m} renders the mPTP to be opened (5, 22, 23), we hypothesized that the mPTP could contribute to the regulation of [Ca\textsuperscript{2+}]\textsubscript{i} in the situation of ΔΨ\textsubscript{m} dissipation. Therefore, in this series of experiments, we investigated whether ΔΨ\textsubscript{m} dissipation by FCCP induces mPTP
opening by measuring calcein leakage from the mitochondria. The confocal images in Fig. 4A show that calcein fluorescence in a permeabilized myocyte decreased after 15 min of FCCP perfusion (0.3 μM). In contrast, when cells were pretreated with the mPTP inhibitor cyclosporin A (CsA; 0.1 μM), the reduction in the calcein signal by FCCP was not inhibited (Fig. 4B). Figure 4C shows the time courses of the changes in calcein signals after perfusion of a control solution ([Ca\(^{2+}\)]\(_c\) = 300 nM), CsA (0.1 μM), FCCP (0.3 μM), and FCCP plus CsA, and Fig. 4D summarizes the calcein intensities after a 15-min perfusion of each solution. The application of FCCP significantly decreased the calcein signal to 83 ± 3% of the baseline level (FCCP, P < 0.01 vs. 95 ± 1% of control; n = 5), whereas the decrease in the calcein signal by FCCP was inhibited in the presence of CsA (FCCP plus CsA, 93 ± 1%; P < 0.01 vs. FCCP; n = 5). These results suggest that ∆Ψ\(_m\) dissipation by FCCP opened the mPTP in a CsA-sensitive manner.

**Contribution of mPTP opening to [Ca\(^{2+}\)]\(_m\):** To investigate whether there is an mPTP-related mitochondrial Ca\(^{2+}\) flux, we examined the effects of CsA on the changes in [Ca\(^{2+}\)]\(_m\) when ∆Ψ\(_m\) was dissipated by FCCP. As shown in Fig. 5A, in the cells pretreated with 0.1 μM CsA, the rhod-2 signal after perfusion of 0.3 μM FCCP decreased more than that of FCCP alone. When the mPTP was inhibited by pretreatment with CsA, the rhod-2 signal after a 15-min FCCP perfusion was 25 ± 6% of the level before FCCP perfusion (P < 0.01; 53 ± 1% of FCCP alone; n = 4), which indicates that there was a CsA-sensitive decrease in [Ca\(^{2+}\)]\(_m\). These results suggest that mPTP inhibition reduces Ca\(^{2+}\) influx into the mitochondria during conditions of ∆Ψ\(_m\) dissipation.

**Ca\(^{2+}\) efflux via mitoNCX when ∆Ψ\(_m\) is dissipated:** Next we focused on the properties of mitochondrial Ca\(^{2+}\) efflux after ∆Ψ\(_m\) dissipation. Because previous studies using isolated mitochondria indicated that mitoNCX is a main Ca\(^{2+}\) efflux pathway in cardiac myocytes (6, 32), we examined the effects of mitoNCX inhibition on [Ca\(^{2+}\)]\(_m\) under conditions of dissipated ∆Ψ\(_m\). Figure 5B shows the changes in [Ca\(^{2+}\)]\(_m\) after FCCP perfusion (0.3 μM) in a normal-Na\(^+\)-extra-mitochondrial Na\(^+\) concentration ([Na\(^+\)]\(_i\), 10 mM) or a Na\(^+\)-free ([Na\(^+\)]\(_i\), 0 mM) internal solution. To eliminate the effects of mPTP-related Ca\(^{2+}\) flux, myocytes were pretreated with 0.1 μM CsA. When cells were perfused with FCCP, [Ca\(^{2+}\)]\(_m\) declined rapidly in the normal-Na\(^+\) solution, whereas [Ca\(^{2+}\)]\(_m\) did not decrease in the Na\(^+\)-free solution. When mitoNCX was inhibited by a Na\(^+\)-free solution, the rhod-2 signal after a 15-min FCCP perfusion was 84 ± 8% compared with before FCCP perfusion ([Na\(^+\)]\(_i\) = 0 mM; P < 0.01 vs. 25 ± 6% of [Na\(^+\)]\(_i\) = 10 mM; n = 4), which indicates that the extrusion of mitochondrial Ca\(^{2+}\) was almost abolished. These results suggest that even after complete ∆Ψ\(_m\) dissipation, mitoNCX extrudes Ca\(^{2+}\) from mitochondria.

Finally, we investigated the effects of [Na\(^+\)]\(_i\) on the rates of Ca\(^{2+}\) extrusion by mitoNCX in both normal and dissipated ∆Ψ\(_m\). In this series of experiments, to exclude the contribution of Ca\(^{2+}\) influx into mitochondria, we measured the [Ca\(^{2+}\)]\(_m\) decline when extramitochondrial Ca\(^{2+}\) was removed (from 300 to 0 nM) with different [Na\(^+\)]\(_i\) (varied from 0 to 50 mM) as a function of mitochondrial Ca\(^{2+}\) extrusion. Figure 6A demonstrates the Na\(^+\)-dependent declines in [Ca\(^{2+}\)]\(_m\) under normal ∆Ψ\(_m\). The higher [Na\(^+\)]\(_i\) caused faster declines in [Ca\(^{2+}\)]\(_m\).

**Fig. 4.** Imaging of mitochondrial permeability transition pore (mPTP) opening with calcein in permeabilized myocytes. A: calcein images of permeabilized myocytes were obtained before and after 15-min FCCP perfusion (0.3 μM). B: after 5-min pretreatment with cyclosporin A (CsA, 0.1 μM; left), FCCP was applied in the presence of CsA (right). C: time courses of the changes in calcein signals during perfusion of a control internal solution (C, control, [Ca\(^{2+}\)]\(_i\) = 300 nM), CsA (C, 0.1 μM), FCCP (●, 0.3 μM), and FCCP in the presence of CsA (●, FCCP + CsA). *P < 0.05 vs. control; †P < 0.01 vs. control by ANOVA. D: calcein intensities after a 15-min perfusion of each solution were summarized. Data are presented as the percentage of baseline calcein signal (before perfusion). *P < 0.01 vs. control; †P < 0.01 vs. FCCP by ANOVA.
Because the declines in $[\text{Ca}^{2+}]_{m}$ were slowed by the mitoNCX inhibitors diltiazem (100 µM; $n = 4$) and clonazepam (100 µM; data not shown) with 10 mM $[\text{Na}^+]_{c}$, it is likely that a $\text{Na}^+$-mediated $\text{Ca}^{2+}$ efflux occurred via the mitoNCX. The value of $t_{1/2}$ (the time required for the rhod-2 signal to decrease to one-half of maximal amplitude) was used to evaluate the rate of $[\text{Ca}^{2+}]_{m}$ decline. Figure 6B shows that $[\text{Na}^+]_{c}$, which yields a half-maximal rate of decrease ($k_{0.5}$) for $[\text{Ca}^{2+}]_{m}$, was 3.6 mM with a Hill coefficient of 1.8 for the normal $\Delta\Psi_m$ condition. Figure 6C demonstrates the $\text{Na}^+$-dependent declines in $[\text{Ca}^{2+}]_{m}$ when $\Delta\Psi_m$ was dissipated by 0.3 µM FCCP. To eliminate the contribution of the mPTP, cells were pretreated with 0.1 µM CsA. Although the reduction in $[\text{Ca}^{2+}]_{m}$ was slowed during dissipated $\Delta\Psi_m$ compared with normal $\Delta\Psi_m$, mitochondria certainly extrude $\text{Ca}^{2+}$ in a $\text{Na}^+$-dependent manner. The $k_{0.5}$ of $[\text{Na}^+]_{c}$ was 7.6 mM with a Hill coefficient of 1.7 during $\Delta\Psi_m$ dissipation (Fig. 6D).

**DISCUSSION**

In this study, we measured $[\text{Ca}^{2+}]_{m}$ in chemically skinned cardiac myocytes and investigated the regulation of $[\text{Ca}^{2+}]_{m}$ in both normal and dissipated $\Delta\Psi_m$. Our important experimental findings include the following: 1) the mitochondrial $\text{Ca}^{2+}$ uniporter accumulates $\text{Ca}^{2+}$ in a $\Delta\Psi_m$-dependent manner in the physiological ranges of $[\text{Ca}^{2+}]_{c}$; 2) $\Delta\Psi_m$ dissipation opens the mPTP and results in $\text{Ca}^{2+}$ influx into mitochondria via the mPTP; and 3) although $\Delta\Psi_m$ dissipation reduces mitoNCX activity, mitoNCX extrudes $\text{Ca}^{2+}$ from the matrix even after $\Delta\Psi_m$ dissipation.

Measuring $[\text{Ca}^{2+}]_{m}$ using rhod-2 signals. Rhod-2-AM is used to measure mitochondrial $\text{Ca}^{2+}$, because rhod-2 itself has a net positive charge and is selectively loaded into mitochondria, which have a negative $\Delta\Psi_m$ (1, 35). However, when cells were simply loaded by the membrane-permeate AM-ester form, it was difficult to monitor the mitochondrial rhod-2 signal while avoiding signal contamination from the cytosolic dyes. To improve this problem, further ingenuities such as employing cold-warm loading (1), using dihydro-rhod-2 (35), or permeabilizing the sarcosomal membrane (32) were required. In the present study, the following evidence allowed us to conclude that the rhod-2 signal in the permeabilized myocytes indeed represented $[\text{Ca}^{2+}]_{m}$: 1) rhod-2 signal distribution in a permeabilized myocyte showed complete overlap with Mito Tracker Green (see Fig. 1A); 2) rhod-2 intensity increased in response to $[\text{Ca}^{2+}]_{c}$ (see Fig. 1, B and C); and 3) rhod-2 intensity did not increase when the $\text{Ca}^{2+}$ uniporter was inhibited by RuR or FCCP (see Fig. 2, B–D).

Even in permeabilized myocytes, rhod-2 fluorescence could distribute to the sarcoplastic reticulum (SR), and thus the changes in rhod-2 signals in permeabilized myocytes might be contaminated by SR $\text{Ca}^{2+}$. To eliminate this possibility, we repeated our $[\text{Ca}^{2+}]_{m}$ studies in the presence of thapsigargin (5 µM), which is an inhibitor of SR $\text{Ca}^{2+}$-ATPase. The results were quite similar to those obtained without thapsigargin (data not shown). Thus we determined that the contribution of the SR $\text{Ca}^{2+}$ concentration was negligible in our experimental conditions.

**Mitochondrial $\text{Ca}^{2+}$ uptake via the $\text{Ca}^{2+}$ uniporter.** Although mitochondria could accumulate a significant amount of $\text{Ca}^{2+}$ from the cytosol during intracellular $\text{Ca}^{2+}$ signaling, previous studies that used isolated mitochondria indicated a very low affinity of the mitochondrial $\text{Ca}^{2+}$ uniporter for $\text{Ca}^{2+}$; that is, an apparent $K_m$ of 4.7–10 μM (3, 4, 16). Recently, Kirichok et al. (21) showed an extremely high $\text{Ca}^{2+}$ affinity ($K_m$ ≤ 2 nM) and a high $\text{Ca}^{2+}$ selectivity of the mitochondrial $\text{Ca}^{2+}$ uniporter by patch-clamping the inner mitochondrial membrane of mitoplasts. Here we have shown that mitochondrial $\text{Ca}^{2+}$ influx via the $\text{Ca}^{2+}$ uniporter occurred even in the physiological $[\text{Ca}^{2+}]_{c}$ range, where the bulk $[\text{Ca}^{2+}]_{c}$ was 177–600 nM.
In this study, [Ca\(^{2+}\)]\(_m\) decreased in response to the changes in partially depolarized \(\Delta \Psi_m\) (see Fig. 3B). These results are in good correspondence with those obtained from isolated mitochondria (10, 11). Because Ca\(^{2+}\) entry into the mitochondria directly reflects the activity of the Ca\(^{2+}\) uniporter (see Fig. 2), and Ca\(^{2+}\) efflux from mitochondria by mitoNCX is not accelerated by \(\Delta \Psi_m\) depolarization (see Fig. 6), the reduction in [Ca\(^{2+}\)]\(_m\) could be due to the decreased driving force for the Ca\(^{2+}\) uniporter by the \(\Delta \Psi_m\) depolarization. In contrast, when \(\Delta \Psi_m\) was completely dissipated, [Ca\(^{2+}\)]\(_m\) remained at one-half of the control level (see Fig. 3C). Because [Ca\(^{2+}\)]\(_m\) did not increase after pretreatment with 0.3 \(\mu\)M FCCP in our experimental procedures (see Fig. 2C), Ca\(^{2+}\) influx via the Ca\(^{2+}\) uniporter was inhibited after dissipation of the \(\Delta \Psi_m\). These results indicate that pathways other than the Ca\(^{2+}\) uniporter might be involved in regulation of [Ca\(^{2+}\)]\(_m\). Montero et al. (28) have demonstrated that when \(\Delta \Psi_m\) was depolarized, the action of the Ca\(^{2+}\) uniporter was reversed, and Ca\(^{2+}\) could be released via the Ca\(^{2+}\) uniporter. However, the simultaneous application of 0.3 \(\mu\)M FCCP plus 1 \(\mu\)M RuR did not alter [Ca\(^{2+}\)]\(_m\) in our experiments (data not shown), which indicates that there is little contribution of the Ca\(^{2+}\) uniporter to [Ca\(^{2+}\)]\(_m\) regulation after \(\Delta \Psi_m\) dissipation.

\textit{Dissipation of \(\Delta \Psi_m\) opening of mPTP, and [Ca\(^{2+}\)]\(_m\).} The mPTP opening and closing are strictly regulated by multiple factors such as reactive oxygen radicals, matrix pH, [Ca\(^{2+}\)]\(_m\), and \(\Delta \Psi_m\) (8, 12, 13, 24). It has also been reported (4, 10, 11) that the mPTP serves as a mitochondrial Ca\(^{2+}\) flux pathway during pathophysiological conditions. Earlier we reported (20) a method for assessing mPTP opening by monitoring mitochondrial calcein signals whereby cells were loaded with calcein-AM, and cytosolic fluorescence was quenched with Co\(^{2+}\). Here we advanced this method using permeabilized myocytes and showed that the complete dissipation of \(\Delta \Psi_m\) accelerated calcein leakage from mitochondria in a CsA-sensitive manner (see Fig. 4). To confirm that there is calcein leakage when mitochondrial permeability is altered, we tested the effects of the pore-forming antibiotic alamethicin (3 \(\mu\)M) on changes in calcein signals. As was found by Petronilli et al. (30), alamethicin-induced pore formation caused abrupt and complete loss of calcein fluorescence from the matrix within 5 min (data not shown). Because CsA is a well-known inhibitor of the mPTP (8, 12, 13, 24), it is suggested that the mPTP opened when \(\Delta \Psi_m\) was completely dissipated by FCCP. Importantly, the calcein leakage from mitochondria by FCCP was almost abolished in the Ca\(^{2+}\)-free solution (data not shown), which indicates that the opening of the mPTP strongly depends on matrix Ca\(^{2+}\). We also showed that the application of CsA alone did not alter calcein leakage (see Fig. 4, C and D), which indicates that mPTP opening is not significant in energized (fully polarized) mitochondria. Although the Ca\(^{2+}\)-activated protein phosphatase calcineurin is also inhibited by CsA, the effect of CsA on calcineurin might be negligible, because the sarcolemmal membrane was permeabilized, and cytosolic signal proteins were released in our experiments.
The opening of the mPTP was originally described (14, 17) in isolated mitochondria as a massive mitochondrial swelling under the conditions of matrix Ca\(^{2+}\) overload, oxidative stress, and depleted adenine nucleotide contents. In those studies, an extremely high concentration of extramitochondrial Ca\(^{2+}\) was administrated that caused mitochondrial Ca\(^{2+}\) release as a result of mPTP opening, with an apparent Km of 16 \(\mu\)M at pH 7.0 (14). In this study, we showed that there was an mPTP-related Ca\(^{2+}\) influx into mitochondria after the \(\Delta\Psi_m\) dissipation (see Fig. 5A). Because the opening of the mPTP allows mitochondrial Ca\(^{2+}\) release, it might not be accelerated so much as it conquers excessive matrix [Ca\(^{2+}\)], which suggests that the opening of the mPTP allows mitochondrial Ca\(^{2+}\) release.

Aions (where [Na\(^+\)]/H\(^{+}\)) that achieved a half-maximal \([\text{Ca}^{2+}]_{\text{im}}\) were dependent on [Na\(^+\)]. Allen DG and Xiao XB. Role of the cardiac Na\(^+\)/H\(^{+}\) exchanger during ischemia and reperfusion. Cardiovasc Res 57: 934–941, 2003.


This work was supported by Japan Grants-in-Aid 13670703 (to H. Katoh) and 50135258 (to H. Hayashi) and by a grant-in-aid from the Center of Excellence from the Ministry of Education, Culture, Sports, Science, and Technology.

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