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Different Actions of Cardioprotective Agents on Mitochondrial Ca^{2+} Regulation in a Ca^{2+} Paradox-Induced Ca^{2+} Overload

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Background Mitochondrial Ca^{2+} overload is a major cause of irreversible cell injury during various metabolic stresses. The protective effects of various agents that affect mitochondrial function against Ca^{2+} overload during Ca^{2+} paradox were investigated in rat ventricular myocytes.

Methods and Results On Ca^{2+} repletion following Ca^{2+} depletion, $[Ca^{2+}]_i$ increased rapidly, and 90 of 210 cells (43%) died. In viable cells, the increase in $[Ca^{2+}]_i$ was lower than in dead cells. KB-R7943 prevented the increase in $[Ca^{2+}]_i$, and completely inhibited cell death. Ruthenium red (RuR), diazoxide (Dz) or cyclosporin A (CsA) prevented cell death (15%, 26% and 17%, respectively; $p<0.05$), and the protective effect of Dz was abolished by 5-hydroxydecanoate. These agents did not reduce the increase in $[Ca^{2+}]_i$ in viable cells or the rate of initial increase in $[Ca^{2+}]_i$ in all cells. RuR and Dz decreased $[Ca^{2+}]_m$ in skinned myocytes, but CsA did not affect $[Ca^{2+}]_m$. Dz reduced NADH fluorescence, whereas RuR and CsA did not.

Conclusions The protective effects of RuR and Dz could be ascribed to altered Ca^{2+} regulation by decreasing $[Ca^{2+}]_m$, and Dz could have an additional effect on oxidative phosphorylation. The protective effect of CsA could be directly associated with the mitochondrial permeability transition pore. (*Circ J* 2005; **69:** 1132–1140)

Key Words: Ca^{2+} overload; Cardiac myocyte; Mitochondria; Mitochondrial permeability transition pore; Mitochondrial redox state

Cellular Ca^{2+} overload is responsible for irreversible myocardial damage in ischemia/reperfusion, hypoxia/reoxygenation and digitalis toxicity, but the precise mechanisms remain unclear. The Ca^{2+} overload precipitates structural damage and functional failure in cardiac myocytes, including (1) spontaneous Ca^{2+} release from the sarcoplasmic reticulum (SR) that can generate delayed afterdepolarization and triggered activity, (2) hypercontraction and membrane disruption, (3) degradation of myofilaments and cytoskeleton by Ca^{2+}-activated proteases (calpains), and (4) exhaustion of high-energy phosphates by the myofilaments and Ca^{2+}-ATPase[1,2] Mitochondrial Ca^{2+} overload has been recently proposed as a major cause of irreversible cell injury[3–5]

The mitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_m$) is important for the maintenance of cellular functions, including ATP production by oxidative phosphorylation and the interaction with SR Ca^{2+} content[6] In addition, mitochondrial Ca^{2+} plays a pivotal role in pathophysiological conditions such as generation of reactive oxygen species (ROS) and cell apoptosis. In Ca^{2+}-overloaded cells, mitochondria have to accumulate and buffer large amounts of Ca^{2+} through a Ca^{2+} uniporter driven by the mitochondrial membrane potential. The massive increase in $[Ca^{2+}]_m$ induces mitochondrial depolarization, NADH oxidation and hydrolysis of ATP, resulting in mitochondrial dysfunction. A proteinaceous pore in the mitochondrial membrane (ie, the mitochondrial permeability transition pore; mPTP) also opens, which increases the permeability of the mitochondrial membrane. This irreversible opening of the mPTP ultimately leads to cell death[3,7–9]

Thus, in order to protect myocytes from Ca^{2+} overload, it is necessary to prevent excessive Ca^{2+} accumulation and/or prevent the mPTP opening. Indeed, a blocker of the mitochondrial Ca^{2+} uniporter, ruthenium red (RuR) and an opener of the mitochondrial K_{ATP} (mitoK_{ATP}) channels, diazoxide (Dz), are known to protect myocytes from various stresses by reducing mitochondrial Ca^{2+} accumulation[4,8,10–12] Cyclosporin A (CsA), a potent inhibitor of the mPTP, has been shown to also improve postischemic cardiac function[13] However, there has not been a comparison of the protective effects of these agents under the same experimental conditions.

To clarify the importance of mitochondrial protection against Ca^{2+} overload, we made a model of cellular Ca^{2+} overload by a Ca^{2+} paradox protocol, and examined the effects of the agents that affect mitochondrial function. For this purpose, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the rate of cell death were monitored during a Ca^{2+} paradox protocol in isolated rat myocytes. The $[Ca^{2+}]_m$ was also measured by laser scanning confocal microscope with rhod-2 in saponin-permeabilized myocytes.

Methods

Myocyte Isolation

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Male Sprague-Dawley rats (220–300 g) were killed by a blow to the head followed by cervical dis-

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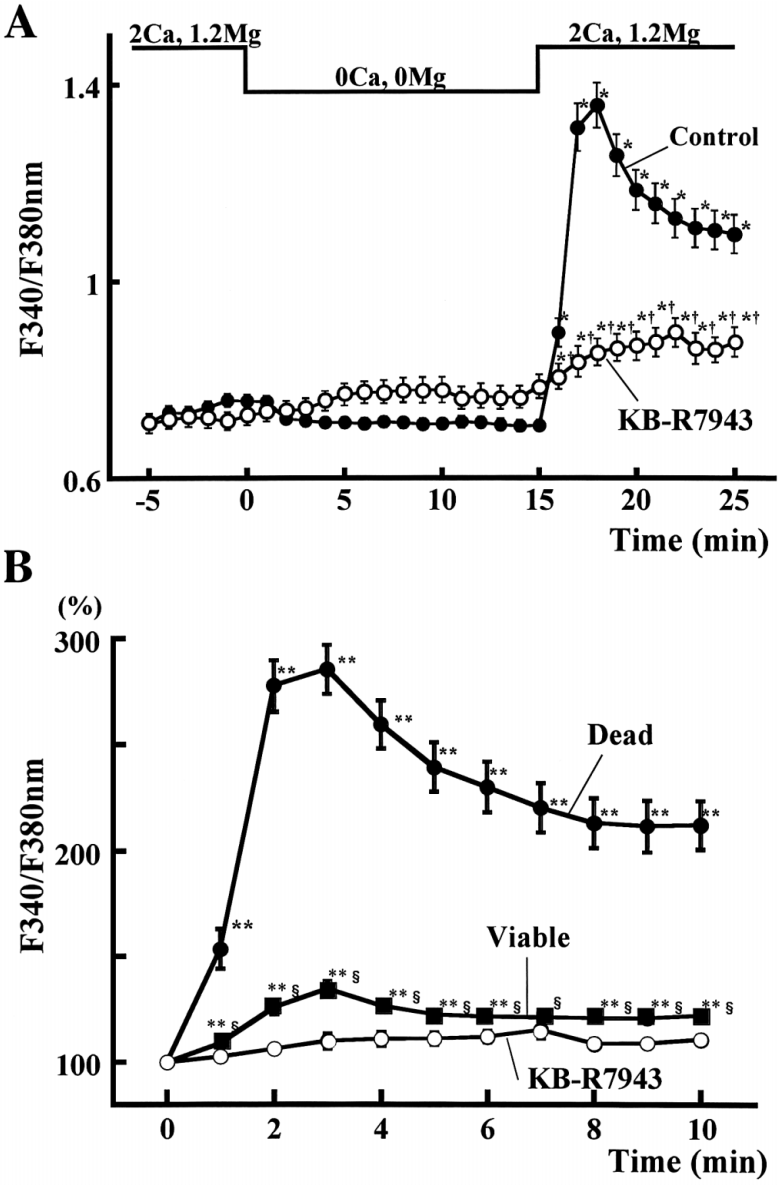

Fig 1. Ca^{2+} overload and cell injury by the Ca^{2+} paradox protocol. (A) The time course of the changes in $[Ca^{2+}]_i$ in rat ventricular myocytes. For the Ca^{2+} paradox protocol, the perfusate was changed from standard Tyrode's solution (2 mmol/L Ca^{2+}: –5–0 min) to a Ca^{2+}-, Mg^{2+}-free solution for 15 min (Ca^{2+} depletion: ~0–15 min), and then returned to standard Tyrode's solution (Ca^{2+} repletion: ~15–25 min) at 37°C. The change in $[Ca^{2+}]_i$ was expressed as the increase in the fura-2 ratio (F340/F380nm). On Ca^{2+} repletion, 90 of 210 cells (43%) died and the fura-2 ratio increased rapidly and decreased gradually (Control: ●). An inhibitor of the Na^+/Ca^{2+} exchange (NCX), KB-R7943 (10 μmol/L), significantly attenuated the increase in the fura-2 ratio and completely abolished cell death (KB-R7943: ○). *$p<0.05$ vs data at 15 min, and †$p<0.05$ vs data of Control by two-way repeated ANOVA. (B) The percent changes in the fura-2 ratio against that at the end of Ca^{2+} depletion. In viable cells (■), the increases in the fura-2 ratio were smaller than those in dead cells (●), but were still larger than those in cells treated with KB-R7943 (○). Data are means ± SE from 90 myocytes for dead cells, 120 cells for viable cells and 30 cells for KB-R7943. **$p<0.05$ vs data of KB-R7943, and §$p<0.05$ vs data of dead cells by two-way repeated ANOVA.

location. The heart was rapidly removed and mounted on a Langendorff apparatus. Perfusion with nominally Ca^{2+}-free Tyrode's solution for 5 min was followed by perfusion with the same solution containing 0.18 mg/ml collagenase (Yakult Pharmaceuticals, Tokyo, Japan) at 36°C for approximately 10 min. After enzymatic digestion, the tissue was dispersed and filtered, and isolated ventricular myocytes were stocked in modified Kraftbrühe (KB) solution as described previously until use.[14]

Measurement of $[Ca^{2+}]_i$

The myocytes were loaded with fura-2 by exposure to the acetoxymethyl ester, fura-2/AM (2 μmol/L), for 30 min at room temperature and incubated for a further 30 min for complete hydrolysis of the dye. Fluorescent signals were imaged using a silicon-intensified target camera (SIT camera; model C2400, Hamamatsu Photonics, Hamamatsu, Japan), with the output digitized to a resolution of 512×512 pixels by an image analysis system (Argus 50, Hamamatsu Photonics). The cells were excited via an epifluorescence illuminator from a 100 W xenon lamp equipped with an interference filter. The exciting wavelengths were 340 nm and 380 nm, and the emission signal at 520 nm was detected using an SIT camera and stored in a digital imaging processor. Each image was accumulation of 8 (30/s) successive video frames and was obtained every 10–60 s. The fluorescence intensity was measured in a defined cell area, avoiding the nuclei. Fluorescence ratios were obtained by dividing, pixel by pixel, the 340 nm image by the 380 nm image after each background subtraction. Exposure to excitation light was limited to the time of actual data collection (0.27 s/each collection) by an electrically controlled shutter, and neutral density filters were placed in the exciting light paths to prevent photobleaching.

Experimental Protocol

After fura-2 was loaded, a small aliquot of myocytes was placed in an experimental chamber mounted on the stage of an inverted microscope (TMD, Nikon, Tokyo, Japan), and perfused with standard Tyrode's solution at 37°C. In our experiments, 50–60% of all myocytes were rod-shaped with clear sarcomeres, and were well stained with fura-

Table 1 Effects of Various Agents on the Ca^{2+} Paradox-Induced Cell Death and the Increase in $[Ca^{2+}]i$

	Control	*KBR*	*RuR*	*Dz*
Cell number	*210*	*30*	*81*	*125*
Survival rate (%)	*57*	*100**	*85**	*74**
ΔR				
Total	*0.81±0.05*	*0.14±0.02**	*0.51±0.06**	*0.60±0.06**
Viable	*0.33±0.02*	*0.14±0.02**	*0.32±0.03*	*0.31±0.02*
Dead	*1.47±0.06†*		*1.57±0.16†*	*1.43±0.14†*
ΔR/dt	*0.25±0.04*	*0.02±0.01**	*0.13±0.03*	*0.34±0.11*

*ΔR, increase in the fura-2 ratio (F340/F380 nm); ΔR/dt, rate of increase in the fura-2 ratio at the initial 30 s of Ca^{2+} repletion. Data are means ± SE from indicated number of myocytes. *p<0.05 vs Control by chi-square test or one-way ANOVA followed by Fisher's PLSD, †p<0.05 vs Viable by unpaired t-test.*

2/AM. These cells were selected, and the changes in $[Ca^{2+}]_i$ and cell shape during the Ca^{2+} paradox protocol were examined. For the Ca^{2+} paradox protocol, the perfusate was changed from standard Tyrode's solution (2 mmol/L Ca^{2+}) to a Ca^{2+}-, Mg^{2+}-free solution for 15 min (Ca^{2+} depletion), and then returned to the standard Tyrode's solution (Ca^{2+} repletion). After the Ca^{2+} paradox protocol, the myocytes were perfused with a hypotonic staining solution. Cells permeable to trypan blue were counted and expressed as the percentage cell death. Because almost all of the hypercontracted cells (shortened to less than 60% of the initial longitudinal length) after Ca^{2+} repletion were stained with trypan blue, the cells that hypercontracted during the Ca^{2+} paradox protocol were judged to be dead in some experiments.

Measurement of $[Ca^{2+}]_m$ in Permeabilized Myocytes

In another series of experiments, isolated rat ventricular myocytes were loaded with 20 μmol/L rhod-2/AM at room temperature for 60 min. To remove cytosolic rhod-2, the sarcolemmal membrane was permeabilized by the perfusion of 0.05 mg/ml saponin in a Ca^{2+}-free internal solution.[15,16] After the sarcolemmal membrane was permeabilized, the free Ca^{2+} concentration of the internal solution ($[Ca^{2+}]_c$) was increased to 300 nmol/L. $[Ca^{2+}]_c$ was obtained by mixing EGTA and $CaCl_2$, calculated by a computer program (Win MAXC version 2.05, kindly provided by Stanford University, USA).

Experiments were performed using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Germany) coupled to an inverted microscope (Axiovert S100, Zeiss, Germany) with a ×63 water-immersion objective lens (numerical aperture = 1.3; Zeiss). Cells were excited with a 514 nm line of an argon laser, and images were acquired thorough a >560 nm long-pass filter. To localize the mitochondria, some myocytes were co-loaded with rhod-2/AM and Mito Tracker Green (which specifically stains mitochondria), excited at 488 nm and the emission signal was monitored through a 505–530 nm band pass.[17]

Measurement of NADH Fluorescence

The mitochondrial redox state was assessed by the fluorescence of NADH. Endogenous NADH fluorescence images were recorded by excitation of intact myocytes with 340 nm (emission wavelength at 520 nm). The NADH fluorescence was calibrated by sodium cyanide (2 mmol/L) for complete inhibition (set to 100%) and by FCCP (2 μmol/L) for maximum acceleration (set to 0%) of oxidative phosphorylation.[18,19]

Reagents and Solutions

The standard Tyrode's solution contained (in mmol/L): 137 NaCl, 4 KCl, 1.2 $MgSO_4$, 10 HEPES, 10 glucose, and 2 $CaCl_2$ (pH 7.4 with NaOH). In the Ca^{2+}-, Mg^{2+}-free solution, $CaCl_2$ and $MgSO_4$ were omitted, and 3 mmol/L EGTA was added. The pH of all solutions was adjusted to 7.4 with NaOH. The modified KB solution contained (in mmol/L): 70 KOH, 40 KCl, 20 KH_2PO_4, 3 $MgCl_2$, 50 glutamic acid, 10 glucose, 10 HEPES, and 0.5 EGTA (pH 7.4 with KOH). The staining solution contained (in mmol/L): 2.68 KCl, 1.8 $CaCl_2$, 0.42 NaH_2PO_4, 11.9 $NaHCO_3$, 0.83 $MgSO_4$, 5.55 glucose, 3.3 amobarbital, and 0.5% glutaraldehyde, 0.5% trypan blue. The Ca^{2+}-free internal solution contained (in mmol/L): 50 KCl, 80 potassium asparate, 4 sodium pyruvate, 20 HEPES, 3 $MgCl_2$–$6H_2O$, 3 Na_2ATP, 5.8 glucose, 3 EGTA (pH 7.3 with KOH). Fura-2/AM and rhod-2/AM were obtained from Molecular Probes Inc. RuR, Dz and CsA were purchased from Sigma (St Louis, MO, USA). RuR was dissolved in water, and Dz and CsA were dissolved in dimethyl sulfoxide and added to the solution immediately before use.

Statistical Analyses

Results are expressed as means ± SE for the indicated number (n) of myocytes from at least 3 rats. Chi-square test, Student's t-test and one- or two-way analysis of variance (ANOVA) were used for statistical analyses. Multiple group comparison was carried out using Fisher's protected least significant difference, and the probability was considered significant at $p<0.05$.

Results

Ca^{2+} Overload and Cell Injury by Ca^{2+} Paradox

Fig 1A shows the time course of the changes in $[Ca^{2+}]_i$ during the Ca^{2+} paradox protocol in rat ventricular myocytes. During 15 min of Ca^{2+} depletion, there was a small decline in the fura-2 ratio, suggesting increased Ca^{2+} extrusion and/or decreased Ca^{2+} influx. On Ca^{2+} repletion, the fura-2 ratio increased rapidly and then decreased gradually. Ninety of 210 cells (43%) died, and spontaneous contractile activities were observed in some cells. A well-known inhibitor of Na^+/Ca^{2+} exchange (NCX), KB-R7943 (10 μmol/L), significantly inhibited the increase in the fura-2 ratio and completely prevented cell death (see also Table 1). Thus, the Ca^{2+} paradox protocol induced a rapid Ca^{2+} influx via NCX and caused cell injury from Ca^{2+} overload.

Next, we compared the changes in $[Ca^{2+}]_i$ after Ca^{2+} repletion between viable and dead cells. Fig 1B demonstrates the % changes in the fura-2 ratio on Ca^{2+} repletion

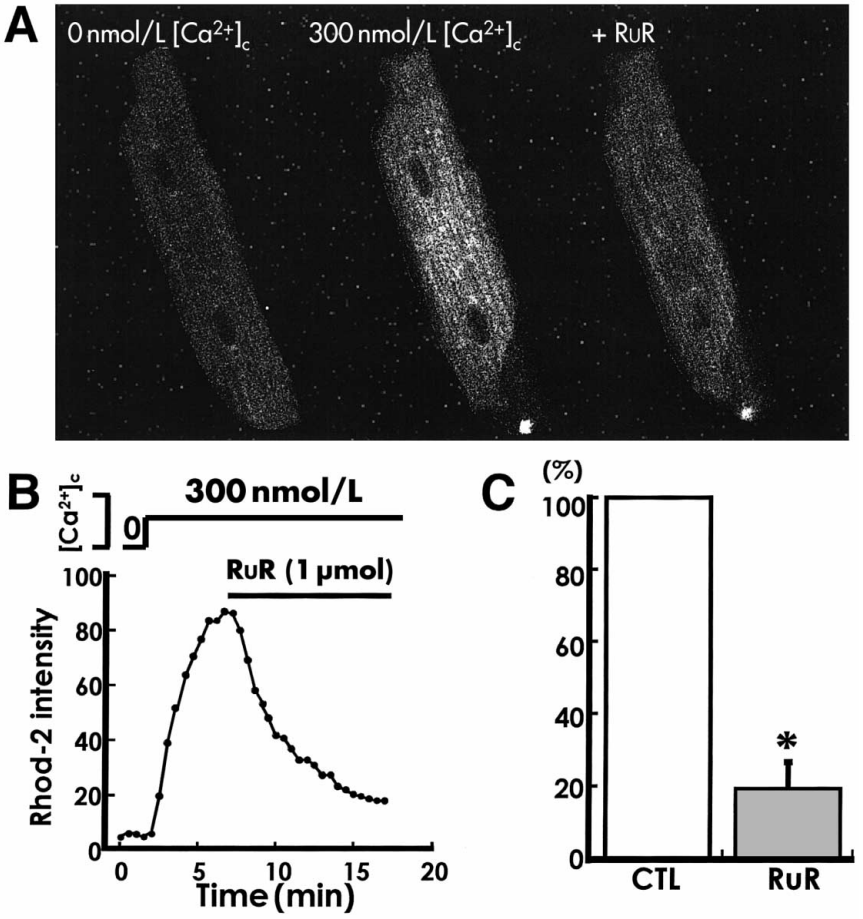

Fig 2. Imaging of mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) and the modulation of $[Ca^{2+}]_m$ by ruthenium red (RuR). (A) Representative images of $[Ca^{2+}]_m$ in a saponin-permeabilized rat myocyte loaded with rhod-2. The changes in $[Ca^{2+}]_m$ before (Left: $[Ca^{2+}]_o$=0 mV) and after increasing $[Ca^{2+}]_o$ to 300 nmol/L (Middle), and after the addition of RuR (1 μmol/L) (Right) were obtained with a laser scanning confocal microscopy. (B) The time course of $[Ca^{2+}]_m$ was expressed as the changes in rhod-2 intensity. After increasing the perfusate Ca^{2+} to 300 nmol/L, the rhod-2 intensity increased rapidly by ~18-fold, and the addition of RuR reduced the signal. (C) The relative changes in $[Ca^{2+}]_m$ by RuR. RuR at 1 μmol/L decreased the rhod-2 intensity by ~80%. Data are means±SE from 4 myocytes. *$p<0.05$ vs before the addition of RuR (CTL) by paired t test.

against that at the end of Ca^{2+} depletion. In dead cells, the fura-2 ratio increased significantly and hypercontracture occurred. The morphological changes were accompanied by decreases in fura-2 fluorescence intensity and ratio, indicating a disruption of the cell membrane. In viable cells, the increases in the fura-2 ratio were smaller than those in the dead cells, but still larger than those in cells treated with KB-R7943. Table 1 summarizes the mean values of the maximum increases in the fura-2 ratio (ΔR) in viable and dead cells, and the rate of the initial increase increased fura-2 ratio (ΔR/dt). Both the ΔR in viable cells and the ΔR/dt in all cells were significantly higher than those in cells treated with KB-R7943.

Effects of Agents That Target Mitochondria

Because most of the cellular Ca^{2+} regulatory systems are energy-dependent, and the mitochondria themselves buffer cytosolic Ca^{2+} via the Ca^{2+} uniporter, it is likely that inhibition of mitochondrial Ca^{2+} overload and/or the preservation of mitochondrial function could protect myocytes against Ca^{2+} overload caused by the Ca^{2+} paradox.

Table 1 indicates the rate of cell death and the increase in $[Ca^{2+}]_i$ in the presence of RuR (10 μmol/L), a blocker of the mitochondrial Ca^{2+} uniporter, and Dz (100 μmol/L), an opener of the mitoKATP channels, which were perfused throughout the Ca^{2+} paradox protocol. The changes in the fura-2 ratio during Ca^{2+} depletion were not affected by these agents (data not shown). Both agents reduced the rate of cell death and blunted the ΔR in all cells significantly. The protective effect of Dz was also abolished by the co-administration of 5-hydroxydecanoate (100 μmol/L), an inhibitor of the mitoKATP channels (survival rate: 50%, $p<0.05$ vs Dz by chi-square test). However, in contrast to KB-R7943, these agents did not reduce the ΔR in viable cells nor the ΔR/dt in all cells (Table 1). Therefore, it is most likely that both RuR and Dz protected myocytes against Ca^{2+} overload by targeting the mitochondria rather than by reducing the Ca^{2+} influx via the NCX.

Changes in Mitochondrial Ca^{2+} Concentration

The next series of experiments were planned to investigate how RuR and Dz modified mitochondrial function. First, we measured $[Ca^{2+}]_m$ in saponin-permeabilized rat myocytes loaded with rhod-2. Fig 2A,B shows the representative images and the time course of the changes in $[Ca^{2+}]_m$ before and after the addition of RuR (1 μmol/L). The rhod-2 signal was distributed homogeneously, and after increasing the Ca^{2+} concentration of the perfusate to 300 nmol/L, the rhod-2 intensity increased rapidly by approximately 18-fold and the cell length shortened, suggesting that 300 nmol/L Ca^{2+} was higher than the resting $[Ca^{2+}]_i$ in intact cells (ie, Ca^{2+} overload). The addition of RuR reduced the rhod-2 intensity to 19±7% of the control (Fig 2C, $p<0.05$ by paired t-test).

After the Ca^{2+} concentration of the perfusate was increased to 300 nmol/L, the addition of Dz (50 μmol/L) reversibly decreased $[Ca^{2+}]_m$ (Fig 3A). Fig 3B indicates that Dz reduced $[Ca^{2+}]_m$ in a concentration-dependent manner, and at 50 μmol/L it reduced $[Ca^{2+}]_m$ to 70±16% of the control.

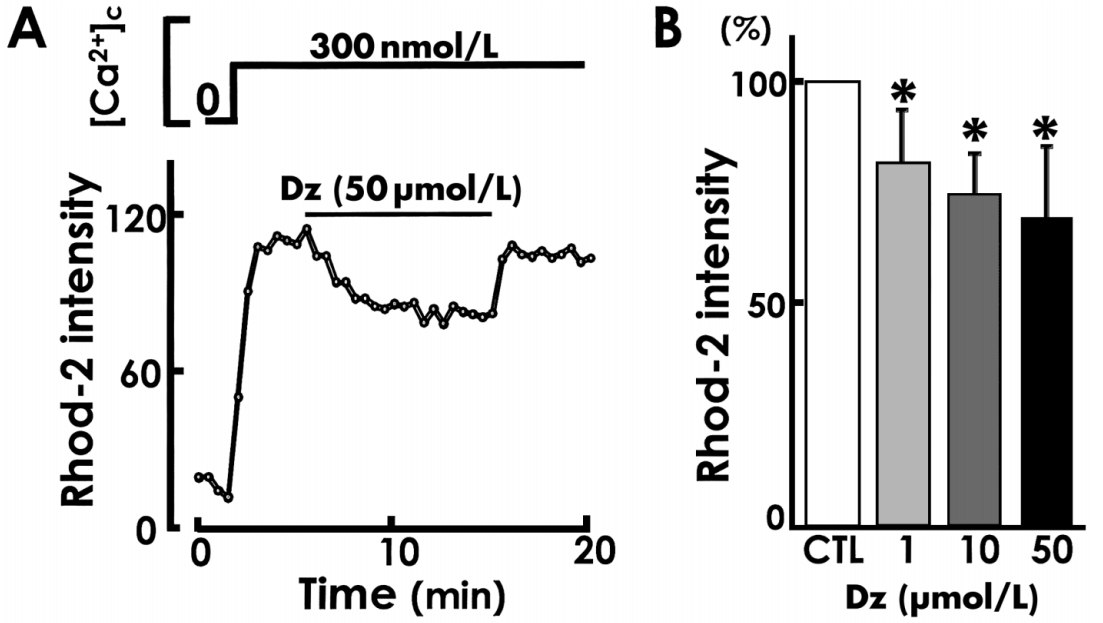

Fig 3. Changes in $[Ca^{2+}]_m$ by Dz. (A) The time course of $[Ca^{2+}]_m$ before and after increasing the perfusate Ca^{2+} to 300 nmol/L, and after the addition of Dz (50 μmol/L). (B) The relative changes in $[Ca^{2+}]_m$ by Dz. Dz reduced the rhod-2 intensity in a concentration-dependent manner. Data are means ± SE from 6 or 7 myocytes. *p<0.05 vs before the addition of Dz (CTL) by paired t test.

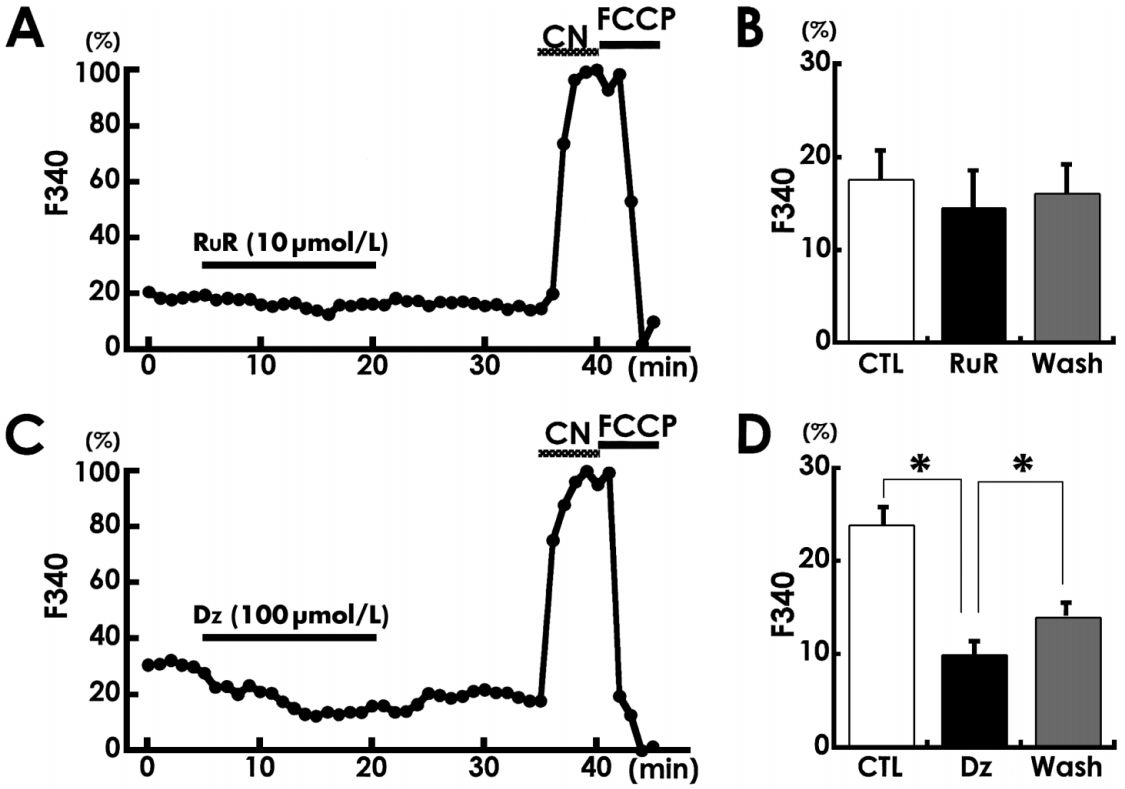

Fig 4. Changes in NADH fluorescence by RuR and Dz. (A) Representative recording of the changes in NADH fluorescence (F340 nm) by RuR (10 μmol/L) in an intact myocyte. Each signal was calibrated as 100% for sodium cyanide (CN: 2 mmol/L)-induced complete reduction and as 0% for FCCP (2 μmol/L)-induced maximal oxidation. (B) The relative changes in the NADH fluorescence by RuR. Bars indicated the relative fluorescence intensities just before (CTL), at 15 min during perfusion of RuR (RuR), and at 15 min after the washout (Wash). (C) Representative recording of the change in NADH fluorescence by Dz (100 μmol/L) in an intact myocyte. (D) The relative changes in the NADH fluorescence before (CTL), at 15 min during perfusion of Dz (Dz), and at 15 min after the washout (Wash). Dz reduced NADH fluorescence, whereas RuR had no significant effect. Data are means ± SE from 8 (for RuR) and 11 (for Dz) myocytes. *p<0.05 vs CTL by one-way repeated ANOVA.

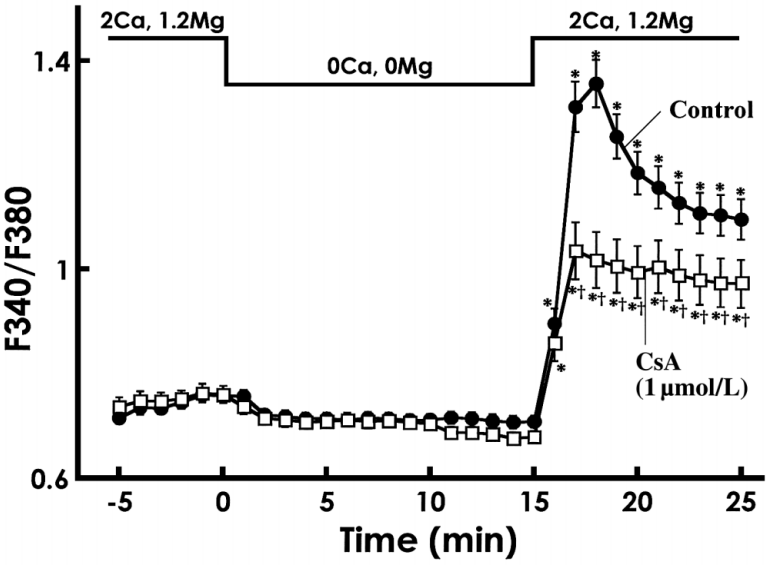

Fig 5. Effects of cyclosporin A (CsA) against Ca^{2+} overload in the Ca^{2+} paradox protocol. The time course of the changes in $[Ca^{2+}]_i$ in the presence of CsA (1 μmol/L) (□). CsA inhibited the increase in $[Ca^{2+}]_i$, and significantly reduced the % cell death (19 of 109 cells, 17.4%, $p<0.05$ vs 43% in Control by chi-square test). Data are means ± SE from 210 myocytes for Control and 109 myocytes for CsA. *$p<0.05$ vs data at 15 min, and †$p<0.05$ vs data of Control by two-way repeated ANOVA.

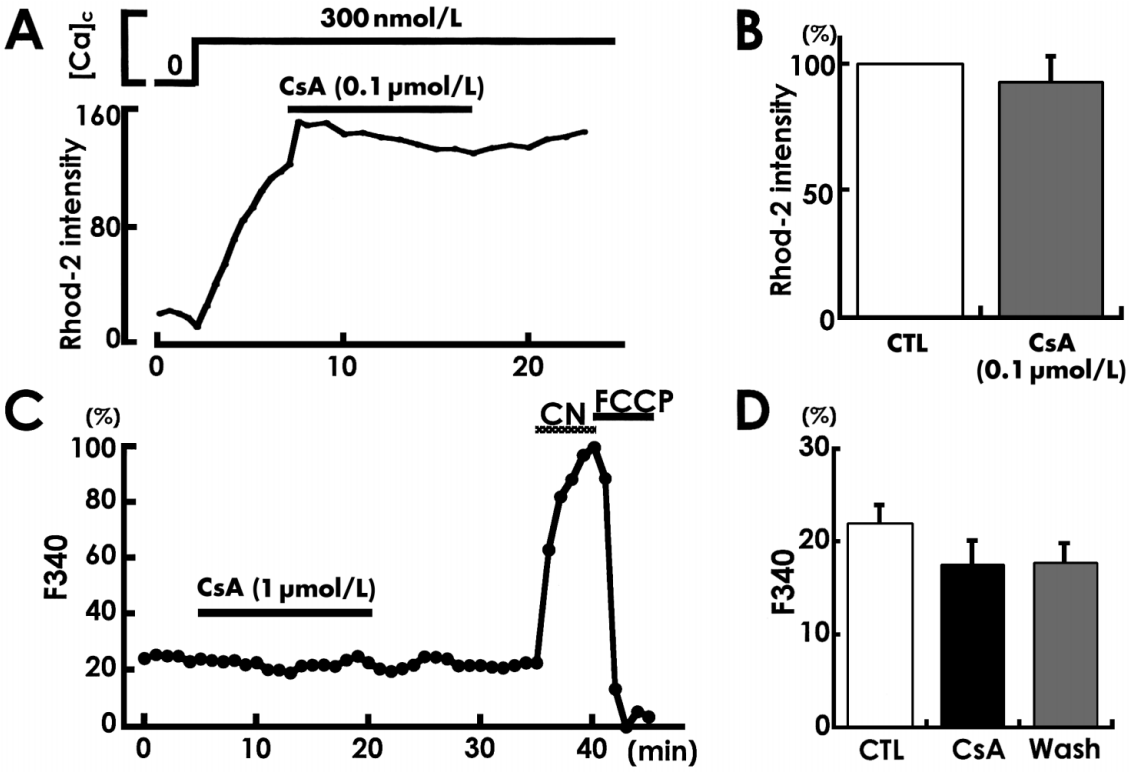

Fig 6. Changes in $[Ca^{2+}]_m$ and NADH fluorescence by cyclosporin A (CsA). (A) Representative change in $[Ca^{2+}]_m$ by CsA in a saponin-permeabilized myocyte. After the increase of $[Ca^{2+}]_m$, the addition of CsA (0.1 μmol/L) transiently raised $[Ca^{2+}]_m$, which then decreased and reached a plateau. (B) Relative changes in $[Ca^{2+}]_m$ by CsA. (C) Representative recording of the change in NADH fluorescence by CsA (1 μmol/L) in an intact myocyte. (D) Relative changes in the NADH fluorescence (F340 nm) before (CTL), at 15 min during perfusion of CsA (CsA), and at 15 min after the washout (Wash). CsA did not significantly change $[Ca^{2+}]_m$ or NADH fluorescence. Data are means±SE from 11 myocytes for both experiments.

These results suggest that both RuR and Dz are likely to have protected the myocytes by preventing mitochondrial Ca^{2+} overload.

Changes in NADH Fluorescence

To investigate the possible link between these agents and mitochondrial oxidative phosphorylation, we measured NADH fluorescence as an index of the mitochondrial redox state in intact myocytes. Fig 4A shows that RuR (10 μmol/L) did not alter NADH fluorescence, but the following addition of sodium cyanide (2 mmol/L) increased it markedly and FCCP (2 μmol/L) attenuated it. As shown in Fig 4B, there were no significant changes in the relative values of NADH fluorescence after perfusion of RuR (n=8,

p=NS).

On the other hand, NADH fluorescence apparently decreased during perfusion of Dz (100μmol/L), and recovered after the washout (Fig 4C). As indicated in Fig 4D, the relative values of NADH fluorescence were 23.8±1.5% before the perfusion of Dz, 9.9±1.4% at 15 min after perfusion and 13.9±1.3% at 15 min after the washout (n=11, p<0.05 by one-way repeated ANOVA).

Effects of CsA

Mitochondrial Ca^{2+} overload causes an irreversible opening of the mPTP, resulting in cell death. In the final experiment, we investigated the role of the mPTP on Ca^{2+} paradox-induced cell injury. Fig 5 shows the time course of the changes in $[Ca^{2+}]_i$ during the Ca^{2+} paradox protocol in the presence and absence of CsA (1μmol/L), an inhibitor of the mPTP. CsA inhibited the increase in $[Ca^{2+}]_i$, and significantly reduced the %cell death (19 of 109 cells, 17%, p<0.05 vs 43% in control by chi-square test). However, CsA did not reduce the ΔR in viable cells (0.28±0.04 vs 0.33±0.02 in control) or the ΔR/dt in all cells (0.32±0.07 vs 0.25±0.04 in control).

Fig 6A indicates the changes in $[Ca^{2+}]_m$ by CsA in a saponin-permeabilized myocyte. After the increase in $[Ca^{2+}]_m$ with 300 nmol/L Ca^{2+} perfusion, the addition of CsA (0.1μmol/L) transiently raised the $[Ca^{2+}]_m$, then it decreased slightly, and reached a plateau. The rhod-2 intensity at 10 min after the perfusion of CsA was 93.6±8.9% of that just before the perfusion (Fig 6B, n=11, p=NS). Fig 6C shows that the perfusion of CsA (1μmol/L) did not alter the NADH fluorescence in an intact myocyte. The relative values of NADH fluorescence were 21.9±1.8% just before the perfusion of CsA and 17.5±2.5% at 15 min after the perfusion (Fig 6D, n=11, p=NS). This indicated that the protective effect of CsA was not mediated by reducing the $[Ca^{2+}]_m$ or by acceleration of NADH oxidation.

Discussion

The results of the present study demostrated that (1) the Ca^{2+} paradox protocol induced a rapid Ca^{2+} influx via the NCX and caused cell injury because of Ca^{2+} overload, (2) in viable cells, the increase in $[Ca^{2+}]_i$ after Ca^{2+} repletion was lower than that in dead cells, (3) RuR, Dz and CsA prevented cell death without significant changes in Ca^{2+} entry because there were no changes in the rate of increase in $[Ca^{2+}]_i$ after Ca^{2+} repletion, (4) in permeabilized myocytes, the addition of RuR or Dz decreased $[Ca^{2+}]_m$, whereas the reduction was not significant in CsA-treated cells, and (5) Dz accelerated NADH oxidation whereas RuR nor CsA did not alter it. From these results, we conclude that the agents targeting mitochondria can confer cardioprotection against Ca^{2+} overload, although the protective mechanisms differ.

Ca^{2+} Overload by a Ca^{2+} Paradox Protocol and the Role of NCX

In isolated myocytes, perfusion with a Ca^{2+}- and Mg^{2+}-free solution causes Na^+ loading by a Na^+ influx via the sarcolemmal Ca^{2+} channels. This cellular Na^+ loading in turn activates a Ca^{2+} influx via the NCX following Ca^{2+} repletion, resulting in a lethal Ca^{2+} overload[20] This scheme was supported by our result that KB-R7943 attenuated the Ca^{2+} overload. This scenario could also be applied to ischemia/reperfusion injury in which cellular Na^+ loading is an important prerequisite[2,21] Although KB-R7943 completely blocked cell death in the present Ca^{2+} paradox protocol, the effectiveness of direct or indirect inhibition of the NCX in ischemia/reperfusion injury remains equivocal[2,22,23] After ischemia and reperfusion, the functional recovery of many Ca^{2+} regulatory proteins, including SR Ca^{2+}-ATPase and the ryanodine receptors, and the increase in Ca^{2+} influx via L-type Ca^{2+} channels (by recovery of the action potential) could elevate the cytosolic Ca^{2+} concentration, resulting in mitochondrial Ca^{2+} overload. A burst of ROS generation and pH_i recovery also cause mitochondrial function to deteriorate[3,7] Therefore, an additional strategy is needed to protect myocytes against Ca^{2+} overload. Because many cardiac proteins are energy dependent, the preservation of mitochondrial function is critical.

Measurements of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$

In this study, the techniques we used to monitor $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$, respectively, were fura-2 in intact myocytes and rhod-2 in saponin-permeabilized myocytes. First, we have to consider possible factors that may perturb the measurement $[Ca^{2+}]_i$. These include: (1) compartmentalization of fura-2, (2) incomplete hydrolysis of intracellular accumulated fura-2/AM, and (3) photobleaching of fura-2 fluorescence. The residual fluorescence intensity of fura-2 after treatment with 20μmol/L digitonin was approximately 10% of the pre-exposed control level[24] Myocytes were incubated for a further 30 min after dye loading to allow formation of the hydrolyzed fluorescent form of the dye. Illumination from the xenon lamp was eliminated with neutral-density filters.

Second, rhod-2/AM is used for the measurement of mitochondrial Ca^{2+} because it has a net positive charge and is selectively loaded into mitochondria that have the negative $\Delta\Psi_m$[25,26] However, when cells were simply loaded with the membrane-permeate AM ester form, it was difficult to monitor the mitochondrial rhod-2 signal without signal contamination from the cytosolic dye. Using a low concentration of saponin specifically permeabilizes cholesterol-rich membranes such as the sarcolemma and can keep the mitochondrial membrane from being affected. Previous studies have shown intact mitochondrial morphology and function after saponin permeabilization[15,16] Thus, removing the cytosolic indicator by permeabilizing the plasma membrane meant the fluorescence measurements originated solely from the mitochondrial matrix[17] The merits of this method are the ability to monitor $[Ca^{2+}]_m$ without changing the intracelluar architecture and to precisely control the extra-mitochondrial medium. We have confirmed that this method can indicate $[Ca^{2+}]_m$, because (1) the distribution of the rhod-2 intensity in permeabilized myocytes showed complete overlap with the mitochondrial specific fluorescent dye Mito Tracker Green, (2) the rhod-2 intensity increased in response to the extra-mitochondrial Ca^{2+} concentration, (3) the rhod-2 intensity did not increase when the membrane potential was completely dissipated by FCCP[17]

Cellular Protection by Various Agents That Target Mitochondria

In this study, RuR, Dz and CsA all effectively prevented Ca^{2+} paradox-induced cell death. Reduction in Ca^{2+} entry via the sarcolemma was unlikely because these agents did not reduce either the ΔR or the ΔR/dt in viable cells. Therefore, it is possible that these agents preserved mitochondrial

function and maintained the ability of cellular Ca^{2+} regulatory proteins to eliminate the excessive Ca^{2+}. However, the modulation of the mitochondria by each of these agents might differ.

RuR at 100 nmol/L completely inhibited Ca^{2+} uptake into isolated mitochondria![11] Miyamae et al demonstrated that contractile dysfunction following post-ischemic reperfusion correlated with the degree of $[Ca^{2+}]_m$ and that RuR attenuated the increase in $[Ca^{2+}]_m$ and improved the recovery of left ventricular function[4] Ban et al reported that the Ca^{2+} paradox caused mitochondrial depolarization, NADH oxidation and ATP hydrolysis, and that RuR prevented or delayed them![12] Our result that RuR at 1 μmol/L largely reduced $[Ca^{2+}]_m$ after cytosolic Ca^{2+} loading (300 nmol/L) suggests that inhibition of the mitochondrial Ca^{2+} uniporter is a possible mechanism for protecting myocytes from injury by Ca^{2+} overload. However, complete blockade of mitochondrial Ca^{2+} uptake and also the other effects of RuR on the SR Ca^{2+} release channels may increase $[Ca^{2+}]_i$! Furthermore, because $[Ca^{2+}]_m$ stimulates key enzymes for NADH regulation, the reduced $[Ca^{2+}]_m$ may inhibit oxidative phosphorylation. Although we did not find any difference in the increase of $[Ca^{2+}]_i$ (Table 1) or NADH fluorescence (Fig 4) in the present study, these possibilities may limit the effectiveness of this agent.

It has been reported that Dz opens the mitoK$_{ATP}$ channels and dissipates mitochondrial membrane potential, leading to a decreased driving force for the Ca^{2+} uniporter. In addition, Liu et al showed that Dz increased flavoprotein fluorescence in rabbit cardiomyocytes and they proposed that flavoprotein oxidation after the application of Dz was a consequence of depolarization the mitochondrial inner membrane![10] Therefore, both reduction of $[Ca^{2+}]_m$ and changes in the mitochondrial redox state could be involved in the protective mechanisms of Dz. In fact, the mitoK$_{ATP}$ channels have been considered to be a mediator of ischemic preconditioning, and the clinical use of mitoK$_{ATP}$ channel modulators, such as nicorandil and sarpogrelate, has had beneficial effects in patients with coronary artery disease![27,28]

In the present study Dz reduced $[Ca^{2+}]_m$ in a concentration-dependent manner in saponin-permeabilized myocytes and also significantly decreased the NADH fluorescence in intact cells. We and other researchers have shown that Dz does not depolarize the mitochondrial membrane potential![9,29] Hanley et al demonstrated that Dz inhibited succinate oxidation and succinate dehydrogenase activity, suggesting that a Dz-induced increase in flavoprotein fluorescence was caused by inhibition of succinate-supported respiration[29] We could not determine whether the activation of NADH oxidation by Dz was caused by depolarization of the mitochondrial inner membrane or just reflected compensation for inhibited succinate oxidation. Therefore, at the present time, the protective mechanisms of Dz are still unknown, and we also have to consider other mechanisms, such as reduction of ROS generation, inhibition of nucleotide degradation or induction of the mPTP (low-conductance mode)![9,30,31] On this issue, we previously reported a difference between ischemic preconditioning and pharmacological preconditioning in the cardioprotective mechanism of Dz![32]

The increase in $[Ca^{2+}]_m$ and the generation of ROS promote the opening of the mPTP. The irreversible opening of mPTP (high-conductance mode) causes dissipation of the mitochondrial membrane potential, matrix swelling and uncoupling of oxidative phosphorylation, and plays a key role in apoptosis by releasing cytochrome c![3,33] It is also known that in ischemia/reperfusion injury, some cells undergo apoptosis as opposed to necrosis and that the opening of the mPTP may act as the trigger for apoptosis. In this regard, inhibiting the irreversible opening of the mPTP would protect myocytes from various metabolic stresses. In fact, Javadov et al showed that the cardioprotection by ischemic preconditioning was associated with inhibition of the opening of the mPTP and stimulation of its closure![34]

In this study, the RuR- and Dz-induced protection may involve indirect inhibition of the opening of the mPTP through the reduction in $[Ca^{2+}]_m$ and/or the activation of NADH oxidation. CsA is a high-affinity inhibitor of mPTP at the concentration of 0.1–0.5 μmol/L, which inhibits cyclophilin D![35] After the increase in $[Ca^{2+}]_m$, the addition of CsA (0.1 μmol/L) transiently raised the $[Ca^{2+}]_m$, but the plateau level of $[Ca^{2+}]_m$ did not differ significantly from that before the addition. The reason for the transient increase in $[Ca^{2+}]_m$ is unknown, but may reflect a reduction of Ca^{2+} efflux from the mitochondria by inhibition of the low-conductance mode of the mPTP![19] Although a very recent report showed that CsA at relatively high concentrations (>1 μmol/L) decreased $[Ca^{2+}]_m$ by inhibiting the mitochondrial Ca^{2+} uniporter,[36] we could not find any decrease in $[Ca^{2+}]_m$ at 0.1–1 μmol/L of CsA. Because CsA did not reduce $[Ca^{2+}]_m$ after Ca^{2+} loading nor affect NADH oxidation, direct inhibition of the mPTP might be its protective mechanism against Ca^{2+} overload. The inhibition of the opening of the mPTP would prevent dissipation of the mitochondrial membrane potential and enable the mitochondria to keep producing ATP, thereby maintaining cellular Ca^{2+} homeostasis and/or repairing the damage to cellular components from the Ca^{2+} overload. In fact, several studies have shown that CsA prevents lethal cell injury in a variety of models including ischemia/reperfusion, oxidative stress, and Ca^{2+} overload![12,36,37] However, a specific caution should be made of the other unexpected effects of CsA on the mitochondrial membrane potential and some Ca^{2+} regulatory proteins. Indeed, reports have shown that inhibition of calcineurin, which is another target of CsA, increased Ca^{2+} transients by modifying Ca^{2+} currents, SR Ca^{2+} release channels and the NCX![25,38]

Conclusion

We have shown the effectiveness of RuR, Dz and CsA against Ca^{2+} paradox-induced Ca^{2+} overload. All these agents significantly prevented cell injury, but the mechanisms for their cardioprotective effects differ and include reducing $[Ca^{2+}]_m$ (by RuR and Dz), changes in the mitochondrial redox state (by Dz), and possibly preventing the opening of the mPTP (by CsA). Because cellular Ca^{2+} overload is responsible for irreversible myocardial damage in ischemia/reperfusion injury, the preservation of both $[Ca^{2+}]_m$ and the cellular energy status is essential in maintaining the cell's tolerance to metabolic stress. The combination of these agents that protect mitochondria a NCX blocker could augment the efficacy. To clarify these issues, more detailed investigations are necessary.

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