Mitochondrial ATP-Sensitive Potassium Channels Attenuate Matrix Ca\textsuperscript{2+} Overload During Simulated Ischemia and Reperfusion

Possible Mechanism of Cardioprotection

Mitsushige Murata, Masaharu Akao, Brian O’Rourke, Eduardo Marbán

Abstract—Mitochondrial ATP-sensitive potassium (mitoK\textsubscript{ATP}) channels play a key role in ischemic preconditioning of the heart. However, the mechanism of cardioprotection remains controversial. We measured rhod-2 fluorescence in adult rabbit ventricular cardiomyocytes as an index of mitochondrial matrix Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{m}), using time-lapse confocal microscopy. To simulate ischemia and reperfusion (I/R), cells were exposed to metabolic inhibition (50 minutes) followed by washout with control solution. Rhod-2 fluorescence gradually increased during simulated ischemia and rose even further with reperfusion. The mitoK\textsubscript{ATP} channel opener diazoxide attenuated the accumulation of [Ca\textsuperscript{2+}]\textsubscript{m} during simulated I/R (EC\textsubscript{50}=18 \textmu mol/L). These effects of diazoxide were blocked by the mitoK\textsubscript{ATP} channel antagonist 5-hydroxydecanoate (5HD). In contrast, inhibitors of the mitochondrial permeability transition (MPT), cyclosporin A and bongkrekic acid, did not alter [Ca\textsuperscript{2+}]\textsubscript{m} accumulation during ischemia, but markedly suppressed the surge in rhod-2 fluorescence during reperfusion. Measurements of mitochondrial membrane potential, \(\Delta \Psi_m\), in permeabilized myocytes revealed that diazoxide depolarized \(\Delta \Psi_m\) (by 12% at 10 \textmu mol/L, \(P<0.01\)) in a 5HD-inhibitable manner. Our data support the hypothesis that attenuation of mitochondrial Ca\textsuperscript{2+} overload, as a consequence of partial mitochondrial membrane depolarization by mitoK\textsubscript{ATP} channels, underlies cardioprotection. Furthermore, mitoK\textsubscript{ATP} channels and the MPT differentially affect mitochondrial calcium homeostasis: mitoK\textsubscript{ATP} channels suppress calcium accumulation during I/R, while the MPT comes into play only upon reperfusion. (Circ Res. 2001;89:891-898.)

Key Words: mitochondrial calcium overload ■ cardioprotection ■ ischemia

Ischemic preconditioning (IPC)\textsuperscript{1} is the endogenous mechanism whereby brief periods of ischemia paradoxically protect the myocardium against the damaging effects of subsequent prolonged ischemia. IPC exists in all species examined, including humans.\textsuperscript{2} Mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) channels feature prominently in the mechanism of cardioprotection\textsuperscript{3–9}; however, their precise role remains controversial. A decrease in the extent of mitochondrial Ca\textsuperscript{2+} overload during ischemia and reperfusion (I/R) has been proposed to prevent or delay cell death,\textsuperscript{10,11} and activation of the mitochondrial permeability transition (MPT)\textsuperscript{12} during ischemia and MPT contributing to mitochondrial Ca\textsuperscript{2+} homeostasis during reperfusion.

I/R and separately measured mitochondrial membrane potential in response to mitoK\textsubscript{ATP} channel mediators. Our findings reveal that activation of mitoK\textsubscript{ATP} channels suppresses mitochondrial Ca\textsuperscript{2+} overload during simulated I/R and depolarizes \(\Delta \Psi_m\), which might be cardioprotective against I/R injury. We have also discovered that the effects of mitoK\textsubscript{ATP} channels and the MPT on [Ca\textsuperscript{2+}]\textsubscript{m} are distinctly different, with mitoK\textsubscript{ATP} channel opener modulating mitochondrial calcium accumulation during ischemia and MPT contributing to mitochondrial Ca\textsuperscript{2+} homeostasis during reperfusion.

Materials and Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).

Materials

All of the chemicals were purchased from Sigma, unless otherwise stated. Diazoxide, pinacidil, cyclosporin A, rhod-2, and tetramethylrhodamine ethyl ester (TMRE) were dissolved in DMSO before added into experimental solutions. The final concentration of DMSO was <0.1%.

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891
Isolation of Adult Rabbit Ventricular Myocytes

Rabbit ventricular myocytes were isolated enzymatically from adult rabbit hearts as described previously. In brief, hearts were excised from anesthetized (30 mg/kg pentobarbital IV) New Zealand White rabbits (1 to 2 kg; Robinson Inc, Clemmons, NC) and mounted on a Langendorff apparatus. The heart was perfused with normal Tyrode solution composed of (mmol/L) NaCl 140, KCl 5, MgCl₂ 1, HEPES 10, CaCl₂ 1, and glucose 10. The perfusate was bubbled with 100% O₂ and maintained at 37°C. After 5 minutes of perfusion, hearts were perfused without Ca²⁺ for another 5 minutes, after which the perfusion solution was switched to one containing collagenase (1.0 mg/mL, Worthington type II) and protease (0.1 mg/mL, Sigma). The perfusion pressure was monitored, and the flow rate was adjusted to maintain perfusion pressure at ~75 mm Hg. After 10 minutes of collagenase and protease perfusion, hearts were perfused with Ca²⁺-free Tyrode solution for another 5 minutes, removed from the perfusion apparatus, and the atria were trimmed away. The ventricles were minced in solution composed of (mmol/L) K-glutamate 120, KCl 25, MgCl₂ 1, HEPES 10, EGTA 1, glucose 10 and were then filtered through nylon mesh and washed several times with M199 medium. Cells were stored in normal Tyrode solution before experiments.

Experimental Protocols for Measuring [Ca²⁺]ᵢ

Experiments were performed on myocytes isolated the same day. Myocytes were randomly assigned to one of four groups (Figure 1B). All experiments were performed at room temperature.

Group 1: control (C). Myocytes were incubated in control solution, which contained (mmol/L) NaCl 140, KCl 5, MgCl₂ 1, HEPES 10, CaCl₂ 2, and glucose 10 (pH 7.4) during the entire experimental period.

Group 2: metabolic inhibition (MI). Myocytes were incubated with MI solution, which contained (mmol/L) NaCl 140, KCl 15, MgCl₂ 1, HEPES 10, CaCl₂ 2, and NaCN 1, 2-deoxyglucose 20 (pH 6.5) for 50 minutes followed by 10 minutes of washout with control solution.

Group 3: metabolic inhibition plus diazoxide (MI+DZ). Myocytes were incubated with MI solution for 50 minutes followed by 10 minutes of washout with control solution. Diazoxide was included at various concentrations in external solution from 20 minutes before MI application to the end of MI.

Group 4: metabolic inhibition plus diazoxide plus 5HD (MI+DZ+5HD). Myocytes were incubated with MI solution for 50 minutes followed by 10 minutes of washout with control solution. Diazoxide and 5HD (500 μmol/L) were included in external solution from 20 minutes before MI application to the end of MI.

Confocal Microscopy

Single myocytes loaded with fluorescent probes were imaged with a laser-scanning confocal microscope (PCM-2000 + Diaphot 300; Nikon Inc). Probes were excited at 488 nm with an argon laser or at 543 nm with a helium-neon laser. Fluorescence emissions were separated by a 565-nm dichroic mirror into green (520±15 nm) and...
red (605±16 nm) emission signals. Experiments were analyzed using simple32 software (Compix Inc.).

For colocalization studies, MitoTracker Green was excited with the 488-nm line of an argon laser and emission monitored through a 520-nm (30-nm bandpass) barrier filter. Signal bleed-through of either probe was determined on cells loaded with only one probe and imaged using identical settings (gain, iris, and black level), concentrations, and incubation times to those used in colocalization studies.

**Time-Lapse Analysis of \([Ca^{2+}]_m\)**

To monitor \([Ca^{2+}]_m\), isolated ventricular cardiomyocytes were incubated with rhod-2-AM (Molecular Probes, 3 μmol/L).13,16 for at least 3 hours at room temperature. The \(Ca^{2+}\)-sensitive fluorescent indicator rhod-2 was excited at 543 nm, with emission monitored through a 605-nm (32-nm bandpass) barrier filter. At the beginning of each experiment, 1 to 5 cells with good staining conditions were selected per field. Confocal images (1024×1024 pixels) were acquired every 30 seconds. We evaluated \([Ca^{2+}]_m\) by drawing regions around individual cells, and red fluorescence (100 F/F₀, where F is the measured rhod-2 fluorescence, and F₀ is that at the beginning of the experiment).

**Time-Lapse Analysis of Mitochondrial Membrane Potential (Δ\(Ψ_m\))**

To monitor Δ\(Ψ_m\), isolated ventricular cardiomyocytes were incubated with a Δ\(Ψ_m\) indicator, tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, 100 nmol/L) for 30 minutes. Throughout the experiments, 100 nmol/L TMRE was included in the perfusion solution. In preliminary experiments on intact cells, we found no significant effects of diazoxide (100 μmol/L) on TMRE fluorescence (data not shown). However, small changes in the whole-cell fluorescence of intact cells may be obscured by redistribution of the TMRE from the mitochondria to the cytosolic compartment.17 Therefore, we used saponin-permeabilized cells to exclude this possibility. Moreover, the resultant direct access to the cytosol improved the kinetics of drug application while eliminating any confounding effects of an intact sarcolemma. Rabbit ventricular myocytes were first loaded with fluorescent dyes and then cells attached to coverslips were transferred to a bath filled with a solution containing (mmol/L) KCl 125, EGTA 0.5, MgCl₂ 1, HEPES 10, glutamate-K Ë- 5, malate-K Ë- 5, and ATP 1 (pH 7.2 with N-Me-D-glucamime). The plasma membrane was permeabilized by the addition of saponin (0.05 mg/mL). TMRE was excited at 543 nm with a helium-neon laser. Twenty cells were randomly selected in each scan by drawing regions around individual cells, and red fluorescence intensity was sequentially monitored every 90 seconds, using ×20 objective lens. We assessed Δ\(Ψ_m\) by calculating relative TMRE fluorescence (100 F/F₀, where F is the measured TMRE fluorescence, and F₀ is that at the beginning of the experiment).

**Data Analysis**

All quantitative data are presented as mean±SEM, and the number of cells or experiments is shown as n. Statistical analysis was performed using one-way ANOVA with Fisher’s least-significant difference as the post hoc test. A level of \(P<0.05\) was accepted as statistically significant.

**Results**

Adult rabbit ventricular myocytes loaded with rhod-2 exhibited a regular, banded fluorescence pattern, typical of the cardiac mitochondrial distribution, which enabled us to monitor \([Ca^{2+}]_m\). Figure 1A compares the intracellular distribution of rhod-2 with that of the mitochondrial-specific probe MitoTracker Green, a molecule that covalently binds to the inner mitochondrial membrane and fluoresces independently of Δ\(Ψ_m\) and \([Ca^{2+}]_m\). Figure 1A shows the localization of rhod-2 (Figure 1A-a) and MitoTracker Green (Figure 1A-b); colocalization was confirmed by merging the two images (Figure 1A-c).

To simulate ischemia/reperfusion (I/R), we exposed cells to metabolic inhibition followed by washout with control solution (MI/W, described in Materials and Methods). Figure 1C shows representative time courses of rhod-2 fluorescence from individual cells under control conditions and in the standard metabolic inhibition protocol. Rhod-2 fluorescence remained steady in the control myocyte. In contrast, approximately 20 minutes after the onset of MI, rhod-2 fluorescence started to increase, followed by a gradual steady rise as MI was continued. The increase of relative rhod-2 fluorescence at 50 minutes after exposure to MI was 40% in this cell. After reperfusion, the fluorescence fell transiently and then showed a rapid overshoot followed by a partial recovery. The maximum relative rhod-2 fluorescence (overshoot) during reperfusion (10 minutes) was 73%.

We tested the effect of diazoxide, a mitoK\(_{ATP}\) channel opener, on the mitochondrial calcium accumulation during simulated I/R. Figure 2A shows the effect of diazoxide on rhod-2 fluorescence. In the MI group, relative rhod-2 fluorescence increased to a peak value of 40.7±7.3% (n=14) at the end of MI, whereas in the MI+DZ group, the rise was blunted (25.8±3.5%, n=14; \(P<0.05\)). Moreover, diazoxide inhibited the rhod-2 fluorescence after washout (10 minutes) compared with no drug (43.1±4.9% increase over basal level, n=14 versus 78.6±9.0% with no drug, n=14; \(P<0.05\)).
Next, we investigated whether the mitoK$_{ATP}$ channel inhibitor, 5-hydroxydecanoate (5HD) could block the effect of diazoxide on rhod-2 fluorescence. The attenuation of mitochondrial calcium accumulation by diazoxide during MI was completely blocked by 5HD ($37.9\pm7.8\%$, $n=10$; $P=NS$ versus $40.7\pm7.3\%$ with MI group, $n=14$). These results indicate that mitoK$_{ATP}$ channels inhibit mitochondrial calcium accumulation during simulated I/R. The EC$_{50}$ for inhibition of mitochondrial calcium accumulation during simulated ischemia was $18.0\,\mu$mol/L, as shown in Figure 2B. There was no obvious difference in the time courses of mitochondrial calcium accumulation in the MI and MI+DZ groups (data not shown).

Cyclosporin A (CsA) is an inhibitor of the MPT. To examine the role of the MPT on mitochondrial calcium accumulation during simulated I/R, we incubated cells with CsA (0.2 $\mu$mol/L) using the same drug application protocol as for diazoxide (see Materials and Methods). Figure 3A shows the effect of CsA on relative rhod-2 fluorescence. There was no significant difference between the increase of relative rhod-2 fluorescence in the absence of CsA and that in the presence of CsA during MI ($41.7\pm4.7\%$, $n=9$; $P=NS$ versus $40.7\pm7.3\%$ with MI group, $n=14$). On the other hand, CsA completely suppressed the overshoot of rhod-2 fluorescence after washout ($52.8\pm4.8\%$ versus $78.6\pm9.0\%$ with no drug; $P<0.05$). The differences are readily apparent in cell-by-cell plots of the percentage increase of rhod-2 fluorescence after washout relative to the values at the end of MI (Figure 3B). Pooled data in Figure 3C reveal that CsA significantly inhibited the reflow-associated increase in rhod-2 fluorescence ($1.3\pm0.1$, $n=9$ versus $2.3\pm0.5$ with no drug, $n=14$; $1.8\pm0.1$ with diazoxide, $n=14$; $P=0.05$). To confirm the involvement of the MPT in mitochondrial calcium homeostasis, we examined the effects of another MPT inhibitor, bongkrekic acid (BA, Calbiochem, 50 $\mu$mol/L), during simulated I/R. The results reproduced those with CsA: there was no significant difference between the increase of relative rhod-2 fluorescence in the absence of BA and that in the presence of BA during MI ($43.5\pm3.0\%$, $n=9$; $P=NS$ versus $40.7\pm7.3\%$ with MI group, $n=14$), but BA suppressed the overshoot of rhod-2 fluorescence after washout ($50.1\pm2.8\%$ versus $78.6\pm9.0\%$ with no drug; $P<0.05$). These results suggest that the overshoot of rhod-2 fluo-
mechanism whereby diazoxide may limit mitochondrial Ca\(^{2+}\)/H9004/H9023 matrix calcium.19,20 This apparent paradox was investigated the idea that the MPT would serve to release accumulated reperfusion was suppressed by CsA was incompatible with independent of MPT.

MI/H11001 plus bongkrekic acid (50
/H9262 mol/L) plus cyclosporin A (0.2
/H11001 mol/L); MI
/H11001 CsA, metabolic inhibition plus cyclosporin A (0.2
/H9262 mol/L); MI
/H11001 BA, metabolic inhibition plus bongkrekic acid (50
/H9262 mol/L); and MI
/H11001 PINA, metabolic inhibition plus pinacidil (100
/H9262 mol/L). P<0.05 vs MI.

Figure 4. Summarized data for the increase of relative rhod-2 fluorescence at the end of metabolic inhibition and after reperfusion, respectively. MI indicates metabolic inhibition; MI+DZ, metabolic inhibition plus diazoxide (100
/H9262 mol/L); MI+DZ+5HD, metabolic inhibition plus diazoxide (100
/H9262 mol/L) plus 5HD (500
/H9262 mol/L); MI+CsA, metabolic inhibition plus cyclosporin A (0.2
/H9262 mol/L); MI+BA, metabolic inhibition plus bongkrekic acid (50
/H9262 mol/L); and MI+PINA, metabolic inhibition plus pinacidil (100
/H9262 mol/L). P<0.05 vs MI.

cence after reperfusion is mediated by MPT, while the gradual accumulation of mitochondrial Ca\(^{2+}\) during MI is independent of MPT.

The observation that the increase in rhod-2 fluorescence on reperfusion was suppressed by CsA was incompatible with the idea that the MPT would serve to release accumulated matrix calcium.19,20 This apparent paradox was investigated further by comparing images taken just after reperfusion in the presence and absence of CsA. As shown in Figure 3D, the pattern of rhod-2 distribution remained localized to mitochondria in the presence of CsA; however, without CsA, rhod-2 partially redistributed to the cytoplasm, as evidenced by the diffuse pattern of fluorescence after reperfusion. Thus, the increased whole-cell signal on reperfusion was likely due to Ca\(^{2+}\) overload and binding to rhod-2 in the cytoplasm. Inhibition of MPT opening preserves mitochondrial inner membrane integrity and prevents the subcellular redistribution of rhod-2 on reperfusion. These data further support the conclusion that the MPT opens during reperfusion, but not during the simulated ischemia.

Diazoxide, at high concentrations, has been reported to have actions independent of mitoK\(_{\text{ATP}}\) channel opening, including an inhibition of mitochondrial respiration that cannot be blocked by K\(_{\text{ATP}}\) channel antagonists.21 To confirm the effect of mitoK\(_{\text{ATP}}\) channel opening on mitochondrial calcium accumulation, we also used pinacidil, a chemically unrelated mitoK\(_{\text{ATP}}\) channel opener. As summarized in Figure 4, 100
/H9262 mol/L pinacidil similarly attenuated the mitochondrial calcium accumulation, not only during MI (22.3±5.2%, n=12 versus 40.7±7.3% with no drug, n=14, P<0.05) but also after washout (41.3±7.4% versus 78.6±9.0% with no drug; P<0.05).

A greater depolarization of the mitochondrial membrane potential (ΔΨ\(_{\text{m}}\)) during ischemia has been hypothesized as a mechanism whereby diazoxide may limit mitochondrial Ca\(^{2+}\) overload.4,19 To test whether diazoxide could directly depolarize ΔΨ\(_{\text{m}}\), we examined the concentration-dependent effects of diazoxide on ΔΨ\(_{\text{m}}\) using TMRE. In intact cells, spatial inhomogeneities of a mitochondrial response and redistribution of fluorescent dyes among subcellular compartments often obscure small changes in signal when whole-cell average intensity is measured. Therefore, for these experiments we used saponin-permeabilized myocytes to disrupt the sarcolemmal membrane. Figure 5A shows representative time courses of ΔΨ\(_{\text{m}}\) at various concentrations of diazoxide, indicating that diazoxide decreased the TMRE fluorescence in permeabilized cells (eg, at 10 μmol/L diazoxide, 88.2±1.6% versus control 101.9±2.4%; P<0.05, both quantified at 15 minutes). These effects were reproducible even when 1 mmol/L phosphate was added to the intracellular bathing solution (data not shown).

In addition, we find that the effects of diazoxide in permeabilized cells are antagonized by the mitoK\(_{\text{ATP}}\) channel blocker 5HD (500 μmol/L), as shown in Figure 5B (88.2±1.6%; 10 μmol/L diazoxide versus 97.5±3.4%; 10 μmol/L diazoxide+5HD; P<0.05, both quantified at 15 minutes). On the other hand, 500 μmol/L 5HD could not block the effect of 100 μmol/L diazoxide (80.5±4.1%; 100 μmol/L diazoxide versus 81.5±2.3%; 100 μmol/L diazoxide+5HD; P=NS, both quantified at 15 minutes), suggesting that this concentration may be having nonspecific effects in the permeabilized myocyte preparation. The pooled data in Figure 5C demonstrate the consistency of the findings. To “calibrate” the effect of diazoxide, we determined the effect of 2,4-dinitrophenol (DNP), a potent uncoupler of mitochondrial respiration, on TMRE fluorescence. Figure 5D compares the dose-response inhibitory effects of DNP and diazoxide on TMRE fluorescence, indicating that the effect of 10 μmol/L diazoxide on ΔΨ\(_{\text{m}}\) corresponds to that of less than 1 μmol/L DNP. Thus, while diazoxide depolarizes the inner mitochondrial membrane, its effects are much more modest than those of a potent uncoupling agent.

Discussion

In this study, we have demonstrated the following: (1) The mitoK\(_{\text{ATP}}\) channel opener diazoxide attenuates the accumulation of mitochondrial Ca\(^{2+}\) during simulated I/R in a concentration-dependent manner. (2) The mitoK\(_{\text{ATP}}\) channel blocker 5HD abolishes the effects of diazoxide. (3) CsA and BA, but not diazoxide, selectively inhibit the overshoot of rhod-2 fluorescence after reperfusion, principally due to suppression of redistribution of the dye into the cytoplasm. (4) Diazoxide depolarizes ΔΨ\(_{\text{m}}\) at cardioprotective concentrations in permeabilized cells.

Role of mitoK\(_{\text{ATP}}\) Channels in Cardioprotection

The cardioprotective effects of IPC are mimicked by agonists of mitoK\(_{\text{ATP}}\) channels and abolished by antagonists of mitoK\(_{\text{ATP}}\) channels, implying that mitoK\(_{\text{ATP}}\) channels play an important role in IPC.4,22,23 We hypothesize that mitoK\(_{\text{ATP}}\) channel opening partially depolarizes ΔΨ\(_{\text{m}}\) and might attenuate mitochondrial calcium accumulation during ischemia by decreasing the driving force for Ca\(^{2+}\) uptake.4 In agreement with our hypothesis, Holmuhamedov et al19 have also reported that diazoxide causes a decrease of ΔΨ\(_{\text{m}}\) and blunts mitochondrial calcium accumulation in isolated mitochondria.
and intact cells. Diazoxide also depolarizes mitochondrial membrane potential in rat permeabilized hippocampal homogenates. Importantly, we have confirmed that the effect of diazoxide on mitochondrial Ca\(^{2+}\) exposed to simulated ischemia can be inhibited by 5HD, arguing against an effect mediated by nonspecific inhibition of the respiratory chain.

While the diazoxide-induced changes in \(\Delta \Psi_m\) are modest relative to the associated blunting of mitochondrial calcium overload, mathematical modeling of mitochondrial ion homeostasis reveals a nonlinear dependence of [Ca\(^{2+}\)]\(_m\) on \(\Delta \Psi_m\), suggesting that even small changes in \(\Delta \Psi_m\) may have large effects on Ca\(^{2+}\) accumulation during ischemia (S. Cortassa, M. Aon, E. Marbán, R. Winslow, and B. O’Rourke, unpublished data, 2001). On the other hand, Kowaltowski et al have demonstrated in isolated mitochondria that the effects of diazoxide on \(\Delta \Psi_m\) and Ca\(^{2+}\) uptake are caused not by mitoK\(_{ATP}\) channel opening but by the intrinsic uncoupling and inhibitory properties of this compound when used at concentrations in excess of those required to open mitoK\(_{ATP}\) channels (>100 \(\mu\)mol/L). In contrast, our data indicate that low concentrations of diazoxide, comparable to those that have been considered to be cardioprotective, partially depolarize the mitochondrial inner membrane in a 5HD-inhibitable manner, providing direct evidence for mitoK\(_{ATP}\) channel activation. However, we also observed that higher concentrations of diazoxide lead to further mitochondrial depolarization in a 5HD-independent manner, implying that the effects of a supratherapeutic dose might be mediated by “toxic” properties of diazoxide. The finding that the effects of 100 \(\mu\)mol/L diazoxide could be blocked by 5HD in intact cells, but not in permeabilized myocytes, can be rationalized by the difference in accessibility of the drug to the intracellular active site. Diazoxide is a weak acid that is negatively charged under physiological conditions; the negative electrical potential across the sarcolemma favors anion exclusion, so that any given concentration applied directly to the mitochondria is likely to be much higher than that attained when diazoxide is added to intact cells.

Since the measurement of the diazoxide-induced change in \(\Delta \Psi_m\) was performed in permeabilized myocytes due to limitations in the methodology for measuring small differences in \(\Delta \Psi_m\) in intact cells during simulated ischemia (see Results), we could not directly link the effects of diazoxide on matrix Ca\(^{2+}\) to changes in \(\Delta \Psi_m\); however, the results clearly demonstrate the plausibility of the hypothesis. The greater
challenge of making the connection to the specific mechanism of protection also awaits further investigation.

Recently, Wang et al.27 reported that pretreatment with diazoxide mimics either IPC or its effects on mitochondrial Ca\(^{2+}\) overload during I/R in perfused rat hearts. Ylitalo et al.28 reported that the decrease in mitochondrial membrane potential during prolonged ischemia was faster in the preconditioned heart, with no difference during reperfusion. Terzic and his colleagues reported that inhibition of oxidative phosphorylation by calcium overload was restored by diazoxide, contributing to the cardioprotective effects of this drug in I/R injury.29,30 These reports support our hypothesis, despite fundamental differences in methodology.

Mitochondrial Ca\(^{2+}\) Overload During Simulated I/R: Involvement of the MPT

In the present study, we applied chemical hypoxia (metabolic inhibition) to the cells to simulate I/R. As in previous reports,31,32 mitochondrial calcium accumulation could be observed during simulated I/R. After reperfusion, rhod-2 fluorescence further increased, producing an overshoot that was blocked by the MPT inhibitors CsA and BA. However, the MPT is a large pore, potentially enabling Ca\(^{2+}\) release from mitochondria and a resultant decrease in [Ca\(^{2+}\)]\(_{in}\). As shown in Figure 3D, CsA inhibited the diffusion of rhod-2 from mitochondria to cytosol, implying that rhod-2 translocates from mitochondria to the cytosol through MPT upon reperfusion. It has been argued that MPT may be responsible for reperfusion injury,33,34 and that mitochondrial Ca\(^{2+}\) overload after reperfusion activates MPT opening. In this sense, these concepts are supported by our finding that CsA and BA completely suppressed the surge of rhod-2 fluorescence after reperfusion, whereas it did not alter [Ca\(^{2+}\)]\(_{in}\) during ischemia. On the other hand, diazoxide did not selectively block the surge in rhod-2 fluorescence after reperfusion, indicating that it does not directly block MPT. Recent studies revealed that apoptosis contributes to ischemic injury in the heart,35,36 and that diazoxide suppresses apoptosis induced by oxidative stress.37 Taken together, we propose that diazoxide may be cardioprotective against ischemic injury by preventing apoptosis in addition to necrosis, as a consequence of attenuation of mitochondrial calcium accumulation and inhibition of the opening of MPT. However, further studies are required to evaluate the full ramifications of this hypothesis.

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