Opening of Mitochondrial K\textsubscript{ATP} Channels Attenuates the Ouabain-Induced Calcium Overload in Mitochondria

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We tested whether opening of mitochondrial ATP-sensitive K\textsuperscript{+} (mitoK\textsubscript{ATP}) channels depolarizes mitochondrial membrane potential (\(\Delta\Psi\text{m}\)) and thereby prevents the mitochondrial Ca\textsuperscript{2+} overload. With the use of a Nipkow disk confocal system, the mitochondrial Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{m}) and \(\Delta\Psi\text{m}\) in rat ventricular myocytes were measured by loading cells with Rhod-2 and JC-1, respectively. Exposure to ouabain (1 mmol/L) for 30 minutes produced mitochondrial Ca\textsuperscript{2+} overload, and the intensity of Rhod-2 fluorescence significantly increased to 173±16% of baseline (\(P<0.001\)). Treatment of myocytes with the mitoK\textsubscript{ATP} channel opener diazoxide (100 \(\mu\text{mol/L}\)) blunted the ouabain-induced mitochondrial Ca\textsuperscript{2+} overload (131±10% of baseline; \(P<0.001\) versus ouabain). Moreover, diazoxide significantly depolarized \(\Delta\Psi\text{m}\) and reduced the intensity of JC-1 fluorescence during application of ouabain to 89±2% of baseline (\(P<0.05\)). These effects of diazoxide were blocked by the mitoK\textsubscript{ATP} channel blocker 5-hydroxydecanoate (500 \(\mu\text{mol/L}\)). These results indicate that opening of mitoK\textsubscript{ATP} channels prevents a mitochondrial Ca\textsuperscript{2+} overload in association with \(\Delta\Psi\text{m}\) depolarization and thereby protects myocardium against ischemic damage.

Mitochondrial ATP-sensitive K\textsuperscript{+} (mitoK\textsubscript{ATP}) channels are thought to play a key role in cardioprotection,\textsuperscript{1} but the crucial question remains as to why the opening of mitoK\textsubscript{ATP} channels can be so protective. Liu et al\textsuperscript{2} originally hypothesized that the K\textsuperscript{+} entry through mitoK\textsubscript{ATP} channels depolarizes the \(\Delta\Psi\text{m}\), which reduces the driving force for Ca\textsuperscript{2+} influx and, hence, results in the prevention of mitochondrial Ca\textsuperscript{2+} overload. Later on, Holmuhamedov et al\textsuperscript{3} demonstrated that diazoxide reduced the [Ca\textsuperscript{2+}]\textsubscript{m} in isolated cardiac mitochondria and neonatal cardiomyocytes. In contrast, Kowaltowski et al\textsuperscript{4} have shown that diazoxide has minimal effects on [Ca\textsuperscript{2+}]\textsubscript{m} and \(\Delta\Psi\text{m}\). Thus, investigations addressing the original hypothesis have yielded conflicting results. The present study therefore was aimed to carefully determine, using adult rat ventricular myocytes, whether opening of mitoK\textsubscript{ATP} channels depolarizes \(\Delta\Psi\text{m}\) and attenuates mitochondrial Ca\textsuperscript{2+} overload.

Materials and Methods

Cell Preparation

Adult rat ventricular myocytes were isolated by collagenase digestion, as previously described.\textsuperscript{5} Once isolated, the cells were resuspended in a culture medium composed of 5% fetal calf serum, 47.5% M199, and 47.5% modified Tyrode’s solution containing (mmol/L) NaCl 137, KCl 5.4, MgCl\textsubscript{2} 1, HEPES 5, dextrose 22, taurine 20, creatine 5, and sodium pyruvate 5 (pH 7.4) at room temperature until use.

Confocal Fluorescence Imaging of [Ca\textsuperscript{2+}]\textsubscript{m} and \(\Delta\Psi\text{m}\)

The Ca\textsuperscript{2+} fluorophore Rhod-2 was used to measure changes of [Ca\textsuperscript{2+}]\textsubscript{m}. Myocytes were loaded with 10 \(\mu\text{mol/L}\) Rhod-2 acetoxymethyl ester (Molecular Probes) for 120 minutes at 4°C and then incubated for 30 minutes at 37°C in the culture medium. This two-step cold loading/warm incubation protocol achieves exclusive loading of Rhod-2 into the mitochondria.\textsuperscript{6} The \(\Delta\Psi\text{m}\) was monitored with a fluorescent probe, JC-1 (Molecular Probes). Myocytes were incubated with 0.5 \(\mu\text{mol/L}\) JC-1 for 10 minutes at 37°C. We verified that the subcellular distribution of fluorescence arising from Rhod-2 and JC-1 was virtually identical to that observed by loading myocytes with the mitochondrial dye rhodamine-123.

Myocytes loaded with Rhod-2 and JC-1 were perfused with a HEPES-buffered physiological solution (37°C) containing (mmol/L) NaCl 123, KCl 5, CaCl\textsubscript{2} 2.7, MgCl\textsubscript{2} 1, HEPES 5, and glucose 5.5 (pH 7.4) and imaged with a Nipkow disk confocal system (CSU10, Yokogawa, Japan), as previously described.\textsuperscript{5} Rhod-2 was excited at 488 nm by an argon ion laser, with emission collected above 515 nm through a long-pass barrier filter. JC-1 was excited at 488 nm and the red emission fluorescence was detected using a long-pass filter of 580 nm. The emission light was imaged through a relay lens to an intensified CCD camera. Images were recorded on a computer (Macintosh 8500/120) at video rate and analyzed with NIH Image 1.62f software.

Statistical Analysis

Data are expressed as mean±SEM. Intergroup comparisons are made by Student’s t test for two groups and by ANOVA followed by Tukey’s test for multiple groups. A value of \(P<0.05\) was regarded as significant.

Results

Confocal images of rat ventricular myocytes loaded with Rhod-2, obtained before and after application of diazoxide, are shown in Figure 1A. The resting [Ca\textsuperscript{2+}]\textsubscript{m} in intact cells was low as revealed by the dim baseline signal of Rhod-2 fluorescence (Figure 1A, a). Subsequent exposure to diazoxide (100 \(\mu\text{mol/L}\)) did not affect the [Ca\textsuperscript{2+}]\textsubscript{m} and the Rhod-2 fluorescence remained unchanged (Figure 1A, b). However, we occasionally observed the cells with high intensity of Rhod-2 fluorescence (Figure 1A, c; shown in pink), which might have damage caused by the isolation procedure. In such cells with mitochondrial Ca\textsuperscript{2+} overload, exposure to diazoxide dramatically decreased the intensity of Rhod-2 fluorescence (Figure 1A, d). As summarized in Figure 1B, diazoxide significantly decreased the [Ca\textsuperscript{2+}]\textsubscript{m} to 41±9% of baseline.
(P<0.05) only when the resting [Ca\textsuperscript{2+}]\textsubscript{m} was high. Here, the intensity of Rhod-2 fluorescence of baseline in Ca\textsuperscript{2+}-overloaded cells was 3-fold greater than that of the intact cells (Figure 1B, inset). The effect of diazoxide was antagonized by 5-hydroxydecanoate (500 \textmu mol/L).

In the next series of experiments, cells were exposed to the Na\textsuperscript{+}/K\textsuperscript{−}-ATPase inhibitor ouabain in an attempt to produce mitochondrial Ca\textsuperscript{2+} overload. Treatment of myocytes with 1 mmol/L ouabain increased the intensity of Rhod-2 fluorescence, suggesting that Ca\textsuperscript{2+} overload in mitochondria was evoked (Figure 1C, a and b). Treatment with diazoxide (100 \textmu mol/L) attenuated the mitochondrial Ca\textsuperscript{2+} overload during exposure to ouabain (Figure 1C, c and d). As summarized in Figure 1D, Rhod-2 fluorescence after 15 minutes and 30 minutes of exposure to ouabain significantly increased to 130\% and 173\% of baseline (P<0.001), respectively. Diazoxide significantly attenuated the ouabain-induced mitochondrial Ca\textsuperscript{2+} overload (116\% at 15 minutes, 131\% at 30 minutes; P<0.001), and the effect was antagonized by 5-hydroxydecanoate (500 \textmu mol/L). 5-Hydroxydecanoate alone slightly increased the Rhod-2 fluorescence (123\% at 30 minutes, n=4; P=NS versus control), so that the Rhod-2 fluorescence in the ouabain+diazoxide+5-hydroxydecanoate group was insignificantly higher than that of the ouabain group. Like diazoxide, nicorandil (100 \textmu mol/L), a potent mitoK\textsubscript{ATP} channel opener, significantly reduced the ouabain-induced Ca\textsuperscript{2+} overload (see Figure in the online data supplement, available at http://www.circresaha.org). These results indicate that opening of mitoK\textsubscript{ATP} channels attenuates the ouabain-induced Ca\textsuperscript{2+} overload in mitochondria.

We then examined whether the protective effect of diazoxide on the ouabain-induced Ca\textsuperscript{2+} overload is associated with the depolarization of ΔΨ\textsubscript{m}. Diazoxide (100 \textmu mol/L) alone reduced the intensity of JC-1 fluorescence to 93\% of baseline (n=9), but this change was not statistically significant. In the experiments shown in Figure 2A, although ouabain alone did not affect the JC-1 fluorescence (Figure 2A, a and b), diazoxide apparently reduced the intensity of JC-1 fluorescence during application of ouabain (Figure 2A, c and d). As summarized in Figure 2B, diazoxide signifi-
cantly reduced the intensity of JC-1 fluorescence to 89±2% of baseline (P<0.05), and the effect was antagonized by 5-hydroxydecanoate (500 μmol/L). These results suggest that diazoxide attenuates the mitochondrial Ca\(^{2+}\) overload in association with the depolarization of \(\Delta \Psi_m\).

### Discussion

Among putative mechanisms of cardioprotection,\(^1\) the hypothesis of mitochondrial Ca\(^{2+}\) handling seems to be plausible. Indeed, Holmuhamedov et al\(^3\) showed that diazoxide inhibited the Ca\(^{2+}\) uptake and depolarized \(\Delta \Psi_m\) in isolated cardiac mitochondria. Their results, however, raise methodological criticism that mitoK\(_{ATP}\) channels would be already open because of the ATP-free assay condition.\(^9\) Conflicting observations in isolated cardiac mitochondria have been reported, showing that diazoxide has little effect on Ca\(^{2+}\) uptake and \(\Delta \Psi_m\).\(^4\) Thus, the results so far obtained from isolated mitochondria remain inconclusive.

The present results demonstrate that diazoxide does not affect the basal [Ca\(^{2+}\)]\(_{im}\) in intact rat ventricular myocytes, which is inconsistent with previous findings observed in neonatal rat cardiomyocytes.\(^3\) Although such disparity may stem from the difference in cell preparations used, our results suggest that the mitoK\(_{ATP}\) channel does not play a significant role in regulating [Ca\(^{2+}\)]\(_{im}\) under normal conditions. However, it should be noted that diazoxide could decrease [Ca\(^{2+}\)]\(_{im}\) when the resting mitochondrial Ca\(^{2+}\) level was raised. In this study, we evoked mitochondrial Ca\(^{2+}\) overload experimentally by exposure to ouabain. Elevation in cytosolic Ca\(^{2+}\) concentration occurs during ischemia, and it eventually results in mitochondrial Ca\(^{2+}\) accumulation.\(^10\) More recently, functional interaction between mitoK\(_{ATP}\) channels and Na\(^+\)/K\(^+\)-ATPase in preconditioned rat hearts has been reported.\(^11\) Accordingly, our results obtained in the presence of ouabain are of interest and notable. The salient finding is that diazoxide could prevent the ouabain-induced Ca\(^{2+}\) overload in mitochondria. Also, nicorandil produced similar effects to diazoxide. Moreover, 5-hydroxydecanoate abolished these effects of diazoxide and nicorandil. Although the pathways for Ca\(^{2+}\) uptake and efflux need to be defined, these results indicate that opening of mitoK\(_{ATP}\) channels attenuates mitochondrial Ca\(^{2+}\) overload in adult rat ventricular myocytes.

It is debatable whether K\(^{+}\) influx through mitoK\(_{ATP}\) channels can depolarize \(\Delta \Psi_m\). Holmuhamedov et al\(^3\) showed that diazoxide depolarized \(\Delta \Psi_m\) by \(\sim 15\) mV, whereas Kowaltowski et al\(^4\) observed only a 1- to 2-mV depolarization of \(\Delta \Psi_m\) in isolated mitochondria. The present study has demonstrated that diazoxide can depolarize \(\Delta \Psi_m\) and notably the degree of depolarization is augmented in the presence of ouabain. These results suggest that the effect of diazoxide on \(\Delta \Psi_m\) seems to depend on Ca\(^{2+}\) concentration. Such Ca\(^{2+}\)-dependent effect of diazoxide has been demonstrated for protection against Ca\(^{2+}\) paradox injury in rat myocardium.\(^12\) Although the Ca\(^{2+}\)-dependent mechanism has not been defined, Ca\(^{2+}\)-mediated signaling cascades including protein kinase C might augment the opening of mitoK\(_{ATP}\) channels by diazoxide. We further found that the reduction of \(\Delta \Psi_m\) by \(\sim 10\%\) resulted in >50% reduction of [Ca\(^{2+}\)]\(_{im}\) after 30 minutes of exposure to ouabain. Because membrane-impermeable Rhod-2 free acids are not excluded from the depolarized mitochondria,\(^6\) the reduction of Rhod-2 fluorescence cannot be ascribed to exclusion of dye from mitochondria, but reflects the reduction of [Ca\(^{2+}\)]\(_{im}\). Therefore, our present results suggest that even small changes in the electrochemical driving force could potentially lead to large differences in total mitochondrial Ca\(^{2+}\) accumulation over long periods of time.

In conclusion, we have shown convincing evidence that opening of mitoK\(_{ATP}\) channels attenuates the Ca\(^{2+}\) overload in mitochondria, a phenomenon associated with the depolarization of \(\Delta \Psi_m\). These results support the original hypothesis and provide insight into the mechanism by which the opening of mitoK\(_{ATP}\) channels confers cardioprotection.

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### References


**Key Words**: mitochondria | calcium | K\(_{ATP}\) channel | cardioprotection
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Effect of Nicorandil on Ouabain-Induced Ca\(^{2+}\) Overload in Mitochondria

Figure shows summarized data for the time course of changes in Rhod-2 fluorescence. Nicorandil (NICO, 100 μmol/L) significantly attenuated the mitochondrial Ca\(^{2+}\) overload induced by exposure to ouabain (OUAB, 1 mmol/L). This effect of nicorandil was antagonized by 5-hydroxydecanoate (5HD, 500 μmol/L). *P<0.001 versus baseline; #P<0.001 versus OUAB; †P<0.001 versus OUAB+NICO.