Opening of Mitochondrial $K_{ATP}$ Channels Attenuates the Ouabain-Induced Calcium Overload in Mitochondria

Hideyuki Ishida, Yuki Hirota, Chokoh Genka, Hiroe Nakazawa, Haruaki Nakaya, Toshiaki Sato

We tested whether opening of mitochondrial ATP-sensitive $K^+$ (mito$K_{ATP}$) channels depolarizes mitochondrial membrane potential ($\Delta \Psi_m$) and thereby prevents the mitochondrial Ca$^{2+}$ overload. With the use of a Nipkow disk confocal system, the mitochondrial Ca$^{2+}$ concentration ([Ca$^{2+}]_m$) and $\Delta \Psi_m$ 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$^{2+}$ overload, and the intensity of Rhod-2 fluorescence significantly increased to 173±16% of baseline ($P<0.001$). Treatment of myocytes with the mito$K_{ATP}$ channel opener diazoxide (100 μmol/L) blunted the ouabain-induced mitochondrial Ca$^{2+}$ overload (131±10% of baseline; $P<0.001$ versus ouabain). Moreover, diazoxide significantly depolarized the $\Delta \Psi_m$ 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 mito$K_{ATP}$ channel blocker 5-hydroxydecanoate (500 μmol/L). These results indicate that opening of mito$K_{ATP}$ channels prevents a mitochondrial Ca$^{2+}$ overload in association with $\Delta \Psi_m$ depolarization and thereby protects myocardium against ischemic damage.

Mitochondrial ATP-sensitive $K^+$ (mito$K_{ATP}$) channels are thought to play a key role in cardioprotection, but the crucial question remains as to why the opening of mito$K_{ATP}$ channels can be so protective. Liu et al originally hypothesized that the K$^+$ entry through mito$K_{ATP}$ channels depolarizes the $\Delta \Psi_m$, which reduces the driving force for Ca$^{2+}$ influx and, hence, results in the prevention of mitochondrial Ca$^{2+}$ overload. Later on, Holmuhamedov et al demonstrated that diazoxide reduced the [Ca$^{2+}]_m$ in isolated cardiac mitochondria and neonatal cardiomyocytes. In contrast, Kowaltowski et al have shown that diazoxide has minimal effects on [Ca$^{2+}]_m$ and $\Delta \Psi_m$. 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 mito$K_{ATP}$ channels depolarizes $\Delta \Psi_m$ and attenuates mitochondrial Ca$^{2+}$ overload.

Materials and Methods

Cell Preparation

Adult rat ventricular myocytes were isolated by collagenase digestion, as previously described. 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, MgCl2 1, HEPES 5, and glucose 22, taurine 20, creatine 5, and sodium pyruvate 5 (pH 7.4) at room temperature until use.

Confocal Fluorescence Imaging of [Ca$^{2+}]_m$ and $\Delta \Psi_m$

The Ca$^{2+}$ fluorophore Rhod-2 was used to measure changes of [Ca$^{2+}]_m$. Myocytes were loaded with 10 μmol/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. The $\Delta \Psi_m$ was monitored with a fluorescent probe, JC-1 (Molecular Probes). Myocytes were incubated with 0.5 μmol/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, CaCl2 2.7, MgCl2 1, HEPES 5, and glucose 5.5 (pH 7.4) and imaged with a Nipkow disk confocal system (CSU10, Yokogawa, Japan), as previously described. 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$^{2+}]_m$ in intact cells was low as revealed by the dim baseline signal of Rhod-2 fluorescence (Figure 1A, a). Subsequent exposure to diazoxide (100 μmol/L) did not affect the [Ca$^{2+}]_m$, 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$^{2+}$ 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$^{2+}]_m$ to 41±9% of baseline.
(P<0.05) only when the resting [Ca\(^{2+}\)]\(_{\text{m}}\) was high. Here, the intensity of Rhod-2 fluorescence of baseline in Ca\(^{2+}\)-overloaded cells was \(\approx 3\)-fold greater than that in intact cells (Figure 1B, inset). The effect of diazoxide was antagonized by 5-hydroxydecanoate (500 \(\mu\)mol/L).

In the next series of experiments, cells were exposed to the Na\(^+/K^+\)-ATPase inhibitor ouabain in an attempt to produce mitochondrial Ca\(^{2+}\) overload. Treatment of myocytes with 1 \(\text{mmol/L}\) ouabain increased the intensity of Rhod-2 fluorescence, suggesting that Ca\(^{2+}\) overloading in mitochondria was evoked (Figure 1C, a and b). Treatment with diazoxide (100 \(\mu\)mol/L) attenuated the mitochondrial Ca\(^{2+}\) 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 \(\pm\) 3\% and 173 \(\pm\) 16\% of baseline (\(P<0.001\)), respectively. Diazoxide significantly attenuated the ouabain-induced mitochondrial Ca\(^{2+}\) overload (116 \(\pm\) 7\% at 15 minutes, 131 \(\pm\) 10\% at 30 minutes; \(P<0.001\)), and the effect was antagonized by 5-hydroxydecanoate (500 \(\mu\)mol/L). 5-Hydroxydecanoate alone slightly increased the Rhod-2 fluorescence (123 \(\pm\) 3\% at 30 minutes, \(n=4\); \(P=\text{NS}\) versus control), so that the Rhod-2 fluorescence in the ouabain + diazoxide + 5-hydroxydecanoate group was insignificantly higher than that in the ouabain group. Like diazoxide, nicorandil (100 \(\mu\)mol/L), a potent mitoK\(_{ATP}\) channel opener, significantly reduced the ouabain-induced Ca\(^{2+}\) overload (see Figure in the online data supplement, available at http://www.circresaha.org). These results indicate that opening of mitoK\(_{ATP}\) channels attenuates the ouabain-induced Ca\(^{2+}\) overload in mitochondria.

We then examined whether the protective effect of diazoxide on the ouabain-induced Ca\(^{2+}\) overload is associated with the depolarization of \(\Delta\Psi_{\text{m}}\). Diazoxide (100 \(\mu\)mol/L) alone reduced the intensity of JC-1 fluorescence to 93 \(\pm\) 2\% 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 Ca2+ overload in association with the depolarization of ΔΨm.

**Discussion**

Among putative mechanisms of cardioprotection,1 the hypothesis of mitochondrial Ca2+ handling seems to be plausible. Indeed, Holmuhamedov et al3 showed that diazoxide inhibited the Ca2+ uptake and depolarized ΔΨm in isolated cardiac mitochondria. Their results, however, raise methodological criticism that mitoKATP 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 Ca2+ uptake and ΔΨm.4 Thus, the results so far obtained from isolated mitochondria remain inconclusive.

The present results demonstrate that diazoxide does not affect the basal [Ca2+]m 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 mitoKATP channels do not play a significant role in regulating [Ca2+]m under normal conditions. However, it should be noted that diazoxide could decrease [Ca2+]m when the resting mitochondrial Ca2+ level was raised. In this study, we evoked mitochondrial Ca2+ overload experimentally by exposure to ouabain. Elevation in cytosolic Ca2+ concentration occurs during ischemia, and it eventually results in mitochondrial Ca2+ accumulation.10 More recently, functional interaction between mitoKATP 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 Ca2+ overload in mitochondria. Also, nicorandil produced similar effects to diazoxide. Moreover, 5-hydroxydecanoate abolished these effects of diazoxide and nicorandil. Although the pathways for Ca2+ uptake and efflux need to be defined, these results indicate that opening of mitoKATP channels attenuates mitochondrial Ca2+ overload in adult rat ventricular myocytes.

It is debatable whether K’ influx through mitoKATP channels can depolarize ΔΨm. Holmuhamedov et al3 showed that diazoxide depolarized ΔΨm by ~15 mV, whereas Kowaltowski et al4 observed only a 1- to 2-mV depolarization of ΔΨm in isolated mitochondria. The present study has demonstrated that diazoxide can depolarize ΔΨm and notably the degree of depolarization is augmented in the presence of ouabain. These results suggest that the effect of diazoxide on ΔΨm seems to depend on Ca2+ concentration. Such Ca2+-dependent effect of diazoxide has been demonstrated for protection against Ca2+ paradox injury in rat myocardium.12 Although the Ca2+-dependent mechanism has not been defined, Ca2+-mediated signaling cascades including protein kinase C might augment the opening of mitoKATP channels by diazoxide. We further found that the reduction of ΔΨm by ~10% resulted in >50% reduction of [Ca2+]m 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 [Ca2+]m. Therefore, our present results suggest that even small changes in the electrochemical driving force could potentially lead to large differences in total mitochondrial Ca2+ accumulation over long periods of time.

In conclusion, we have shown convincing evidence that opening of mitoKATP channels attenuates the Ca2+ overload in mitochondria, a phenomenon associated with the depolarization of ΔΨm. These results support the original hypothesis and provide insight into the mechanism by which the opening of mitoKATP channels confers cardioprotection.

**Acknowledgments**

This study was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology (Nos. 13670743, 13670080, and 17670044) and by the Mitsui Life Social Welfare Foundation.

**References**


**Key Words:** mitochondria ■ calcium ■ KATP channel ■ cardioprotection
Opening of Mitochondrial K\textsubscript{ATP} Channels Attenuates the Ouabain-Induced Calcium Overload in Mitochondria

Hideyuki Ishida, Yuki Hirota, Chokoh Genka, Hiroe Nakazawa, Haruaki Nakaya and Toshiaki Sato

Circ Res. 2001;89:856-858; originally published online October 18, 2001;
doi: 10.1161/hh2201.100341

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/89/10/856

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2001/11/06/hh2201.100341.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Online Data Supplement

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.