Diazoxide maintenance of myocyte volume and contractility during stress: Evidence for a non-sarcolemmal $K_{ATP}$ channel location

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Objective: Animal and human myocytes demonstrate significant swelling and reduced contractility during exposure to stress (metabolic inhibition, hyposmotic stress, or hyperkalemic cardioplegia), and these detrimental consequences may be inhibited by the addition of diazoxide (adenosine triphosphate-sensitive potassium channel opener) via an unknown mechanism. Both SUR1 and SUR2A subunits have been localized to the heart, and mouse sarcolemmal adenosine triphosphate-sensitive potassium channels are composed of SUR2A/Kir6.2 subunits in the ventricle and SUR1/Kir6.2 subunits in the atria. This study was performed to localize the mechanism of diazoxide by direct probing of sarcolemmal adenosine triphosphate-sensitive potassium-sensitive potassium channel current and by genetic deletion of channel subunits.

Methods: Sarcolemmal adenosine triphosphate-sensitive potassium channel current was recorded in isolated wild-type ventricular mouse myocytes during exposure to Tyrode’s solution, Tyrode’s +100 μmol/L diazoxide, hyperkalemic cardioplegia, cardioplegia+diazoxide, cardioplegia+100 μmol/L pinacidil, or metabolic inhibition using whole-cell voltage clamp (N = 7–12 cells per group). Ventricular myocyte volume was measured from SUR1(-/-) and wild-type mice during exposure to control solution, hyperkalemic cardioplegia, or cardioplegia +100 μmol/L diazoxide (N = 7–10 cells per group).

Results: Diazoxide did not increase sarcolemmal adenosine triphosphate-sensitive potassium current in wild-type myocytes, although they demonstrated significant swelling during exposure to cardioplegia that was prevented by diazoxide. SUR1(-/-) myocytes also demonstrated significant swelling during exposure to cardioplegia, but this was not altered by diazoxide.

Conclusions: Diazoxide does not open the ventricular sarcolemmal adenosine triphosphate-sensitive potassium channel but provides volume homeostasis via an SUR1-dependent pathway in mouse ventricular myocytes, supporting a mechanism of action distinct from sarcolemmal adenosine triphosphate-sensitive potassium-sensitive potassium channel activation. (J Thorac Cardiovasc Surg 2010;140:1153-9)
via the $s_{K_{ATP}}$ channel, the $m_{K_{ATP}}$ channel, or a $K_{ATP}$ channel-independent mechanism. $K_{ATP}$ channels are composed of a Kir inward rectifier channel-forming subunit and a sulfonylurea sensitive regulatory subunit, SUR (Figure 1). It is clear that $s_{K_{ATP}}$ channels require both Kir6.2 and SUR2A subunits in the ventricle, while Kir6.2 and SUR1 in the atria. Both SUR1 and SUR2A are expressed in mouse heart, and there is evidence that SUR2 and SUR1 subtypes are both present in cultured neonatal rat ventricular myocytes. Mice lacking the SUR1 subunit seem to tolerate ischemia/reperfusion injury better than wild-type (WT) mice in a model of left anterior descending coronary artery ligation. Although there is some evidence for SUR1 subunits in the ventricle of various species, $K_{ATP}$ currents in mouse ventricle are unaltered in mice lacking the SUR1 subunit.

We initially hypothesized that the mechanism of action of DZX might involve opening the $s_{K_{ATP}}$ channel (SUR2A/Kir6.2). Previous work documented a beneficial effect of $K_{ATP}$ channel opener DZX in ventricular myocytes that was unaltered by the pharmacologic inhibition of the $K_{ATP}$ channel. The first part of this study was designed to definitively investigate the action of DZX by the \textit{direct} measurement of $s_{K_{ATP}}$ channel current in WT mice using whole-cell voltage clamp.

After demonstrating that $s_{K_{ATP}}$ (SUR2A/Kir6.2) channel activity was not observed in ventricular myocytes from WT mice during exposure to DZX, and knowing that SUR1 subunits have been documented in ventricular tissue, we next hypothesized that DZX might act via SUR1 subunits of an alternative $K_{ATP}$ channel or some other channel in ventricular cells. The second part of this study was therefore designed to determine whether ventricular myocytes lacking the SUR1 subunit would be responsive to DZX during stress.

This study was designed to elucidate the location of action of DZX by the \textit{direct} measurement of $s_{K_{ATP}}$ channel activity (in WT mice) and by response to stress in ventricular myocytes from WT mice and mice lacking the SUR1 subunit. The elucidation of DZX’s mechanism of action in this model will facilitate its future clinical use.

**MATERIALS AND METHODS**

**Myocyte Isolation**

All animal procedures were approved by the Animal Studies Committee at Washington University School of Medicine, and all animals received humane care in compliance with the “Guide to Care and Use of Laboratory Animals.”

Ventricular myocytes were used for all experiments and isolated from adult mice (either WT or SUR1 knockout [KO], either sex, 6 weeks to 5 months, 25–30 g body weight) as previously described. Rapid cardiectomy was performed in the anesthetized (2.5% tribromoethanol [Avertin; Sigma, St. Louis, Mo]) mouse, and the aorta was cannulated using a 28-gauge needle. The heart was attached to a Langendorff apparatus, and solution A was perfused through the aorta for 5 minutes. The heart was then perfused at 37°C for 12 minutes with solution B. The left ventricle was removed and transferred into solution C, where it was gently dispersed by glass pipette at room temperature. The cells were allowed to centrifuge by gravity, and serial washings were performed every 10 minutes for a 30-minute period. Cells were used in experiments within 5 hours after isolation. A typical yield of viable myocytes was 65% to 75% per mouse.

Solution A consisted of (in millimoles per liter, except as noted) 116 NaCl, 5.36 KCl, 0.97 NaHPO4, 1.47 KH2PO4, 21.10 HEPES, 11.65 glucose, 26.50 μmol/L phenol red (Sigma); 3.72 MgCl2; 4.40 NaHCO3; essential vitamins (100×, 10 mL; Gibco, Grand Island, NY); and amino acids (50×, 20 mL; Gibco). Solution B consisted of solution A plus 10 μmol/L CaCl2 and 1.2 mg/mL collagenase (Type 2; Worthington Biochemical Corporation, Freehold, NJ), Solution C consisted of solution A plus 5 mg/mL bovine serum albumin (Sigma), 1.25 mg/mL taurine, and 150 μmol/L CaCl2.

The DZX (7-chloro-3-methyl-1,2,4-benzothiadiazine-1,1-dioxide; Sigma) dose of 100 μmol/L was used because it was effective in ameliorating cell swelling secondary to stress (hyperkalemic CPG, hypotonic stress, and metabolic inhibition [MI]) in previous studies. A stock solution of DZX was made by dissolving the reagent in dimethyl sulfoxide at a concentration dimethyl sulfoxide has no effect on cell volume. DZX was made by dissolving the reagent in 0.1% dimethyl sulfoxide.

Cells were selected for viability using the following criteria: normal rod shape, smooth edges, sharp borders and clear striations, absence of vacuoles or blebbing, and lack of spontaneous beating. After isolation, acceptable myocytes were randomly assigned to a test solution group. A maximum of 2 cells were used per each animal.

**Electrophysiology in Wild-Type Myocytes**

After isolation, WT myocytes were placed in a recording chamber containing normal Tyrode’s solution (NT). Macroscopic currents in isolated ventricular myocytes were recorded using standard whole-cell voltage-clamp recording techniques. Patch-clamp electrodes (1–3 MΩ when filled with electrode solution) were fabricated from soda lime glass microhematocrit tubes (Kimble 73813, Kimble Glass Co, Vineland, NJ). Electrode solution contained the following (in millimoles/liter): 140 KCl, 10 HEPES, and 10 EGTA (pH 7.3–7.4). Cell capacitance and series resistance were determined using a 5–to 10-mV hyperpolarizing square pulse from a holding potential of –70 mV after establishment of the whole cell recording configuration. PCclamp 9.2 software and DigiData 1322 (both from Molecular Devices, Sunnyvale, Calif) were used to generate command pulses and collect data. Data were filtered at 5 kHz. A 4-second ramp from –110 and 40 mV was used to isolate and detect $s_{K_{ATP}}$ channel current only.

**Experimental protocol**

Isolated WT myocytes were exposed to NT for baseline measurement for 1 to 2 minutes, followed by exposure to test solution (5–10 minutes), and followed by NT for 5 to 10 minutes. Test solutions included NT (n = 8 cells), NT + 100 μmol/L DZX (n = 7 cells), hyperkalemic CPG in the form of St Thomas’ solution (CPG, Plegisol, Abbott Laboratories, North...
Chicago, Ill) (n = 11 cells), CPG + 100 μmol/L DZX (n = 8 cells), CPG + 100 μmol/L pinacidil (Sigma) (n = 12 cells), and MI (n = 8 cells). NT consisted of the following (in millimoles/liter): 137 NaCl, 5.4 KCl, 25 NaH₂PO₄, 10 glucose, 0.5 MgCl₂, and 3 NaHCO₃ (pH 7.3–7.4). St Thomas’ solution consisted of (in millimoles/liter) 110 NaCl, 10 NaHCO₃, 16 KCl, 32 MgCl₂, and 2.4 CaCl₂, and was equilibrated with 95% O₂, 5% CO₂ and titrated to correct to pH 7.3. MI solution consisted of (in millimoles/liter) 137 NaCl, 5.4 KCl, 3 NaHCO₃, 0.16 NaH₂PO₄, 10 2-deoxyglucose, 0.5 MgCl₂, 5 HEPES, 5% oligomycin, and 3 NaHCO₃ (pH 7.3–7.4). Isolated sKATP channel activity and cellular capacitance were recorded during exposure to NT and compared with channel activity during test solution exposure.

MI was used as a positive control to document an increase in sKATP channel current. Myocytes that demonstrated an increase in potassium current were subsequently exposed to glibenclamide (10 μmol/L, a known sKATP channel blocker; Sigma) to confirm that the increase in potassium current during exposure to MI was through sKATP channels.

Myocyte Volume Imaging in Wild-Type and SUR1 (-/-) Myocytes

An aliquot of isolated myocytes was placed in a glass-bottomed chamber on an inverted microscope stage (Leitz, Wetzlar, Germany) equipped with Hoffman modulation optics (Modulation Optics, Greenvale, NY). After a 5-minute stabilization period, the chamber was perfused at a rate of 3 mL/min with 37°C NT (in millimoles/liter) 130 NaCl, 5 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 24 NaHCO₃, 1.75 Na₂HPO₄, and 10 glucose, buffered to a pH of 7.4 using 95% O₂, 5% CO₂. Chamber temperature was controlled by a waterbath system (Thermo Haake, Karlsruhe, Germany). Cell images were displayed on a video monitor using a charge-coupled device camera (KPM1U; Hitachi Denshi, Tokyo, Japan). Digital images of cells were captured at a rate of 120 frames per second using a video-frame grabber (Scion Corp, Frederick, Md) and manually traced using Scion Image software (Scion Corp). Length, width, and area were measured and recorded. To calculate cell volume, it was assumed that changes in cell width and thickness were proportional, and relative cell volume change was determined by the following formula:15

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\frac{\text{volume}_t}{\text{volume}_c} = \frac{(\text{area}_t \times \text{width}_t)}{(\text{area}_c \times \text{width}_c)}
\]

where t and c refer to test and control, respectively. On the basis of repeated measurements of single images and measurements of multiple images of a cell, this methodology for estimating cell volume has been shown to be reproducible with an error of less than 1%.15

SUR1(-/-) mice were created by removal of the 1-kbp gene segment containing both promoter and exon 1 sequences of SUR1 gene by re-mediated recombination.18 Genotype was confirmed by polymerase chain reaction analysis.18 This deletion is not lethal, and the SUR1 (-/-) mice lack pancreatic β cell KATP channels (SUR1/Kir6.2) and glucose-stimulated insulin secretion, and are mildly glucose intolerant.18

Experimental protocol

Myocytes were not subjected to ischemia or modified ischemia in this protocol to delineate changes caused by the stress of exposure to CPG alone. Myocytes from WT and KO mice were perfused with 37°C NT for 5 minutes to obtain baseline measurements. Any baseline changes in cell volume secondary to the isolation or imaging protocol would be evident during this period. Myocytes were then perfused for 5 minutes with test solution, followed by glibenclamide (10 μmol/L) before re-perfusion with NT.
Sarcolemmal K\textsubscript{ATP} Channel Current in Wild-Type Myocytes

WT ventricular myocytes exposed to NT exhibited no increase in sarcolemmal adenosine triphosphate-sensitive potassium current throughout the experiment (Figure 2, A), and the addition of DZX to NT was not associated with an increase in potassium current (Figure 2, A). sK\textsubscript{ATP} current increased significantly in WT cells exposed to MI (P = .002 vs Tyrode’s) (Figure 2, B) and was inhibited by the addition of glibenclamide (Figure 2, A).

WT myocytes exposed to CPG alone after exposure to NT exhibited no increase in sK\textsubscript{ATP} current (Figure 3, A), and the addition of DZX to CPG had no effect on the potassium current when compared with CPG alone (Figure 3, A). The addition of pinacidil in cardioplegic solution was associated with an increase in sK\textsubscript{ATP} current, which returned to baseline when cells were reexposed to NT (Figure 3, A, B). This increase in potassium current was comparable to the potassium current induced by MI.

Statistical Analysis

Data were analyzed using SYSTAT 11 (SYSTAT Software Inc, Point Richmond, Calif). All data are presented as mean value ± standard error of the mean, with N equal to the number of cells in each group. For cell volume measurements, a repeated-measures analysis of variance was used for sequential time-based measurements for each test solution against its own baseline value. By using Fisher’s least significant difference test, post hoc multiple comparisons between different test groups were made separately during the test solution and reexposure periods. For electrophysiology experiments, Student t test was used to compare data.

RESULTS

Sarcolemmal K\textsubscript{ATP} Channel Current in Wild-Type Myocytes

WT ventricular myocytes exposed to NT exhibited no increase in sarcolemmal adenosine triphosphate-sensitive potassium current in WT myocytes. The y-axis represents potassium current (nA), and the x-axis represents time. WT myocytes were initially exposed to NT for baseline potassium current measurement, followed by test solution and reexposure to NT. The first graph (far left) is a representative cell exposed to NT throughout the experiment. The second graph (middle) is a representative cell exposed to NT with DZX during the test period. The addition of DZX to NT did not alter the potassium current. The third graph (far right) is a representative cell exposed to NT followed by MI. MI was associated with an increase in potassium current that was reversed by the addition of glibenclamide. B, DZX does not elicit sK\textsubscript{ATP} current in WT myocytes. Cell conductance is represented in nanoseimens/picofarads (y-axis) and test solution on the x-axis. Test solutions included NT in addition to DZX and MI. When correcting for cell conductance, there was a significant increase in potassium current in cells exposed to MI (*P = .002 vs NT). DZX, Diazoxide; MI, metabolic inhibition; NT, normal Tyrode’s solution; N, number of myocytes; GLIB, glibenclamide.

by a 5-minute reexposure period to 37°C NT. Test solutions included NT (n = 8 cells for KO; n = 7 cells for WT), hyperkalemic CPG in the form of St Thomas’ solution (n = 10 cells for KO; n = 7 cells for WT), or CPG + 100 µmol/L DZX (n = 8 cells for KO; n = 7 cells for WT). Volume measurements were made after the end of each 5-minute period.

Myocyte Volume in Wild-Type and SUR1(-/-) Mice

WT and SUR1(-/-) myocytes exposed to NT had no significance change in volume throughout the experiment...
Diazoxide Requires SUR1 Subunit of the K<sub>ATP</sub> Channel

Diazoxide (DZX) is a K<sub>ATP</sub> channel opener that has been studied for its effect on cellular volume regulation. The mechanism of action of DZX is thought to be mediated through the SUR1 subunit of the K<sub>ATP</sub> channel complex. In this study, the authors investigated whether the addition of DZX could prevent or reverse volume changes observed in myocytes under various stress conditions.

Results:

1. **Hyperkalemic CPG (4 mEq/L K<sup>+</sup>)**
   - Myocyte swelling was observed in isolated ventricular myocytes from WT and SUR1<sup>-/-</sup> mice. The addition of DZX (100 μmol/L) significantly ameliorated the volume change secondary to hyperkalemic CPG, but this effect was independent of sK<sub>ATP</sub> channels.
   - The mechanism of action of DZX in this setting is thought to be mediated through the SUR1 subunit of the K<sub>ATP</sub> channel.

2. **Cardioplegia**
   - Myocyte swelling was also observed in response to cardioplegia. DZX (100 μmol/L) significantly ameliorated cardioplegic swelling in SUR1<sup>-/-</sup> myocytes, suggesting a role for SUR1 in the maintenance of myocyte volume homeostasis.

3. **Hyposmotic Stress**
   - Myocyte swelling was observed in response to hyposmotic stress (6% reduction in extracellular osmolality). DZX (100 μmol/L) significantly ameliorated hyposmotic swelling in both WT and SUR1<sup>-/-</sup> myocytes, consistent with its role in maintaining cellular volume homeostasis.

**DISCUSSION**

Diazoxide Is Not Associated With an Increase in sK<sub>ATP</sub> Channel Current in Wild-Type Ventricular Myocytes

Diazoxide did not induce an sK<sub>ATP</sub> channel current in ventricular myocytes isolated from WT mice, even at concentrations as high as 100 μmol/L. This finding suggests that the effect of DZX on myocyte volume is not mediated through a direct increase in sK<sub>ATP</sub> channel current.

Mechanism of Myocyte Swelling Secondary to Stress

In the present study, myocyte volume derangement after exposure to hyperkalemic CPG alone was evaluated. Myocytes were exposed to hyperkalemic CPG (4 mEq/L K<sup>+</sup>) and DZX (100 μmol/L) for 5 minutes, with volume measurement performed at baseline and after treatment.

Both Pinacidil and Metabolic Inhibition Produce an Increase in sK<sub>ATP</sub> Channel Current in Wild-Type Myocytes

Pinacidil, a nonspecific K<sub>ATP</sub> channel opener, was associated with a significant increase in sK<sub>ATP</sub> channel current, consistent with findings of previous studies. MI (metabolic inhibition) is known to induce a large sK<sub>ATP</sub> channel current, and this is consistent with the results of the present study.

Maintenance of Myocyte Volume Homeostasis by Diazoxide Requires SUR1

Myocytes from mice lacking the SUR1 gene also swelled significantly when exposed to CPG; however, DZX failed to reverse the effect. This finding indicates that DZX is in fact ameliorating cardioplegic swelling through the SUR1 subunit, but that this is independent of sK<sub>ATP</sub> channels.

Location of Action of Diazoxide

The results of the present study suggest that the observed protective mechanism of DZX in isolated myocytes (maintenance of volume homeostasis and contractility during stress) is dependent on the SUR1 subunit, but is independent of the sK<sub>ATP</sub> channel. The mechanism of action may be at the mitochondrial (mK<sub>ATP</sub>) level or at a K<sub>ATP</sub> channel-independent level.
location in the ventricle. Many investigators attribute the myocardial protection provided by K$_{\text{ATP}}$ channel openers to the opening of the purported mK$_{\text{ATP}}$ channel rather than the sK$_{\text{ATP}}$ channel. However, much of the data claimed as support for the existence of an mK$_{\text{ATP}}$ channel have been indirect, including by measurement of mitochondrial flavoprotein oxidation. Critics have noted that DZX inhibits succinate dehydrogenase activity, which in turn will inhibit the tricarboxylic acid cycle leading to oxidation of mitochondrial flavoproteins. Other evidence supported by patch clamping of the inner mitochondrial membrane documenting the presence of mK$_{\text{ATP}}$ channels has been criticized because of its lack of reproducibility and potential contamination by other cellular membranes. The determination of the structure of an mK$_{\text{ATP}}$ channel would thus facilitate localization of DZX’s mechanism of action.

Recent studies have documented RNA expression for SUR1 in left ventricular tissue from failing human hearts. The elucidation of the composition of the K$_{\text{ATP}}$ channel specifically in humans will also increase the knowledge of the exact site of cardioprotection offered by DZX.

**Study Limitations**

This study investigated the action of DZX at the cellular level and in one species. This species was chosen because of the availability of a genetic knockout. Genetically tractable larger animal models are not available, and pharmacologic methods have limitations as discussed. Potassium current at the cellular level was measured to definitively observe any action of DZX at the sK$_{\text{ATP}}$ channel in isolation. Extrapolation to the whole organism level should therefore be taken with caution.
CONCLUSIONS

The inverse relationship previously demonstrated between myocyte volume derangement and contractility in isolated myocytes suggested loss of myocyte volume homeostasis as a potential mechanism of myocardial stunning.\(^4\) The ability of DZX to prevent myocyte swelling and resultant contractile dysfunction secondary to 3 independent stresses in 3 species suggests that its use may be exploited for the reduction of myocardial stunning. Elucidation of the mechanism of action of DZX in the mouse at the cellular and subcellular levels will subsequently facilitate its acceptance and use at the clinical level.

References


Discussion

Dr Friedhelm Beyersdorf (Freiburg, Germany). Swelling of cardiomyocytes is an important aspect of ischemia–reperfusion injury of the heart that leads to impaired myocardial function. You simulate ischemia in your experiments by MI using a specific solution. I have 2 questions for you:

First, you showed that a current in the ATP-sensitive potassium channels is not induced by CPG but by MI. However, both conditions lead to cardiomyocyte swelling. In contrast, again, only swelling caused by MI is prevented by DZX. Could you comment on possible differences between these 2 experimental conditions?

Ms Sellitto. The hyperkalemic CPG is more of a hypo-osmotic stress as opposed to the inhibition of metabolism. Possibly, because it’s a different mechanism of action in the stresses, that would be the difference in the activity of the K\(_{ATP}\) channel.

Dr Beyersdorf. Second, Elrod and coworkers demonstrated in an article in Circulation in 2008 that SUR1 KO mice are protected against ischemia. Their experiments showed reduced infarct size and preserved function after a surgical ligation of the left coronary artery. Could you comment on a positive correlation between your and their results?

Ms Sellitto. The SUR1 unit in the mouse model has been shown to have heterozygotes found predominantly in the atria and not so much in the ventricle. But in humans, SUR1 is more homogeneous, so DZX could have an effect in preventing damage caused by ischemia in the human heart.