Selective Pharmacological Agents Implicate Mitochondrial but Not Sarcolemmal K\(_{\text{ATP}}\) Channels in Ischemic Cardioprotection

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Background—Pharmacological evidence has implicated ATP-sensitive K\(^+\) (K\(_{\text{ATP}}\)) channels as the effectors of cardioprotection, but the relative roles of mitochondrial (mitoK\(_{\text{ATP}}\)) and sarcolemmal (surfaceK\(_{\text{ATP}}\)) channels remain controversial.

Methods and Results—We examined the effects of the K\(_{\text{ATP}}\) channel blocker HMR1098 and the K\(_{\text{ATP}}\) channel opener P-1075 on surfaceK\(_{\text{ATP}}\) and mitoK\(_{\text{ATP}}\) channels in rabbit ventricular myocytes. HMR1098 (30 \(\mu\)mol/L) inhibited the surfaceK\(_{\text{ATP}}\) current activated by metabolic inhibition, whereas the drug did not blunt diazoxide (100 \(\mu\)mol/L)-induced flavoprotein oxidation, an index of mitoK\(_{\text{ATP}}\) channel activity. P-1075 (30 \(\mu\)mol/L) did not increase flavoprotein oxidation but did elicit a robust surfaceK\(_{\text{ATP}}\) current that was completely inhibited by HMR1098. These results indicate that HMR1098 selectively inhibits surfaceK\(_{\text{ATP}}\) channels, whereas P-1075 selectively activates surface K\(_{\text{ATP}}\) channels. In a cellular model of simulated ischemia, the mitoK\(_{\text{ATP}}\) channel opener diazoxide (100 \(\mu\)mol/L), but not P-1075, blunted cellular injury. The cardioprotection afforded by diazoxide or by preconditioning was prevented by the mitoK\(_{\text{ATP}}\) channel blocker 5-hydroxydecanoate (500 \(\mu\)mol/L) but not by the surfaceK\(_{\text{ATP}}\) channel blocker HMR1098 (30 \(\mu\)mol/L).

Conclusions—The cellular effects of mitochondria- or surface-selective agents provide further support for the emerging consensus that mitoK\(_{\text{ATP}}\) channels rather than surfaceK\(_{\text{ATP}}\) channels are the likely effectors of cardioprotection. (Circulation. 2000;101:2418-2423.)

Key Words: mitochondria ■ potassium ■ ischemia ■ preconditioning
activity.3 Cells were superfused with solution containing (in mmol/L) NaCl 140, KCl 5, CaCl2 1, MgCl2 1, and HEPES 10 (pH 7.4 with NaOH) at room temperature (~22°C). Endogenous flavoprotein fluorescence was excited with a xenon arc lamp with a bandpass filter centered at 480 nm. Emitted fluorescence was recorded from 1 cell at a time at 530 nm by a photomultiplier tube and expressed as a percentage of the DNP-induced fluorescence. In some experiments, flavoprotein fluorescence was measured during whole-cell patch-clamp experiments to administer drugs through the pipette (cf Figure 3).

For whole-cell patch recordings, the internal pipette solution contained (in mmol/L) potassium glutamate 120, KCl 25, MgCl2 0.5, K-EGTA 10, HEPES 10, and MgATP 1 (pH 7.2 with KOH). Currents were elicited every 6 seconds from a holding potential of ~80 mV by 2 consecutive steps to ~40 mV (for 100 ms) and 0 mV (for 380 ms). Current amplitude at 0 mV was measured 200 ms into the pulse to quantify surface-K<sub>ATP</sub> channel activity. In some experiments (eg, Figure 6), whole-cell currents and flavoprotein fluorescence were recorded simultaneously, and flavoprotein fluorescence was excited during the 100-ms step to ~40 mV.

Functional Expression of K<sub>ATP</sub> Channels and Electrophysiology

Details of the functional expression of K<sub>ATP</sub> channels in HEK 293 cells have been described previously.1 Plasmid DNA (3 μg total) containing Kir6.1 or Kir6.2 was cotransfected with either SUR2B or SUR2A cDNA into HEK cells by use of lipofectamine (Gibco) 18 hours after the cells were split. Mouse Kir6.1, provided by Prof Y. Kurachi (Osaka University, Japan), and rabbit Kir6.2 (GenBank AF006262) were cloned into vector pGFP-IRES. Rat SUR2A, supplied by Prof S. Seino (Chiba University, Japan), was expressed in the mammalian vector pCMV6. Mouse SUR2B, supplied by Prof Y. Kurachi, was cloned into the expression vector pCDNA3.

Electrophysiological recordings were made 48 hours after transfection with solutions identical to those used in rabbit ventricular myocytes (see above). Voltage ramps from -100 to +60 mV were applied every 100 ms every 6 seconds from a holding potential of ~80 mV. The current at 0 mV was measured to assay K<sub>ATP</sub> channel activity. Experiments were performed at room temperature (~22°C).

Simulated Ischemia and Cellular Injury

A cell-pelleting model of ischemia modified from Vander Heide et al14 was used to quantify myocyte injury. In brief, adult rabbit ventricular cells were washed with incubation buffer: (in mmol/L) NaCl 140, KCl 5, CaCl2 1, MgCl2 1, and HEPES 10 (pH 7.4 with NaOH). Aliquots (0.5 mL) of suspended cells were placed into a microcentrifuge tube and centrifuged for 10 seconds. Approximately 0.25 mL of supernatant was removed to leave a thin fluid layer above the pellet, and 0.2 mL of mineral oil was layered on the top to prevent gaseous diffusion. After 60 minutes or 120 minutes, 5 μL of cell pellet was sampled through the oil layer and mixed with 75 μL of 85 mOsm hypotonic staining solution: (in mmol/L) NaHCO3 11.9, KH2PO4 0.4, KCl 2.7, MgSO4 0.8, and CaCl2 1, with 0.5% glutaraldehyde and 0.5% trypan blue. Cells permeable to trypan blue were counted and expressed as a percentage of the total cells counted (~300 for each sample).

In the control group, cells were pelleted and sampled at 60 or 120 minutes. For the diazoxide-treated or P-1075-treated groups, diazoxide (100 μmol/L) or P-1075 (30 μmol/L) was added to the solution 15 minutes before the pelleting. Cells treated with diazoxide in the presence of 500 μmol/L 5HD or in the presence of 30 μmol/L HMR1098 were likewise pelleted and sampled at 60 minutes. Once applied, drugs were not washed out and thus were present throughout the simulated ischemia.

For the IPC group, the cells pelleted were incubated under oil for 10 minutes and then removed from beneath the oil with a pipette and resuspended in fresh buffer for 30 minutes. Subsequently, the cells were pelleted again and subjected to a prolonged period of simulated ischemia. In the control group, cells were subjected only to prolonged period of simulated ischemia without IPC. For the 5HD-treated and HMR1098-treated groups, either 5HD (500 μmol/L) or HMR1098 (30 μmol/L) was added to the incubation buffer 10 minutes before the IPC. All 4 conditions were tested simultaneously in each of 6 replications.

The small percentage of cells (~18%) that were nonviable at the beginning of the experiment were mostly rounded and had been damaged as a consequence of the enzymatic isolation process. The osmotic fragility of cells induced by ischemia was quantified as percentage of the vital cells at the beginning of each experiment. In nonpelleted control cells suspended in oxygenated buffer with or without drugs, there was no change in the percentage of stained cells measured after 120 minutes of incubation. Pelleting experiments were performed at 37°C.

Chemicals

Diazoxide and DNP were purchased from Sigma Chemical Co. 5HD was purchased from Research Biochemicals International. HMR1098 was a gift from Hoechst Marion Roussel (now Aventis Pharmaceuticals), and P-1075 was a gift from Leo Pharmaceutical Products. Diazoxide and P-1075 were dissolved in DMSO before being added into the experimental solution. The final concentration of DMSO was <0.1%.

Statistical Analysis

All data are presented as mean±SEM, and the number of cells or experiments is shown as n. Statistical analysis was performed with ANOVA combined with the Fisher post hoc test. Values of P<0.05 were considered significant.

Results

Effect of HMR1098 on Surface-K<sub>ATP</sub> and MitoxK<sub>ATP</sub> Channels

We first verified the inhibitory effect of HMR1098 on surface-K<sub>ATP</sub> channels by whole-cell patch clamp. Figure 1A shows surface-K<sub>ATP</sub> current (I<sub>K,ATP</sub>) elicited by exposure to 2,4-dinitrophenol (DNP, 100 μmol/L). Although DNP eventually increased I<sub>K,ATP</sub>, subsequent application of 30 μmol/L HMR1098 suppressed I<sub>K,ATP</sub>. As summarized in Figure 1B, HMR1098 (30 μmol/L) inhibited DNP-induced I<sub>K,ATP</sub> from 2.22±0.89 to 0.52±0.14 nA (P<0.05, n=5).

The effects of HMR1098 on mitoxK<sub>ATP</sub> channels were examined by measuring mitochondrial matrix redox potential. Figure 2A shows the time course of flavoprotein fluorescence in a cell exposed twice to diazoxide, a selective mitoxK<sub>ATP</sub> channel opener in heart cells.8,20 Diazoxide (100 μmol/L) induced reversible oxidation of the flavoproteins. A second application of diazoxide in the presence of HMR1098 suppressed the fluorescence.
Once again increased the flavoprotein fluorescence, and the degree of oxidation was identical to that achieved during the first exposure to diazoxide. As summarized in Figure 2B, diazoxide (100 μmol/L) reversibly increased flavoprotein oxidation to 41±8% of the DNP value (n=4). HMR1098 (30 μmol/L) did not alter the effect of diazoxide (42±9% of the DNP value, n=4).

To verify that the lack of effect of HMR1098 on diazoxide-induced flavoprotein oxidation did not result from inadequate diffusion of the drug to mitochondria, we measured flavoprotein fluorescence after including HMR1098 (30 μmol/L) in the patch pipette. Figure 3A shows that after 10 minutes in the whole-cell configuration, exposure to diazoxide (100 μmol/L) still induced flavoprotein oxidation. This effect of diazoxide could be blocked by 5HD (500 μmol/L), a specific mitoK<sub>ATP</sub> channel inhibitor. Subsequent reapplication of diazoxide in the absence of 5HD once again increased flavoprotein oxidation. Despite the presence of HMR1098 in the pipette, diazoxide increased flavoprotein oxidation to 46±6% of the DNP value (n=5, Figure 3B). This degree of oxidation is comparable to that observed in the absence of HMR1098 (cf Figure 2). The results indicate that HMR1098 selectively inhibits surfaceK<sub>ATP</sub> channels but not mitoK<sub>ATP</sub> channels.

### Effect of P-1075 on SurfaceK<sub>ATP</sub> and MitoK<sub>ATP</sub> Channels

We then examined the effects of the K<sub>ATP</sub> channel opener P-1075<sup>16</sup> on surfaceK<sub>ATP</sub> and mitoK<sub>ATP</sub> channels. P-1075 is a derivative of the cyanoguanidine K<sub>ATP</sub> channel agonist pinacidil, which is known to open both mitoK<sub>ATP</sub> and surfaceK<sub>ATP</sub> channels.<sup>8</sup> Figure 4, A and B, shows that P-1075 (30 μmol/L) significantly increased I<sub>K<sub>ATP</sub></sub> (P<0.01 vs control (CONT) and P-1075+HMR1098. C, Dose-response curve for P-1075 in rabbit ventricular myocytes. Each point constitutes measurements from 4 to 5 cells. D, Dose-dependent effects of P-1075 on Kir6.2+SUR2A (cardiac type) and Kir6.1+SUR2B K<sub>ATP</sub> (vascular smooth muscle–type) channels expressed in HEK cells. Each point constitutes measurements from 5 to 10 cells.

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**Figure 2.** Effect of HMR1098 on diazoxide (DIAZO)-induced flavoprotein oxidation. A, DIAZO (100 μmol/L) reversibly oxidized flavoprotein. HMR1098 (30 μmol/L) did not affect oxidative effect of DIAZO. Lines indicate periods when cell was exposed to drug. B, Summarized data for diazoxide-induced flavoprotein oxidation measured in absence and presence of HMR1098.

**Figure 3.** Effect of diazoxide (DIAZO) on flavoprotein fluorescence measured in whole-cell patch-clamp configuration. Patch pipette contained 30 μmol/L HMR1098. A, DIAZO (100 μmol/L) reversibly increased flavoprotein oxidation. Oxidative effect of DIAZO was inhibited by 5HD (500 μmol/L). B, Summarized pooled data for DIAZO-induced flavoprotein oxidation when HMR1098 was administered via pipette. Intracellular HMR1098 did not alter oxidative effect of diazoxide.

**Figure 4.** Effects of P-1075 on surfaceK<sub>ATP</sub> current. A, Time course of current measured at 0 mV. Lines indicate periods when cell was exposed to P-1075 (30 μmol/L) and HMR1098 (30 μmol/L). B, Summarized data from 4 cells. **P<0.01 vs control (CONT) and P-1075+HMR1098. C, Dose-response curve for P-1075 in rabbit ventricular myocytes. Each point constitutes measurements from 4 to 5 cells. D, Dose-dependent effects of P-1075 on Kir6.2+SUR2A (cardiac-type) and Kir6.1+SUR2B K<sub>ATP</sub> (vascular smooth muscle–type) channels expressed in HEK cells. Each point constitutes measurements from 5 to 10 cells.
Kir6.1+SUR2B (vascular smooth muscle type) K_ATP channels. In cells expressing the cardiac-type Kir6.2+SUR2A channels, P-1075 effectively increased I_KATP in a concentration-dependent manner (EC50 for P-1075 = 2.5 μmol/L). Conversely, P-1075 activated Kir6.1+SUR2B K_ATP channels at nanomolar concentrations (EC50 = 102 nmol/L). Thus, the low-dose effects previously described are unlikely to reflect activation of cardiac surface K_ATP channels.

The effects of P-1075 on mitoK_ATP channels were examined by measurement of mitochondrial redox potential. Figure 5 shows that diazoxide (100 μmol/L) induced reversible oxidation of the mitochondrial matrix to 44±4% of the DNP value (n=5). Subsequent exposure to P-1075 (30 μmol/L) had no effect (2±1% of the DNP value, n=5), whereas diazoxide once again increased flavoprotein oxidation (39±5% of the DNP value, n=4). Even when very high (100 μmol/L) or low (100 nmol/L) concentrations of P-1075 were applied, the drug failed to elicit any flavoprotein response (not shown). These results indicate that P-1075 selectively activates surfaceK_ATP channels without affecting mitoK_ATP channels.

To verify further the specificity of diazoxide and P-1075 for mitoK_ATP and surfaceK_ATP channels, respectively, we measured flavoprotein fluorescence and membrane current simultaneously. Figure 6A and 6B shows the effects of diazoxide and P-1075 in a representative experiment. Diazoxide (100 μmol/L) induced reversible oxidation of flavoproteins but did not affect I_KATP. In contrast, exposure to P-1075 (30 μmol/L) failed to increase flavoprotein oxidation but did elicit I_KATP. As summarized in Figure 6C and 6D, unlike diazoxide, P-1075 activated only I_KATP.

Effects of HM1R098 and P-1075 on Simulated Ischemia and Cellular Injury

Using the mitochondria- or surface-selective agents, we examined the role of mitoK_ATP and surfaceK_ATP channels for ischemic cardioprotection. The mitoK_ATP channel opener diazoxide (100 μmol/L) significantly decreased the percentage of cells stained after 60 minutes of simulated ischemia (from 32±3% to 17±3%, P<0.001, n=5), and this protection was completely prevented by the mitoK_ATP channel blocker 5HD (500 μmol/L) (Figure 7A). In contrast, the surfaceK_ATP channel blocker HM1R098 (30 μmol/L) did not prevent the cardioprotection by diazoxide (from 38±4% to 18±1%, P<0.001, n=4) (Figure 7B). In a separate series of experiments (Figure 7C), simulated ischemia for 60 and 120 minutes stained 35±2% (n=5) and 42±2% (n=5) of cells, respectively. Inclusion of diazoxide (100 μmol/L) significantly decreased the percentage of cells stained, to 18±2%...
Cardioprotection afforded by IPC is mediated by mitoKATP channels. Grover et al. showed that dofetilide attenuates the abbreviation of action potential duration is not necessary for protection. However, this hypothesis cannot account for IPC, because surfaceKATP channels are quite resistant to this drug; the specificity of diazoxide for mitoKATP channels is verified in Figure 6 of the present article. Moreover, we reported that 5HD completely and reversibly blocks the oxidative effect of diazoxide without affecting surfaceKATP channels, indicating that 5HD is an effective and specific blocker of mitoKATP channels (although this may not be the case in other species). When these drugs are used as pharmacological tools to activate or to inhibit mitoKATP channels, growing evidence supports the idea that mitoKATP channels rather than surfaceKATP channels are the dominant players for cardioprotection. Moreover, in animal models in vivo, diazoxide mimics, whereas 5HD abolishes, the infarct size-limiting effect of IPC. These findings motivated us to reevaluate the role of surfaceKATP channels in cardioprotection.

The sulfonylthiourea HMR1098 has been reported to be a cardioselective KATP channel blocker, blocking KATP channels in cardiac muscle cells with 10- to 50-fold higher potency than in pancreatic β-cells with little effect on the coronary vasculature. We demonstrated that HMR1098 inhibited surfaceKATP channels activated by metabolic inhibition (Figure 1) and by the surfaceKATP channel opener P-1075 (Figure 4). However, HMR1098 did not affect diazoxide-induced flavoprotein oxidation (Figure 2). Furthermore, direct application of HMR1098 to the cytoplasm by inclusion in the pipette failed to prevent the oxidizing effect of diazoxide (Figure 3). These results, taken together, indicate that HMR1098 selectively inhibits surfaceKATP channels without affecting mitoKATP channels.

The KATP channel opener P-1075 activated IKATP in rabbit ventricular myocytes; IKATP was blocked by HMR1098 (Figure 4). Moreover, the molecularly defined cardiac surfaceKATP channel (Kir6.2+SUR2A) expressed in HEK cells was effectively activated by P-1075. Indeed, P-1075 showed similar potencies in activating native surfaceKATP channels and expressed Kir6.2+SUR2A channels (EC50 of 13 μmol/L and 2.5 μmol/L, respectively). P-1075 activated Kir6.1+SUR2B (vascular smooth muscle type) channels ∼100-fold more potently than Kir6.2+SUR2A channels (EC50 of 102 nmol/L). Conversely, P-1075 did not affect mitochondrial oxidation state measured with or without invasion by patch pipettes or their contents (Figures 5 and 6). These results indicate that, unlike diazoxide, P-1075 selectively activates surfaceKATP channels but not mitoKATP channels.

We previously reported that diazoxide (50 μmol/L) decreased myocyte death in a cellular model of simulated ischemia. In the present study, we verified the cardioprotective effects of diazoxide (Figure 7). Diazoxide-induced cardioprotection was prevented by the mitoKATP channel blocker 5HD. In contrast, the surfaceKATP channel blocker HMR1098 did not abolish the cardioprotective effect of diazoxide (Figure 7B). Furthermore, we found that the surfaceKATP channel opener P-1075 did not...
produce cardioprotection (Figure 7C). Sargent et al. reported that, in Langendorff-perfused rat hearts, P-1075 increases coronary flow and protects ischemic myocardium at nanomolar concentrations. Consistent with their findings, P-1075 does activate the vascular smooth muscle type (Kir6.1+SUR2B) K<sub>ATP</sub> channel at nanomolar concentrations, whereas the cardiac-type (Kir6.2+SUR2A) K<sub>ATP</sub> channel is affected only in the micromolar range (Figure 4D). However, we found that the surface-K<sub>ATP</sub> channel opener P-1075 (30 μmol/L) did not produce cardioprotection (Figure 7C). Although the reason for this discrepancy is unknown, our results imply that the direct activation of surface-K<sub>ATP</sub> channels in rabbit myocytes does not underlie myocyte cardioprotection.

IPC is present in all species examined, including humans. Compelling evidence suggests that mito-K<sub>ATP</sub> channels rather than surface-K<sub>ATP</sub> channels may serve as end effectors of IPC. Logical evidence that surface-K<sub>ATP</sub> channels are not involved in preconditioning. These results divorce the cardioprotective effect of IPC from activation of surface-K<sub>ATP</sub> channels.

In conclusion, our observations with diazoxide, P-1075, 5HD, and HMR1098 provide the first definitive pharmacological evidence that surface-K<sub>ATP</sub> channels are not involved in cardioprotection in isolated rabbit myocytes. By extension, the present data provide further support for the emerging consensus that mito-K<sub>ATP</sub> channels are the end effectors of preconditioning specifically in rabbit heart.

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