Kir6.2 limits Ca$^{2+}$ overload and mitochondrial oscillations of ventricular myocytes in response to metabolic stress

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The cardiovascular system operates under a broad range of demands, varying between periods of rest to bursts of intense exercise. During ischemic heart disease, the cardiac muscle experiences an extreme and pathological metabolic challenge. There is exquisite control of mitochondrial metabolism so that the ATP supply is balanced for the wide range of physiological workloads. However, any mismatch in ATP supply and demand can have fatal consequences (4). The maintenance of cardiac workloads is predicted to be diabetic (37).

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Address for reprint requests and other correspondence: N. M. Storey, Dept. of Cell Physiology and Pharmacology, College of Medicine, Biological Sciences and Psychology Univ. of Leicester, Maurice Shock Medical Sciences Bldg., University Rd., PO Box 138, Leicester LE1 9HN, UK (e-mail: ns140@le.ac.uk).

Kir6.2 limits Ca$^{2+}$ overload and mitochondrial oscillations of ventricular myocytes in response to metabolic stress. Am J Physiol Heart Circ Physiol 305: H1508–H1518, 2013. First published September 6, 2013; doi:10.1152/ajpheart.00540.2013.—ATP-sensitive K⁺ (KATP) channels are abundant membrane proteins in cardiac myocytes that are directly gated by intracellular ATP and form a signaling complex with metabolic enzymes, such as creatine kinase. KATP channels are known to be essential for adaption to cardiac stress, such as ischemia; however, how all the molecular components of the stress response interact is not fully understood. We examined the effects of decreasing the KATP current density on Ca$^{2+}$ and mitochondrial homeostasis and ischemic preconditioning. Acute knockdown of the pore-forming subunit, Kir6.2, was achieved using adenoviral delivery of short hairpin RNA targeted to Kir6.2. The acute nature of the knockdown of Kir6.2 accurately shows the effects of Kir6.2 depletion without any compensatory effects that may arise in transgenic studies. We also investigated the effect of reducing the KATP current while maintaining KATP channel protein in the sarcolemmal membrane using a nonconducting Kir6.2 construct. Only 50% KATP current remained after Kir6.2 knockdown, yet there were profound effects on myocyte responses to metabolic stress. Kir6.2 was essential for cardiac myocyte Ca$^{2+}$ homeostasis under both baseline conditions before any metabolic stress and after metabolic stress. Expression of nonconducting Kir6.2 also resulted in increased Ca$^{2+}$ overload, showing the importance of K⁺ conductance in the protective response. Both ischemic preconditioning and protection during ischemia were lost when Kir6.2 was knocked down. KATP current density was also important for the mitochondrial membrane potential at rest and prevented mitochondrial membrane potential oscillations during oxidative stress. KATP channel density is important for adaption to metabolic stress.

The damage to cardiac muscle by ischemia-reperfusion injury is caused, in part, by Ca$^{2+}$ overload (38). KATP channels play an adaptive role during metabolic stress by opening when cellular ATP levels are low, as in ischemia (34). This hyperpolarizes the sarcolemmal membrane potential in an attempt to prevent Ca$^{2+}$ overload (3, 30). High intracellular Ca$^{2+}$ is deleterious to ventricular myocytes because in addition to preventing correct contractile function, it can trigger opening of the mitochondrial permeability transition pore (mPTP), which is an irreversible step toward myocyte death particularly during ischemia-reperfusion injury (16, 23).

Ischemic preconditioning (IPC) is a cardioprotective phenomenon in which short periods of ischemia reduce the impact of a prolonged ischemic event (33). KATP channels have also been proposed to play a key role in this intrinsic protective mechanism of the heart. KATP channel openers have been shown to mimic the protective effects of IPC (51), whereas KATP channel blockers ablated the effects of preconditioning (21).

The importance of KATP channels in adaptation to stress has also been highlighted in a number of transgenic studies. IPC is lost in mice lacking functional KATP channels (22). Furthermore, Kir6.2 knockout mice develop ventricular arrhythmia and sudden death because they have lost their ability to adapt to exercise or sympathetic challenge (54). Although the ATP sensitivity of KATP channels underlies their protective function during ischemia, stressed hearts of Kir6.2 knockout and control mice do not show a decrease of total cellular ATP concentration (54), suggesting that there may be other mechanisms that account for the role of KATP channels in cardioprotection. The full extent of the role of KATP channels may not be revealed by transgenic studies due to compensatory effects that are known to occur since the Kir6.2 knockout adult mouse shows only mild impairment of glucose homeostasis despite being predicted to be diabetic (37).

Sarcolemmal KATP channels are sensitive to mitochondrial ATP production through signaling complexes with metabolic enzymes important for cellular energy homeostasis, including adenylate kinase and creatine kinase (1, 27, 41). This creates a phosphotransfer bridge from the sarcolemmal membrane to the mitochondria, which allows metabolic fine tuning of excitability for the wide range of physiological workloads. How these signaling complexes influence KATP channel opening during ischemia is not fully understood. The aim of this study was to...
investigate the contribution of K\textsuperscript{+} flux and the signaling complex on Ca\textsuperscript{2+} and mitochondrial homeostasis.

Gene knockout and transgenic approaches are powerful tools but have some significant limitations. In addition to genetic background-dependent variations in phenotype, developmental compensation has the potential to obscure or distort "true" phenotypes. This is illustrated by the remodeling of excitation-contraction coupling seen in mice expressing an ATP-insensitive K\textsubscript{ATP} channel (12). The function of K\textsubscript{ATP} channels in ischemia remains to be unequivocally explained. It is estimated that opening of as few as 1% of K\textsubscript{ATP} channels would be sufficient to shorten action potential duration and thus be protective, suggesting considerable redundancy, yet increased channel expression has been reported to be cardioprotective (9).

In this study, we used we used RNA interference to probe the effects of acute reductions in channel expression and investigate the role of the K\textsubscript{ATP} channel subunit Kir6.2 in IPC and Ca\textsuperscript{2+} homeostasis. In addition, dominant negative, non-conducting Kir6.2 was expressed to distinguish between the roles of K\textsubscript{ATP} channels in ion conduction or as part of a metabolic signaling complex. The effect of decreasing K\textsubscript{ATP} channel density on mitochondrial function, particularly mPTP opening, was also investigated.

**MATERIALS AND METHODS**

**Adenovirus construction.** Recombinant adenovirus encoding two separate rat RNA interfering short hairpin sequences [short hairpin (sh)RNA] for knockdown of Kir6.2 were generated using pAdEasy (25).

Cassettes expressing shRNAs were created by ligating the following oligonucleotides into pSilencer adenov 1.0 CMV (Ambion): 6.2 shRNA-A, 5'-TCAAGGAAAAGCAGCTCGATCGAAGAAAGGGAACGGTGTTCCCGAATTA-3' and 5'-CTAGTAAAGTTCAAGAGACTTTAACGGTGTTCCCGAATTA-3'; 6.2 shRNA-B, 5'-TCAAGGAAAAGCAGCTCGATCGAAGAAAGGGAACGGTGTTCCCGAATTA-3' and 5'-CTAGTAAAGTTCAAGAGACTTTAACGGTGTTCCCGAATTA-3'.

The control shRNA sequence (Ambion) was a nontargeting shRNA with limited sequence similarity to known genes in the rat. For the nonconducting Kir6.2 construct, pore loop mutated (GFG to AFA) dominant negative Kir6.2 (29) CDNA was generated by overlap PCR and cloned into pIRE2-DS-Red2 (Clontech). An insert including the mutant Kir6.2, IRES, and DsRed2 sequences was subcloned into pENTR-1A (Invitrogen) and recombined with pAd-CMV-DEST (Invitrogen) to produce recombinant adenovirus.

These cassettes included a modified cytomegalovirus promoter, shRNA insert, and polyA terminator and were subcloned into the linearized shuttle vector PmeI, Invitrogen. In this assay, TMRM sequesters into the mitochondria, where fluorescence is autoquenched. TMRM-loaded myocytes were then continuously illuminated at 546 nm, generating ROS, and emission was measured using a Digidata 1322A interface, and records were acquired and analyzed using pCLAMP 9.2 software (Molecular Devices, Sunnyvale, CA). Patch pipettes were made from thick-walled borosilicate glass, filled with a solution containing (in mM) 140 KCl, 2 ATP, 0.1 ADP, 0.1 GTP, 1 MgCl\textsubscript{2}, 10 HEPES, and 5 BAPTA titrated to pH 7.2 with NaOH, and had resistances of 5–6 M\textOmega. Cells were superfused with normal Tyrode solution containing (in mM) 135 NaCl, 5 KCl, 1.33 NaH\textsubscript{2}PO\textsubscript{4}, 5 Na-pyruvate, 10 glucose, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, and 10 HEPES titrated to pH 7.4 with NaOH. P1075 and glibenclamide were from Tocris Bioscience (Abingdon, UK), and all other reagents were from Sigma-Aldrich (Dorset, UK).

**Measurement of contractile activity and intracellular Ca\textsuperscript{2+} concentration.** Myocytes were placed in a 500-μl chamber on the stage of a Nikon inverted microscope, continuously superfused at 5 ml/min with Tyrode solution or metabolic inhibition Tyrode solution [which contained NaCN (2 mM) and iodoacetic acid (1 mM) without glucose or pyruvate], and stimulated at 1 Hz by electrical field stimulation. Contractile activity was determined from observation of fields containing 8–15 cells using a charge-coupled device camera (Orca2, Hamamatsu, Cairn Research, Kent, UK). To measure intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i), myocytes were loaded with 5 μM indo-1 AM or fura-2 AM (Invitrogen, Paisley, UK). Indo-1 was excited at 340 nm, the emitted light was split with a 440-nm dichroic filter, and the resulting images passed through 485- and 405-nm bandpass filters, respectively. The indo-1 ratio (405-450-nm fluorescence ratio), corresponding to Ca\textsuperscript{2+} bound/Ca\textsuperscript{2+} free, was calculated using AQM Advance 6 software (Kinetic Imaging, Cairn Research). For fura-2 experiments, single myocytes were excited alternately with 340- and 380-nm light from a monochromator (deltaRAM, Photon Technology, West Sussex, UK). Fluorescence intensity was collected at 510 nm with a photomultiplier tube and analyzed with a video imaging system (Photon Technology). Free Ca\textsuperscript{2+} values were estimated using in vitro calibration for all treatment groups. The KD may differ in the cell, so the values given are intended to be an estimated indication of [Ca\textsuperscript{2+}], rather than the precise intracellular concentration.

**Measurement of mitochondrial membrane potential.** Ventricular myocytes in Tyrode solution were loaded with the fluorescent dye tetramethylrhodamine methyl ester (TMRM; 5 μM, Invitrogen). In this assay, TMRM sequesters into the mitochondria, where fluorescence is autoquenched. TMRM-loaded myocytes were then continuously illuminated at 546 nm, generating ROS, and emission was measured using a long-pass filter of >560 nm at a rate of 0.5 Hz (Orca2, Hamamatsu, Cairn Research). Accumulation of ROS induced mPTP opening. This allows TMRM to leave the mitochondria and dequench in the cytosol, which is observed as an increase in fluorescence. An increase in TMRM fluorescence is used as an index of mitochondrial membrane potential, which, in these circumstances, reflects the opening of the mPTP (24). Regions of interest were placed around all viable myocytes in the field of view, and fluorescence changes were plotted against time (Kinetic Imaging, Cairn Research).

**Ischemic pelleting.** Freshly isolated myocytes were infected with adenovirus and cultured for 24 h as described above. Myocytes were placed in 1.8-ml tubes and allowed to settle under gravity into a pellet. To simulate ischemia, excess supernatant was removed, and mineral...
oil was layered on top to exclude gaseous exchange. Samples were incubated at 37°C in a 5% CO2 incubator. To simulate reperfusion, the oil was removed, fresh medium 199 was added, and myocytes were incubated for a further 60 min. After reperfusion, myocytes were resuspended in Tyrode solution containing sodium amytal (3 mM), trypan blue (0.4%), and paraformaldehyde (0.5%). Myocytes were counted, and trypan blue exclusion was used as an index of survival.

Western blot analysis. A sample of cardiac myocytes infected with control or Kir6.2 shRNA adenovirus was taken and lysed. Equal amounts of protein, measured by the Bradford assay, from each treatment group were loaded in each well. Samples were subjected to SDS-PAGE and transferred to nitrocellulose using a wet transfer cell. The Kir6.2 antibody (a kind gift from Dr Norman, University of Leicester), which has been previously characterized (43), was used to detect Kir6.2 protein levels and was normalised to total ERK protein (Cell Signaling Technology, Hitchin, UK). Densitometry was determined using Felix software (Phosphor Technology).

Statistics. Data are presented as means ± SE from at least three independent experiments. Statistical significance was calculated using Student’s t-test and one-way ANOVA with Tukey’s post hoc test where appropriate. P values of <0.05 were accepted as significant.

RESULTS

Decrease of KATP channel protein and current by silencing shRNA targeted to Kir6.2. The cellular consequences of reduced expression of Kir6.2 in cardiac myocytes were investigated by RNA interference with shRNA. An adenoviral system for delivering shRNA sequences to adult rat cardiac myocytes was developed. Two independent Kir6.2 subunit-specific sequences, designated shRNA-A and shRNA-B, were used to knock down Kir6.2. In addition, a control shRNA containing an RNA interfering sequence with limited sequence similarity to known genes in the rat was used. Whole cell sarcolemmal KATP current was evoked by the KATP channel opener P1075 (10 μM) from myocytes voltage clamped to 0 mV, and the current was blocked by the specific KATP channel inhibitor glibenclamide (10 μM; Fig. 1A). Currents were normalized for cell size to show KATP current density. In contrast to the control, expression of Kir6.2 shRNA-A or Kir6.2 shRNA-B sequences for 24 h resulted in a significant decrease in KATP current density (Fig. 1, A and C).

To exclude the possibility of a general reduction of ion channel function, L-type Ca2+ channel currents were evoked by a voltage step from -40 to 0 mV and recorded from cardiac myocytes infected with Kir6.2 shRNA virus (Fig. 1B). Mean normalized Ca2+ current amplitudes showed no significant differences between control, Kir6.2 shRNA-A, or Kir6.2 shRNA-B virus-infected myocytes (Fig. 1D).

In addition to the specific and significant decrease in KATP channel current density, Western blot analysis confirmed that KATP channel protein levels were significantly decreased in cardiac myocytes expressing either Kir6.2 shRNA-A or shRNA-B (Fig. 1, E and F).

Protection by IPC is abolished after knockdown of Kir6.2. To investigate the effect of acute Kir6.2 knockdown on the protective effect of IPC, the ischemia-pelleting technique was used (8). Myocytes were incubated under a layer of mineral oil to create an ischemic environment for 60 min. When the oil layer was removed, myocytes were resuspended in fresh medium to simulate reperfusion for 60 min. IPC was induced by incubating a pellet of myocytes for 10 min under oil and 10 min of reperfusion before the prolonged ischemia. Control myocytes were maintained in medium 199 throughout. The protocols used are shown in Fig. 2A. The population of live and dead myocytes was assessed by the ability to exclude trypan blue at the end of reperfusion (Fig. 2B). In contrast to the control virus-infected population of myocytes, the survival of those expressing Kir6.2 shRNA-A was not increased by IPC (Fig. 2C). Furthermore, knockdown of Kir6.2 resulted in a significant decrease in the survival of myocytes subjected to ischemia (32 ± 4%, n = 21 experiments) compared with myocytes infected with the control virus (42 ± 4%, n = 25 experiments, P < 0.05). In summary, control virus-infected myocytes showed significant protection by IPC; however, the protection was completely lost in Kir6.2 knockdown myocytes. Also, after ischemia, the percentage of dead myocytes was greater in Kir6.2 knockdown myocytes compared with control myocytes.

Effect of Kir6.2 knockdown on Ca2+ homeostasis. The ability of cardiac myocytes to maintain Ca2+ homeostasis during ischemia-reperfusion is central to the recovery of contractile function and myocyte survival. To investigate the effect of Kir6.2 knockdown on Ca2+ homeostasis, the Ca2+ indicator indo-1 was used. To simulate the decrease in cellular ATP that occurs in ischemia, myocytes were challenged with metabolic inhibition solution, which inhibits glycolysis and oxidative phosphorylation and thus reduces cellular ATP production. Myocytes were perfused with metabolic inhibition solution (4 min) and reperfused with Tyrode solution for 10 min in a contracting myocyte model of ischemia-reperfusion injury. Ventricular myocytes infected for 24 h with either control, shRNA-A, or shRNA-B adenovirus were stimulated to contract synchronously with electrical field stimulation at 1 Hz. Initially, control myocytes responded to field stimulation; shortly after metabolic inhibition solution, myocytes stopped responding to field stimulation, and at the end of reperfusion, the majority of myocytes regained their contractile function, although with elevated intracellular Ca2+ (Fig. 3, A and C). Initially, all Kir6.2 knockdown myocytes responded to field stimulation, but in contrast to control myocytes, they developed much higher intracellular Ca2+ at the end of reperfusion, and many did not regain their contractile function (Fig. 3, B and C). Interestingly, the baseline estimated [Ca2+] from Kir6.2 knockdown myocytes measured before the addition of metabolic inhibition solution was significantly elevated compared with control myocytes. Figure 3D shows the percentage of cells that did not recover Ca2+ homeostasis (indicated by estimated intracellular Ca2+ > 350 nM) after metabolic inhibition solution followed by 10 min of reperfusion. After metabolic inhibition solution and reperfusion, 76% of myocytes infected with control shRNA recovered contractile activity; in contrast, only 33% myocytes infected with Kir6.2 shRNA-A recovered contractile function (Fig. 3E). The results from experiments using shRNA-B to knock down Kir6.2 were very similar to those for shRNA-A virus-infected myocytes (data not shown).

In summary, acute knockdown of Kir6.2 resulted in a significant increase in estimated cellular Ca2+ levels at the beginning of the experiment (baseline) and at the end of reperfusion (after 10 min of reperfusion). The proportion of myocytes that could regain contractile function was significantly decreased by Kir6.2 knockdown.
**Effect of dominant negative Kir6.2 on Ca\(^{2+}\) homeostasis.**

The metabolic enzymes that couple with K\(_{\text{ATP}}\) channels are known to influence the cardiac myocytes response to stress and the pathophysiology of ischemic heart disease (2). The role of the metabolic signaling complex was assessed by expressing a nonconducting Kir6.2 (dominant negative) subunit in adult ventricular myocytes using an adenoviral vector. In this way, the effect of prevention of the ion conduction properties of the K\(_{\text{ATP}}\) channels while leaving the scaffolding properties of the ion channel intact was tested.

Untreated and control virus-infected myocytes were held at 0 mV. Current was observed upon application of the K\(_{\text{ATP}}\) channel opener P1075, and the current was blocked by application of the specific K\(_{\text{ATP}}\) inhibitor glibenclamide. Expression of Kir6.2 shRNA-A or Kir6.2 shRNA-B sequences. Currents were recorded at 0 mV, and P1075 (10 \(\mu\)M) and glibenclamide (10 \(\mu\)M) were bath applied, as indicated by the horizontal bars. **Fig. 1.** Short hairpin (sh)RNA knockdown of ATP-sensitive K\(^+\) (K\(_{\text{ATP}}\)) currents in cardiac myocytes. A: whole cell recordings of K\(_{\text{ATP}}\) currents from isolated ventricular myocytes after 24-h infection with adenovirus expressing either control shRNA, Kir6.2 shRNA-A, or Kir6.2 shRNA-B sequences. Currents were recorded at 0 mV, and P1075 (10 \(\mu\)M) and glibenclamide (10 \(\mu\)M) were bath applied, as indicated by the horizontal bars. B: whole cell voltage-clamp recordings of voltage-gated Ca\(^{2+}\) currents from single ventricular myocytes after 24-h infection with either control shRNA, Kir6.2 shRNA-A, or Kir6.2 shRNA-B adenoviruses. Ca\(^{2+}\) currents were evoked by a 240-ms voltage step from −40 to 0 mV. C: peak K\(_{\text{ATP}}\) currents normalized for cell size (in pA/pF) of myocytes infected with control shRNA \((n = 17)\), Kir6.2 shRNA-A \((n = 12)\), or Kir6.2 shRNA-B \((n = 5)\). **P < 0.01 vs. control. D:** mean peak Ca\(^{2+}\) currents normalized for myocyte size (in pA/pF) recorded from cardiomyocytes infected with either control shRNA, Kir6.2 shRNA-A, or Kir6.2 shRNA-B adenoviruses \((n = 3\) for each). E: representative Western blots showing Kir6.2 protein from isolated ventricular myocytes after 24-h infection expressing control shRNA, Kir6.2 shRNA-A, or Kir6.2 shRNA-B. Kir6.2 shRNA-A, or Kir6.2 shRNA-B. Total ERK was used as a loading control. **F:** average densitometry showing the effects of Kir6.2 shRNA on Kir6.2 protein levels. **P < 0.01.** Stats were done on raw densitometry values.

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of nonconducting Kir6.2 for 24 h resulted in a decrease of ~50% of the K<sub>ATP</sub> current compared with GFP-expressing control virus-infected myocytes (Fig. 4, A and B). Expression of either dominant negative Kir6.2 or Kir6.2-targetting shRNA resulted in comparable patterns of intracellular Ca<sup>2+</sup> changes both before and after metabolic inhibition solution and reperfusion challenge; intracellular Ca<sup>2+</sup> levels were significantly higher both at baseline and after reperfusion compared with control virus-infected myocytes.

**Effect of Kir6.2 knockdown on mitochondrial membrane potential.** The metabolic signaling complex that couples to the K<sub>ATP</sub> channel forms a phsophotransfer bridge to the mitochondria, allowing for high-fidelity coupling of mitochondrial ATP to the excitability of ventricular myocytes. To test the effect of knockdown of Kir6.2 on mitochondrial function, adenovirus-infected cardiac myocytes were loaded with a high concentration of TMRM (5 µM) to access mitochondrial membrane potential. For myocytes infected
Fig. 3. Effect of Kir6.2 knockdown on Ca^{2+} homeostasis and contractile recovery after metabolic stress/reperfusion. A: simultaneous recordings of the indo-1 ratio from six control virus-infected myocytes within a field of view during 5-min perfusion with normal Tyrode solution followed by 4 min of metabolic inhibition (MI) solution and 10 min of reperfusion in normal Tyrode solution. Each trace is the ratio recorded from a different myocyte. Fields of myocytes were stimulated at 1 Hz, and images were sampled at 0.1 Hz. These Ca^{2+} transients ceased when the stimulator was turned off at the end of 10 min of reperfusion. B: recordings of the indo-1 ratio from six Kir6.2 shRNA-A virus-infected myocytes, as in A. C: averaged estimated Ca^{2+} concentrations for the protocol described in A. For the myocyte populations infected with control (n = 41, 7 experiments) or Kir6.2 shRNA-A virus (n = 77, 11 experiments). D: percentage of myocytes with an estimated diastolic intracellular Ca^{2+} concentration ([Ca^{2+}]_i) of >350 nM before the bath application of MI solution (baseline) and after 10 min of reperfusion with Tyrode solution. ***Statistical significance at P < 0.001 between control and Kir6.2 RNA-A virus-infected myocytes measured at the end of reperfusion. E: percentage of myocytes recovering a contractile response to electrical field stimulation (1 Hz) after 4 min of metabolic inhibition followed by 10 min of reperfusion. Cardiac myocytes were infected for 24 h with either control virus (n = 228 cells, 14 experiments), Kir6.2 shRNA-A virus (n = 79 cells, 10 experiments), or Kir6.2 shRNA-B virus (n = 126, 17 experiments). **P < 0.01 vs. control.
with the control virus, the fluorescence recorded was initially low because the TMRM fluorescence was quenched in the mitochondria; however, after a period of continuous illumination, ROS accumulation caused a depolarization of the mitochondrial membrane potential, which was observed as an increase in fluorescence. Typical TMRM images from this assay are shown in Fig. 5A. The warmer colors (yellow and red) illustrate high-intensity fluorescence, which occurs on mitochondrial membrane potential depolarization. In contrast, knockdown of Kir6.2 resulted in oscillations in the fluorescence intensity before the final increase in fluorescence intensity. Figure 5B shows typical fluorescence traces of TMRM-loaded myocytes under continuous illumination over the time. Knockdown of Kir6.2 resulted in a number of changes in mitochondrial function. First, the baseline TMRM fluorescence of Kir6.2 shRNA virus-infected myocytes was significantly higher compared with control virus-infected myocytes (Fig. 5, B and C), indicating a reduced mitochondrial membrane potential. Second, knockdown of Kir6.2 significantly increased the number of myocytes that had mitochondrial membrane potential oscillations before the final and irreversible mitochondrial depolarization (Fig. 5, B and D).

**DISCUSSION**

The K$_{ATP}$ channel pore-forming subunit Kir6.2 was knocked down in isolated rat ventricular myocytes using adenoviral transfer of silencing RNA targeted to Kir6.2. This resulted in a decrease in total Kir6.2 protein compared with control after 24 h and a significant decrease (~50%) in sarcolemmal K$_{ATP}$ current. Ca$_{2+}$ currents were unaffected by Kir6.2 shRNA adenoviral infection, clarifying specificity to K$_{ATP}$.

To investigate whether knockdown of Kir6.2 affected IPC in ventricular myocytes, the ischemic pelleting technique was used as this has been suggested to provide a realistic model of IPC in isolated myocytes (8). The IPC response was abolished when Kir6.2 was knocked down in cardiac myocytes. While this is consistent with previous knockout studies (22, 44), it shows that even a modest reduction in channel expression is sufficient to block this effect. In the present study, the knockdown of Kir6.2 was acute, allowing little possibility for compensation, which may occur in transgenic mice. Interestingly, knockdown of Kir6.2 markedly reduced the proportion of myocytes surviving ischemia. This, again, is consistent with the inability of Kir6.2 knockout mice to adapt to stress situations such as ischemia, exercise exertion, or β-adrenergic challenge (54), but in this study with acute rather than chronic...
Kir6.2 is required for Ca^{2+} and mitochondrial homeostasis

Fig. 5. Changes in mitochondrial membrane potential of myocytes infected with control or Kir6.2 shRNA-A adenovirus. A: example time series showing fluorescence images of representative ventricular myocytes loaded with 5 μM tetramethylrhodamine methyl ester (TMRM) subjected to laser-induced oxidative stress. Time 0 is before oxidative stress. Mitochondrial depolarization is indicated by the increase in the intensity of fluorescence (increase in warm colors, e.g., red indicating the highest and blue the lowest fluorescence intensity). B: typical TMRM fluorescence intensity traces (in arbitrary units) recorded from myocytes infected with control (solid line) or Kir6.2 shRNA-A (shaded line) adenovirus for 24 h. C: baseline TMRM fluorescence (in arbitrary units) recorded at the beginning of the experiment from myocytes infected with control (n = 157) and Kir6.2 shRNA-A (n = 174) for 24 h. **P < 0.001. D: percentage of myocytes that displayed mitochondrial membrane potential oscillations before the final depolarization for myocytes infected with control adenovirus (n = 77 myocytes, 20 experiments) compared with myocytes infected with Kir6.2 shRNA-A (n = 84 myocytes, 19 experiments) for 24 h. **P < 0.01.

and partial rather than total ablation of K_{ATP} channels. However, it is in contrast to the observation that whole hearts from Kir6.2 knockout mice yielded the same recovery of function and had the same infarct size in response to global ischemia as littermate controls (48). This suggests there is more to understand about the role of K_{ATP} channels in response to physiological and pathophysiological stresses.

During metabolic stress, such as ischemia-reperfusion injury, myocytes become overloaded with Ca^{2+} and maintaining Ca^{2+} homeostasis becomes increasingly energy consuming. The opening of the sarcolemmal K_{ATP} channels has been proposed to oppose this Ca^{2+} overload by hyperpolarization of the membrane potential (13, 36). K_{ATP} channels are an abundant protein in cardiac myocytes, and <1% of K_{ATP} channels may need to open to hyperpolarize the membrane potential and thus prevent Ca^{2+} loading (35, 46). This would suggest that only very extreme changes in K_{ATP} channel expression would result in altered myocyte responses to stress. However, despite the Kir6.2 silencing RNA only reducing the K_{ATP} current by ~50% in this study, Ca^{2+} overload was increased in response to metabolic inhibition solution and reperfusion. This may be because the reduction in K_{ATP} channel number decreases the channel density in caveolin-rich microdomains, which could affect responses to local changes in ATP concentration (17).

It is interesting to note that there was a significant increase in the basal estimated [Ca^{2+}], before any metabolic challenge in the Kir6.2 knockdown myocyte population. This suggests that basal Ca^{2+} homeostasis is perturbed by a decrease in K_{ATP} channel density and that, in addition to their pronounced effects during metabolic stress, sarcolemmal K_{ATP} channels may play a role in Ca^{2+} homeostasis under more normal physiological conditions. This increased basal [Ca^{2+}], may be a result of increased action potential duration, as both Kir6.2 knockout mouse hearts (20) and hearts from normal mice treated with the K_{ATP} blocker tolbutamide (10) show increased action potential duration. Elevated [Ca^{2+}], may also account for the upregulation of calcineurin- and Ca^{2+}-dependent transcription factors in Kir6.2 knockout mice (49). Indeed, it has been suggested that transgenic mice expressing an ATP-insensitive Kir6.2 mutant that is more active in vivo than wild type compensate for the change in K_{ATP} activity by increasing Ca^{2+} current density (12). However, in this study, we found no change in the Ca^{2+} current density.
Despite increased baseline estimated [Ca\(^{2+}\)], all myocytes showed contractile function at the beginning of the experiments, which is consistent with observations in other Kir6.2 knockout studies (44, 52). This might be because the increase in [Ca\(^{2+}\)] was not sufficient to inhibit contraction. During metabolic inhibition solution, the Kir6.2 knockout myocyte population continued contracting for ~2 min longer than the control population due to the loss of K\(_{\text{ATP}}\) channel-dependent loss of excitability, which is also consistent with the prolonged contractility reported for Kir6.2 knockout hearts (39).

In addition to conducting K\(^+\) efflux, K\(_{\text{ATP}}\) channels form a signaling complex with creatine kinase and adenylate kinase, which forms a phosphotransfer bridge connecting the mitochondria to the sarcolemmal membrane (1, 41). It is possible that a decrease in K\(_{\text{ATP}}\) channel density might disrupt this phosphotransfer bridge (6, 45). Indeed, knockout of creatine kinase from cardiac myocytes causes uncoupling of metabolic signals such that K\(_{\text{ATP}}\) channels open significantly earlier in response to metabolic stress (41). This may also occur in heart failure, when the ability of K\(_{\text{ATP}}\) channels to respond to metabolic challenge is lost due to deficits in the creatine kinase network (26). However, it is unclear whether K\(^+\) current is necessary for the adaptive response to stress or whether the K\(_{\text{ATP}}\) channel and surrounding metabolic signaling complex is sufficient for protection (6). To address this issue, a nonconducting dominant negative mutant of Kir6.2 was delivered by adenovirus to adult rat cardiac myocytes. Expression of the nonconducting dominant negative mutant of Kir6.2 resulted in a ~50% reduction of K\(_{\text{ATP}}\) current density. The response to metabolic inhibition solution and reperfusion was like that of shRNA knockdown myocytes in that there was a significant increase in baseline intracellular Ca\(^{2+}\) in addition to increased Ca\(^{2+}\) overload after metabolic inhibition solution and reperfusion. This suggests that K\(^+\) flux is important in the prevention of Ca\(^{2+}\) overload, presumably by hyperpolarizing the membrane potential. Another study (52) with cardiac myocytes expressing dominant negative Kir6.2 showed K\(_{\text{ATP}}\) current reduction by ~85%, which resulted in a slowed rate of action potential duration shortening, again highlighting a role for K\(_{\text{ATP}}\) channels in adaptation to metabolic stress.

The opening of the mPTP is the point of no return in myocyte death after ischemia-reperfusion injury (24). To investigate whether mitochondrial responses to stress were altered by Kir6.2 knockdown, a well-established assay using a high concentration of TMRM and oxidative stress to induce mPTP opening was used (11, 24). First, this experiment showed that TMRM fluorescence was significantly higher in Kir6.2-downregulated myocytes. This suggests a more depolarized mitochondrial membrane potential compared with control myocytes and shows a link between K\(_{\text{ATP}}\) channels and mitochondrial membrane potential under baseline conditions. Second, this experiment showed that knockdown of Kir6.2 resulted in a significant increase in the number of myocytes that showed mitochondrial membrane potential oscillations before the final and irreversible mitochondrial depolarization compared with control myocytes. This has also recently been shown to occur in intact hearts in response to ischemia-reperfusion injury (31).

The oscillations occur in metabolic stress because to maintain a hyperpolarized mitochondrial membrane potential, F\(_{1}\)/F\(_{0}\)-ATPase reverses and consumes ATP (28), but when all the ATP is consumed, the mitochondrial membrane potential depolarizes (5, 47, 50). The glycolytic enzyme phosphofructokinase is sensitive to ATP and exaggerates the oscillations in the cellular ATP-to-ADP ratio until all the glucose is exhausted (50). This has been shown in cardiac myocytes near anoxia when the mitochondrial membrane potential oscillates in synchrony with glycolysis (15). The downregulation of K\(_{\text{ATP}}\) channels could lead to a loss in the finely balanced connection between energy homeostasis and excitability afforded by the metabolic signaling complex. However, it is also possible that the increase in basal [Ca\(^{2+}\)], observed in Kir6.2 knockdown myocytes could increase mitochondrial Ca\(^{2+}\) loading and depolarizes the mitochondrial membrane potential by activation of Ca\(^{2+}\)-sensitive respiratory enzymes (7, 19, 28). Inhibition of K\(_{\text{ATP}}\) channels has also been shown to cause mitochondrial Ca\(^{2+}\) loading (32) and enhance mPTP opening (42).

In addition to sarcolemmal K\(_{\text{ATP}}\) channels, a role for mitochondrial K\(_{\text{ATP}}\) channels has been proposed in preconditioning through K\(^+\) influx into the matrix of the mitochondria (18). However, the present study was unable to distinguish whether the effects are due to loss of one or both the mitochondrial and sarcolemmal K\(_{\text{ATP}}\) channels. Recently, the mitochondrial K\(_{\text{ATP}}\) channel has been found to be composed of ROMK1 as the pore-forming subunit rather than Kir6.1 or Kir6.2, which lends support to the notion that the effects observed here are due to a decrease in sarcolemmal rather than mitochondrial K\(_{\text{ATP}}\) channel density (14).

In summary, this study shows that K\(_{\text{ATP}}\) channel density is important for Ca\(^{2+}\) homeostasis both before and after metabolic stress. Kir6.2 plays a role in the protective effect of IPC and protection during ischemia, as both were lost when Kir6.2 was knocked down. Kir6.2 influences mitochondrial membrane potential at rest and prevents mitochondrial membrane potential oscillations during oxidative stress. The K\(_{\text{ATP}}\) channel plays a central role in IPC and Ca\(^{2+}\) and mitochondrial homeostasis during stress, and the impact of decreasing channel number is deleterious to myocyte survival.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

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