Inhibition of inward rectifier K⁺ channels under ischemic conditions may contribute to electrophysiological consequences of ischemia such as cardiac arrhythmia. Ischemia causes metabolic inhibition, and the use of metabolic inhibitors is one experimental method of simulating ischemia. The effects of metabolic inhibitors on the activity of inward rectifier K⁺ channels Kir2.1, Kir2.2, and Kir2.3 were studied by heterologous expression in Xenopus oocytes and two-electrode voltage clamp. 10 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) inhibited Kir2.2 and Kir2.3 currents but was without effect on Kir2.1 currents. The rate of decline of current in FCCP was faster for Kir2.3 than for Kir2.2. Kir2.3 was inhibited by 3 mM sodium azide (NaN₃), whereas Kir2.1 and Kir2.2 were not. Kir2.2 was inhibited by 10 mM NaN₃. All three of these inward rectifiers were inhibited by lowering the pH of the solution perfusing inside-out membrane patches. Kir2.3 was most sensitive to pH (pK = 6.9), whereas Kir2.1 was least sensitive (pK = 5.9). For Kir2.2 the pK was 6.2. These results demonstrate the differential sensitivity of these inward rectifiers to metabolic inhibition and internal pH. The electrophysiological response of a particular cell type to ischemia may depend on the relative expression levels of different inward rectifier genes.

Inward rectifier K⁺ channels are known to be involved in the electrophysiology of several different tissues, including the heart (1). In fast response myocardial tissue they are responsible for the relatively negative value of the resting membrane potential and the late rapid repolarization phase of the action potential (2). Their characteristic property of inward rectification allows the myocardial cell to generate long lasting action potentials while minimizing ion fluxes (3).

Ischemia produces electrophysiological changes that can lead to the generation of cardiac arrhythmias (4) or cerebral excitoxicity (5). Simulated ischemia has been shown to inhibit an inwardly rectifying current in isolated cardiac myocytes (6). This effect may contribute to arrhythmogenesis and is therefore worthy of further study. Three closely related inward rectifier genes, Kir2.1, Kir2.2, and Kir2.3, are expressed in myocardium (7, 8), so each of these could potentially be involved in the aforementioned ischemic response in the heart. These genes are also expressed in the brain (9–11). In this report we have simulated ischemia by applying metabolic inhibitors to Xenopus oocytes expressing these three inward rectifier K⁺ channels. We show that Kir2.1, Kir2.2, and Kir2.3 are affected differently by metabolic inhibition. We also show that the sensitivity of Kir2.2 to internal pH is intermediate between that of Kir2.1 and Kir2.3.

**EXPERIMENTAL PROCEDURES**

**Subcloning and in Vitro Transcription**—Complementary DNAs encoding Kir2.1 (IRK1) (9), Kir2.2 (MB-IRK2) (11), and Kir2.3 (MB-IRK3) (10) were subcloned into the Xenopus expression vector pGEMHE (12). Plasmids were linearized with NheI and RNA was transcribed in vitro with T7 RNA polymerase (mMessage mMachine, Ambion, Austin, TX). RNA yield and integrity were assessed by agarose-ethidium bromide gel electrophoresis.

**Oocyte Isolation**—Stage V–VI oocytes were surgically removed from Xenopus laevis frogs (Nasco) under anesthesia (0.03% benzocaine for 10–15 min) and incubated with 1.3 mg/ml collagenase (Worthington, type CLS3) for 2 h at 22 °C in 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.4, with agitation to remove connective tissue. Oocytes were then washed several times with 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.4, and stored in the same solution at 18 °C. Both solutions also contained 100 μg/ml streptomycin and 60 μg/ml ampicillin. Between 16 and 24 h later, oocytes were injected (Nanoliter; World Precision Instruments) with amounts of RNA that gave approximately equal expression levels (0.2 ng of Kir2.1, 4 ng of Kir2.2, and 4 ng of Kir2.3) in 50 nl of nuclease-free water.

**Two-electrode Voltage Clamp**—Inward rectifier currents were recorded 1–2 days after injection using a TEC-03 amplifier (npi, Germany) controlled by Pulse 8.4 software (Heka, Germany) via an ITC-16 computer interface (Instrutech, Long Island, NY). Currents were filtered at 500 Hz and digitized at 1 kHz. Data were analyzed using Pulse 8.4 and Prism 3.02 (GraphPad). Microelectrode pipettes had resistances of 0.5–1.5 MΩ. Oocytes were placed in a 100-μl volume recording chamber that was continuously perfused at a rate of ~1.2 ml/min. Different solutions entered the chamber via a common outlet with a dead space of ~50 μl. Under control conditions oocytes were perfused with “90K” solution: 90 mM KCl/KOH, 3 mM MgCl₂, 5 mM HEPES, pH 7.4. Sodium azide (NaN₃) and BaCl₂ were dissolved in aliquots of this solution as solid. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (ICN, Costa Mesa, CA) was dissolved in dimethyl sulfoxide (Me₂SO) to 100 mM. This stock solution was diluted into 90K.

**Patch Clamp Recording**—Inward rectifier currents were recorded in “giant” inside-out patches from Xenopus oocytes (13) 3–9 days after injection with Kir2.2x RNA. The patch clamp amplifier was an Axopatch 200B (Axon Instruments). Currents were filtered at 1 kHz, and data were acquired at 4.76 kHz with a Digidata 1320A computer interface and pClamp 8 software (Axon Instruments). Data were analyzed using pClamp 8 and Prism 3.02 (GraphPad). Patch pipettes had inner tip diameters of 20–25 μM and contained a “FVPP” type solution (14) (in mM): 40 KCl, 75 potassium gluconate, 5 KF, 0.1 mM NaVO₃, 10 sodium pyrophosphate, 1 mM EGTA, 10 glucose, 0.1 spermine, 10 PIPES, pH 7.4, with HCl. The recording chamber contained this same solution. Inside-out patches were perfused with this solution and with aliquots of

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Inward Rectifiers and Metabolic Inhibitors

**RESULTS**

**Effects of FCCP on Inward Rectifier Currents in Intact Oocytes**—Inward rectifier currents were monitored by means of a continuous two-electrode voltage clamp protocol. Membrane potential was held at 0 mV and at 20-ms intervals was stepped to 50 mV for 350 ms. Expression of inward rectifier channels was confirmed by brief application of 20 mM BaCl₂, which completely blocks Kir2.1, Kir2.2, and Kir2.3. Inward rectifier currents were recorded by two-electrode voltage clamp as in Figs. 1 and 2. The current recorded at −50 mV was used for this analysis. Oocytes were perfused with 90K plus 10 μM FCCP or 90K alone (plus 0.01% Me₂SO) for 15 min. The perfusate was then switched to 90K plus 20 mM BaCl₂. The current remaining in the presence of BaCl₂ was subtracted from currents recorded at the beginning and end of the 15-min interval. The BaCl₂-sensitive current at the end of the 15-min interval was normalized to the BaCl₂-sensitive current at the beginning of the 15-min interval. A standard t test was used to compare pairs of data groups. p values are as follows: p < 0.005 for column 1 versus column 2, column 2 versus column 3, and column 2 versus column 4; p < 0.0001 for column 3 versus column 5. Bars represent means ± S.E. from 6 oocytes.

In Fig. 1. To rule out the unlikely possibility that the decline of Kir2.2 and Kir2.3 currents was because of spontaneous “run-down,” we perfused oocytes expressing these clones with 90K (plus 0.01% Me₂SO) for prolonged periods without switching to FCCP. As shown by the examples in Fig. 2, spontaneous rundown did not occur. Indeed, in some cases (as in Fig. 2) the current had a tendency to slowly increase. This was not because of an increase in leak current, as demonstrated by applying 20 mM BaCl₂.

Because the rate of decrease of inward rectifier currents in FCCP was quite slow, we chose an arbitrary time point of 15

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**FIG. 1.** Examples of FCCP application to *Xenopus* oocytes expressing Kir2.1, Kir2.2, or Kir2.3. FCCP (10 μM) was applied in 90K solution following 1 min, 40 s of perfusion with 90K alone (plus 0.01% Me₂SO). Inward rectifier current was monitored by two-electrode voltage clamp. At 20-s intervals the membrane potential was stepped from the holding potential of 0 mV to 50 mV for 75 ms and then to −50 mV for 350 ms. The data in panel A represent the current recorded at the end of the −50 mV step. After 15 min of FCCP application the oocytes were briefly exposed to 20 mM BaCl₂, which was added to the 90K solution as BaCl₂. Panels B, C, and D show current traces obtained at the times indicated by the letters a, b, and c, for Kir2.1, Kir2.2, and Kir2.3, respectively. The horizontal line indicates zero current level.

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**FIG. 2.** Examples of prolonged recordings of Kir2.2 and Kir2.3 currents in the continued presence of 90K (plus 0.01% Me₂SO). The voltage clamp protocol was the same as in Fig. 1. The data in panel A represent the current recorded at −50 mV. The oocytes were briefly exposed to 20 mM BaCl₂ at the times indicated. Panels B and C show current traces obtained at the times indicated by the letters a, b, and c, for Kir2.2 and Kir2.3, respectively. The horizontal line indicates zero current level.

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**FIG. 3.** Comparison of the effects of FCCP on Kir2.1, Kir2.2, and Kir2.3. Currents were recorded by two-electrode voltage clamp as in Figs. 1 and 2. The current recorded at −50 mV was used for this analysis. Oocytes were perfused with 90K plus 10 μM FCCP or 90K alone (plus 0.01% Me₂SO) for 15 min. The perfusate was then switched to 90K plus 20 mM BaCl₂. The current remaining in the presence of BaCl₂ was subtracted from currents recorded at the beginning and end of the 15-min interval. The BaCl₂-sensitive current at the end of the 15-min interval was normalized to the BaCl₂-sensitive current at the beginning of the 15-min interval. A standard t test was used to compare pairs of data groups. p values are as follows: p < 0.005 for column 1 versus column 2, column 2 versus column 3, and column 2 versus column 4; p < 0.0001 for column 3 versus column 5. Bars represent means ± S.E. from 6 oocytes.
 Effects of Sodium Azide on Inward Rectifier Currents in Intact Oocytes—To gain further evidence that the effect of FCCP was because of metabolic inhibition, we tested another metabolic inhibitor, sodium azide (NaN₃) (16, 17). Voltage clamp experiments were carried out using the same protocol as that used for the FCCP experiments. As shown in Fig. 4, Kir,2,3 currents decreased significantly during 15 min of exposure to 3 mM NaN₃, whereas Kir,2,1 and Kir,2,2 currents were unaffected by this treatment. However, Kir,2,2 currents were inhibited by 10 mM NaN₃.

Effects of Internal pH on Inward Rectifier Currents in Inside-out Patches—It has been reported that metabolic inhibitors decrease intracellular pH (18, 19). It has also been reported that Kir,2,3 is more sensitive than Kir,2,1 to decreased intracellular pH (14). Therefore the sensitivity of Kir,2,3 to metabolic inhibitors compared with Kir,2,1 is consistent with a decrease in intracellular pH caused by metabolic inhibition. To investigate whether the intermediate sensitivity of Kir,2,2 to metabolic inhibitors could be explained in terms of pH sensitivity, we compared the dose response of the three inward rectifiers to internal pH in inside-out membrane patches. As shown in Fig. 5, the sensitivity of Kir,2,2 to decreasing pH was intermediate between that of Kir,2,1 and Kir,2,3.

**DISCUSSION**

Acute ischemia produces many changes in the intracellular environment. These changes affect the activity of several different ion channels, leading to cellular electrophysiological alterations that can produce arrhythmias in the heart (4) or excitotoxicity in the brain (5). Inward rectifier K⁺ channels play an important role in the electrophysiology of the cardiac myocyte (2, 20), and inhibition of their activity will have a significant and possibly arrhythmogenic effect (4). Acute ischemia produces a shortening of the action potential because of activation of ATP-sensitive K⁺ channels preceded in some cases by a lengthening. The lengthening has been attributed to inhibition of the transient outward current accompanied by inhibition of the inward rectifier current in some preparations (6) but not others (21). Further studies are required to determine the contribution of inward rectifier channels to changes in action potential duration during ischemia relative to other channel types.

The results presented here suggest that the effect of ischemia on inward rectifier current will depend on the molecular identity of the channels. We have demonstrated that the inward rectifiers Kir,2,1, Kir,2,2, and Kir,2,3 are differentially sensitive to inhibition of cellular metabolism. Because cellular metabolism is inhibited in ischemia (4), our experimental conditions simulate some of the conditions prevailing during ischemia. Therefore it seems reasonable to propose that of these three related inward rectifiers Kir,2,3 will be inhibited the most under ischemic conditions, whereas Kir,2,1 will be inhibited the least.

One of the intracellular changes that occurs during ischemia and metabolic inhibition is a decrease in pH (4, 18, 19). In this...
study, the order of sensitivity of Kir2.1, Kir2.2, and Kir2.3 to metabolic inhibition was the same as the order of sensitivity to lowering internal pH. However, the relative sensitivity to metabolic inhibition cannot be accounted for by a change in internal pH alone. For example, the degree of inhibition of Kir2.2 after 15 min of exposure to 10 μM FCCP (15%; Fig. 3, column 2) corresponds to an internal pH of 6.5 (Fig. 5D), whereas the degree of inhibition of Kir2.3 after 15 min of exposure to 10 μM FCCP (38%; Fig. 3, column 3) corresponds to an internal pH of 7.0 (Fig. 5D). Therefore our results suggest the influence of other intracellular consequences of metabolic inhibition. For example, metabolic inhibitors have been shown to deplete intracellular ATP in Xenopus oocytes (22).

Experimental data and simulations indicate that a change of a few nanosiemens in the K+ conductance of a cardiac myocyte can significantly affect action potential duration (23). Therefore, inhibition of only a fraction of the ~150 nanosiemens inward rectifier conductance in the cardiac myocyte (24) may have a significant effect in ischemia. In the heart the inward rectifier current (I_K1) is probably because of expression of both Kir2.1 and Kir2.2 with a small or no contribution from Kir2.3, depending on the species (7–11, 25). However, the relative functional expression levels of these different channels are not known accurately. Inward rectifier mRNA levels have been measured in human heart, but they may not reflect expression at the functional level (8). Differences in the relative expression levels of these genes in different anatomical locations in the heart may produce spatial disparities in the refractory period in response to ischemia. Such situations are proarrhythmic (4). Details of the spatial distributions of inward rectifier gene expression in the heart are unknown at present. As an added complication, it is possible that Kir2.2 exists as a heteromultimer with Kir2.1 (25). Although Kir2.3 may not contribute much to I_K1 in the heart, its location in neurons (26, 27) raises the possibility of a role in ischemia-induced excitotoxicity.

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