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Abstract—Mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channels have been suggested as triggers and end effectors in myocardial ischemic preconditioning. However, the intracellular mechanism regulating mitoK$_{ATP}$ channels remains unclear. In the present study, mitoK$_{ATP}$ channels from bovine ventricular myocardium were reconstituted using planar lipid bilayers, and the effect of superoxide ($O_2^{-\cdot}$) on the activity of these reconstituted channels was examined. After incorporation, a potassium-selective current was recorded. The mean conductance of this current was 56 pS at 150 mmol/L KCl, which was substantially inhibited by 1 mmol/L MgATP. 5-Hydroxydecanoate (5-HD, 10 to 100 μmol/L), a selective mitoK$_{ATP}$ antagonist, reduced the open state probability (NPo) of these channels in a concentration-dependent manner, whereas diazoxide (10 μmol/L), a selective mitoK$_{ATP}$ agonist, significantly increased channel activity. HMR-1098 (100 μmol/L), a selective sarcolemmal K$_{ATP}$ antagonist, had no effect on the activity of reconstituted channels. Addition of xanthine/xanthine oxidase (100 μmol/L per 0.038 U/mL), an $O_2^{-\cdot}$-generating system, resulted in a marked activation of mitoK$_{ATP}$ channels; the NPo of the channels was increased from 0.60±0.10 to 1.94±0.02. This $O_2^{-\cdot}$-induced channel activation was completely abolished by pretreatment with 5-HD (100 μmol/L) or a sulfhydryl alkylating compound, *N*-ethylmaleimide (2 mmol/L). It is concluded that myocardial mitoK$_{ATP}$ channels can be reconstituted into lipid bilayers and that $O_2^{-\cdot}$ activates these channels. The effect of $O_2^{-\cdot}$ may be associated with its direct action on the sulfhydryl groups of the channel protein. **(*Circ Res*. 2001;89:1177-1183.)**

Key Words: ATP-sensitive K^+ channel ■ mitochondria ■ superoxide ■ heart ■ channel reconstitution

Single or multiple brief periods of myocardial ischemia confer protection against infarction produced by a subsequent prolonged ischemia, a phenomenon termed ischemic preconditioning (IPC).[1] Although a variety of intracellular signaling pathways and molecules have been implicated in the protective effect of IPC, there is substantial evidence suggesting that the adenosine 5′-triphosphate–sensitive potassium (K$_{ATP}$) channel is an important component of this phenomenon and may serve as a distal effector in this process.[2] Cardiac myocytes contain 2 distinct forms of K$_{ATP}$ channels, as follows: the sarcolemmal K$_{ATP}$ (sarcK$_{ATP}$) channel[3] and the mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel.[4] The mitoK$_{ATP}$ channels are present in the mitochondrial inner membrane and modulate mitochondrial function by regulating mitochondrial membrane potential, ion homeostasis, and matrix volume.[5] Recent studies suggest that mitoK$_{ATP}$ channels more importantly contribute to IPC-induced cardioprotection compared with sarcK$_{ATP}$ channels.[6,7] It has been demonstrated that diazoxide, a selective mitoK$_{ATP}$ agonist,[8] mimicked the beneficial effect of IPC to reduce infarct size or cell killing of cardiomyocytes and that sodium 5-hydroxydecanoate (5-HD), a selective mitoK$_{ATP}$ antagonist,[9] blocked the protection produced by IPC and diazoxide.[10–12] In contrast, selective sarcK$_{ATP}$ channel agonists or antagonists were without effect on IPC.[13,14]

Previous work has shown that IPC-induced protection is associated with an early increase in reactive oxygen species (ROS) during the preconditioning period in hypoxic cardiomyocytes.[15,16] Exogenous oxidants have been found to induce preconditioning in the intact heart,[17] whereas antioxidants can block the protective effect of IPC.[18–20] These results suggest that ROS may be involved in the activation of IPC. However, it remains unknown whether the role of ROS in IPC is attributed to the activation of mitoK$_{ATP}$ channels. Given that the mitoK$_{ATP}$ channel may serve as a trigger and distal effector for various intracellular signaling pathways in IPC, we hypothesize that ROS may participate in IPC through activation of mitoK$_{ATP}$ channels. The present study was designed to characterize myocardial mitoK$_{ATP}$ channels using a lipid bilayer reconstitution technique and to determine the effect of ROS on the activity of these reconstituted channels.

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Materials and Methods

Preparation of Submitochondrial Membrane Vesicles

Mitochondria were isolated from bovine hearts by differential centrifugation as reported by Holmuhamedov et al.[5] Briefly, a piece of left ventricular wall was rapidly dissected from fresh bovine hearts obtained from a local abattoir and immersed in ice-cold isolation buffer containing the following (in mmol/L): sucrose 50, mannitol 200, KH_2PO_4 5, EGTA 1, and MOPS 5, and 0.1% BSA (pH 7.15 adjusted with KOH). This myocardial section was transported immediately to the laboratory, weighed, and minced into small pieces. The minced tissue was rinsed clear of blood with cold isolation buffer and transferred to a glass Potter-Elvehjem homogenizing vessel on ice. Three 20-second homogenization cycles were performed on ice, and then the tissue suspension was centrifuged at 750*g* for 10 minutes to remove cellular debris and nuclei. The supernatant containing the mitochondrial fraction was further centrifuged at 8000*g* for 10 minutes, and the pellet was washed twice by resuspension in isolation buffer followed by centrifugation at 8000*g* for 10 minutes. To further purify the mitochondrial preparation, an aliquot of mitochondrial suspension (0.5 mL) was layered on top of 15 mL of a solution consisting of 30% Percoll, 0.25 mol/L sucrose, 1 mmol/L EDTA, and 10 mmol/L HEPES (pH 7.4), followed by centrifugation at 35 000*g* for 30 minutes.[21] The major mitochondrial layer was separated from less-dense contaminants and broken mitochondria, collected with a pipette, and washed twice in 5 mL of the isolation buffer by centrifugation at 8000*g* for 5 minutes. The final mitochondrial pellet was resuspended at 10 to 20 mg protein/mL in the isolation buffer (without EGTA). To prepare the submitochondrial membrane vesicles, the above mitochondrial suspension was sonicated at 55 W for eight 15-second intervals on ice.[22] The membrane preparations were aliquoted, frozen in liquid N_2, and stored at −80°C until used. All procedures were performed at 4°C, and protein concentrations were determined using the Bio-Rad protein assay with BSA as a standard.

Reconstitution of mitoK$_{ATP}$ Channels Into Lipid Bilayers

The mitoK$_{ATP}$ channels in mitochondrial membrane vesicles were reconstituted into a planar lipid bilayer as described by Yarov-Yarovoy et al[23] and our laboratory.[24] A lipid solution of phosphatidylethanolamine and phosphatidylserine (1:1 dissolved in *n*-decane to 20 mg/mL) was used for channel reconstitution. Briefly, lipid bilayers were painted via a glass rod across an aperture (250 μm in diameter) in the wall of a 1.5-mL Delrin cup that was inserted into a cutaway polyvinyl chloride (PVC) block. The cup formed the *trans* compartment, and the remainder of the PVC block formed the *cis* compartment. The *cis* and *trans* compartments were initially filled with 50 mmol/L KCl in 20 mmol/L Tris-HCl buffer, pH 7.2. After bilayer formation, an asymmetric KCl gradient (150 mmol/L KCl *cis* and 50 mmol/L *trans*) was established by replacing an aliquot of 50 mmol/L KCl with 1.25 mol/L KCl, and the submitochondrial membrane vesicles (50 to 100 μg protein) were added into the *cis* side. This KCl gradient was used to facilitate the fusion of channel protein into the lipid bilayer. Fusion was induced by application of 40 to 50 mV across the membrane without $CaCl_2$ in solutions. When channel currents were detected, KCl in the *trans* side was quickly raised to 150 mmol/L to collapse the chemical gradient and prevent further fusion of vesicles into the lipid bilayer.

Recording of mitoK$_{ATP}$ Currents

An integrating bilayer clamping amplifier (model BC-525C, Warner Instrument Corp) was used to record single-channel currents. The *cis* compartment was the voltage control side connected by Ag/AgCl electrode in agar salt bridges to the head stage of the amplifier, while the *trans* side was held at virtual ground. The amplifier output signals were filtered at 1 kHz using an eight-pole Bessel filter (Frequency Devices). Currents were digitized at a sampling rate of 10 kHz and acquired and stored with an IBM PC equipped with a DigiData 1200 AD/DA interface and pClamp 7.0 software (Axon Instruments). Channel open probability (NPo) was determined from recordings of 3 to 5 minutes as described previously in our bilayer clamping studies.[24] A positive current reflects the flow of cations from the *cis* to *trans* compartment or the flow of anions in the opposite direction. The holding potential (V_m) is defined as the electric potential of the *cis* with reference to the *trans* (ground) compartment.

To establish current-voltage relations of reconstituted channels, the lipid bilayer was exposed to a symmetrical (150/150 mmol/L KCl for *cis*/*trans*) or asymmetrical potassium (150/50 mmol/L KCl for *cis*/*trans*) solution. *N*-methyl-D-glucamine was used to compensate for the osmolarity changes of the *trans* solution. The single-channel currents were then recorded while holding potentials were varied from −40 to +40 mV in steps of 20 mV. Na_2ATP with $MgCl_2$ (MgATP, 0.1 to 1 mmol/L); GTP (0.1 to 1 mmol/L); and, in μmol/L, 5-HD (10 to 100), glibenclamide (10 to 100), and diazoxide (10) were used as mitoK$_{ATP}$ activators or inhibitors to characterize reconstituted channels in the lipid bilayer. HMR-1098, a selective sarcK$_{ATP}$ antagonist,[14] was used to distinguish mitoK$_{ATP}$ from sarcK$_{ATP}$ channels. To determine the effect of $O_2^{\cdot-}$ on the activity of mitoK$_{ATP}$ channels, xanthine (100 μmol/L)/xanthine oxidase (0.038 U/mL), a commonly used $O_2^{\cdot-}$-generating system, was used in the absence or presence of 5-HD (100 μmol/L), glibenclamide (100 μmol/L), MgATP (1 mmol/L), or the sulfhydryl alkylating compound *N*-ethylmaleimide (2 mmol/L). Unless otherwise stated, all compounds used were added to the *cis* solution, and the bilayer potential was held at −40 mV. All experiments were performed at room temperature (≈20°C).

Statistics

Data are presented as mean±SEM; the significance of the differences in mean values between and within multiple groups was examined using ANOVA for repeated measures followed by a Duncan multiple-range test. The Student *t* test was used to evaluate statistical significance of differences between two paired observations. $P<0.05$ was considered statistically significant.

Results

Activity of Reconstituted Myocardial mitoK$_{ATP}$ Channels in Lipid Bilayer

Figure 1A shows representative recordings of reconstituted K^+ currents in lipid bilayers under symmetrical K^+ (150/150 mmol/L for *cis*/*trans*) at a holding potential of −40 mV. Under these conditions, a negative voltage was applied to the *cis* side and K^+ moved from *trans* solution to *cis* solution, thereby forming an outward current. This outward current indicates the movement of K^+ from the inside of mitochondria into the cytosol. As shown in Figure 1B, the majority of these K^+ currents (≈85%) had amplitudes of 1.7 to 3.0 pA. Some smaller channel currents (0.7 to 1.6 pA, ≈10%) and bigger channel currents (3.1 to 4.0 pA, ≈5%) were also observed. By determining the voltage dependence of these K^+ currents, it was found that the majority of these currents exhibited rectifying properties (Figure 1C). The amplitude of these currents increased linearly with voltage in the negative range, with a mean slope conductance of 56 pS. In some experiments, small conductance channels (ie, 18 pS) and big conductance channels (ie, 100 pS) were observed at 150 mmol/L KCl. Because these currents were rare in our bilayer preparation, we did not carefully characterize them. The following experiments were focused on the 56-pS channels. In the presence of asymmetrical K^+ (150/50 mmol/L for *cis*/*trans*), the currents exhibited cation selectivity as evidenced by positive currents when the holding potential was at

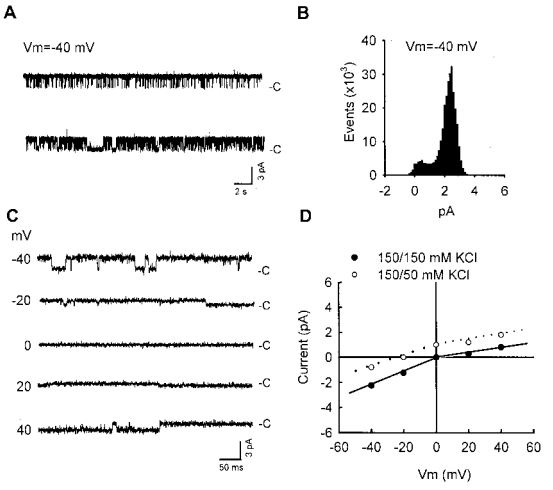

Figure 1. Representative recordings and current-voltage relationship of reconstituted myocardial mitoK$_{ATP}$ channels. A, Recordings of high (NPo=0.96, upper trace) and low (NPo=0.58, lower trace) channel activity in the presence of 150/150 mmol/L KCl. V_m=−40 mV. B, Representative amplitude histogram at V_m=−40 mV. C, Recordings of mitoK$_{ATP}$ currents in the presence of 150/150 mmol/L KCl and at various holding potentials from −40 to 40 mV. D, Current-voltage relations in the presence of 150/150 or 150/50 mmol/L KCl. n=7. C indicates channel closed.

0 mV. The reversal potential, estimated by linear fitting to the zero current, was ≈−23 mV in the presence of 150/50 mmol/L K^+ (Figure 1D). Given the equilibrium potential for K^+ (E_K) of −29 mV, this indicates that this channel obtained from the mitochondrial membrane is primarily permeable to K^+. The NPo of these 56-pS K^+ channels was usually <0.8. Occasionally, the channels were fully open with an NPo >0.95.

Pharmacological Characteristics of Reconstituted Myocardial mitoK$_{ATP}$ Channels

Several K$_{ATP}$ channel agonists or antagonists were used to further characterize and identify the K^+ currents in lipid bilayers as mitoK$_{ATP}$ channels. First, the sensitivity of K^+ currents to ATP and GTP was examined. Figure 2A depicts representative recordings of mitochondrial K^+ currents before and after the sequential addition of MgATP and GTP into the *trans* solution. MgATP at 1 mmol/L resulted in a marked inactivation of the channels within 2 minutes, and the subsequent addition of 1 mmol/L GTP reactivated the channels. MgATP had no significant effect on the channel activity when added to the *cis* solution. Figure 2B summarizes the results of these experiments. The NPo was decreased from 0.76±0.08 in control to 0.20±0.06 in the presence of 1 mmol/L ATP, and then restored to 0.77±0.06 by 1 mmol/L GTP. Lower concentrations of ATP (0.5 mmol/L) or GTP (0.1 to 0.5 mmol/L) were also found to decrease or increase channel activity, respectively (data not shown). Figure 2C shows that ATP (1 mmol/L) inhibited the 1.7- to 3.0-pA currents but not the smaller currents. GTP (1 mmol/L) restored the 1.7- to 3.0-pA currents.

The effects of 5-HD and diazoxide on channel activity were examined. As shown in Figures 3A and 3B, 5-HD (100 μmol/L) added to the *cis* solution resulted in inactivation of the channels within 3 minutes, and channels could be reactivated by diazoxide (10 μmol/L). 5-HD (10 to 100 μmol/L) concentration-dependently decreased the NPo of the reconstituted channels from 0.66±0.11 to 0.09±0.05, and the NPo was restored to 0.71±0.11 by addition of 10 μmol/L diazoxide (Figure 3B). Diazoxide also significantly activated the channels in the absence of 5-HD (data not shown). Like ATP, 5-HD (100 μmol/L) inhibited the 1.7- to 3.0-pA currents, which were restored by diazoxide (10 μmol/L, Figure 3C).

To confirm myocardial mitoK$_{ATP}$ activity of reconstituted channels, the effect of HMR-1098, a selective sarcK$_{ATP}$ antagonist, was examined. HMR-1098 at a concentration up to 100 μmol/L had no significant effect on the activity of the reconstituted channels. The NPo values in the absence and presence of HMR-1098 were 0.63±0.11 and 0.67±0.11, respectively. However, the nonselective K$_{ATP}$ antagonist glibenclamide (10 to 100 μmol/L) inhibited the activity of the reconstituted channels in a concentration-dependent manner; these channels were also reactivated by addition of 10 μmol/L diazoxide (Figure 4A). Figure 4B shows that HMR-1098 (100 μmol/L) had no effect on the 1.7- to 3.0-pA currents. However, glibenclamide (100 μmol/L) inhibited these currents, which were again restored by diazoxide (10 μmol/L, Figure 4C).

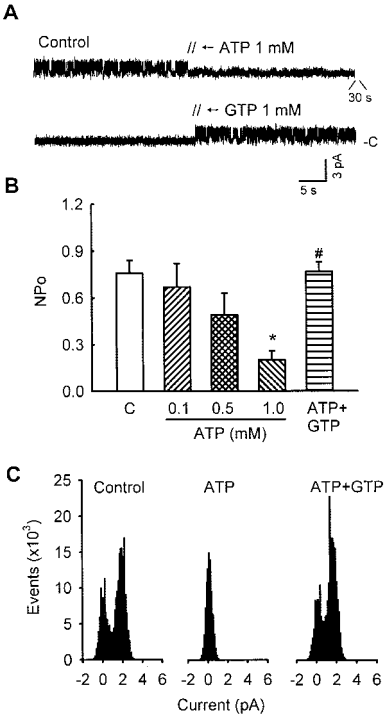

Figure 2. Effect of MgATP and GTP on activity of reconstituted myocardial mitoK$_{ATP}$ channels. A, Representative recordings of mitoK$_{ATP}$ currents under control conditions, after addition of 1 mmol/L MgATP, and after subsequent addition of 1 mmol/L GTP. V_m=−40 mV. B, Summarized data showing NPo of reconstituted mitoK$_{ATP}$ channels in the absence or presence of MgATP (0.1 to 1 mmol/L) or MgATP followed by GTP (1 mmol/L). C, Corresponding amplitude histograms of the same current recordings. *P<0.05 vs control; #P<0.05 vs MgATP. n=8 from 4 animals.

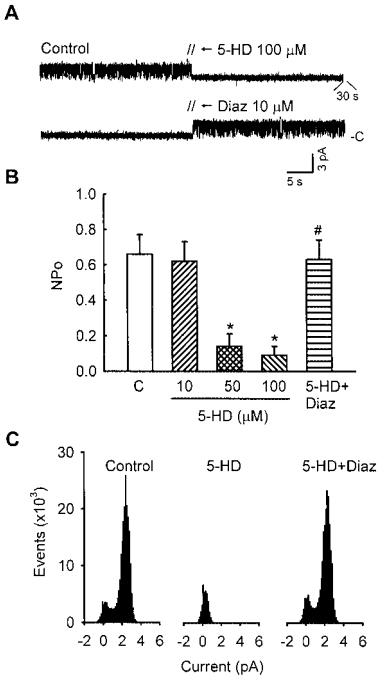

Figure 3. Effect of 5-HD and diazoxide on activity of reconstituted myocardial mitoK$_{ATP}$ channels. A, Representative recordings of mitoK$_{ATP}$ currents under control conditions, after addition of 100 μmol/L 5-HD, and after subsequent addition of 10 μmol/L diazoxide (Diaz). $V_m = -40$ mV. B, Summarized data showing NPo of reconstituted mitoK$_{ATP}$ channels in the absence or presence of 5-HD (10 to 100 μmol/L) or 5-HD followed by diazoxide (10 μmol/L). C, Corresponding amplitude histograms of the same current recordings. *$P<0.05$ vs control; #$P<0.05$ vs 5-HD. n=8.

Effect of $O_2^{-\cdot}$ on the Activity of Reconstituted Myocardial mitoK$_{ATP}$ Channels

Representative recordings depicting the effect of $O_2^{-\cdot}$ on the activity of reconstituted mitoK$_{ATP}$ channels are presented in Figure 5A. Addition of xanthine/xanthine oxidase (100 μmol/L per 0.038 U/mL) to the *cis* solution resulted in a rapid activation of the channels within 1 minute. A stacked opening of these channels was observed in xanthine/xanthine oxidase–treated patches. The NPo of the reconstituted channels was increased from 0.60±0.10 to 1.94±0.02 (Figure 5B). However, neither xanthine nor xanthine oxidase alone had an effect on channel activity. In addition, xanthine/xanthine oxidase had no significant effect on the conductance of the lipid membrane in the absence of channel protein incorporation. Figure 5C shows that xanthine/xanthine oxidase induced large conductance currents (≈4.0 pA). As indicated in Figure 5A, these currents represent the stacked opening of two mitoK$_{ATP}$ channels instead of the opening of a new channel.

Modulation of $O_2^{-\cdot}$ Action on Reconstituted Myocardial mitoK$_{ATP}$ Channels by Antagonists and *N*-Ethylmaleimide

To explore the mechanism of action of $O_2^{-\cdot}$ on mitoK$_{ATP}$ channel activity, mitoK$_{ATP}$ antagonists and *N*-ethylmaleimide were used to modulate $O_2^{-\cdot}$-induced activation of mitoK$_{ATP}$. As shown in Figure 6A, 5-HD (100 μmol/L) completely abolished xanthine/xanthine oxidase–induced activation of mitoK$_{ATP}$ channels. The NPo values in the presence of 5-HD and the subsequent xanthine/xanthine oxidase were 0.14±0.05 and 0.14±0.05, respectively. *N*-Ethylmaleimide (2 mmol/L) had no significant effect on basal channel activity. However, pretreatment with *N*-ethylmaleimide resulted in a complete blockade of xanthine/xanthine oxidase–induced activation of mitoK$_{ATP}$ channels. It was also found that glibenclamide (100 μmol/L) completely blocked mitoK$_{ATP}$ activation by xanthine/xanthine oxidase. In contrast, MgATP (1 mmol/L) was less effective in blocking xanthine/xanthine oxidase–induced channel activation. The NPo in the presence of MgATP and the subsequent xanthine/xanthine oxidase was 0.10±0.08 and 1.66±0.13, respectively. Figure 6B shows that 5-HD (100 μmol/L) and glibenclamide (100 μmol/L) abolished xanthine/xanthine oxidase–activated 1.7- to 3.0-pA currents and the stacked opening of these currents. *N*-Ethylmaleimide (2 mmol/L) also blocked stacked opening of mitoK$_{ATP}$ channels induced by xanthine/xanthine oxidase.

Discussion

In the present study, we first reconstituted mitoK$_{ATP}$ channels from bovine ventricular myocardium into a planar lipid bilayer and examined their electric and pharmacological properties. These channels displayed an electric rectifying

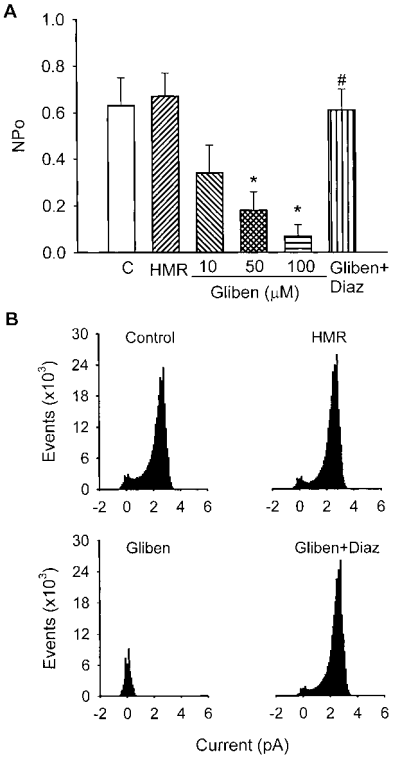

Figure 4. Effect of HMR1098, glibenclamide, and diazoxide on activity of reconstituted myocardial mitoK$_{ATP}$ channels. A, Summarized data showing NPo of reconstituted myocardial mitoK$_{ATP}$ channels in the absence or presence of HMR1098 (HMR, 100 μmol/L), glibenclamide (Gliben, 10 to 100 μmol/L) or glibenclamide followed by diazoxide (10 μmol/L). B, Amplitude histograms of current recordings under control conditions and after addition of HMR 1098 (10 μmol/L), glibenclamide (100 μmol/L), or glibenclamide followed by diazoxide (10 μmol/L). *$P<0.05$ vs control; #$P<0.05$ vs glibenclamide. n=8.

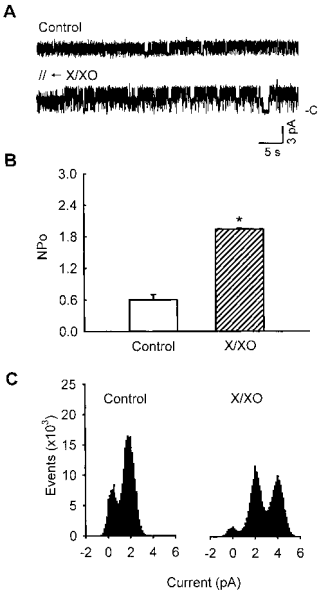

Figure 5. Effect of $O_2^{-\cdot}$ on activity of reconstituted myocardial mitoK$_{ATP}$ channels. A, Representative recordings of mitoK$_{ATP}$ currents under control conditions and after addition of xanthine (X, 100 μmol/L)/xanthine oxidase (XO, 0.038 U/mL). B, Summarized data showing NPo of reconstituted myocardial mitoK$_{ATP}$ channels in the absence or presence of xanthine/xanthine oxidase. C, Corresponding amplitude histograms of the same current recordings. *$P<0.05$ vs control. n=8.

property with a mean conductance of 56 pS under symmetrical KCl (150 mmol/L). The rectifying property of the channels resembled that of mitoK$_{ATP}$ previously isolated from rat liver,[25] but the channels had a larger conductance compared with the mitoK$_{ATP}$ observed in intact liver mitoplasts (9.7 pS)[4] and from rat liver (10 pS at 100 mmol/L KCl).[25,26] In previous studies, the variation in mitoK$_{ATP}$ channel conductance was assumed to be associated with the incorporation of multiple channels to form a cluster and the simultaneous switching of these channels in lipid bilayers.[4,25–27] We also found that the reversal potential of these mitochondrial channels at asymmetrical KCl (150/50 mmol/L) was close to the equilibrium potential for K^+, indicating that these channels are primarily permeable to K^+.

In agreement with the results of previous studies,[4,25–27] the mitoK$_{ATP}$ channel was active under control conditions. In some cases, the channel was fully open. However, there is evidence that mitoK$_{ATP}$ channels are in a closed state in vivo under physiological conditions.[2] The inner mitochondrial membrane potential is estimated to be ≈ -180 mV. To prevent excessive accumulation of cations in the mitochondria, the mitoK$_{ATP}$ channel must be finely regulated in vivo, presumably by ATP or other nucleotides, or long-chain acyl–coenzyme A (CoA) esters.[28] The activity of mitoK$_{ATP}$ channels would control the concentrations of K^+ in the mitochondria and regulate the function of the mitochondria. In response to the ATP decrease, mitoK$_{ATP}$ channels may be activated, which would consequently result in an increase in ATP production in the mitochondria.[2] Therefore, the activation of mitoK$_{ATP}$ channels may represent an important cellular adaptive mechanism. Accordingly, the high activity of mitoK$_{ATP}$ channels observed under control conditions may be due to the absence and lack of inhibition by ATP or acyl-CoA esters in the reconstituted bilayer system.

To further characterize reconstituted mitoK$_{ATP}$ currents, we tested the effects of different K$_{ATP}$ antagonists and agonists on channel activity. It was found that the activity of reconstituted channels was inhibited by ATP in the presence of Mg^{2+} ions and reactivated by GTP, indicating that these mitochondrial channels are ATP and GTP sensitive. These results are consistent with those of previous studies using fluorescence measurements and proteoliposomes containing reconstituted mitoK$_{ATP}$ from rat liver mitochondria.[23] ATP-induced inhibition of channel activity represents an important feature of mitoK$_{ATP}$ channels as reported previously.[4,25–27] In agreement with previous studies,[25,26] ATP was effective only when added to the *trans* side of our bilayer preparations. This indicates that the regulatory site of ATP is only on one side of the channel protein. Given the general agreement that the *cis* side represents the cytosolic side of reconstituted channel proteins, it is likely that ATP binds to the matrix side of mitoK$_{ATP}$ channels in our preparations. In previous studies, using K^+ flux assays in proteoliposomes containing reconstituted mitoK$_{ATP}$ channels, however, ATP has been reported to act on the cytosolic side of the channel protein.[23] We do not know why there is a difference in the location of ATP action observed in bilayer and proteoliposome studies. It is possible that mitoK$_{ATP}$ channels are somehow incorporated into the

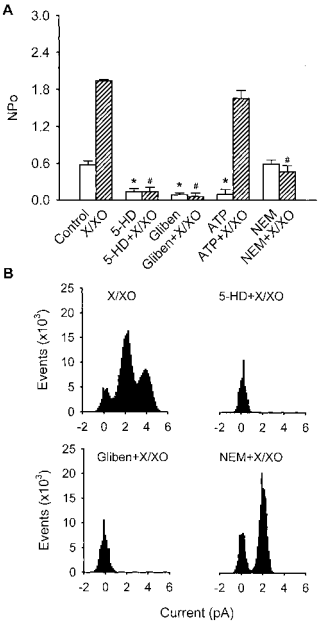

Figure 6. Effect of 5-HD, glibenclamide, MgATP, and *N*-ethylmaleimide on $O_2^{-\cdot}$-induced activation of reconstituted myocardial mitoK$_{ATP}$ channels. A, Summarized data showing NPo of reconstituted myocardial mitoK$_{ATP}$ channels. Xanthine (100 μmol/L)/xanthine oxidase (0.038 U/mL) was added to *cis* solution in the absence or presence of 5-HD (100 μmol/L), glibenclamide (Gliben, 100 μmol/L), MgATP (1 mmol/L), or *N*-ethylmaleimide (NEM, 2 mmol/L). B, Amplitude histograms of current recordings after addition of xanthine/xanthine oxidase (X/XO) in the absence or presence of 5-HD, glibenclamide, or *N*-ethylmaleimide. *$P<0.05$ vs control; #$P<0.05$ vs xanthine/xanthine oxidase. n=8.

bilayer with the ATP binding site oriented to the *trans* side. This controversy regarding the sidedness of mitoK$_{ATP}$ channels in the bilayer and the location of action of the channel modulating ligands remains to be clarified.[4,23]

Using proteoliposomes containing reconstituted myocardial mitoK$_{ATP}$ or sarcK$_{ATP}$ channels or intact cardiomyocytes, diazoxide opened the mitoK$_{ATP}$ channel with an EC$_{50}$ of 3 to 27 μmol/L, which was 2000-fold less than that required to open sarcK$_{ATP}$ channels.[8,11] Similarly, 5-HD selectively inhibits the activity of mitoK$_{ATP}$ channels but not sarcK$_{ATP}$ channels.[9,12] Using these specific inhibitors or activators of mitoK$_{ATP}$ channels, the present study demonstrated that reconstituted mitochondrial K^+ channels were also inhibited by 5-HD and activated by diazoxide. The inhibition of K$_{ATP}$ channels by channel antagonists (ie, glibenclamide) under basal conditions and subsequent reactivation by channel openers has also been reported previously using a bilayer clamping technique.[29] In some experiments, there were some remaining small conductance currents after ATP or 5-HD treatment, which was also observed previously in liver mitoK$_{ATP}$ channels.[4,25–27] The identity of these currents is currently unknown; however, they could be due to a fast flickering of mitoK$_{ATP}$ channels, which was filtered during recording.

With regard to the inhibitory effect of 5-HD, a previous study has shown that 5-HD did not inhibit mitoK$_{ATP}$ channels in both intact mitochondria and reconstituted proteoliposomes containing mitoK$_{ATP}$ channels unless ATP and a channel opener (ie, diazoxide) were present.[30] The reason for this discrepancy is not clear. It is possible that an important regulatory factor of mitoK$_{ATP}$ channels targeted by 5-HD was lost during the reconstitution process in that previous study.[30] It is also possible that mitoK$_{ATP}$ channels need to be in a specific conformation or open state for 5-HD to be effective, a condition that may occur under the present experimental conditions (open actively) but not in intact and respiring mitochondria. In support of this view, glibenclamide, a structurally unrelated K$_{ATP}$ antagonist, also inhibited mitoK$_{ATP}$ activity under basal conditions, in which it is ineffective in the intact mitochondria.[30]

To exclude the possibility that the ATP-sensitive K^+ current is derived from sarcK$_{ATP}$ channels, we examined the effect of HMR-1098, a specific sarcK$_{ATP}$ antagonist,[14,31] on the activity of reconstituted channels. As expected, HMR-1098 had no significant effect on channel activity. Previous studies have shown that HMR-1098, at similar or lower concentrations, blocked the activity of sarcK$_{ATP}$ channels.[14,31] The failure of HMR-1098 to block K^+ channel activity in the present study further confirms that the K^+ currents recorded represent mitoK$_{ATP}$ channels.

The present studies used submitochondrial membrane vesicles instead of purified proteins as reported previously.[25–27] This experimental system is more analogous to patch-clamp studies on liver mitoplasts.[4] Apart from the mitoK$_{ATP}$ channel, we observed several other ATP-insensitive currents in some experiments, as also reported previously on mitoplasts.[4] However, these channels could be generally differentiated from mitoK$_{ATP}$ channels by their current-voltage relations, conductance, channel kinetics, or drug sensitivity. None of these ATP-insensitive channels had the characteristic rectifying properties of mitoK$_{ATP}$ channels. A channel with a conductance of ≈100 pS resembles the voltage-sensitive anion-selective current recorded in liver mitoplasts.[4] Another channel with a conductance of ≈250 pS was similar to the Ca^{2+}-activated K^+ channel recently reported in the mitochondrial inner membrane.[32] Another displayed "spiky" activity and several conductance levels. Because these channels were rare and inconsistent, the present study did not characterize them.

Superoxide anions or other ROS have been reported to activate sarcK$_{ATP}$ channels in inside-out membrane patches of ventricular myocytes.[33–35] The effect of $O_2^{-\cdot}$ on sarcK$_{ATP}$ channels has been implicated in myocardial ischemia/reperfusion injury. In the present study, we demonstrated that addition of xanthine/xanthine oxidase markedly increased the NPo of reconstituted mitoK$_{ATP}$ channels, whereas xanthine or xanthine oxidase alone had no effect on the activity of these channels. The apparent increase in current amplitude (≈2-fold) may be due to the "stack" or simultaneous opening of two or three channels of the same type instead of an increase in channel conductance or nonspecific ion permeability, given that it can be blocked by 5-HD and glibenclamide. In addition, the activation of mitoK$_{ATP}$ channels by $O_2^{-\cdot}$ could not be due to a nonspecific effect of $O_2^{-\cdot}$ on the lipid environment, given that xanthine/xanthine oxidase had no significant effect on the conductance of the lipid membrane in the absence of mitoK$_{ATP}$ channel incorporation. To our knowledge, these results provide the first direct evidence that $O_2^{-\cdot}$ activates myocardial mitoK$_{ATP}$ channels.

Because mitoK$_{ATP}$ channels were reconstituted in lipid bilayers, it is possible that $O_2^{-\cdot}$can directly act on the channel protein and result in channel activation. To test this hypothesis, *N*-ethylmaleimide, a sulfhydryl alkylating agent, was used to determine whether a modification of channel protein by sulfhydryl alkylation alters the effect of $O_2^{-\cdot}$ on the activity of these channels. We found that $O_2^{-\cdot}$-induced channel activation was completely blocked after treatment with *N*-ethylmaleimide, suggesting that sulfhydryl groups in channel protein may be the target for the action of $O_2^{-\cdot}$. Previous studies have shown that switching of the neighboring sulfhydryls from the oxidized to the reduced state or vice versa is able to modulate channel conformation and channel gating.[36] A number of redox active compounds have been shown to modulate the activity of reconstituted mitoK$_{ATP}$ channels from rat liver, such as *p*-diethylaminoethylbenzoate and pelargonidine. These compounds may act as electron donors or acceptors and target sulfhydryl groups of mitoK$_{ATP}$ channel protein.[37] Our findings further support the view that redox status may regulate the activity of mitoK$_{ATP}$ channels in the myocardium. Furthermore, $O_2^{-\cdot}$-induced activation of mitoK$_{ATP}$ channels is substantially blocked by a specific mitoK$_{ATP}$ channel antagonist, 5-HD. This indicates that $O_2^{-\cdot}$ mainly alters the gating mechanism of mitoK$_{ATP}$ channels and that the functional activation due to modification of the channel protein by $O_2^{-\cdot}$ can be blocked by a selective mitoK$_{ATP}$ inhibitor. However, the activation of mitoK$_{ATP}$ channels induced by $O_2^{-\cdot}$ was not blocked by ATP, thus suggesting that $O_2^{-\cdot}$ activates mitoK$_{ATP}$ channels through a different mechanism from the sensitivity of these channels to ATP.

The present study did not attempt to address the significance of $O_2^{-\cdot}$-induced activation of mitoK$_{ATP}$ channels in IPC or ischemia/reperfusion injury. ROS have been shown to

serve as important intracellular signaling molecules in the activation of IPC.[15–20] However, the precise intracellular mechanism for the action of ROS remains unclear. Although there is evidence suggesting that mitoK$_{ATP}$ channel activation promotes ROS production,[38] recent studies demonstrated that 5-HD did not affect the oxidant generation during preconditioning, suggesting that ROS formation during preconditioning did not likely result from mitoK$_{ATP}$ activation.[16] The results of the present study demonstrate that $O_2^{-\cdot}$ is a potent activator of myocardial mitoK$_{ATP}$ channels. Therefore, ROS, such as $O_2^{-\cdot}$ generated during IPC, may activate mitoK$_{ATP}$ channels, thereby leading to a cardioprotective effect.

In summary, the present study reconstituted and characterized myocardial mitoK$_{ATP}$ channels in planar lipid bilayers. The mitoK$_{ATP}$ channels were inhibited by MgATP and 5-HD and activated by diazoxide. $O_2^{-\cdot}$ significantly increased channel activity, which was associated with modification of the sulfhydryl groups of the channel protein. It is suggested that activation of myocardial mitoK$_{ATP}$ channels by $O_2^{-\cdot}$ may represent an important intracellular pathway in mediating the protective effect associated with IPC and potassium channel openers.

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