ABSTRACT

Bepridil, which is clinically useful in the treatment of arrhythmias, has been reported to inhibit sarcolemmal ATP-sensitive K⁺ (sarcKATP) channels. However, the effect of bepridil on mitochondrial ATP-sensitive K⁺ (mitoKATP) channels remains unclear. The objective of the present study was to determine whether bepridil activates mitoKATP channels and confers cardioprotection. SarcKATP channels composed of Kir6.2/SUR2A channel current expressed in HEK 293 cells were examined using the patch-clamp technique. Flavoprotein fluorescence in guinea pig ventricular cells was measured to assay mitoKATP channel activity. Mitochondrial Ca²⁺ concentration ([Ca²⁺]ₘ) was measured using cells with rhod-2 fluorescence. Coronary-perfused guinea pig ventricular muscles were subjected to 35-min no-flow ischemia followed by 60-min reperfusion. Bepridil (10 μM) completely inhibited the pinacidil-induced Kir6.2+/SUR2A channel current expressed in HEK 293 cells. Bepridil reversibly oxidized the flavoprotein and increased mitochondrial matrix volume in a concentration-dependent manner. Furthermore, bepridil significantly attenuated the ouabain-induced increase of [Ca²⁺]ₘ. Pretreatment with bepridil for 5 min before ischemia improved the recovery of developed tension measured after 60 min of reperfusion. These effects of bepridil were abolished by the mitoKATP channel blocker 5-hydroxydecanoate (500 μM) and by the nonselective K⁺ ATP channel blocker glisoxepide (10 μM). Our results indicate that bepridil is an opener of mitoKATP channels but an inhibitor of sarcKATP channels and exerts a direct cardioprotective effect on native cardiac myocytes. This is the first report of a unique modulator of K⁺ ATP channels; bepridil would be expected to mitigate ischemic injury while blunting arrhythmias.
class IV antiarrhythmic drug, was originally developed as an antiarrhythmic drug with calcium channel-blocking effects, but the drug has multiple ion channel-blocking effects (Yatani et al., 1986; Cohen et al., 1992; Gill et al., 1992; Wang et al., 1999) similar to amiodarone and is expected to be effective for the termination of arrhythmias. Li et al. (1999) have previously demonstrated that bepridil inhibits sarcKATP channel activity in guinea pig ventricular myocytes. However, it still remains unclear whether or not bepridil modulates the cardiac mitoKATP channel activity. The results presented here show that bepridil is a mixed mitoKATP channel opener/sarcKATP channel blocker and confers cardioprotection.

Materials and Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (National Institutes of Health publication 85-23, revised 1985).

Functional Expression of SarcKATP Channels and Electrophysiology. Human embryonic kidney (HEK) 293 cells were transiently cotransfected with pcDNA3.1 vectors (Invitrogen, Carlsbad, CA) containing the coding sequence of cloned cDNAs of rat SUR2A and human Kir6.2 subunits (both gifts of Dr. S. Seino, Kobe University, Kobe, Japan) and pEGFP-C1 (BD Biosciences, San Jose, CA) using LipofectAMINE (Invitrogen). The cells that expressed green fluorescent protein were identified by fluorescence microscopy and were used for electrophysiology. Electrophysiological recordings were made 18 to 48 h after transfection. Membrane currents were recorded using the whole-cell patch configuration, with bath solution containing 143 mM NaCl, 5.4 mM KCl, 0.42 mM NaHCO3, 5.4 mM MgCl2, 0.5 mM HEPES, 5.5 mM glucose, and 5 mM HEPES, adjusted to pH 7.4 with NaOH. The pipette solution contained 110 mM NaH2PO4, 0.5 mM MgCl2, 5.5 mM glucose, and 5 mM HEPES, pH 7.2.

[Ca2+]i Measurement. The Ca2+ fluorophore rhod-2 was used to measure changes of mitochondrial Ca2+ concentration ([Ca2+]M). For rhod-2 loading, guinea pig ventricular myocytes were plated on uncoated 35-mm Falcon culture dishes with a medium based on a 1:1 mixture of Dulbecco's modified Eagle's medium and HEPES-buffered Tyrode's solution, supplemented with 5% fetal calf serum. Then, cells were loaded with rhod-2 acetoxymethyl ester (10 μM) for 120 min at 4°C. After cold loading, cells were incubated for 30 min at 37°C. This two-step cold loading/warm incubation protocol achieves exclusive loading of rhod-2 into the mitochondria (Trollinger et al., 2000). Cells loaded with rhod-2 were perfused with a HEPES-buffered Tyrode's solution containing 2.7 mM CaCl2 at 37°C. Rhod-2 fluorescence was excited at 540 nm (for 100 ms), with emission monitored through a 55-nm bandpass barrier filter. The imaging of rhod-2 was analyzed for average pixel intensities of regions of interest drawn to include whole cell, following correction for background, using an Aquacosmos image-processing system (Hamamatsu Corporation).

Coronary-Perfused Right Ventricular Myocardium. The isolated coronary-perfused guinea pig right ventricular free wall was prepared as described previously (Shigematsu et al., 1995). The preparation was mounted in the recording chamber and pinned to the floor of the chamber. The coronary artery was perfused with oxygenated Tyrode's solution containing 136.7 mM NaCl, 11.9 mM NaHCO3, 5.4 mM KCl, 0.42 mM NaHPO4, 0.5 mM MgCl2, 1.8 mM CaCl2, and 11 mM glucose, pH 7.35 to 7.40 (when gassed with 95% O2 and 5% CO2). The flow rate was maintained at 1.0 ± 0.2 ml/min/g wet weight using a roller pump (MP-3; Tokyo Rikakikai, Tokyo, Japan). The surface of the preparation was superfused with glucose-free hypoxic Tyrode's solution (10 ml/min) to minimize direct O2 diffusion from the surface of the preparations into the muscles. The composition of the hypoxic Tyrode's solution was the same as above, except that it contained no glucose and was gassed with 95% N2 and 5% CO2. The temperatures of these solutions were maintained at 37 ± 0.5°C. The basal portion of the preparation was stimulated at 3.3 Hz throughout the experiment, and contractile tension was recorded using a force transducer (TB-612T; Nihon Kohden, Tokyo, Japan) connected to the apical end of the preparation. Resting tension was adjusted to obtain the optimal developed tension. The contractile tension was monitored on a multibeam oscilloscope (VA-
9A; Nihon Kohden) and recorded on a multichannel thermal array-recorder (WT-645G; Nihon Kohden).

**Experimental Protocol.** After equilibration for 60 min, the coronary-perfused ventricular muscle preparations were assigned to the study groups: CONT, the preparations were subjected to 35 min of no-flow ischemia followed by 60 min of reperfusion; BP(1), the preparations were treated for 5 min with 1 μM bepridil followed by a 10-min washout before no-flow ischemia; BP(10), the preparations were treated for 5 min with 10 μM bepridil followed by a 10-min washout before no-flow ischemia; BP(10)+GX, pretreatment with glisoxepide (GX, 10 μM) starting 5 min prior to and continued during bepridil (10 μM) treatment; and BP(10)+5HD, pretreatment with the mitoK<sub>ATP</sub> channel blocker 5-hydroxydecanate (5HD; 500 μM) starting 5 min prior to and continued during bepridil (10 μM) treatment.

**Chemicals.** Bepridil was a kind gift from Sankyo Pharmaceutical (Omiya, Japan). Glisoxepide was a kind gift from Aventis (Strasbourg, France). Diazoxide, sodium 5HD, glibenclamide, ouabain, and tetraphenylphosphonium were purchased from Sigma-Aldrich (St. Louis, MO). DNP was purchased from Wako Pure Chemicals (Osaka, Japan). Rhod-2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR). Glibenclamide and glisoxepide were dissolved in dimethyl sulfoxide at a concentration of 100 mM. Ouabain, 5HD, and DNP were dissolved in the perfusate.

**Data Analysis.** Data are presented as mean ± S.E.M., and the number of cells or experiments is shown as n. Intergroup comparisons are made by Student’s t test for two groups and by analysis of variance followed by Fisher’s post hoc test for multiple groups. A value of p < 0.05 was regarded as significant.

## Results

### Effect of Bepridil on Expressed SarcK<sub>ATP</sub> Channel Current.

Bepridil has been reported to block sarcK<sub>ATP</sub> channel current in guinea pig ventricular myocytes (Li et al., 1999). We therefore examined the effect of bepridil on Kir6.2+SUR2A (cardiac-type sarcK<sub>ATP</sub>) channel current expressed in HEK 293 cells. Figure 1A shows the representative current traces elicited by ramp pulses. Pinacidil (100 μM), a nonselective K<sub>ATP</sub> channel opener (Liu et al., 1998), evoked an outward current. The pinacidil-induced current was completely suppressed by the subsequent application of bepridil (10 μM). As summarized in Fig. 1B, pinacidil significantly increased the membrane current measured at the membrane potential of 0 mV from 0.53 ± 0.22 nA (control, n = 5) to 3.15 ± 1.20 nA (n = 5, p < 0.01 versus control). Bepridil (10 μM) significantly blocked the pinacidil-induced currents to 0.87 ± 0.42 nA (n = 5, p < 0.01 versus pinacidil). These results indicate that bepridil is a blocker of cardiac sarcK<sub>ATP</sub> channels.

### Effect of Bepridil on Flavoprotein Fluorescence.

The effects of bepridil on mitoK<sub>ATP</sub> channel currents were evaluated by measuring flavoprotein fluorescence. Figure 2, A and B, show the time course of flavoprotein fluorescence in a cell exposed to 5HD (10 μM)-induced flavoprotein oxidation. Figure 2A shows the time course of flavoprotein fluorescence in a cell exposed to 5HD (10 μM)-induced flavoprotein oxidation. Bepridil (10 μM) failed to increase flavoprotein fluorescence (Fig. 2B). Bepridil (10 μM) prevented changes in mitochondrial Ca<sup>2+</sup> volume due to opening and closing of mitoK<sub>ATP</sub> channels in rat heart mitochondria. Mitochondrial matrix swelling was inhibited by addition of ATP (0.2 mM) as previously described (Beavis et al., 1985; Jaburek et al., 1998). Concomitant addition of bepridil (0.3 μM) restored matrix volume to control values. 5HD (300 μM) prevented changes in mitochondrial matrix volume induced by bepridil. As summarized in Fig. 3B, analysis of the matrix volume steady state at 120 s in the presence of various concentrations of bepridil showed that activation of mitoK<sub>ATP</sub> channels by bepridil was dose-dependent, with ED<sub>50</sub> values of 27.5 ± 2.3 nM.

### Effect of Bepridil on Mitochondrial Ca<sup>2+</sup> Overload.

We previously reported that the mitoK<sub>ATP</sub> channel openers attenuate the mitochondrial Ca<sup>2+</sup> overload in ventricular myocytes (Ishida et al., 2001, 2004). We then examined the effect of bepridil on mitochondrial Ca<sup>2+</sup> overload. As summarized in Fig. 4, treatment of myocytes with ouabain (1 mM) evoked the elevation of mitochondrial Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>m</sub>), and the intensity of rhod-2 fluorescence after 30 min significantly increased to 249.5 ± 16.2% of baseline (n = 9, p < 0.001). Bepridil (10 μM) alone did not increase the [Ca<sup>2+</sup>]<sub>m</sub> (102.0 ± 1.3% of baseline, n = 8). Bepridil at concentrations of 1 and 10 μM significantly attenuated the ouabain-induced elevation of [Ca<sup>2+</sup>]<sub>m</sub> to 173.4 ± 8.9% of baseline (n = 7, p < 0.05) and 150.3 ± 10.7% of baseline (n =
7, \( p < 0.05 \), respectively. The effect of bepridil was antagonized both by glisoxepide (10 \( \mu M \), 247.9 \( \pm \) 21.7\% of baseline, \( n = 7 \)) and by 5HD (500 \( \mu M \), 223.4 \( \pm \) 15.9\% of baseline, \( n = 7 \)). These results indicate that opening of mitoK\(_{ATP}\) channel by bepridil attenuates the ouabain-induced Ca\(_{\text{2+}}\) overload in mitochondria.

**Effect of Bepridil on Contractile Function during Ischemia/Reperfusion.** To test whether bepridil confers cardioprotection in guinea pig hearts, coronary-perfused right ventricular preparations were subjected to 35-min no-flow ischemia, followed by 60-min reperfusion. Figure 5 shows the time courses of developed tension during ischemia/reperfusion. In the control group, the developed tension measured after 60 min of reperfusion was decreased to 24.9 \( \pm \) 2.2\% of preischemic values (5). Pretreatment with 1 and 10 \( \mu M \) bepridil prior to ischemia significantly and dose dependently improved the recovery of contractility after 60 min of reperfusion to 48.6 \( \pm \) 7.4\% (\( n = 5 \), \( p < 0.05 \)) and 60.2 \( \pm \) 5.2\% (\( n = 5 \), \( p < 0.05 \)), respectively. This cardioprotective effect of bepridil was blocked by glisoxepide (29.4 \( \pm 

**Discussion**

**Bepridil Blocks SarcK\(_{ATP}\) Channel.** A previous study by Li et al. (1999) has provided electrophysiological evidence that bepridil inhibits the sarcK\(_{ATP}\) channel activity in guinea pig ventricular myocytes, with apparent IC\(_{50}\) value of 6.6 to 10.5 \( \mu M \). To confirm these findings in a mammalian expression system, cardiac-type sarcK\(_{ATP}\) channel (Kir6.2+SUR2A) was expressed in HEK 293 cells. We found that bepridil (10 \( \mu M \)) completely inhibited the pinacidil-induced Kir6.2+SUR2A channel currents (Fig. 1), suggesting bepridil is a potent blocker of cardiac sarcK\(_{ATP}\) channel. It is generally accepted that the activation of sarcK\(_{ATP}\) channels during ischemia shortens action potential duration and refractoriness, which may result in reentrant ventricular arrhythmias (Wilde and Janse, 1994). Our previous report in Kir6.2-deficient mice, however, has demonstrated that opening of sarcK\(_{ATP}\) channels is by no means proarrhythmic and may...
rather reduce the incidence of arrhythmias (Saito et al., 2005). Since configuration of action potentials varies from species to species, the relative impact of sarcKATP channel activation on the development of ischemic arrhythmia may differ between mouse hearts and those of larger mammals. In a dog model of ischemia/reperfusion, nonselective KATP channel opener pinacidil increased the potential for the development of ventricular fibrillation (Chi et al., 1990), and the selective sarcKATP channel blocker HMR 1883 effectively prevented ischemia-induced ventricular fibrillation (Billman et al., 1998; Sato et al., 2000b). Therefore, sarcKATP channel blockade by bepridil appears to be effective against ischemic arrhythmias by preventing the action potential shortening.

**Bepridil Activates MitoKATP Channel.** To assay the function of mitoKATP channels in intact cells, we measured flavoprotein fluorescence (Liu et al., 1998; Sato et al., 1998) and found that bepridil oxidized flavoprotein in a concentration-dependent manner (Fig. 2). These oxidative effects of bepridil were inhibited by 5HD, a potent mitoKATP channel blocker. However, the method of measuring flavoprotein oxidation has some limitations; an uncoupling effect of drug, without relation to mitoKATP channel, results in flavoprotein oxidation. In fact, bepridil exerts an uncoupling effect at high concentrations (A. D. T. Costa and K. D. Garlid, unpublished data), in agreement with a previous report (Leblondel and Allain, 1984). Moreover, it has been claimed that 5HD may be converted to 5HD-CoA (Hanley et al., 2002). To rule out possible mitoKATP channel-independent effects of bepridil, we carried out experiments in which several mitoKATP channel blockers other than 5HD were examined on the bepridil-induced flavoprotein oxidation. Although glibenclamide alone uncouples mitochondria and independently causes flavoprotein oxidation (Hu et al., 1999), glisoxepide alone did not oxidize flavoprotein, suggesting this sulfonylurea has no uncoupling effect. Glisoxepide significantly inhibited the oxidative effect of bepridil. Furthermore, the potent mitoKATP channel blocker tetrathenylphosphonium (Mironova et al., 2004) prevented the bepridil-induced flavoprotein oxidation.
To further verify that the bepridil-induced flavoprotein oxidation reflects the activation of mitoK\textsubscript{ATP} channel, we measured the mitochondrial matrix volume, a robust indicator of mitoK\textsubscript{ATP} channel opening (Beavis et al., 1985; Jaburek et al., 1998). As in the case of the mitoK\textsubscript{ATP} channel opener diazoxide (Jaburek et al., 1998), bepridil increased K\textsuperscript{+} flux and caused mitochondrial swelling (Fig. 3). Therefore, it is reasonable to assume that bepridil is an opener of mitoK\textsubscript{ATP} channels.

In isolated mitochondria, matrix swelling could be induced by lower concentrations of bepridil (EC\textsubscript{50} = 27.5 nM). The difference in sensitivity of bepridil in isolated mitochondria compared with intact myocytes, which is common to most pharmacological attempts to correlate mitochondrial ion channels with a response, may be explained by the higher concentration of ATP and diffusional barriers in the intact cells. Whatever this difference is, the effect of bepridil on the mitoK\textsubscript{ATP} channel would be expected in clinical settings because the therapeutic clinical concentrations were reported to be \~3 μM (Hollingshead et al., 2000).

**Mechanisms of Cardioprotection.** The experimental model of ouabain-induced mitochondrial Ca\textsuperscript{2+} overload was used in our earlier study, in which we showed that in rat cardiomyocytes, the mitoK\textsubscript{ATP} channel opener diazoxide attenuated the mitochondrial Ca\textsuperscript{2+} overload and such effect associated with the depolarization of mitochondrial membrane potential (Ishida et al., 2001, 2004). The present study demonstrated that bepridil prevented the mitochondrial Ca\textsuperscript{2+} overload in guinea pig ventricular myocytes (Fig. 4). Moreover, these effects of bepridil were abolished both by 5HD and by glisoxepide. These results suggest that attenuation of mitochondrial Ca\textsuperscript{2+} overload might potentially be contributed to the mechanism of cardioprotection afforded by bepridil. Recent study has shown that opening of mitoK\textsubscript{ATP} channels results in reactive oxygen species formation and acts as trigger mechanisms to preconditioning (Pain et al., 2000; Forbes et al., 2001). In the present study, we found that brief exposure to bepridil before ischemia significantly improved the postischemic functional recovery (Fig. 5). These effects of bepridil were again abolished both by 5HD and by glisoxepide. Although we did not examine the effect of reactive oxygen species scavenger, these results suggest that opening of mitoK\textsubscript{ATP} channels by bepridil acts as a trigger of cardioprotection.

Sasaki et al. (2003) reported that MCC-134, a novel vasorelaxing agent, opened sarcK\textsubscript{ATP} channels but blocked mitoK\textsubscript{ATP} channels; therefore, the drug abolished cardioprotection in ischemic hearts. In the present study, despite the blockade of sarcK\textsubscript{ATP} channels, bepridil conferred cardioprotection via opening of mitoK\textsubscript{ATP} channels. Therefore, the results of studies of both MCC-134 and bepridil support the concept that mitoK\textsubscript{ATP} channels rather than sarcK\textsubscript{ATP} channels are the key players in cardioprotection.

**Conclusions**

As described above, the selective mitoK\textsubscript{ATP} channel openers are effective in protecting hearts, whereas the selective sarcK\textsubscript{ATP} channel blockers are effective in preventing arrhythmias. In the context of this theory, a mixed sarcK\textsubscript{ATP} channel blocker/mitoK\textsubscript{ATP} channel agonist may be a perfect modulator of K\textsubscript{ATP} channels. Unfortunately, a mixed agonist/blocker MCC-134 has the opposite profile (Sasaki et al., 2003) and may blunt cardioprotection and favor ischemic arrhythmias. The present study demonstrates that bepridil, which has been utilized clinically in arrhythmias, represents the first known mixed mitoK\textsubscript{ATP} channel opener/sarcK\textsubscript{ATP} channel blocker. Besides inhibiting sarcK\textsubscript{ATP} channels, bepridil has been shown to block multiple ion channels (Yatani et al., 1986; Cohen et al., 1992; Gill et al., 1992; Wang et al., 1999). Such diverse actions of bepridil are probably responsible for the mechanism of prevention of arrhythmias, and further studies are required to distinguish between these mechanisms. However, it seems reasonable to conclude that bepridil is of advantage both in preventing ischemic damage and in management of ischemic arrhythmias.

**Acknowledgments**

We thank S. Seino (Kobe University, Kobe, Japan) for providing us the cDNAs of rat SUR2A and human Kir6.2. We also thank M. Tamagawa, Y. Reien, and I. Sakashita for excellent technical and secretarial assistance.

**References**


Li Y, Sato T, and Arita M (1999) Bepridil blunts the shortening of action potential duration caused by metabolic inhibition via blockade of ATP-sensitive K\textsuperscript{+} channels and Na\textsuperscript{+}-activated K\textsuperscript{+} channels. *J Pharmacol Exp Ther* 291:562–568.


Address correspondence to: Dr. Toshiaki Sato, Department of Pharmacology, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. E-mail: tsato@faculty.chiba-u.jp