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Involvement of the phosphatidylinositol kinase pathway in augmentation of ATP-sensitive K⁺ channel currents by hypo-osmotic stress in rat ventricular myocytes

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Abstract

The objective of this study was to investigate the mechanisms of increase in efficacy of ATP-sensitive K⁺ channel (K\text{ATP}) openings by hypo-osmotic stress. The whole-cell K\text{ATP} currents (I\text{K,ATP}) stimulated by 100µM pinacidil, a K⁺ channel-opening drug, were significantly augmented during hypo-osmotic stress (189 mOsm) compared to normal condition (303 mOsm). The EC\text{50} and E\text{max} value for pinacidil-activated I\text{K,ATP} (measured at 0 mV) was 154 μM and 844 pA in normal and 16.6 μM and 1266 pA in hypo-osmotic solution. Augmentation of I\text{K,ATP} during hypo-osmotic stress was attenuated by wortmannin (50 µM), an inhibitor of phosphatidylinositol 3- and 4-kinases, but not by (a) phalloidin (30 µM), an actin filament stabilizer, (b) absence of Ca\textsuperscript{2+} from the internal and external solutions, and (c) presence of creatine phosphate (3 mM) which affects creatine kinase regulation of the K\text{ATP} channels. In the single-channel recordings, inside-out patch was made after about 5 minute exposure of the myocyte to hypo-osmotic solution. However, the IC\text{50} value for ATP under such condition was not different from that obtained in normal osmotic solution. In conclusions, hypo-osmotic stress could augment cardiac I\text{K,ATP} through the intracellular mechanisms involving the phosphatidylinositol kinases pathway.

Key words: Hypo-osmotic stress, ATP-sensitive K⁺ channels, Wortmannin, Phosphatidylinositol kinases, K⁺ channel-opening drug, Cardiomyocytes
Introduction

The ATP-sensitive K\textsuperscript{+} channel (K\textsubscript{ATP}) is a potassium channel that opens when the intracellular ATP concentration is decreased. This channel is activated during myocardial ischemia, shortens the action potential duration and prevents intracellular calcium overload, thereby protecting cardiac myocytes from ischemia (Yokoshiki et al. 1998; Proks and Ashcroft 2009). However, the IC\textsubscript{50} value for ATP in cardiac K\textsubscript{ATP} channel is 20 – 100μM (Yokoshiki et al. 1998), that is extremely low compared to the intracellular ATP concentration during ischemia, i.e., about 1mM (Elliott et al. 1989).

The accumulation of metabolites under ischemia could change the osmotic pressure in the cell (Jennings and Ganote 1974). It has been reported that, in rat atrial (Van Wagoner 1993; Baron et al. 1999) and guinea-pig ventricular (Priebe and Beuckelmann 1998; Kocic et al. 2004) myocytes, cell swelling induced by hypotonic solution evoked the outward current that was inhibited by 1μM glibenclamide, a blocker of K\textsubscript{ATP} channels (Van Wagoner 1993; Priebe and Beuckelmann 1998; Baron et al. 1999), or enhanced efficacy of rilmakalim, an opener of K\textsubscript{ATP} channels (Kocic et al. 2004b; Kocic et al. 2007). Thus, hypotonic cell swelling and/or membrane stretch may contribute to facilitation of K\textsubscript{ATP} channel openings in ischemic myocardium.

Van Wagoner (1993) demonstrated that stretch of the atrial cell membrane produced by negative pressure or by hypotonic swelling caused a reversible increase in K\textsubscript{ATP} channel activity and that not all increases in channel activity immediately return to baseline on release of these mechanical stimuli. As a mechanism of enhanced efficacy of K\textsubscript{ATP} channels by hypo-osmotic stress, Kocic et al. (2004b) proposed a cytoskeletal regulation in terms of absence of hypotonic augmentation of K\textsubscript{ATP} channel current
(I$_{K,ATP}$) by pretreatment with phalloidin, an actin filament stabilizer. On the other hand, the several signal transduction pathways including phosphorylation by protein kinases, intracellular Ca$^{2+}$, and inositol phosphates are known to be involved in hypo-osmotic cell swelling and subsequent regulatory volume decrease (Furst et al. 2002) as well as in the regulation of K$_{ATP}$ channels (Yokoshiki et al. 1998; Proks and Ashcroft 2009). These findings thus indicated that, in addition to direct conformational changes of the channel or cellular membrane elements such as cytoskeleton, the indirect intracellular pathway could also be involved in the K$_{ATP}$ channel activation by hypo-osmotic cell swelling and/or stretch. The present study was aimed to identify the major intracellular mechanism of hypo-osmotic augmentation of the outward current stimulated by pinacidil, an opener of K$_{ATP}$ channels.
Materials and methods

The study was approved by our institutional animal research committee and conformed to the animal care guidelines for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine.

Cell isolation

Freshly-isolated single cells were prepared from ventricles of young adult male (200 – 400 g) rats (Wistar Kyoto Rats) as previously described (Shimokawa et al. 2007). In brief, hearts were excised after anesthesia with inhalation of diethyl ether and peritoneal injection of heparin sodium (400IU/kg). The heart was mounted on a Langendorff apparatus and was retrogradely perfused with Tyrode’s solution (37°C) containing (mM) NaCl 143, KCl 5.4, NaH₂PO₄ 0.33, HEPES 5, glucose 5.5, MgCl₂ 0.5, CaCl₂ 1.8 (pH 7.4 using NaOH) gassed with 100% O₂ until the beating rate became stable (3–5 min). The perfusate was then changed to nominally Ca²⁺-free Tyrode’s solution (otherwise identical to above) for 10 minutes, resulting in the cessation of the heart beating. The quiescent heart was perfused with the nominally Ca²⁺-free Tyrode’s solution containing 0.5 mg/ml collagenase (Wako Chemicals, Osaka, Japan) and 0.1 mg/ml protease (Sigma Chemical Co., St. Louis, USA) for 35 to 40 minutes. At the end of the digestion by collagenase, the perfusate was changed to the nominally Ca²⁺-free Tyrode’s solution in order to wash the collagenase / protease solution off. After removing the atria and the right ventricle, small pieces of the left ventricular tissues were dissected with fine scissors. The tissue pieces in modified “Kraftbrühe” (KB) solution containing (mM) KOH 70, L-glutamic acid 50, KCl 40, taurine 20, KH₂PO₄ 20, glucose 10, HEPES 10, EGTA 1, MgCl₂ 3 (pH 7.4 using KOH) were gently stirred, and
filtered through a stainless-steel mesh. The cell suspension was stored in a refrigerator (4°C). The cells were used for the experiments 2–12 h after isolation.

**Electrophysiological recording techniques**

Whole cell currents and single channel currents were recorded using patch clamp method (Shimokawa et al. 2007). The electrode was connected to an input of a current–voltage converter with a feedback resistance of 100 mega ohms for recording whole cell current and 10 giga ohms for recording single channel current. The isolated cells were placed in a perfusion chamber (1 ml volume) attached to an inverted microscope (model IX70, Olympus, Japan). After the cells settled to the chamber bottom, they were perfused with bath solution by a fine tube located close to cells to ensure fast solution exchange with use of pressure-driven perfusion system (Model BPS-8 and Model PR-10, ALA Scientific Instruments, New York, USA). During superfusion with Tyrode's solution containing 1.8 mM CaCl₂, 30-40% of the cells were Ca²⁺ tolerant and rod-shaped. Single rod-shaped cells having smooth surfaces with clearly demarked striations were selected for the electrical measurements. Pipettes were fabricated from 1.5 mm outer diameter to 0.85 mm inner diameter borosilicate glass capillaries (World Precision Instruments Co., Sarasota, Florida, USA) using a multi-stage horizontal puller (model P-97; Sutter Instrument Co., Novato, CA, USA). All experiments were performed at room temperature (22–25°C).

**Whole cell currents recordings**

Voltage-clamp experiments were performed by applying voltage ramp to measure whole-cell currents using pipettes with tip resistances of 2 to 3 mega ohms filled by pipette (intracellular) solution containing (mM) K-glutamate 100, KCl 40, NaOH 10, HEPES 5, EGTA 5, and Mg-ATP 1, pH 7.2 using KOH (or HCl) when
needed. When inhibiting creatine kinase regulation of the \( \text{K}_{\text{ATP}} \) channels (Bienengraeber et al. 2000; Zingman et al. 2001), creatine phosphate-dipotassium salt (CrP-K\(_2\)) 3 mM was included the pipette solution (Fig. 6). Cell membrane capacitance (\( C_m \)) was determined from the amplitude of the current elicited by hyperpolarizing voltage ramp pulses from a holding potential of 0 mV to \(-5 \) mV (duration 25 ms at 0.2 V/s); this procedure avoided interference by any time-dependent ionic currents. After this procedure, the Tyrode's solution in the bath was changed to iso-osmotic (ISO) solution containing (mM) D(-)-Mannitol 126, NaCl 80, KCl 5.4, NaH\(_2\)PO\(_4\) 0.33, HEPES 5, glucose 5.5, MgCl\(_2\) 0.5, CaCl\(_2\) 1.8 (pH 7.4 using NaOH). That is, the ISO solution was made by reducing NaCl in the Tyrode's solution from 143 mM to 80 mM, and adding D(-)-Mannitol 126 mM, thereby maintaining the normal osmotic pressure. In order to apply hypo osmotic stress, the bath solution was exchanged to the hypo-osmotic (Hypo) solution, that was made by omitting D(-)-Mannitol 126 mM from the ISO solution. The osmolarities of the hypo-osmotic and iso-osmotic solutions were calculated to be 189 and 303 mOsm, respectively. Ca\(^{2+}\)-free iso-osmotic and hypo-osmotic solution was made by omitting CaCl\(_2\) 1.8 mM and increasing MgCl\(_2\) 0.5 mM to 2.3 mM from the ISO and Hypo solution, respectively. A liquid junction potential between the internal solution and the bath solution (i.e., ISO and Hypo solution) was 4 mV, and it was not corrected.

Whole-cell current signals were filtered at 1 kHz, sampled at 2 kHz, and stored on a personal computer using pCLAMP 6.0/8.0 software (Molecular Devices, Sunnyvale, CA, USA).

**Single channel recordings**

Single-channel currents were recorded by the excised inside-out patch method.
The pipette resistances were around 2-5 mega ohms when filled with pipette (extracellular) solution containing (mM) KCl 150, MgCl₂ 0.5, CaCl₂ 1.8, HEPES 5, pH 7.4 with KOH. The cells were perfused with the bath (intracellular) solution containing (mM) KCl 140, KOH 10, MgCl₂ 2, HEPES 5, EGTA 5, pH 7.3 with KOH. To obtain excised inside-out patch currents, the pipette tip was withdrawn from the cell surface after forming of the cell-attached patch without a decrease in seal resistance. Single channel currents signals were filtered at 2 kHz, sampled at 4 kHz, and stored on a personal computer using AxoScope 1 software (Molecular Devices, Sunnyvale, CA, USA). Channel openings were determined by using 50% threshold criterion. The number of functional channels in the patch was approximated as the maximum number of overlaps of the openings in the absence of ATP. Open state probability (P₀) was estimated using $P_0 = I/(N_i)$, where $I$= the mean patch current, $N$= number of channels in the patch, $i$= the unit amplitude of single channel current.

**ATP sensitivity of the $K_{ATP}$ channels under hypo-osmotic pressure**

For the control experiments, the single $K_{ATP}$ channel currents from inside-out patches of left ventricular myocytes were recorded during exposure of intracellular membrane to the standard bath solution (see above) containing no ATP, 3 mM ATP, 300 µM ATP, 100 µM ATP, 30 µM ATP, successively. In order to apply hypo-osmotic pressure, the standard bath solution was exchanged to the hypo-osmotic (Hypo) solution (see above). The excised inside-out patch was made after about 5 minutes perfusion with the hypo osmotic solution, and it was again exposed to the standard bath solution for obtaining the ATP sensitivity of the channels.

**Statistical Analysis**

All data are expressed as mean ± SE. Simple between–group analyses were
conducted by using a Student's t test. Between-group comparisons of the repeated measurements were made by using two-way repeated-measures analysis of variance (ANOVA), and intergroup comparisons were performed by the adjusted t test within ANOVA (Bonferroni method). Differences with P < 0.05 were considered significant.
Results

Swelling of rat ventricular myocytes induced by hypo-osmotic stress

Isolated single rat ventricular myocyte and the pipette for the patch electrode are shown (Fig. 1). After making the whole-cell configuration in the Tyrode’s solution, the cell was superfused with the normal osmotic (ISO: iso-osmotic) solution (Fig. 1). Using the pressure-driven perfusion system with a fine tube (not shown in the figure) located close to the myocyte, rapid solution exchange with hypo-osmotic solution (Hypo) induced cell swelling (Fig. 1).

Enhanced ATP-sensitive K⁺ (K_{ATP}) channel current by hypo-osmotic stress

Fig. 2A shows superimposed whole-cell current traces evoked by voltage-clamp ramp pulses (-80 mV/s) from +30 mV to -90 mV. Under normal osmotic solution (ISO), current traces (a) and (b) were recorded before (a) and after (b) application of pinacidil 100 μM, a K⁺ channel-opening drug. Application of pinacidil rapidly increased a large outward current, the reversal potential of which was –75 mV, which is close to the calculated equilibrium potential for K⁺ (E_K = -83 mV). In addition, the pinacidil-induced outward currents were abolished by glibenclamide (1 μM), a blocker of K_{ATP} channels (not shown). Therefore, the current produced by pinacidil was defined as a K_{ATP} current (I_{K,ATP}). After washing out pinacidil, the cell was superfused with hypo-osmotic solution (Hypo) (trace c). The outward current was slightly increased in about half of the cells, and intersected with the current trace under the normal osmotic solution (trace a) at about -50 mV in this myocyte. Since the calculated equilibrium potential for Cl⁻ was –21 mV, the current stimulated by Hypo might be composed of a Cl⁻ current and a K⁺ current. Pinacidil-induced outward current was
further increased during the superfusion with hypo-osmotic compared to normal osmotic solution (trace d). An example of the time course of the currents measured at 0 mV is shown in Fig. 2B. The letters (a, b, c and d) correspond to the time points at which the currents in panel A were recorded.

**Dose-response relationships of pinacidil-induced $I_{\text{K,ATP}}$ in normal vs. hypo-osmotic conditions**

Fig. 3 gives the dose-response relations of pinacidil-induced $I_{\text{K,ATP}}$ in normal and hypo-osmotic solutions. Currents were evoked by ramp pulses, and measured at 0 mV when the currents were maximally activated. Difference in currents between the maximal activated and control (before application of pinacidil) were plotted against each concentration. Numbers of cells studied are shown in parentheses. Data points were fitted by the Hill equation: Current increase = \( \left[ x^{\text{nH}} / (x^{\text{nH}} + EC_{50}^{\text{nH}}) \right] \times E_{\text{max}} \), where \( E_{\text{max}} \) is the maximal stimulatory effect, \( n_H \) is the Hill coefficient, and \( EC_{50} \) is the concentration for half-maximal effect. The \( EC_{50} \) value was 154 \( \mu \text{M} \) in normal osmotic solution (ISO) and 16.6 \( \mu \text{M} \) in hypo-osmotic solution (Hypo). The Hill coefficient (\( n_H \)) value was 0.90 in ISO and 1.43 in Hypo. The \( E_{\text{max}} \) was calculated to be 844 pA in ISO and 1266 pA in Hypo.

**Effects of the regulators of $K_{\text{ATP}}$ channels on pinacidil-induced $I_{\text{K,ATP}}$ in hypo-osmotic conditions**

An example of the time-course of pinacidil-induced $I_{\text{K,ATP}}$ (at 0 mV) in normal osmotic and hypo-osmotic conditions is given (Fig. 4A). The concentration of pinacidil was 100 \( \mu \text{M} \). The whole-cell currents were evoked (every 6 s) by voltage-clamp ramps (-80 mV/s) between +30 mV to -90 mV (as shown in Fig. 2A, inset). In the presence of
wortmannin (50 μM), an inhibitor of phosphatidylinositol 3- and 4-kinases, augmentation of the pinacidil-induced $I_{K,ATP}$ during hypo-osmotic stress was significantly attenuated (Figs. 4B and 4C). Fig. 4C summarizes the densities of the maximal $I_{K,ATP}$ (at 0 mV) induced by pinacidil during normal osmotic (ISO) and hypo-osmotic (Hypo) conditions in the absence (Control) and presence of the regulators of $K_{ATP}$ channels. Phalloidin (30 μM) (an actin filament stabilizer), and omitting of Ca$^{2+}$ from the internal and external solutions exerted no significant effects on hypo-osmotic augmentation of pinacidil-induced $I_{K,ATP}$.

**Slight inhibition of pinacidil-induced $I_{K,ATP}$ by wortmannin in normal osmotic conditions**

In normal osmotic conditions, direct bath application of wortmannin (50 μM) slightly decreased pinacidil-activated $I_{K,ATP}$ (Figure 5). In four cells, $I_{K,ATP}$ at 0 mV was inhibited by 18.5 ± 2.6 % (P = 0.075). The degree of this inhibition appeared to be small compared to that (about 70 %) under hypo-osmotic conditions which could be estimated from Figure 4C.

**Little effect of creatine kinase regulation on hypo-osmotic activation of pinacidil-induced $I_{K,ATP}$**

$K_{ATP}$ channel openers enhanced the ATPase activity of SUR2A in cardiac membranes, thereby enhancing the channel activation. In addition, opener-induced channel activation was inhibited by the creatine kinase / creatine phosphate system that removes ADP from the channel complex (Bienengraeber et al. 2000; Zingman et al. 2001). To evaluate the involvement of creatine kinase regulation in the hypo-osmotic augmentation of $K_{ATP}$ channel activation, 3 mM creatine phosphate (CrP) was added in the pipette solution (Fig. 6). However, presence of CrP could not prevent the
hypo-osmotic augmentation of pinacidil-induced $I_{\text{K,ATP}}$ in 4 cells. In addition, 0.1 mM 2-4-dinitrofluorobenzene (DNFB), an irreversible creatine kinase inhibitor, did not affect the pinacidil-induced $I_{\text{K,ATP}}$ in the hypo-osmotic condition (Fig. 6).

**Concentration-dependent inhibition of $K_{\text{ATP}}$ channels by ATP in excised inside-out patch recordings under normal osmotic vs. after exposure to hypo-osmotic conditions**

To investigate the effect of hypo-osmotic stress on the ATP sensitivity of single $K_{\text{ATP}}$ channels, the myocytes were exposed to hypo-osmotic solution for about 5 minutes immediately before making inside-out patch configuration. Open circles show the open-state probability under the normal condition and filled circles show under the condition after 5 minutes exposure to hypo-osmotic solution (Fig. 7). The data points were fitted to the Hill equation: relative open probability ($P_o$) = $1/(1+([\text{ATP}]/IC_{50})^n)$, where [ATP] is the ATP concentration, the IC$_{50}$ the concentration required for the half-maximal inhibition and n the Hill’s coefficient. The IC$_{50}$ obtained from fitted curve was 52 µM in normal (n=8) and 58 µM after exposure to hypo-osmotic (n=5) solution. The Hill's coefficient obtained from fitted curve was 2.96 in normal and 1.99 after exposure to hypo-osmotic solution. The relative open probability at 100µM was 0.12 ± 0.12 in normal and 0.25 ± 0.17 after exposure to hypo-osmotic solution ($P = 0.13$).
Discussion

The present study demonstrated that $I_{\text{K,ATP}}$ activated by pinacidil were augmented under the condition of hypo-osmotic stress in rat ventricular myocytes, and that the hypo-osmotic regulation of the $I_{\text{K,ATP}}$ was in part inhibited by wortmannin, an inhibitor of phosphatidylinositol 3- and 4-kinases. In contrast, phalloidin, an actin filament stabilizer (Furukawa et al. 1996; Kocic et al. 2004b), was not effective in reducing the hypo-osmotic augmentation of $I_{\text{K,ATP}}$. In addition, other regulators of $K_{\text{ATP}}$ channels such as $Ca^{2+}$ (Deutsch and Weiss 1993), the creatine kinase / creatine phosphate system (Bienengraeber et al. 2000; Zingman et al. 2001) were not likely to be operative in its mechanism. Therefore, the phosphatidylinositol kinases and phosphatidylinositol-4,5-bisphosphate (PIP2) dependent mechanisms (Hilgemann and Ball 1996; Xie et al. 1999; Ribalet et al. 2000; Song and Ashcroft 2001; Xie et al. 2007) would be involved in stimulation of $K_{\text{ATP}}$ channels of cardiac myocytes during hypo-osmotic swelling.

On the other hand, we could not detect the changes of ATP sensitivity of single $K_{\text{ATP}}$ channels in rat ventricular myocytes exposed to hypo-osmotic solution for about 5 minutes immediately before making inside-out patch configuration. Since the hypo-osmotic stress induces cell swelling and activates several intracellular signaling, the inside-out patch (that is devoid of intracellular structures and swelling) recording of the $K_{\text{ATP}}$ channels would not be suitable for reproducing the hypo-osmotic condition. Furthermore, we should be aware of the technical limitation of the excised inside-out patches in the present study. In the future study, an alternative method for applying stretch on the excised patch continuously by a constant suction (clamp negative...
pressure) would clarify the direct effect of stretch on the ATP sensitivity of single $K_{ATP}$ channels.

It was reported that hypo-osmotic cell swelling increased the Cl$^-$ channel currents ($I_{Cl}$), delayed rectifier K$^+$ currents ($I_{Ks}$), non-selective cation current, Na-K pump current, and $I_{K,ATP}$ in cardiac myocytes (Sasaki et al. 1994; Zhou et al. 1997; Priebe and Beuckelmann 1998; Bewick et al. 1999; Kocic et al. 2001; Kocic et al. 2004a; Kocic et al. 2004b; Kocic et al. 2007; Ren et al. 2008; Piron et al. 2010). Several mechanisms have been proposed for the hypo-osmotic stimulation of these ionic conductance including the activation of tyrosine kinase, phosphatidylinositol 3-kinase (PI-3K) and a serine/threonine protein phosphatase, and the interaction with actin filaments (Zhou et al. 1997;; Bewick et al. 1999; Kocic et al. 2004b; Ren et al. 2008; Piron et al. 2010). For example, it was proposed that in rabbit ventricular myocytes hypo-osmotic swelling activated volume-sensitive Cl$^-$ current ($I_{Cl,swell}$) by stimulation of angiotensin-II type I receptor, thereby evoking the downstream signaling via tyrosine kinase and PI-3K, causing assembly of NADPH oxidase (Ren et al. 2008). This activation of NADPH oxidase could produce H$_2$O$_2$ which may modulate $I_{Cl,swell}$ directory or via a variety of redox sensitive kinases and phosphatases (Ren et al. 2008).

Hypo-osmotic stress altered the level of membrane phospholipids such as PIP2 and PIP (Nasuhoglu et al. 2002; Perera et al. 2004). On the other hand, intracellular Mg$^{2+}$ and positively charged polyamines reduced the $K_{CNQ}$ K$^+$ channel currents expressed in human embryonic kidney tsA-201 cells and acceleration of synthesis of PIP2 by overexpression of phosphatidylinositol 4-phosphate 5-kinase $\gamma$ attenuated the effects of Mg2+ and polyamines, suggesting that cytosolic Mg$^{2+}$ and polyamines electrostatically interact with membrane PIP2 and tonically inhibit the channels by
reducing the amount of free PIP2 available (Suh and Hille 2007). Recently, increasing PIP2 levels with a water-soluble PIP2 analog prevented stimulation of KCNE1-KCNQ1 channels (which generates cardiac I_{Ks}) in hypo-osmotic condition, and intracellular Mg^{2+} removal and polyamines chelation also inhibited the channel osmoregulation (Piron et al. 2010). In addition, direct measurement of intracellular Mg^{2+} variations during osmotic changes indicated that Mg^{2+} participates significantly to the osmoregulation of KCNE1-KCNQ1 channels (Piron et al. 2010). The authors proposed that modulation of the channel-PIP2 interactions by Mg^{2+} and polyamines during cell volume changes could account for the osmoregulation of the channels (Piron et al. 2010). Since the phophatidylinositol kinases dependent osmoregulation has been demonstrated in the present study, the similar mechanisms may apply to the hypo-osmotic regulation of K_{ATP} channels that are sensitive to not only PIP2 but also Mg^{2+} and polyamines (Ribalet et al. 2000; Song and Ashcroft 2001; Xie et al. 2007).

Action potential duration (APD) of guinea-pig ventricular myocytes was gradually abbreviated during superfusion with hypo-osmotic solution (Priebe and Beuckelmann 1998). This abbreviation of APD was associated with appearance of outward currents that were inhibited by glibenclamide, a blocker of K_{ATP} channels (Priebe and Beuckelmann 1998). In contrast, it was reported that, using terikalant (a blocker of inward rectifier K^+ currents (I_{K1})) and glibenclamide, neither I_{K1} nor I_{K,ATP} participated in membrane potential changes induced by hypo-osmotic solution at least with sufficient intracellular ATP (Kocic et al. 2004a). On the other hand, hypo-osmotic stress increased efficacy of K^+-channel opening drugs to activate I_{K,ATP} in guinea-pig ventricular myocytes (Kocic et al. 2007). These findings by Kocic et al. (2004a; 2007) are in agreement with our observations of rat ventricular myocytes in terms of (1) lack
of increase in outward $K^+$ currents during exposure to hypo-osmotic solution in the absence of pinacidil, and (2) hypo-osmotic augmentation of $I_{K,\text{ATP}}$ induced by pinacidil.

In summary, the present study demonstrated that potency of pinacidil to activate cardiac $I_{K,\text{ATP}}$ was augmented during hypo-osmotic swelling, and that it was mediated in part via the phosphatidylinositol kinases dependent pathway. It is plausible that hypo-osmotic stress may facilitate the $K_{\text{ATP}}$ channel openings under the pathophysiological conditions such as mild ischemic myocardium where intracellular ATP was still maintained, i.e., 1 mM and more (Elliott et al. 1989), thereby protecting ischemic myocardial cells.

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Figure legends

Figure 1. Swelling of rat ventricular myocytes produced by hypo-osmotic stress
Isolated rat ventricular myocyte was superfused with the normal osmotic (ISO: iso-osmotic) (left panel) and hypo-osmotic (Hypo) (right panel) solution. The patch pipette attached to the cell is seen on the right.

Figure 2. Enhanced ATP-sensitive K⁺ (K\textsubscript{ATP}) channel current by hypo-osmotic stress
A. Superimposed current traces were evoked by voltage-clamp ramps (-80 mV/s) between +30 mV to -90 mV (inset). The current traces (a) under normal osmotic solution (ISO), (b) in presence of a K⁺-channel opening drug, pinacidil 100 μM, (c) during superfusion with hypo-osmotic solution (Hypo), and (d) in presence of pinacidil (100 μM) under hypo-osmotic stress are given. B. The time course of the currents measured at 0 mV is shown. The letters (a, b, c and d) correspond to the time points at which the currents in panel A were recorded.

Figure 3. Dose-response relationships of pinacidil-induced K\textsubscript{ATP} currents (I\textsubscript{K\textsubscript{ATP}}) in normal vs. hypo-osmotic conditions
Current increases (at 0 mV) by pinacidil, that is I\textsubscript{K\textsubscript{ATP}}, under normal osmotic (ISO) and hypo-osmotic (Hypo) conditions were plotted against each concentration. Numbers of cells studied are given in parentheses. The EC\textsubscript{50} value was 154 μM in normal osmotic solution (ISO) and 16.6 μM in hypo-osmotic solution (Hypo). The Hill coefficient (n\textsubscript{H}) value was 0.90 in ISO and 1.43 in Hypo. The E\textsubscript{max} was calculated to be 844 pA in ISO and 1266 pA in Hypo. See text for details.
Figure 4. Effects of the K$_{ATP}$ channel regulators on pinacidil-induced I$_{K,ATP}$ in hypo-osmotic conditions

A. An example of the time-course of pinacidil (100 μM) -induced I$_{K,ATP}$ (at 0 mV) evoked by voltage-clamp ramps (as shown in Fig. 2, inset) in normal osmotic and hypo-osmotic conditions is given. B. Wortmannin (50 μM), an inhibitor of phosphatidylinositol 3- and 4-kinases, attenuated the augmentation of the pinacidil-induced I$_{K,ATP}$ during hypo-osmotic stress. C. Summary of the densities of the maximal I$_{K,ATP}$ (at 0 mV) induced by pinacidil (100 μM) during normal osmotic (ISO) and hypo-osmotic (Hypo) conditions in the absence (Control) and presence of the regulators of K$_{ATP}$ channels. Phalloidin (30 μM): actin filament stabilizer. Significant difference (*P<0.05 vs. Control) was obtained using two-way repeated-measures ANOVA with Bonferroni method. Numbers of cells studied are shown in parentheses.

Figure 5. Slight inhibition of pinacidil-induced I$_{K,ATP}$ by wortmannin in normal osmotic conditions

In normal osmotic solution (ISO), application of wortmannin (50 μM) slightly inhibited pinacidil-induced I$_{K,ATP}$. The current at 0 mV was decreased by 18.5 % in 4 cells (P = 0.075).

Figure 6. Little effect of creatine kinase regulation on hypo-osmotic activation of pinacidil-induced I$_{K,ATP}$

The involvement of creatine kinase regulation (Bienengraeber et al. 2000; Zingman et al. 2001) in the hypo-osmotic augmentation of I$_{K,ATP}$ was tested by adding 3 mM creatine phosphate (CrP) in the pipette solution. Presence of CrP could not prevent the hypo-osmotic augmentation of pinacidil-induced I$_{K,ATP}$ in 4 cells. Moreover, 0.1 mM 2-4-dinitrofluorobenzene (DNFB), an irreversible creatine kinase inhibitor, did not
affect the pinacidil-induced $I_{\text{K,ATP}}$ in the hypo-osmotic condition.

**Figure 7. Concentration-dependent inhibition of $K_{\text{ATP}}$ channels by ATP in excised inside-out patch recordings under normal osmotic vs. after exposure to hypo-osmotic conditions**

Open circles show the open-state probability under the normal condition and filled circles show after 5 minutes exposure to hypo-osmotic solution. $[\text{ATP}]_i$ indicates the concentration of ATP (μM). The IC$_{50}$ obtained from fitted curve was 52 μM in normal (n=8) and 58 μM after exposure to hypo-osmotic (n=5) solution. The Hill's coefficient obtained from fitted curve was 2.96 in normal and 1.99 after exposure to hypo-osmotic solution. See text for details.