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# Opening of ATP-sensitive $K^+$ Channel by Pinacidil Requires Serine/Threonine Phosphorylation in Rat Ventricular Myocytes

**Yong-Geun Kwak and Soo-Wan Chae**

Department of Pharmacology, and Institute of Cardiovascular Research, Chonbuk National University Medical School, Chonju 560−182, Korea

The influences of specific protein phosphatase and protein kinase inhibitors on the ATP-sensitive $K^+$ ($K_{ATP}$) channel-opening effect of pinacidil were investigated in single rat ventricular myocytes using patch clamp technique. In cell-attached patches, pinacidil (100 $\mu$M) induced the opening of the $K_{ATP}$ channel, which was blocked by the pretreatment with H-7 (100 $\mu$M) whereas enhanced by the pretreatment with genistein (30 $\mu$M) or tyrphostin A23 (10 $\mu$M). In inside-out patches, pinacidil (10 $\mu$M) activated the $K_{ATP}$ channels in the presence of ATP (0.3 mM) or AMP-PNP (0.3 mM) and in a partial rundown state. The effect of pinacidil (10 $\mu$M) was not affected by the pretreatment with protein tyrosine phosphatase 1B (PTP1B, 10 $\mu g\ ml^{-1}$), but blocked by the pretreatment of protein phosphatase 2A (PP2A, 1 U $ml^{-1}$). In addition, pinacidil (10 $\mu$M) could not induce the opening of the reactivated $K_{ATP}$ channels in the presence of H-7 (100 $\mu$M) but enhanced it in the presence of ATP (1 mM) and genistein (30 $\mu$M). These results indicate that the $K_{ATP}$ channel-opening effect of pinacidil is not mediated via phosphorylation of $K_{ATP}$ channel protein or associated protein, although it still requires the phosphorylation of serine/threonine residues as a prerequisite condition.

Key Words: Pinacidil, Phosphorylation, Dephosphorylation, $K_{ATP}$ channel, Serine/threonine, Tyrosine, Rat ventricular myocyte

## INTRODUCTION

Recently, $K^+$ channel openers including pinacidil have received growing attention because they possess therapeutic potential in various clinical conditions including hypertension, acute and chronic myocardial ischemia, or congestive heart failure, as well as in managing bronchial asthma, urinary incontinence, and certain skeletal muscle myopathies (Quast, 1992; Edwards & Weston, 1993; Gopalakrishnan et al, 1993). The primary action of the drugs is to open ATP-sensitive $K^+$ channel ($K_{ATP}$ channel), which is specifically blocked by sulfonylurea derivatives such as tolbutamide and glibenclamide. Several $K^+$ channel openers such as HOE-234 (Terzic et al, 1994), ER-001533 (Shen et al, 1992) and KR-30450 (Kwak et al, 1995) appear to antagonize the intracellular ATP-induced inhibition of $K_{ATP}$ channels. However, activation profiles of other $K^+$ channel openers are more complex, because some of the agents act even in the absence of ATP (Escande et al, 1989), some are only effective in the presence of ADP or other nucleoside diphosphates, or require phosphorylation of $K_{ATP}$ channels (Tung & Kurachi, 1991), and some even inhibit $K_{ATP}$ channels in the absence of intracellular nucleotides (Fan et al, 1990).

The activities of many types of ion channels are modulated by phosphorylation and dephosphorylation (Levitan, 1994). The $K_{ATP}$ channel, which is closed by intracellular ATP (Noma, 1983), appears to be also modulated by phosphorylation and dephosphorylation (Findlay & Dunne, 1986; Takano et al, 1990). It is generally agreed that adenosine 3', 5'-cyclic mono-

Corresponding to: Yong-Geun Kwak, Department of Pharmacology, Chonbuk National University Medical School, Chonju 560-182, Korea. (Tel) 82-652-270-3091, (FAX) 82-652-275-2855, (E-mail) ygkwak@moak.chonbuk.ac.kr

phosphate (cyclic AMP)-dependent phosphorylation is necessary to maintain $K_{ATP}$ channel in an operative state and to prevent 'run-down' of the channel in various tissues (Ashcroft & Ashcroft, 1990; Nichols & Lederer, 1991; Terzic & Kurachi, 1995). Recently, several endogenous agents such as calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), prostacyclin, adenosine and $\beta$-adrenergic agonists have been shown to induce the opening of the $K_{ATP}$ channel through a cyclic AMP-dependent process (Standen et al, 1989; Quayle et al, 1994). These studies have focused mainly on the phosphorylation involving serine/threonine protein kinases. Interestingly, evidence suggest that protein phosphorylation is also essential for opening $K_{ATP}$ channels by exogenous $K^+$ channel openers (Tung & Kurachi, 1991). In addition, in our recent investigation, we have found that $K_{ATP}$ channel activity can be inhibited or stimulated by phosphorylation of tyrosine residues and by phosphorylation of serine/ threonine residues, respectively (Kwak et al, 1996). On considering these reports, we hypothesized that $K^+$ channel opener might induce the opening of the $K_{ATP}$ channel through phosphorylation of serine/ threonine residues or dephosphorylation of phosphorylated tyrosine residues. In addition, it has not yet studied which specific phosphorylation sites of the $K_{ATP}$ channel such as tyrosine or serine/threonine residue should be phosphorylated for effects of $K^+$ channel openers.

In this study, we performed experiments to determine which specific phosphorylation sites are involved in the $K_{ATP}$ channel-opening effect of pinacidil using specific enzymes and inhibitors to phosphorylate or dephosphorylate tyrosine residues and serine/ threonine residues. We present evidence that the $K_{ATP}$ channel-opening effect of pinacidil is not mediated via phosphorylation of $K_{ATP}$ channel protein or associatory protein, but require the phosphorylation of serine/threonine residues of the channel.

## METHODS

### *Cell isolation*

Single ventricular cells from adult Sprague-Dawley rat (250~300 g) were prepared by enzymatic digestion as described previously (Kwak et al, 1995) After opening chest cavities, hearts were excised and immersed in Krebs-Henseleit (KH) buffer solution (pH 7.35). Each heart was retrogradely perfused via the aorta in a Langendorff apparatus, with KH solution for 5 min to clear visible blood. Hearts were then perfused with $Ca^{2+}$-free KH solution until they stopped beating, and then they were perfused with $Ca^{2+}$-free KH solution containing 0.075% collagenase (Worthington, New Jersey, USA) for 30 minutes. After enzymatic digestion, ventricular muscle was removed and placed in $Ca^{2+}$-free KH solution containing 1% bovine serum albumin, cut into small pieces and mechanically dissociated into single cells. All cells used for experiments were rod-shaped with clear striations. KH solution contained (in mmol/L) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 10 HEPES, 25 $NaHCO_3$, 10 pyruvate, 11 dextrose and 1 $CaCl_2$.

Tyrphostin A23 (Calbiochem, California, USA), genistein (Sigma, St. Louis, USA), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7; Sigma, St. Louis, USA), pinacidil (Neo, Netherlands) and glibenclamide (RBI, MA, USA) were dissolved in dimethylsulphoxide (DMSO), and added to the bath solution. Protein phosphatase 2A (PP2A) and protein tyrosine phosphatase 1B (PTP1B) were purchased from UBI (Lake placid, NY, USA).

### *Electrical recording and data acquisition*

Gigaseals were formed with Sylgard-coated pipettes (borosilicate, Kimax) with 4~5 megaohm resistance and single-channel currents were recorded using inside-out and cell-attached configurations of patch clamp technique (Hamill et al, 1981). The channel currents were recorded with an Axopatch 1D patch clamp amplifier (Axon Instruments, CA, USA) and stored on videotapes via a pulse code modulator (SONY, PCM-501ES, Tokyo, Japan) for computer analysis later. Electrical signals were digitized and stored on hard disk (Hyundai, 40486 DX4, Seoul, Korea) at a sampling rate of 330 kHz using an analog-to-digital converter (Axon Instruments, Digidata 1,200, CA, USA). The pClamp software (Axon Instruments, V5.7.2, CA, USA) was used for data acquisition and analysis. All experiments were carried out at $22\pm2^{\circ}C$. The standard bath and pipette solutions contained (in mmol/L) 140 KCl, 2 $MgCl_2$, 5 EGTA and 10 HEPES (pH 7.2).

*Data analysis*

Fifty percent threshold method was used to detect events. The open probability ($P_o$) was calculated using the equation derived by Spruce et al (1985);

$$P_o = \left(\sum_{j=1}^{N} t_j \cdot j\right)/T_d \cdot N$$

where $t_j$ is the time spent at current levels corresponding to j=0, 1, 2 .....N channels in the open state. $T_d$ is the duration of the recording and N is the number of channels active in the patch. Recordings of 30~60 sec were analyzed for determination of $P_o$. The channel activity was expressed as $N \cdot P_o$. Data are presented as means±SEM. For statistical analysis, we compared means using Student's t-test, and a P value of less than 0.05 was considered significant.

## RESULTS

*Effects of protein kinase inhibitors on pinacidil-induced opening of $K_{ATP}$ channel*

Pinacidil is a well-known $K_{ATP}$ channel opener (Edwards & Weston, 1993) and we have recently reported that phosphorylation of tyrosine or serine/threonine residues could decrease or increase the $K_{ATP}$ channel activity (Kwak et al, 1996). In view of these reports, we hypothesized that $K_{ATP}$ channel opening effect of pinacidil might be due to phosphorylation of serine/threonine residues or tyrosine dephosphorylation. To test this hypothesis, we first examined the effects of several known protein kinase inhibitors on $K_{ATP}$ channel-opening of pinacidil in cell-attached patches (Fig. 1). At −60 mV, no $K_{ATP}$ channel activity was observed in the absence of pinacidil in bath solution. When pinacidil (100 μM) was added to bath solution, the $K_{ATP}$ channels were activated with a unitary conductance of about 70 pS. Following washout of pinacidil, the $K_{ATP}$ channel activity disappeared. Reapplication of pinacidil (100 μM) also induced the opening of the $K_{ATP}$ channels at a similar degree to the first application in the same patches (95.4±11.2% of the channel activity at first application; n=15). This current was abolished by 10 μM glibenclamide. Pinacidil-induced channel activities in the presence of several protein kinase inhibitors were expressed as a relative ratio to the channel activity during the first application of pinacidil in the same patch (Fig. 1b). The controls were obtained in the absence of protein kinase inhibitors. We then examined the effects of H-7 (Hidaka et al, 1984), a serine/threonine kinase inhibitor, and genistein (Akiyama et al, 1987) and tyrphostin A23 (Lyall et al, 1989), tyrosine kinase inhibitors.

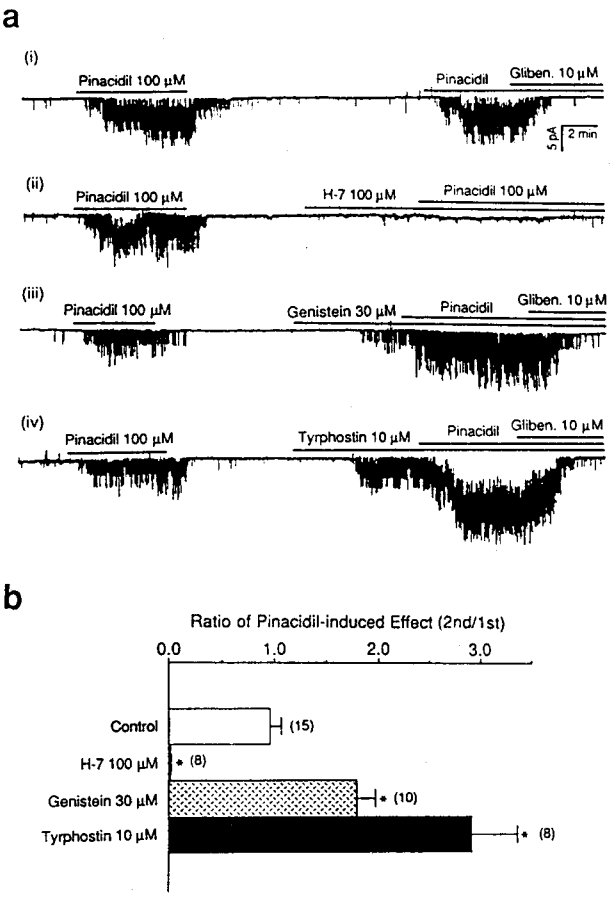


**Fig. 1.** Effects of H-7 (100 μM, ii), genistein (30 μM, iii), and tyrphostin-23 (10 μM, iv) on the pinacidil (100 μM)-induced $K_{ATP}$ channel activity in cell-attached patches. All drugs were added to bath solution. The patch was held at −60 mV, and dotted lines denote the resting current level. (a) Representative tracings show effects of pretreatment with H-7 (100 μM, ii), genistein (30 μM, iii), and tyrphostinA23 (10 μM, iv) on the pinacidil-induced $K_{ATP}$ channel activity. Filter for tracing reproduction was set at 300 Hz. (b) Ratios of pinacidil-induced effects were calculated by dividing the channel activity measured during the second application of pinacidil by that observed during the first pinacidil. Number of myocytes are shown in parenthesis. *$P<0.05$ vs control.

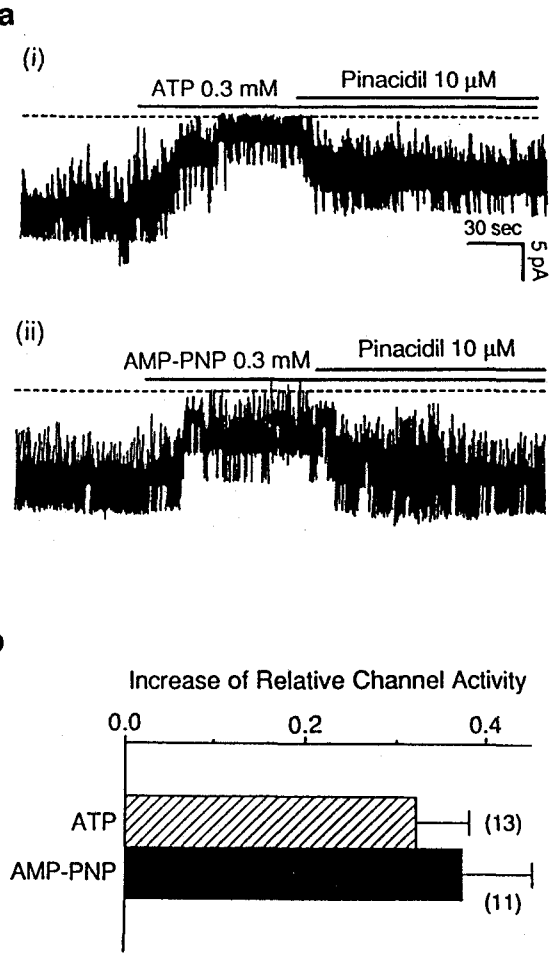


**Fig. 2.** Pinacidil (10 $\mu$M)-induced $K_{ATP}$ channel activity in the presence of AMP-PNP, a nonhydrolysible ATP analogue in inside-out patches. All drugs were added to the bath solution. The patch was held at −60 mV, and dotted lines denote the resting current level. (a) Pinacidil (10 $\mu$M)-induced $K_{ATP}$ channel activity in the presence of ATP (0.3 mM, i) and AMP-PNP (0.3 mM, ii). Filter for tracing reproduction was set at 300 Hz. (b) Relative channel activity was normalized to the channel activity immediately after excision of the patches. The pinacidil-induced channel activities were already subtracted by the channel activity immediately before pinacidil. Number of myocytes in parenthesis.

H-7 (100 $\mu$M) itself did not induce the $K_{ATP}$ channel activity, but prevented the $K_{ATP}$ channel opening by pinacidil (100 $\mu$M) (n=8). Genistein itself (30 $\mu$M) induced the opening of $K_{ATP}$ channel and enhanced the $K_{ATP}$ channel opening effect of pinacidil (100 $\mu$M) to 178.3±18.5% (n=10) which was calculated by subtracting the genistein-induced channel activity from the pinacidil-induced channel activity in the presence of genistein. Similarly, tyrphostin A23 (10 $\mu$M) another tyrosine kinase inhibitor also induced the opening of $K_{ATP}$ channel more potent than genistein (30 $\mu$M) and markedly enhanced the $K_{ATP}$ channel opening effect of pinacidil (100 $\mu$M) to 289.0±45.5% (n=8) which was calculated by subtracting tyrphostin A23 itself-induced channel activity from pinacidil-induced channel activity in the presence of tyrphostin A23. All the channel activities were abolished by 10 $\mu$M glibenclamide.

*Effect of pinacidil in the presence of AMP-PNP or ATP*

To test whether $K_{ATP}$ channel-opening effect of pinacidil was due to phosphorylation of the $K_{ATP}$ channel or an associated regulatory protein, we examined the effect of pinacidil in the presence of AMP-PNP, a nonhydrolysable ATP analogue, and compared it with that in the presence of ATP (Fig. 2). In inside-out patches, pinacidil (10 $\mu$M) added to the bath solution increased the $K_{ATP}$ channel activity in the presence of ATP (0.3 mM) or AMP-PNP (0.3 mM) by 32.1±6.4% (n=13) and 37.0±8.2% (n=11) of the channel activity before ATP or AMP-PNP, respectively, which was calculated by subtracting the channel activity with ATP (0.3 mM) or AMP-PNP (0.3 mM) from the pinacidil-induced channel activity in the presence of ATP or AMP-PNP. There was no significant change observed in $K_{ATP}$ channel-opening effects of pinacidil in the presence of ATP or AMP-PNP, indicating that pinacidil opening of $K_{ATP}$ channel does not involve phosphorylation of the channel.

*Effect of pinacidil on $K_{ATP}$ channel activity in partial rundown state*

We also have examined the effect of pinacidil on the $K_{ATP}$ channel activity in partial rundown state (Fig. 3). Upon formation of an inside-out patch in the ATP-free solution, $K_{ATP}$ channel activity is usually maximally activated but gradually decreases with time. This phenomenon has been referred to as "rundown". A major mechanism for rundown is believed to be $Mg^{2+}$-dependent dephosphorylation of the channel (Ohno-Shosaku et al, 1987; Takano et al, 1990; Kwak et al, 1996), although other mechanisms have been proposed (Furukawa et al, 1994; Terzic & Kurachi, 1996). Therefore, we hypothesized that $K_{ATP}$ channel in partial rundown state was still phos-

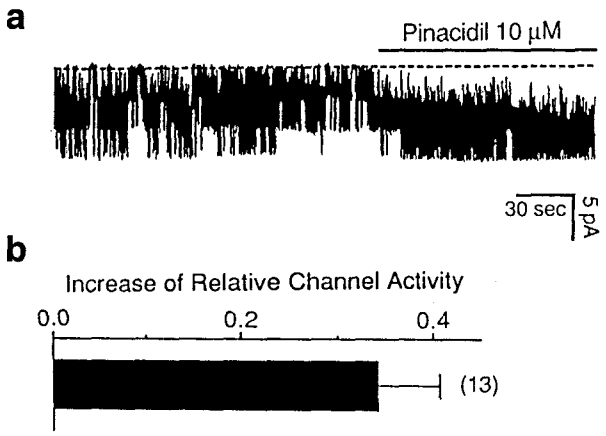


**Fig. 3.** Effect of pinacidil (10 $\mu$M) on the $K_{ATP}$ channel activity in the partial rundown state of inside-out patches. All drugs were added to the bath solution. The patch was held at −60 mV, and dotted lines denote the resting current level. The experiments were performed 30 minutes after the patch was excised. (a) Pinacidil (10 $\mu$M)-induced $K_{ATP}$ channel activity in a partial rundown state. Filter for tracing reproduction was set at 300 Hz. (b) Relative channel activity was normalized to the channel activity immediately after the patch was excised. The pinacidil-induced channel activities were already subtracted by the channel activity before pinacidil. Number of myocytes in parenthesis.

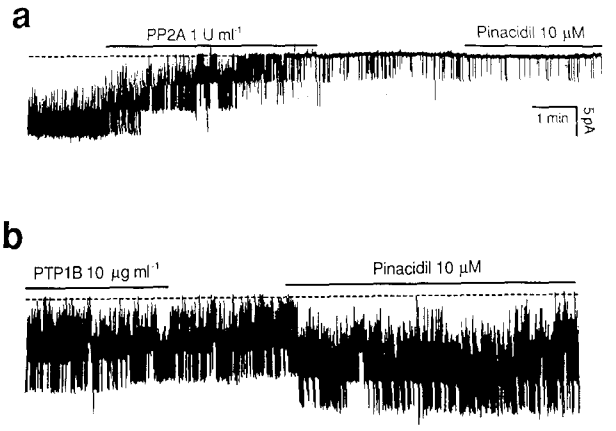


**Fig. 4.** Pinacidil (10 $\mu$M)-induced $K_{ATP}$ channel activity after pretreatment with specific protein phosphatases in inside-out patches. All agents were added to the bath solution. The patch was held at −60 mV, and dotted lines denote the resting current level. Each tracing denotes a representative record from similar observations (n=7) of each group. Filter for tracing reproduction was set at 300 Hz. (a) Protein phosphatase 2A (PP2A, 1 U $ml^{-1}$) was pretreated immediately after excision of patches. (b) The patch was pretreated for 10 minutes with protein tyrosine phosphatase 1B (PTP1B, 10 $\mu$g $ml^{-1}$) which was added 30 minutes after excision of patches. Pinacidil (10 $\mu$M) then was subsequently applied to the bath solution.

phorylated. 30 minutes after excision of patches, $K_{ATP}$ channel activity was decreased to 47.4±7.5% of the channel activity immediately after excision of patches (n=13). Pinacidil (10 $\mu$M) applied to the bath solution increased this channel activity by 34.1±6.5% of that measured immediately after excision of patches (n=13).

*Influences of specific protein phosphatases on the $K_{ATP}$ channel-opening effect of pinacidil*

To determine the phosphorylation site of the $K_{ATP}$ channel involved with $K_{ATP}$ channel-opening effect of pinacidil, we examined the effect of specific protein phosphatases on $K_{ATP}$ channel-opening effect of pinacidil in inside-out patches (Fig. 4). Protein phosphatase 2A (PP2A; 1 U $ml^{-1}$) (Haystead et al, 1989), a serine/threonine phosphatase, produced a rapid decrease in $K_{ATP}$ channel activity, which was not recovered by washout of the drug. Subsequent addition of pinacidil could not increase the $K_{ATP}$ channel activity (Fig. 4a). Similar results were observed in 6 other patches. As we have already shown, protein tyrosine phosphatase 1B (PTP1B) (Frangioni et al, 1992), a specific protein tyrosine phosphatase, had different effects on $K_{ATP}$ channels depending on their rundown state; PTP1B did not affect the channel activity immediately after formation of inside-out patches, whereas it promoted the recovery of the channels from rundown when applied about 30 minutes after formation of inside-out patches (Kwak et al, 1996). Thus, we treated the patches for 10 minutes with PTP1B (10 $\mu$g $ml^{-1}$) 30 minutes after excision of patches, and then used them in these experiments. After treatment with PTP1B, pinacidil (100 $\mu$M) applied to bath solution increased the channel activity by 30.8±7.1% of the channel activity immediately after excision of patches (Fig. 4b; n=7).

*Effect of specific protein kinase inhibitors on the $K_{ATP}$ channel-opening effect of pinacidil in reactivated channel after rundown*

It has been suggested that spontaneous rundown

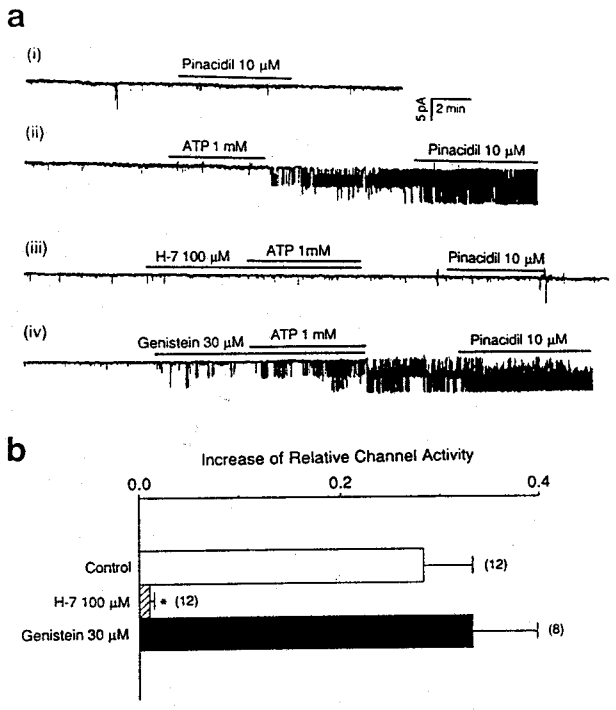


**Fig. 5.** Effects of specific protein kinase inhibitors on the $K_{ATP}$ channel-opening effect of pinacidil (10 $\mu$M) in the reactivated channel after rundown. All experiments were performed after the complete rundown of the $K_{ATP}$ channel. All agents were added to the bath solution. The patch was held at $-60$ mV, and dotted lines denote the resting current level. Filter for tracing reproduction was set at 300 Hz. (a) Effects of pretreatment with H-7 (100 $\mu$M, ii) or genistein (30 $\mu$M, iii) on the pinacidil-induced $K_{ATP}$ channel activity. (b) Relative channel activity was normalized to the channel activity immediately after excision of patches. Number of myocytes are shown in parenthesis. $*P<0.05$ vs control.

and ATP-induced reactivation of the $K_{ATP}$ channel are mainly due to reversible dephosphorylation and phosphorylation of the channel itself or an associated regulatory protein (Ohno-Shosaku et al, 1987; Takano et al, 1990; Kwak et al, 1996), in spite of controversial reports (Furukawa et al, 1994; Terzic & Kurachi, 1996). To further investigate which site of the $K_{ATP}$ channel could be phosphorylated for $K_{ATP}$ channel-opening effect of pinacidil, we tested the $K_{ATP}$ channel-opening effect of pinacidil on specifically phosphorylated channels in the presence of specific protein kinase inhibitors after rundown (Fig. 5). After rundown of the $K_{ATP}$ channels, pinacidil could not increase the channel activity (Fig. 5ai; n=15). Exposure to ATP (1 mM) for 5 minutes followed by washout reactivated the channels to 44.86.3% of the channel activity immediately after excision of patches (Fig. 5aii; n=12). The channel activity was further increased to $73.1\pm6.0\%$ by subsequent pinacidil (10 mM) (Fig. 5aii; n=12). These results indicate that phosphorylation plays an important role in reactivation of the $K_{ATP}$ channel. A serine/threonine kinase inhibitor H-7 (100 $\mu$M) completely prevented the ATP-induced reactivation of the channels (12 of 12 patches; Fig. 5aiii), suggesting that reactivation involves phosphorylation of serine/threonine. Subsequent pinacidil (10 $\mu$M) failed to increase the channel activity in H-7-treated patches. But, genistein (30 $\mu$M) in the presence of ATP (1 mM) slightly increased the $K_{ATP}$ channel activity, and enhanced the ATP-induced reactivation of the channel by $56.6\pm5.4\%$ of the channel activity immediately after excision of patches (Fig. 5aiv; n=8). Pinacidil (10 $\mu$M) increased the channel activity by $33.3\pm6.5\%$ of the channel activity immediately after excision of patches in genistein-treated patches (n=8).

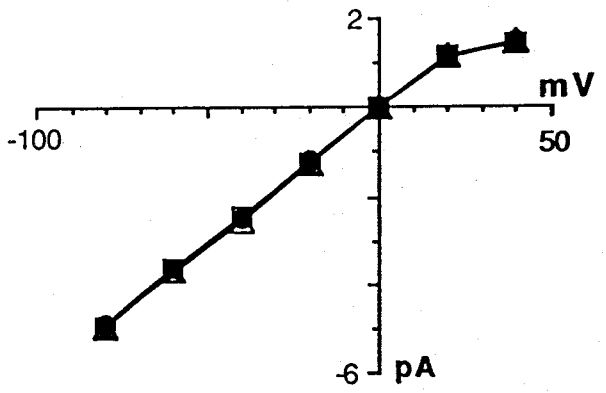


**Fig. 6.** Current-voltage relationships of pinacidil (10 $\mu$M)-induced $K_{ATP}$ channel currents in cell-attached patches (○) pretreated with H-7 (100 $\mu$M, ●), genistein (30 $\mu$M, △), and tyrphostin-23 (10 $\mu$M, □). All drugs were added to bath solution. Each point with vertical bar denotes the mean with SEM from 6 observations.

*Current-voltage relationship of pinacidil-induced $K_{ATP}$ channel currents in cell-attached patches pretreated with protein kinase inhibitors*

To test the possibility that specific phosphorylation could directly affect the gating kinetics and conformational change of $K_{ATP}$ channel itself, we measured current-voltage relationship of pinacidil-induced $K_{ATP}$ channel currents in cell-attached patches pre-

treated with protein kinase inhibitors (Fig. 6). The current-voltage relationship (I-V) curves of pinacidil-induced $K_{ATP}$ channel currents in control patches at negative potentials displayed a linear relationship with slope conductance of $67.3 \pm 2.8$ pS (n=6). In the presence of H-7 (100 $\mu$M), genistein (30 $\mu$M) or tyrphostin A23 (10 $\mu$M), slope conductances of pinacidil-induced $K_{ATP}$ channel currents are $64.3 \pm 3.1$ (n=6), $68.0 \pm 2.9$ (n=6) and $66.2 \pm 3.3$ pS (n=6), respectively (Fig. 6). At potentials positive to +40 mV, the I-V curves displayed inward rectification in all patches examined. The magnitude of inward rectification was not significantly different in the absence or presence of H-7 (100 $\mu$M), genistein (30 $\mu$M) or tyrphostin A23 (10 $\mu$M).

## DISCUSSION

Pinacidil is a well-known $K_{ATP}$ channel opener (Edwards & Weston, 1993), and we have confirmed its effect using patch clamp techniques. In the present study, pinacidil failed to induce the opening of the $K_{ATP}$ channel after H-7 or protein phosphatase 2A, whereas enhanced it after genistein, tyrphostin or protein tyrosine phosphatase 1B. In addition, pinacidil also produced $K_{ATP}$ channel-opening effect in the presence of AMP-PNP and in partial rundown state of the channel. These results indicate that $K_{ATP}$ channel-opening effect of pinacidil was not mediated via phosphorylation, but needs serine/threonine phosphorylation as a prerequisite condition.

Protein phosphorylation is one of the best-characterized mechanisms of ion channel modulation (Levitan, 1994). Ion channel modulation by serine/threonine protein kinases has been studied extensively, and $K^+$ channels are among those subjects to serine/threonine phosphorylation (Busch et al, 1992; Covarrubias et al, 1994; Drain et al, 1994). This holds true also for the $K_{ATP}$ channel. Protein kinase A (PKA) phosphorylates the $K_{ATP}$ channel to induce activation in cardiac myocytes (Tseng & Hoffman, 1990), renal cells (Wang & Giebisch, 1991), pancreatic $\beta$-cells (Dunne & Petersen, 1991) and follicular cells of *Xenopus* oocytes (Honore & Lazdunski, 1993). Protein kinase C also has been shown to regulate $K_{ATP}$ channel activity (Wollheim et al, 1988; de et al, 1989; Light et al, 1995; Hu et al, 1996). In contrast, the role of tyrosine phosphorylation of ion channels has only begun to be investigated (Hopfield et al, 1988; Huang et al, 1993; Wilson & Kaczmarek, 1993; Timpe & Fantl, 1994; Wang & Salter, 1994; Prevarskaya et al, 1995; Holmes et al, 1996; Kwak et al, 1996). In particular, we have found that the $K_{ATP}$ channel activity can be inhibited by tyrosine phosphorylation and stimulated by serine/threonine phosphorylation (Kwak et al, 1996). Recently, calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), prostacyclin, adenosine and $\beta$-adrenergic agonists have been shown to induce the opening of the $K_{ATP}$ channel through a cAMP-dependent process, and $\alpha$-adrenergic agonists (Hu & Nattel, 1995) and adenosine (Liu et al, 1996) activate the $K_{ATP}$ channel through activation of protein kinase C. In contrast, angiotensin II, known to inhibit protein tyrosine phosphatase (Takahasi et al, 1994), blocked $K_{ATP}$ channels in porcine coronary artery smooth muscle cells (Miyoshi & Nakaya, 1991). Thus, we hypothesized that the $K_{ATP}$ channel-opening effect of pinacidil might be mediated via serine/threonine phosphorylation or tyrosine dephosphorylation. However, in the present study, pinacidil caused activation of the $K_{ATP}$ channel even in the presence of AMP-PNP, a nonhydrolyzable ATP analogue, as well as even in the absence of exogenously applied ATP under partial rundown state of the channel. Direct phosphorylation process is, therefore, unlikely to be the part of the mechanism for the $K_{ATP}$ channel-opening effect of pinacidil in cardiac ventricular myocytes.

The modes of action of $K_{ATP}$ channel openers appear to be very complex. In particular, several $K_{ATP}$ channel openers could activate $K_{ATP}$ channel only when the channel is phosphorylated (Tung & Kurachi, 1991; Findlay & Dunne, 1986). It is also generally agreed that phosphorylation is essential in maintaining the $K_{ATP}$ channel activity in an operative state and in preventing 'run-down' of the channel in various tissues (for review see Ashcroft & Ashcroft, 1990; Nichols & Lederer, 1991; Terzic & Kurachi, 1995). In the present study, pinacidil did not affect the $K_{ATP}$ channel in the complete rundown state, but increased the $K_{ATP}$ channel activity in the presence of AMP-PNP, a nonhydrolysable ATP analogue as well as in the absence of ATP under partial rundown state. Furthermore, pinacidil also increased the $K_{ATP}$ channel activity in reactivated state recovered from complete rundown by exposure to ATP. These results support the idea that pinacidil acts only on the $K_{ATP}$ channels already phosphorylated. In addition, pina-

cidil failed to induce the opening of the $K_{ATP}$ channel when the level of serine/threonine phosphorylation was lowered by pretreatment with H-7 or protein phosphatase 2A, whereas enhanced it when the level of tyrosine was decreased by pretreatment with genistein, tyrphostin or protein tyrosine phosphatase 1B. Our recent report (Kwak et al, 1996) and these results support the idea that the $K_{ATP}$ channel activation by pinacidil may depend on phosphorylation level of serine/threonine residues of the channel or associated proteins, regardless of tyrosine phosphorylation.

Furthermore, Wang & Lipsius (1995) demonstrated that PKC activation potentiated acetylcholine-induced opening of the $K_{ATP}$ channel. Liu et al (1996) recently showed that PKC activation enhanced the $K_{ATP}$ current induced by pinacidil or adenosine in myocardial infarction. These reports indicate that PKC-mediated phosphorylation appears to increase the sensitivity of the $K_{ATP}$ channel to $K_{ATP}$ channel openers. Several evidence have been shown to explain the potentiating effect of cAMP-dependent phosphorylation (Tseng & Hoffman, 1990; Linde & Quast, 1995). In particular, Linde & Quast (1995) also showed that PKA activation potentiated the $K_{ATP}$ channel-opening effect of P1075 without any significant change of specific [$^3$H]-P1075 binding. These phosphorylation pathways are well known to involve serine and threonine residues of the $K_{ATP}$ channel or associated proteins. It will thus be possible that a phosphorylated state of the serine/threonine residue of the $K_{ATP}$ channel may provide a conformation preferred for the $K_{ATP}$ channel-opening effect of pinacidil. Thus, the fact that the $K_{ATP}$ channel-opening effect of pinacidil varied considerably among patches, can be explained on the basis of variable degrees of serine/threonine phosphorylation. However, further studies will be required to determine whether the effects of the phosphorylation pathways on the pinacidil response are independent or converge on a common mechanism. And it is not yet clear whether it is the $K_{ATP}$ channels themselves that are directly modified, or whether phosphorylation is simply an early step in a cascade of signal transduction pathway such as levels of receptors or G-proteins that leads ultimately to modulation of $K_{ATP}$ channel activity.

Cloning of $K_{ATP}$ channel has revealed that it is an octameric complex of two proteins which assemble with a 4 : 4 stoichiometry (Shyng & Nichols, 1997). The pore-forming subunit, Kir6.2, is a member of the inwardly rectifying $K^+$ channel family (Inagaki et al, 1995), while the other subunit is an ABC transporter, the sulfonylurea receptors (SUR) (Aguilar-Bryan et al, 1998). The latter endows Kir6.2 with sensitivity to the inhibitory effect of sulfonylurea drugs and to the stimulatory effects of MgADP and $K^+$ channel openers (Nichols et al, 1996; Trapp et al, 1997; Tucker et al, 1997). The $K_{ATP}$ channels are proposed to form a complex of SUR1 and Kir6.2 in pancreatic $\beta$-cells, SUR2A and Kir6.2 in cardiac and skeletal muscles, and SUR2B and Kir6.2 in smooth muscles (Aguilar-Bryan et al, 1995; Inagaki et al, 1995; Aguilar-Bryan et al, 1998). In view of these recent reports and our results that phosphorylation did not affect the conductance of the channel, it could be more evident that phosphorylation sites for $K_{ATP}$ channel-opening effect of pinacidil might be the myocardial sulfonylurea receptor (SUR2A) or other signal transduction cascades, thereby being able to modulate the sensitivity of $K_{ATP}$ channel to pinacidil. Because cloned KATP channel (Kir6.2 and SUR2A) has many potential phosphorylation sites including serine/threonine residues, further studies using site-directed mutagenesis are required to clarify which specific phosphorylation sites among many serine/threonine residues determine the sensitivity to the $K_{ATP}$ channel-opening effect of pinacidil.

In conclusion, the present study provides the evidence to suggest that the $K_{ATP}$ channel-opening effect of pinacidil is not mediated via phosphorylation of $K_{ATP}$ channel protein or associated protein, but requires the phosphorylation of serine/threonine residues of the channel as a prerequisite condition. In various pathophysiological states, phosphorylation level of the $K_{ATP}$ channel, associated protein or intracellular signaling proteins may be altered, and then the sensitivity to endogenous or exogenous $K^+$ channel openers including pinacidil may be changed. For example, serine/threonine phosphorylation level is increased by activation of PKC during myocardial preconditioning (Downey et al, 1994), and the sensitivity of the $K_{ATP}$ channel to pinacidil is expected to be increased which will result in more potent cardioprotective effect of pinacidil. Many substances known to modulate phosphorylation level of the cells, will alter the sensitivities to endogenous or exogenous $K_{ATP}$ channel openers including pinacidil. However, further studies are needed to elucidate what actually affect the sensitivities to endogenous or exogenous $K_{ATP}$ channel openers including pinacidil.

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