ATP-sensitive K⁺ channel activation by nitric oxide and protein kinase G in rabbit ventricular myocytes

JIN HAN,1 NARI KIM,1 HYUN JOO,2 EUIYONG KIM,1 AND YUNG E. EARM3

1Department of Physiology and Biophysics, College of Medicine, Inje University, Busan 614-735; 2Department of Molecular Science and Technology/Life Science, Ajou University, Suwon 442-749; and 3National Research Laboratory for Cellular Signaling and Department of Physiology, College of Medicine, Seoul National University, Seoul 110-799, Korea

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Han, Jin, Nari Kim, Hyun Joo, Euiyong Kim, and Yung E. Earm. ATP-sensitive K⁺ channel activation by nitric oxide and protein kinase G in rabbit ventricular myocytes. Am J Physiol Heart Circ Physiol 283: H1545–H1554, 2002.—The present investigation tested the hypothesis that nitric oxide (NO) potentiates ATP-sensitive K⁺ (KATP) channels by protein kinase G (PKG)-dependent phosphorylation in rabbit ventricular myocytes with the use of patch-clamp techniques. Sodium nitroprusside (SNP; 1 mM) potentiated KATP channel activity in cell-attached patches but failed to enhance the channel activity in either inside-out or outside-out patches. The 8-(4-chlorophenylthio)-cGMP Rp isomer (Rp-CPT-cGMP, 100 μM) suppressed the potentiating effect of SNP. 8-(4-Chlorophenylthio)-cGMP (8-pCPT-cGMP, 100 μM) increased KATP channel activity in cell-attached patches. PKG (5 U/μl) added together with ATP and cGMP (100 μM each) directly to the intracellular surface increased the channel activity. Activation of KATP channels was abolished by the replacement of ATP with ATP-γ-S. Rp-pCPT-cGMP (100 μM) inhibited the effect of PKG. The heat-inactivated PKG had little effect on the KATP channels. Protein phosphatase 2A (PP2A, 1 U/ml) reversed the PKG-mediated KATP channel activation. With the use of 5 nM okadaic acid (a PP2A inhibitor), PP2A had no effect on the channel activity. These results suggest that the NO-cGMP-PKG pathway contributes to phosphorylation of KATP channels in rabbit ventricular myocytes.

A brief period of ischemic preconditioning has been shown to protect the heart against subsequent prolonged ischemia in all species examined to date, including humans (61). In general, ischemic preconditioning appears to play a role in the phenomenon of ischemic preconditioning in the heart, and the activation of these channels may improve recovery of regional contractile function of stunned myocardium by shortening action potential duration and attenuating membrane depolarization, thus decreasing contractility and preserving energy during ischemia. These effects in turn reduce the duration of Ca²⁺ influx through L-type Ca²⁺ channels and increase the time for the Na⁺/Ca²⁺ exchanger to extrude Ca²⁺ from the cell, both of which will prevent intracellular Ca²⁺ overload and myocardial tissue injury (3, 11, 13).

The activation of cardiac muscarinic receptors due to vagal stimulation (35, 60), the release of myocardial nitric oxide (NO) (23, 38), and generation of bradykinin (41, 55) during ischemia have been suggested to play roles in ischemic preconditioning. A common mechanism of these findings is a direct or indirect increase in tissue cGMP content. Furthermore, cGMP has also been shown to contribute to the cardioprotective effect against ischemia-reperfusion injury in various species (7, 40, 58).

NO has been known to activate guanylate cyclase and to generate cGMP (1). A recent study (5) showed that cardiac myocytes express NO synthase, not only the inducible isoform (6) but also the constitutive isoform. In fact, recent studies (38) have shown that the NO level increases dramatically in the ischemic heart to reduce both coronary vascular tone and the extent of the ischemia. Furthermore, NO can protect the heart against ischemia-induced reperfusion injury (22). Thus one would predict that NO modulates the KATP channel during ischemia and reperfusion injury.

Thus it seems reasonable that cGMP-mediated intracellular signal transduction plays an important role in the mechanism of ischemic preconditioning. cGMP is a second messenger that mediates a considerable part of its effects by protein kinase G (PKG) (15, 20, 27, 31, 32, 52, 53). PKG is a serine-threonine protein kinase and has been shown to play a role in the mechanism of cardioprotection during ischemia (39, 41). The KATP channel is activated by phosphorylation of the serine-
threonine residue in rat cardiac myocytes, as shown in a recent study (29). Indeed, there are potential phosphorylation sites, including serine-threonine residues in the cloned $K_{ATP}$ channels (4, 25, 34).

Previous findings raise the intriguing possibility that the myocardial protection afforded by $K_{ATP}$ channel activation may involve phosphorylation of $K_{ATP}$ channels by PKG during ischemia. Accordingly, in this study, we tested the hypothesis that NO-cGMP-PKG-mediated phosphorylation of $K_{ATP}$ channels is involved in the activation of $K_{ATP}$ channels in rabbit ventricular myocytes. We observed that sodium nitroprusside (SNP), a potent stimulator of cGMP formation, and 8-(4-chlorophenylthio)-cGMP (8-pCPT-cGMP), a potent stimulator of PKG, potentiated the pinacidil-induced $K_{ATP}$ channel activity. Furthermore, we observed evidence that PKG activates $K_{ATP}$ channels in the presence of cGMP and ATP and that the PKG-mediated $K_{ATP}$ channel activity is inhibited by 8-pCPT-cGMP Rp isomer (Rp-pCPT-cGMP), a specific blocker of PKG, and protein phosphatase 2A (PP2A). These results suggest that PKG is involved in the phosphorylation of the $K_{ATP}$ channel or an associated protein. Such results may be important in understanding the mechanism by which the NO-cGMP-PKG signaling pathway acts as a link in receptor-mediated increases in $K_{ATP}$ channel activity during ischemic preconditioning.

**MATERIALS AND METHODS**

**Cell isolation.** Single ventricular myocytes were isolated from rabbit hearts by an enzymatic dissociation procedure, as discussed previously (18, 19). Briefly, rabbits weighing 150–200 g were anesthetized by injection of pentobarbital sodium (50 mg/ml, 1 ml/kg body wt) and heparin (300 IU/ml) into the marginal ear vein. Hearts were rapidly removed via thoracotomy with artificial ventilation and the aorta was cannulated. A dissected heart was mounted on a Langendorff apparatus and perfused retrogradely with oxygenated normo Tyrode solution for 5–6 min until all signs of blood were removed with gentle squeezing of the heart. The hearts were then perfused with a normal Ca$^{2+}$-free Tyrode solution for 5 min, followed by perfusion with Ca$^{2+}$-free Tyrode solution containing 0.01% collagenase (5 mg/50 ml, Yakult). After 15–25 min of enzymatic treatment, Kraftrybe (KB) solution was perfused. After being perfused with KB solution for 5 min, the hearts were removed from the cannula, the atria were discarded, and the ventricular walls and septum were cut vertically into 4–6 pieces. They were gently agitated in a small beaker with KB solution to obtain single cells. Isolated ventricular cells were stored in a KB solution at 4°C and used within 12 h. Langendorff column was kept at 37°C during all previous steps.

**Electrophysiological methods.** Single channel currents were measured in the cell-attached, inside-out, and outside-out patch configurations of the patch-clamp technique (17). Channel activity was measured using a patch-clamp amplifier (Axopatch-1D, Axon Instruments; Foster City, CA). Pipettes of 5- to 10-MΩ resistance were pulled from borosilicate glass capillaries (Clark Electrochemical; Pangbourne, UK) using a vertical puller (model PP-83, Narishige; Tokyo, Japan). Their tips were coated with Sylgard and fire polished. Membrane currents were digitized at a sampling rate of 20 kHz and stored in digitized format on digital audiotapes with the use of a recorder (model DTR-1200, Biologic; Grenoble, France). For the analysis of single channel activity, the data were transferred to a personal computer (Pentium III 430, IBM; Busan, Korea) with pCLAMP software (version 6.3; Axon Instruments, Union City, CA) through an analog-to-digital converter interface (Digidata-1200, Axon Instruments).

**Data analysis and quantification of channel activity.** The threshold for judging the open state was set at one-half of the single channel amplitude (12). Open probability ($P_o$) was calculated by using the formula

$$P_o = \frac{\sum_{j=1}^{N} t_j}{T \cdot N}$$

where $t_j$ is the time spent at current levels corresponding to $j = 0, 1, 2, \ldots N$ channels in the open state, $T$ is the duration of the recording, and $N$ is the number of channels active in the patch. The number of channels in a patch was estimated by dividing the maximum current observed during an extended period at zero ATP by the mean unitary current amplitude. When the dose-response relationship between pinacidil and $K_{ATP}$ channel activation was examined at a dose range of 0.1–1,000 μM, we found that the concentration at which $K_{ATP}$ channel activity was maximal for pinacidil was estimated to be 600–800 μM. On the basis of the data, the 1,000 μM pinacidil dose was used for the estimation of the maximum number of $K_{ATP}$ channels in the cell-attached and outside-out patch experiments. $P_o$ was calculated over 30-s records.

**Rundown of $K_{ATP}$ channels.** $K_{ATP}$ channel activity in rabbit ventricular myocytes decreases slowly with time after patches are excised into ATP-free solution. This phenomenon is known as “rundown.” At the time of excision, patches were continuously exposed to ATP (100 μM) except for a brief exposure to zero ATP at the beginning and end of experiments to estimate the number of channels in a patch and the degree of rundown. The data from patches exhibiting >50% rundown were discarded. In experiments designed to test the effects of PKG activation on $K_{ATP}$ channel activity, patches were continuously exposed to 100 μM ATP, unless otherwise stated. This concentration of ATP was chosen to represent a half-maximal inhibition level of ATP while giving a $P_o$ to allow single channel events to be observed.

**Solutions and drugs.** Normal Tyrode solution contained (in mM) 143 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 5.5 glucose, and 5 HEPES (pH 7.4) with NaOH. In the cell-attached and inside-out patch clamp experiments, the pipette solution contained (in mM) 140 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 glucose, and 10 HEPES (pH 7.4) with KOH, whereas the bath solution contained (in mM) 127 KCl, 13 KOH, 1 MgCl$_2$, 5 EGTA, 10 glucose, and 10 HEPES (pH 7.4) with KOH. In outside-out patch-clamp experiments, the pipette and bath solution were opposite to those used in the cell-attached and inside-out patch experiments. The modified KB solution had the following composition (in mM): 25 KCl, 10 KH$_2$PO$_4$, 16 KOH, 50 glucose, 10 taurine, 10 pyruvate, 10 HEPES, and 11 glucose at pH 7.4 adjusted with KOH.

ATP was added to the intracellular solution and glibenclamide was added to the extracellular solutions according to the experimental protocols described in the text. Glibenclamide was dissolved as a 0.2 mM stock solution in 2% dimethyl sulfoxide (DMSO) and diluted into the test solution appropriately before study. The final concentration of DMSO contained in the test solution was <0.01%. We confirmed that DMSO at this concentration had no effect on $K_{ATP}$ channel activity. After drugs were added to the test solution, the pH was readjusted to 7.4 with KOH. Pinacidil (RBI;
Natick, MA) was freshly prepared before experiments and diluted into the test solution to obtain the final concentrations indicated in the text. Okadaic acid (OA) was purchased from RBI. OA was stored at a stock concentration of 100 μM in ethanol at 4°C and used at a final concentration of 5 nM. PP2A was purchased from UBI (Lake Placid, NY). PKG was obtained from Promega (Madison, WI). 8-CPT-cGMP and Rp-pCPT-cGMP were obtained from Biolog Life Science Institute (Bremen, Germany). Unless noted otherwise, the agents were obtained from Sigma (St. Louis, MO). The experiments were performed at a room temperature of 25 ± 2°C.

**Solution exchange system.** In most experiments, we used a superfusion system (DAD-12, Adams and List; New York, NY) to change the bath solution and drugs. The system was designed to simplify the application of various concentrations of drugs and solutions to cells. The system takes advantage of the “sewer pipe effect.” When pointed at the cell to be studied, if the cell remains within the stream of the solution, the cell was essentially immersed completely in the solution that was applied. We could stop and start the flow quickly and change it with up to 12 variables by using the system.

**Statistics.** Data are presented as means ± SE when appropriate. Student’s unpaired t-test was used to calculate statistical significance. P ≤ 0.05 was considered significant.

**RESULTS**

Effect of SNP on $K_{ATP}$ channel activity of rabbit ventricular myocytes. To test the hypothesis that $K_{ATP}$ channels are involved in the action of NO, the effects of the NO donor on $K_{ATP}$ channels in cell-attached patches were investigated (Fig. 1, A and B). NO is well known as a stimulator of soluble guanylate cyclase and produces its effects by increasing intracellular cGMP concentrations, leading to an activation of PKG (1). We used sodium nitroprusside (SNP), a potent stimulator of cGMP formation, which has been known to be a NO donor (49). After gigaseal formation, the bath solution was switched from normal Tyrode solution to a high-K⁺ solution. At the pipette potential of −40 mV, unitary currents through the inward rectifier K⁺ channel were recorded, which was identified from its current-voltage ($I$-$V$) relations showing a slope conductance of −33 pS for the inward current. Under these conditions, $K_{ATP}$ channels were inactive even in the presence of 1 mM SNP, but subsequent bath application of pinacidil (50 μM) opened $K_{ATP}$ channels (Fig. A).

![Fig. 1](http://ajpheart.physiology.org/)

The effect of sodium nitroprusside (SNP) on the ATP-sensitive K⁺ ($K_{ATP}$) channel activity in rabbit ventricular myocytes. SNP (1 mM), pinacidil (50 μM), ATP (1 mM), and glibenclamide (30 μM) were added to the bath solution for the periods indicated by the thin bars. A: effect of SNP on the activation of $K_{ATP}$ channels in the absence of pinacidil in cell-attached patches. B: reversible activating effect of SNP on the pinacidil-induced $K_{ATP}$ channel activity in cell-attached patches. The pipette potential was held at −40 mV in the cell-attached patch experiments. C: effect of SNP on the $K_{ATP}$ channel activity in inside-out patches. D: effect of SNP on the $K_{ATP}$ channel activity in outside-out patches held at −40 mV. Data were sampled at 20 kHz and filtered at 1 kHz. Dashed line indicates the zero current level.
The addition of SNP (1 mM) to the bath failed to enhance the channel activity at −40 mV. In six patches, the average $P_o$ was $0.184 \pm 0.055$ mM before and $0.176 \pm 0.071$ mM during the addition of SNP ($P > 0.05$, $n = 6$ patches). Application of SNP (1 mM) to the extracellular surface of outside-out patch failed to enhance the channel activity (Fig. 1C): the average $P_o$ was $0.135 \pm 0.081$ mM before and $0.141 \pm 0.069$ mM during the addition of SNP ($P > 0.05$, $n = 3$ patches). Addition of ATP (1 mM, Fig. 1B) and glibenclamide (30 μM, Fig. 1D) immediately suppressed this channel activity confirming that observed openings were due to K+ flowing through KATP channels.

To investigate whether SNP facilitates the pinacidil-induced KATP channel activity via a cGMP/PKG-dependent mechanism, we applied Rp-pCPT-cGMP, a selective and membrane-permeant inhibitor of PKG in cell-attached patches (Fig. 2). The potentiating effect of SNP on KATP channel activity was suppressed by Rp-pCPT-cGMP (100 μM) in a reversible manner in all of the cells tested (Fig. 2A); the average $P_o$ was $0.237 \pm 0.045$ before and $0.092 \pm 0.053$ during the addition of Rp-pCPT-cGMP ($P < 0.05$, $n = 5$ patches, Fig. 2B).

**Effects of membrane-permeable cGMP analog on KATP channels.** To evaluate more directly whether the cGMP/PKG-dependent mechanism produces effects similar to those of the application of SNP, we examined effects of the potent PKG activator 8-pCPT-cGMP on the pinacidil-induced single channel activity in cell-attached patches. 8-pCPT-cGMP has the following advantages over other membrane-permeable analogs of cGMP: 1) it has higher lipophilicity so that it should permeate the cell membrane at higher rates, 2) it has a high degree of specificity of the PKG, 3) it has little effect on cGMP-regulated phosphodiesterases, and 4) it is resistant to degradation by phosphodiesterases. In the experiment shown in Fig. 3A, the pinacidil-induced KATP channel activity was reversibly facilitated by the addition of 8-pCPT-cGMP (100 μM). In six patches, the average $P_o$ increased $2.13 \pm 0.12$ times by 8-pCPT-cGMP ($P_o = 0.170 \pm 0.049$) when compared with the $P_o$ ($0.079 \pm 0.028$) recorded before the addition of 8-pCPT-cGMP ($P < 0.05$, $n = 6$). The pinacidil-induced single channel activity was inhibited by subsequent application of 30 μM glibenclamide ($P_o = 0.007 \pm 0.005$). These data are summarized in Fig. 3B.

**Effect of PKG activation on KATP channel in excised inside-out patches.** Because SNP and 8-pCPT-cGMP enhance the KATP channel activity in the cell-attached patches, as described above, these data support the idea that a cGMP-PKG-dependent mechanism can activate KATP channels. To evaluate more directly the involvement of a cGMP-PKG-dependent mechanism in activation of the KATP channel, we applied PKG to the intracellular side of the KATP channel in excised inside-out patches. In the experiments shown in Fig. 4A, after excision of the patch, ATP (100 μM) inhibited spontaneous KATP channel openings, reducing $P_o$ from 0.148 to 0.003. In the continuous presence of ATP, PKG (5 U/μl) with cGMP (100 μM), added to the intracellular surface, enhanced the channel activity ($P_o = 0.239$). Such an increase in channel activity by PKG with cGMP in the presence of ATP was observed in six patches, in which the average $P_o$ of KATP channels increased from $0.008 \pm 0.005$ to $0.250 \pm 0.083$ ($P < 0.05$, $n = 6$ patches). In the experiments described in Fig. 4, B and C, a maximal response to PKG activation
The continuous line in the graph is the curve of the observed ATP concentration. In Fig. 4, the ATP concentration increased, the responses were progressively smaller. In Fig. 4D, the channel activity for the ATP concentration used was normalized using the equation \( y = (P_o - P_{o,min})/(P_{o,max} - P_{o,min}) \), where \( y \) is the relative \( P_o \), \( P_{o,max} \) is the \( P_o \) at a given concentration of 100 \( \mu \)M ATP, and \( P_{o,min} \) is the \( P_o \) at 1,000 \( \mu \)M ATP. The continuous line in the graph is the curve fitted to the Hill equation using the least-squares method \( P_o = (P_{o,max} - P_{o,min})K^n_d/[K^n_d + ([ATP]^n)] + P_{o,min} \), where [ATP] is each ATP concentration, \( K_d \) is the concentration of ATP at the half-maximal inhibition of the channel, and \( n \) is the Hill coefficient. The \( K_d \) value for this inhibitory effect was 384 ± 29 \( \mu \)M (\( n = 7 \) patches), cGMP alone (100 \( \mu \)M; \( P < 0.05 \), \( n = 3 \) patches), cGMP and ATP together (100 \( \mu \)M each; \( P > 0.05 \), \( n = 3 \) patches), PKG alone (5 \( \mu \)M; \( P > 0.05 \), \( n = 3 \) patches), and PKG and cGMP together (5 \( \mu \)M and 100 \( \mu \)M each; \( P > 0.05 \), \( n = 3 \) patches) had no effect on the channel activity (data not shown).

The above results would indicate that PKG is effective only in the presence of cGMP and ATP, which in turn suggests that PKG acts through phosphorylation of the K\textsubscript{ATP} channel or an associated protein. To verify this hypothesis, we replaced ATP with ATP\textsubscript{γS}, a non-hydrolyzable analog of ATP, under experimental conditions similar to that shown in Fig. 4A. In the record shown in Fig. 4E, K\textsubscript{ATP} channels were inhibited by ATP\textsubscript{γS} (100 \( \mu \)M), and \( P_o \) was reduced from 0.094 to 0.002. However, the subsequent addition of PKG (5 \( \mu \)M) along with cGMP (100 \( \mu \)M) failed to enhance the channel activity (\( P_o = 0.003 \)). Similar effects were observed in three other patches.

To confirm the specificity of the PKG in stimulating the channel activity, we repeated the same experiment with heat-inactivated PKG. A stock solution containing only PKG was incubated in a hot water bath (100°C) for 20 min, and this heated PKG was used for the internal solution. Figure 5A shows a representative result obtained in an inside-out patch exposed to ATP (100 \( \mu \)M) at the intracellular surface. In the continuous presence of 100 \( \mu \)M ATP at the intracellular surface (\( P_o = 0.0016 \)), application of heated PKG (5 \( \mu \)M) along with cGMP (100 \( \mu \)M) to the intracellular surface failed to enhance the channel activity (\( P_o = 0.0018 \)). Similar results were observed in four other patches; the average \( P_o \) was 0.003 ± 0.002 \( \mu \)M before and 0.004 ± 0.003 \( \mu \)M during the addition of heated PKG (5 \( \mu \)M) and cGMP (100 \( \mu \)M) (\( P > 0.05 \), \( n = 5 \) patches).

To reaffirm the specificity of the PKG in stimulating the channel activity, another experiment was performed with Rp-pCPT-cGMP, a selective and membrane-permeant inhibitor of PKG. Figure 5B illustrates the effects of applying Rp-pCPT-cGMP (100 \( \mu \)M) in the presence of PKG activation in an excised inside-out patch. PKG (5 \( \mu \)M) caused an increase in K\textsubscript{ATP} channel activity in the presence of 100 \( \mu \)M ATP and 100 \( \mu \)M cGMP. Addition of Rp-pCPT-cGMP (100 \( \mu \)M) resulted in a reversal of the PKG-mediated K\textsubscript{ATP} channel activation, reducing the \( P_o \) from 0.090 to 0.005. Similar results were observed in five of the six patches examined; the average \( P_o \) was 0.133 ± 0.032 \( \mu \)M before and 0.006 ± 0.002 \( \mu \)M during the addition of Rp-pCPT-cGMP (100 \( \mu \)M) (\( P < 0.05 \), \( n = 5 \) patches).

PKG-induced activation of K\textsubscript{ATP} channel is reversed by protein phosphatase. In the present study, we observed that the activation of the K\textsubscript{ATP} channel by PKG occurred in the presence of cGMP and ATP. The result suggests that the cGMP stimulates PKG, which in turn activates the channel by phosphorylation. We sought to investigate whether exogenous PP2A, applied intracellularly during PKG activation, could impair the PKG-induced K\textsubscript{ATP} channel activation. Figure 6A illustrates the effects of applying PP2A (1 \( \mu \)M) in the presence of PKG activation in an excised inside-out patch. After the patch was excised, 100 \( \mu \)M ATP inhibited spontaneous K\textsubscript{ATP} channel openings and \( P_o \) was reduced from 0.163 to 0.012. In the continuous presence of ATP, PKG (5 \( \mu \)M) and cGMP (100 \( \mu \)M), added to the intracellular surface of the patch, enhanced the channel activity (\( P_o = 0.122 \)). Application of exogenous PP2A inhibited the PKG-mediated K\textsubscript{ATP} channel activity (\( P_o = 0.032 \)). Such a decrease in channel activity by PP2A was observed in five other patches (Fig. 6B).

To investigate whether the PKG-mediated phosphorylation of the K\textsubscript{ATP} channel underlies the PKG-induced activation of K\textsubscript{ATP} channels, further experiments were performed with OA, a potent inhibitor of type 1 protein phosphatase and PP2A (Fig. 7A). OA was used at a low concentration (5 \( \text{nm} \)) to specifically block the activity of
PP2A in excised inside-out patches (21). Application of PKG in the presence of ATP and cGMP caused an increase in the channel activity ($P_o = 0.268$). When ATP, cGMP, and PKG were then washed out and OA was applied to the patches, $K_{ATP}$ channel activity remained unchanged ($P_o = 0.275$). In the presence of OA, PP2A did not alter the channel activity ($P_o = 0.274$).

The results were observed in six of seven patches examined (Fig. 7B).

**DISCUSSION**

Numerous local agents or systemic factors that elevate myocardial cGMP have been reported to release...
The present study provided evidence that the activation of K\textsubscript{ATP} channels in rabbit ventricular myocytes can occur through a signal transduction pathway involving stimulation of guanylate cyclase, increased production and accumulation of cGMP, and activation of PKG, which phosphorylates and activates the K\textsubscript{ATP} channel. First, SNP facilitated the pinacidil-induced K\textsubscript{ATP} channel activity in cell-attached patches (Fig. 1). The potentiating effect of SNP was probably mediated by NO, as it is known to be a potent NO donor (48). The role of NO in modulating the K\textsubscript{ATP} channel is further supported by the study by Shinbo and Iijima (47), who demonstrated that NO could potentiate the effects of K\textsuperscript{+} channel opener in cardiac cells, although they could not explain the underlying mechanism for the potentiation of NO. It seems unlikely that NO directly activates K\textsubscript{ATP} channels because in the present study SNP (NO) failed to facilitate the K\textsubscript{ATP} channel activity in inside-out and outside-out patches. Second, SNP-induced K\textsubscript{ATP} channel activity was reduced by Rp-pCPT-cGMP, a selective inhibitor of PKG (Fig. 2). This finding provided direct evidence that the potentiating effect of SNP (NO) is due to an activation of PKG. This excludes the possibility that the effect of SNP (NO) is mediated via the activation of PKA by cross activation (26) or by cGMP-inhibited phosphodiesterases. Third, 8-pCPT-cGMP, a potent stimulator of PKG, potentiated the pinacidil-induced K\textsubscript{ATP} channel activity (Fig. 3). Fourth, PKG, added together with cGMP and ATP directly to the intracellular surface of inside-out patches, also increased the channel activity (Fig. 4). Finally, the effect of PKG was prevented by Rp-pCPT-cGMP, and heat-inactivated PKG had little effect on the channel activity (Fig. 5).

Such results would predict that PKG acts through phosphorylation of K\textsubscript{ATP} channels or some associated protein. Our present data support this notion in the several ways. First, the PKG-mediated activation of K\textsubscript{ATP} channels required both cGMP and ATP. This was further confirmed by the fact that under similar experimental conditions, replacement of ATP by ATP-S, a nonhydrolyzable analog of ATP, abolished the effect of PKG (Fig. 4). Second, application of exogenous PP2A reversed the PKG-induced activation of K\textsubscript{ATP} channels,

during ischemic preconditioning via cGMP-related signal transduction mechanism like PKG (15, 39). Therefore, it is particularly important to know whether there is any interaction between the cGMP-PKG signaling pathway and K\textsubscript{ATP} channels in the heart. The present study showed, for the first time to our knowledge, that K\textsubscript{ATP} channels can be opened through the cGMP-PKG signaling pathway in rabbit ventricular myocytes.

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Such results would predict that PKG acts through phosphorylation of K\textsubscript{ATP} channels or some associated protein. Our present data support this notion in the several ways. First, the PKG-mediated activation of K\textsubscript{ATP} channels required both cGMP and ATP. This was further confirmed by the fact that under similar experimental conditions, replacement of ATP by ATP-S, a nonhydrolyzable analog of ATP, abolished the effect of PKG (Fig. 4). Second, application of exogenous PP2A reversed the PKG-induced activation of K\textsubscript{ATP} channels,
and OA, a potent inhibitor of type 1 protein phosphatase and PP2A, abolished the effects of PP2A (Figs. 6 and 7). Finally, OA prevented the spontaneous reversal of the PKG-induced activation of K\textsubscript{ATP} channels (Fig. 7). The results also suggested that an endogenous membrane-associated PP2A is responsible for the reversal of PKG-mediated activation of K\textsubscript{ATP} channels. We therefore speculate that K\textsubscript{ATP} channels are under the control of both PKG and PP2A in rabbit ventricular myocytes. The processes of phosphorylation and dephosphorylation thus may regulate the K\textsubscript{ATP} channel activity in rabbit ventricular myocytes, proving a mechanism by which cellular excitability can be reversibly controlled.

The present results obtained from rabbit ventricular myocytes are compatible with others, demonstrating the regulation of K\textsubscript{ATP} channels by a cGMP-related signaling mechanism in vascular smooth muscle cells and follicle-enclosed Xenopus oocytes. Kubo et al. (28) have demonstrated that both atrial natriuretic factor (ANF) and isosorbide dinitrate (ISDN), activators of particulate and soluble guanylate cyclase, respectively, activate K\textsubscript{ATP} channels via an increase of intracellular cGMP in the cultured rat thoracic aorta. They also showed that 8-bromo-cGMP (8-BrcGMP) activated K\textsubscript{ATP} channels and had effects similar to those of ANF and ISDN, suggesting that the modulation of K\textsubscript{ATP} channels by ANF and ISDN is mediated by cGMP. In addition, SNP has been found to hyperpolarize rabbit mesenteric arteries by activating K\textsubscript{ATP} channel with accumulation of cGMP as an intermediate step (36). In follicle-enclosed Xenopus oocytes, ANF potentiated K\textsubscript{ATP} currents via the activation of guanylate cyclase and consequent accumulation of cGMP (45) and similar potentiating effects of 8-BrcGMP were observed for the K\textsubscript{ATP} channel activity-induced by K\textsuperscript{+} channel openers (46). These results suggest that activation of the K\textsubscript{ATP} channel may occur through a cGMP-related signaling mechanism more likely due to the activation of PKG. In contrast to the preceding studies, 8-BrcGMP has been found to inhibit the K\textsubscript{ATP} channel activity in guinea pig ventricular cells (47) and mouse pancreatic \(\beta\)-cells (44). On the other hand, SNP has been reported to have no effect on the K\textsubscript{ATP} currents in guinea pig coronary arterial smooth muscle (57). In addition, Tsuura et al. (49) have found that SNP does not affect the K\textsubscript{ATP} channel activity in rat ventricular myocytes. Thus it is likely that the effects of cGMP and PKG on K\textsubscript{ATP} channel function are tissue specific and depend on the signaling pathway to which PKG activation is linked.

Protein phosphorylation has been invoked as a putative effector mechanism in the infarct size-limiting effect of ischemic preconditioning (43), a phenomenon where a brief period of ischemia and reperfusion can protect the heart against subsequent prolonged ischemia and reperfusion injury (37). Indeed, it has been shown that phosphorylation levels are decreased during ischemia, whereas they are enhanced during ischemic preconditioning (56). A number of cellular proteins have been proposed as potential phosphorylation targets in the ischemic preconditioned heart, including the K\textsubscript{ATP} channel, stress proteins, and cytoskeletal proteins (2, 10, 16). To date, considerable evidence has been provided to suggest that the modulation of K\textsubscript{ATP} channel activity by phosphorylation play an important role in the cardioprotective effects of ischemic preconditioning (50, 59). The majority of the studies on the contribution of phosphorylation to K\textsubscript{ATP} channel activity have been centered on the role of PKC in the heart. It has been shown that PKC may act as a link in one or more receptor-mediated pathways to increase K\textsubscript{ATP} channel activity by phosphorylation and lead to ischemic preconditioning (30).

It is of interest that other newly described kinases like PKG may also play a role in the mechanism of ischemic preconditioning (15, 39). Iliodromitis et al. (24) reported that cardiac tissue cGMP is higher in the ischemic preconditioned than the nonpreconditioned regions of the heart. A rationale exists for a protective role for the cGMP-PKG signaling pathway against ischemia-reperfusion injury. PKG may act by the modulation of Ca\textsuperscript{2+} availability (32, 53) or myofilament sensitivity to Ca\textsuperscript{2+} (51). This together with the effect of the cGMP-PKG signaling pathway to which PKG activation is linked. Protein phosphorylation has been invoked as a putative effector mechanism in the infarct size-limiting effect of ischemic preconditioning (43), a phenomenon where a brief period of ischemia and reperfusion can protect the heart against subsequent prolonged ischemia and reperfusion injury (37). Indeed, it has been shown that phosphorylation levels are decreased during ischemia, whereas they are enhanced during ischemic preconditioning (56). A number of cellular proteins have been proposed as potential phosphorylation targets in the ischemic preconditioned heart, including the K\textsubscript{ATP} channel, stress proteins, and cytoskeletal proteins (2, 10, 16). To date, considerable evidence has been provided to suggest that the modulation of K\textsubscript{ATP} channel activity by phosphorylation play an important role in the cardioprotective effects of ischemic preconditioning (50, 59). The majority of the studies on the contribution of phosphorylation to K\textsubscript{ATP} channel activity have been centered on the role of PKC in the heart. It has been shown that PKC may act as a link in one or more receptor-mediated pathways to increase K\textsubscript{ATP} channel activity by phosphorylation and lead to ischemic preconditioning (30).
phosphotransfer reaction through creatine kinase and adenylate kinase, promoting delivery of mitochondrial signals to the channel site (9, 42, 62). Such signal transduction cascades provide a novel pathway for integration of cellular energetics with membrane electrical events believed to be critical in ischemic preconditioning.

KATP channel activation has been shown to be involved in cardioprotection by a variety of stimuli, including brief ischemia in the heart or remote organs and nonischemic stimuli in the heart such as ventricular pacing, stretch, and heat stress. Moreover, pharmacological agents that open KATP channels also produce cardioprotection. Although the exact mechanism by which KATP channel activation protects is still in dispute, current evidence suggests that mitochondrial KATP channels mediate cardioprotection in ischemic preconditioning (35). However, our results do not provide any experimental evidence to support this interesting possibility. It is not yet known whether the cardioprotection of ischemic preconditioning is due to mitochondrial KATP channels or sarcosomal KATP channels or to a mixture of both. The mitochondrial KATP channel is regulated by every ligand that regulates sarcosomal KATP channels. Our finding that the NO-cGMP-PKG signaling pathway can potentiate the KATP channel in rabbit ventricular myocytes raises the intriguing possibility that the NO-cGMP-PKG signaling pathway can modulate mitochondrial KATP channels in the heart. Such a hypothesis is supported by recent studies (8, 14) that addressed a possible role for NO in mediating late ischemic preconditioning.

In conclusion, our data show that rabbit ventricular myocytes may have a phosphorylation sites associated with the KATP channel that can be phosphorylated by a NO-cGMP-PKG signaling mechanism. These sites, when phosphorylated, increase the KATP channel activity, suggesting that this mechanism may contribute, at least in part, to the preconditioning-induced cardioprotection.

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