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# Hypoxic preconditioning upregulates $K_{ATP}$ channels through activation of protein kinase C in rat ventricular myocytes[1]

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**KEY WORDS** potassium channels; protein kinase C; patch-clamp techniques; anoxia; myocardium

## ABSTRACT

**AIM**: To test: 1) if hypoxic preconditioning upregulates the activity of $K_{ATP}$ channels in isolated adult rat cardiac myocytes; 2) if the upregulation involves protein kinase C (PKC). **METHODS**: By the whole-cell patch-clamp recording technique, $K_{ATP}$ channel currents ($I_{K_{ATP}}$) were measured in the cardiomyocytes with no pretreatment (CON), hypoxic exposure preceding reoxygenation (HPC), phorbol 12-myristate 13-acetate (PMA), and chelerythrine addition with HPC (CH + HPC), respectively. **RESULTS**: At 0 mV and 5 min after 2, 4-dinitrophenol (DNP) perfusion, the membrane currents for the CON, HPC, PMA, and CH + HPC were (3.5 ± 1.9), (7.7 ± 1.5), (7.5 ± 3.3), (4.6 ± 2.4) nA, respectively. Compared with the CON, the $I_{K_{ATP}}$ in the HPC and PMA were significantly augmented ($P < 0.01$) while the $I_{K_{ATP}}$ in the CH + HPC remained similar to the CON ($P > 0.05$). **CONCLUSION**: 1) hypoxic preconditioning stimulated the activity of PKC and markedly enhanced the activity of $K_{ATP}$ channels in the isolated rat cardiac myocytes; 2) PKC activation was involved in the upregulation of $K_{ATP}$ channels.

## INTRODUCTION

ATP sensitive potassium channels ($K_{ATP}$ channels) are closed during physiological conditions but are activated by a decrease in intracellular ATP concentration[1], thus they provide a link between the metabolic and electrophysiological states of the myocardium. Their proposed role as an endogenous cardioprotectant has received particular attention in the cardiovascular research in recent years. Accumulating results have strongly suggested that the activity of $K_{ATP}$ channels can be an end-effector both in the immediate and delayed preconditioning-induced protection. However, the mechanism concerning the modulation of $K_{ATP}$ channels is still unclear, although a number of factors, including protein kinase C (PKC), have been proposed as the channel regulators[2-5]. Also, few studies have directly measured the $K_{ATP}$ channel activity in the preconditioned cells while most of them employed the $K_{ATP}$ openers and/or inhibitors to control the channel activities and considered the infarct size, tissue functional recovery, cells viability, or anti-arrhythmic effects as the standards of protection[6-8]. By whole-cell patch-clamp recording technique, we directly measured the $K_{ATP}$ channel currents of the isolated adult rat cardiac myocytes in order to elucidate the relationship between PKC, a proposed key modulator in the signaling cascade of preconditioning, and the $K_{ATP}$ channel activities.

## MATERIALS AND METHODS

**Preparation of ventricular myocytes** Single ventricular myocytes from adult male Sprague-Dawley rats ($n = 12$, Grade Ⅱ, Certificate № HD-156) weighing 250 - 300 g were prepared by enzymatic dissociation as previously described[9] with small modifications. In brief, the heart was excised and the aorta was cannulated before it was retrogradely perfused on a Langendorff apparatus at 37 ℃ with oxygenated $Ca^{2+}$-free Tyrode's solution for 5 min followed by low $Ca^{2+}$ (50 μmol/L) Tyrode's solution containing collagenase 110 kU/L and 1 % bovine serum albumin (BSA) for 12 min. The ventricles were cut, minced, and gently triturated with a pipette in the low-$Ca^{2+}$ Tyrode's solution containing BSA at 37 ℃ for 10 min. The cell suspension was filtered

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through 200-$\mu$m nylon mesh, resuspended in the Tyrode's solution in which the $Ca^{2+}$ concentration gradually increased to 1.0 mmol/L. Isolated cells were stabilized for more than 6 h at the room temperature before being studied. Only the cells with a rod shape and clear cross striation were used for experiments.

**Grouping and preconditioning** Isolated cardiac myocytes were randomly assigned to the following four groups with different pretreatments. 1) CON: with no pretreatment; 2) Hypoxic exposure preceding reoxygenation (HPC): exposed to 95 % $N_2$ + 5 % $CO_2$ in a small chamber (gas phase oxygen fraction < 0.5 %) at 37 ℃ for 20 min and then reoxygenated with room air for 20 – 60 min; 3) PMA: phorbol 12-myristate 13-acetate (PMA) 100 nmol/L was added to the external solution for 20 min and then washed; 4) CH + HPC: the same as the HPC group except that the external solution was pre-added with chelerythrine chloride (CH) 5 $\mu$mol/L.

**Chemicals and solutions** BSA, collagenase (type Ⅱ), taurine, HEPES, glibenclamide, PMA, and CH were purchased from Sigma Co. 3-(*N*-morpholino)-propanesulfonic acid (MOPS) was purchased from Shanghai Boao Biotech Co. CH was dissolved in distilled water to make a 500 $\mu$mol/L stock solution and PMA was dissolved in ethanol to make a 500 $\mu$mol/L stock solution. The maximal concentration of ethanol in the perfusate was 0.02 %.

The composition of the $Ca^{2+}$-free Tyrode's solution was: NaCl 100, KCl 10, $NaH_2PO_4$ 1.2, $MgSO_4$ 5.0, glucose 20, taurine 10, MOPS 10 mmol/L; pH was adjusted with KOH to 7.2. Test solution for $K_{ATP}$ current recording was composed of NaCl 137, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 1.8, $NaH_2PO_4$ 0.33, HEPES 5, glucose 10 mmol/L; pH was adjusted with NaOH to 7.4. The electrode internal solution for $K_{ATP}$ current recording contained: KCl 140, $MgCl_2$ 0.5, egtazic acid 10, HEPES 10 mmol/L; pH was adjusted with KOH to 7.4.

**Potential and current recording** Myocytes were placed in a 500 $\mu$L chamber on the stage of an inverted microscope (Olympus CK2). The chamber was continuously superfused with test solutions 2 mL/min at 23 ℃. Membrane currents were recorded using the whole-cell patch-clamp techniques with a patch-clamp amplifier (CEZ 2300, Nihon Kohden, Japan). Patch electrodes were pulled using a vertical puller (PB-7, Narishige, Tokyo, Japan) and had a resistance of 2 – 3 M$\Omega$ when filled with electrode internal solution. After gigaseal was produced and patch was ruptured, the currents were recorded in a voltage-clamp mode. Experimental protocols, data acquisition and storage were accomplished with Pclamp 5.6 (Axon Instrument, USA) running on a personal computer.

Whole cell currents were recorded under a voltage-clamp mode with the holding potential held at – 80 mV. A ramp-shaped command potential from + 60 mV to – 100 mV with the duration of 5 s was used to elicit the membrane currents. $K_{ATP}$ currents were induced by adding 2,4-dinitrophenol (DNP) 50 $\mu$mol/L to the test solution. Currents were recorded before the addition of DNP, 2, 5, 7 min after the DNP perfusion and 2 min after the washout of DNP.

**Statistics** All data are expressed as $\bar{x} \pm s$. The changes of $K_{ATP}$ currents among groups were compared by the ANOVA with repeated tests, followed by the Turkey's multiple comparisons. Differences were considered significant when $P < 0.05$.

## RESULTS

In pilot studies, we included glibenclamide 10 $\mu$mol/L in the test solution to inhibit the $K_{ATP}$ channels activity. DNP perfusion 50 $\mu$mol/L was not able to induce a significant change in the membrane currents in the presence of this $K_{ATP}$ channel inhibitor. We also measured the reversal potentials of the DNP-induced currents at potassium concentrations 5.4, 8.1, and 10.8 mmol/L in the test solutions. The results were – 76.8, – 67.9, and – 61.6 mV respectively, which were in accordance with the changes of potassium equilibrium potentials calculated by the Nernst equation. Thus we had reasons to believe that the massive increment in the membrane currents induced by the DNP addition in our experiments was the result of $K_{ATP}$ channel opening.

Inclusion of DNP 50 $\mu$mol/L in the test solution significantly increased $I_{K_{ATP}}$ with a rapid rise phase around 3 min after DNP perfusion and with a platform phase from 5 to 7 min following the DNP perfusion in our experiments. Washout of DNP inversed the $I_{K_{ATP}}$ increment, but the recovery was not complete in most of the cells studied (Fig 1). In accordance with the massive increase in $I_{K_{ATP}}$, the durations of action potential (APD) were markedly shortened and partially recovered upon the diminution of $I_{K_{ATP}}$ (data not shown). Compared with the $I_{K_{ATP}}$ of the CON myocytes, the $I_{K_{ATP}}$ was markedly enlarged in the cells preconditioned with 20-min hypoxia followed by reoxygenation. PMA 100 nmol/L treatment showed the similar extent of effects as the hypoxic preconditioning.

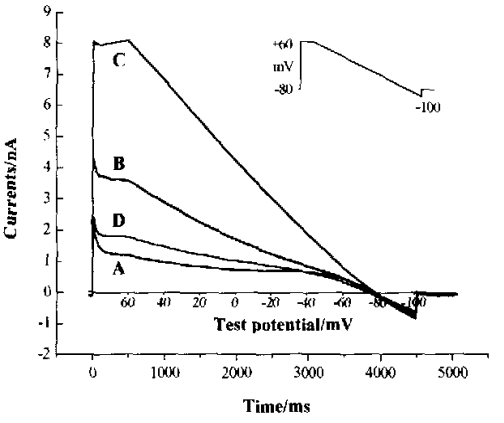


**Fig 1. Typical traces of $K_{ATP}$ currents of a myocyte induced by addition of 50 μmol/L DNP perfusion. The inlet panel shows the test potential waveform. Trace A, B, C, and D are currents recorded before DNP addition, 3 and 6 min after DNP addition, and 4 min after washout, respectively.**

In the presence of CH, however, the effects to enhance the opening of $K_{ATP}$ channels by hypoxic preconditioning were almost completely abolished (Tab 1, Fig 2). The differences in the $I_{K_{ATP}}$ among the groups were more prominent at 3 – 5 min after the DNP perfusion, and thereafter run-down of the currents occurred in the HPC and PMA myocytes to some extent, which made the differences less prominent.

## DISCUSSION

The present study showed that the activity of $K_{ATP}$ channels was markedly enhanced in the isolated rat cardiomyocytes preconditioned with 20-min anoxia followed by 20 – 60 min-reoxygenation and in those pretreated with selective PKC activator, PMA. In the presence of selective PKC inhibitor CH, however, those effects of hypoxic preconditioning were abolished. The results indicated that hypoxic preconditioning activated PKC in the isolated rat cardiac myocytes and the activity of $K_{ATP}$ channels in the hypoxically preconditioned cells was upregulated. The upregulation was possibly modulated by PKC.

Protein kinase C, a kinase family that consists of 12 related Ser/Tre kinases and plays important roles in the cellular growth, differentiation, and immediate regulation of effector functions, has been implicated as a primary cellular mediator of ischemic preconditioning[10], although controversy exists[6]. $K_{ATP}$ channels, a set of proposed target proteins that PKC phosphorylates upon activation, have also been implicated to be involved in the cardioprotection of preconditioning possibly as the

**Tab 1. Comparisons of $K_{ATP}$ currents (nA) of cardiac myocytes with and without pretreatments. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs CON*. $^eP<0.05$, $^fP<0.01$ *vs CH+HPC*.**

| | Test potential (mV) | CON ($n=16$) | HPC ($n=8$) | PMA ($n=8$) | CH+HPC ($n=8$) |
|---|---|---|---|---|---|
| DNP0 | -100 | -0.5±0.2 | -0.7±0.2 | -0.6±0.3 | -0.5±0.2 |
| | 0 | 0.4±0.2 | 0.5±0.2 | 0.4±0.1 | 0.3±0.1 |
| | 60 | 1.2±0.3 | 1.3±0.3 | 1.4±0.4 | 1.1±0.3 |
| DNP2 | -100 | -0.6±0.3 | -1.3±0.5$^{cf}$ | -1.1±0.7$^{be}$ | -0.6±0.3 |
| | 0 | 1.4±1.2 | 4.5±3.0$^{be}$ | 4.0±3.1$^{b}$ | 1.9±1.4 |
| | 60 | 2.8±2.1 | 8.2±5.1$^{c}$ | 7.3±5.3$^{b}$ | 3.8±2.7 |
| DNP5 | -100 | -0.7±0.4 | -1.7±0.4$^{ce}$ | -1.7±0.8$^{b}$ | -1.0±0.6 |
| | 0 | 3.5±1.9 | 7.7±1.5$^{ce}$ | 7.5±3.3$^{c}$ | 4.6±2.4 |
| | 60 | 6.3±3.3 | 13.5±2.5$^{ce}$ | 13.2±5.5$^{c}$ | 8.3±4.1 |
| DNP7 | -100 | -0.7±0.3 | -1.5±0.8$^{b}$ | -1.4±0.8 | -1.0±0.6 |
| | 0 | 3.4±1.5 | 6.7±2.8$^{b}$ | 7.0±3.9$^{b}$ | 4.9±2.5 |
| | 60 | 6.3±2.7 | 12.3±4.3$^{b}$ | 12.2±6.8$^{b}$ | 8.8±4.1 |
| Wash2 | -100 | -0.4±0.1 | -0.6±0.2 | -0.6±0.5 | -0.4±0.2 |
| | 0 | 1.0±0.9 | 3.0±1.3 | 3.5±3.4$^{b}$ | 1.5±1.4 |
| | 60 | 2.2±1.6 | 5.8±2.2 | 6.3±6.0$^{b}$ | 2.8±2.6 |

DNP0, DNP2, DNP5, DNP7, and Wash2 represent before DNP addition, 2, 5, 7 min after DNP perfusion, and 2 min after the DNP washout, respectively.

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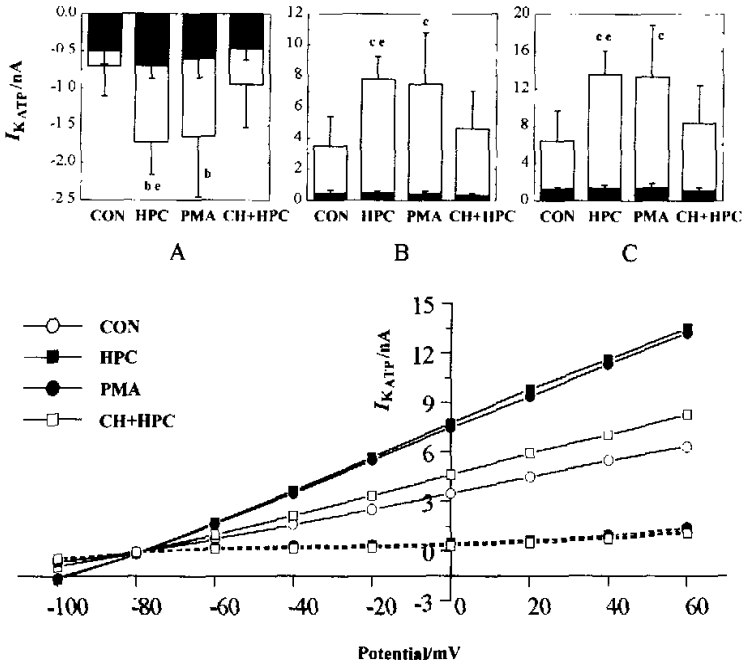


**Fig 2. Comparison of $I_{K_{ATP}}$ at 5 min after DNP 50 μmol/L perfusion. In the lower panel, the dashed line shows the currents before DNP addition, whereas the solid lines are currents after 5 min DNP perfusion. The upper panel A, B, C are the changes of currents at test potentials of −100, 0, and +60 mV, respectively. Solid bars are currents before DNP addition and the open bars are the currents after 5 min DNP perfusion. $^bP<0.05$, $^cP<0.01$ *vs* CON; $^eP<0.05$ *vs* CH+HPC.**

end-effectors[7,8,10]. The modulation of $K_{ATP}$ channel activity by PKC and possibly adenosine has been suggested in recent studies. In excised rabbit ventricular membrane patches, Hu *et al*[4] found that external adenosine (100 μmol/L) significantly increased $K_{ATP}$ activity at $[ATP]_i$ between 5 and 50 μmol/L by decreasing channel sensitivity to $[ATP]_i$. However, when the highly selective PKC blocker bisindolylmaleimide was included in the internal solution, the $K_{ATP}$-stimulating action of adenosine was prevented. Thus they concluded that PKC mediated the adenosine activation of $K_{ATP}$ channels. Liang[7] determined the signaling role of the adenosine receptor, PKC, and the $K_{ATP}$ channel and the temporal sequence of activation of these three mediators in preconditioning of cardiac myocytes and he came to a different conclusion that $K_{ATP}$ channel, not the adenosine receptor, is the effector down-stream from PKC in initiating PKC-mediated preconditioning. Several other studies[2,8] also demonstrated the similar results supporting the direct regulation by PKC of $K_{ATP}$ channel activity.

The results of the present study showing that PKC was upstream of the $K_{ATP}$ channels in the preconditioning were from indirect evaluation of the channel activity by applying the $K_{ATP}$ openers and/or inhibitors and by considering the infarct size, tissue functional recovery, cells viability, and/or anti-arrhythmic effects as the endpoints of protection[6-8]. To our knowledge, so far no studies, have conducted experiments to study the temporal sequence of PKC and $K_{ATP}$ activation by direct measurement of $K_{ATP}$ current changes in the ischemically or hypoxically preconditioned cells[2-5].

Thus our study confirmed the proposition that the activation of PKC upregulated the $K_{ATP}$ channel activity in the hypoxically preconditioned rat ventricular myocytes, and provided more direct evidences for the $K_{ATP}$ channel modulation and its possible roles, thus promoting our understanding of the physiology and pathophysiology of heart.

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## 低氧预处理通过蛋白激酶 C 激活上调大鼠心室肌细胞 $K_{ATP}$通道[1]

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**关键词** 钾通道；蛋白激酶 C；膜片箝技术；缺氧；心肌

**目的**：研究：1）在大鼠心室肌细胞，低氧预处理是否上调 $K_{ATP}$通道活动；2）是否蛋白激酶 C（PKC）参与了调节. **方法**：使用标准的全细胞膜片箝方法. 记录经过不同预先处理的心肌细胞 2,4-dinitrophenol DNP 诱发的 $K_{ATP}$通道电流. 细胞预先处理分别为对照（CON），低氧复氧（HPC），佛波酯（PMA）和 Chelerythrine + 低氧复氧（CH + HPC）. **结果**：在测试电位 0 mV 加入 DNP 5 分钟时 CON，HPC，PMA 和 CH + HPC 组的膜电流值分别为：（3.5 ± 1.9），（7.7 ± 1.5），（7.5 ± 3.3），（4.6 ± 2.4）nA. 与 CON 相比，HPC 和 PMA 组的 $I_{K_{ATP}}$显著增高（$P < 0.01$），而 CH + HPC 和 CON 相比则无显著意义（$P > 0.05$）. **结论**：1）低氧预处理激活 PKC 并显著加强心肌细胞 $K_{ATP}$通道活动；2）PKC 的激活参与了 $K_{ATP}$通道的上调.

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