British Journal of Pharmacology (2000) 131, 1097–1104 © 2000 Macmillan Publishers Ltd All rights reserved 0007–1188/00 $15.00

www.nature.com/bjp

# Effects of acidosis and NO on nicorandil-activated $K_{ATP}$ channels in guinea-pig ventricular myocytes

[1,2]Gustavo A. Moncada, [1]Yukio Kishi, [1]Fujio Numano, [3]Masayasu Hiraoka & *,[2]Tohru Sawanobori

[1]The Third Department of Internal Medicine – Cardiology Division, Graduate School of Medicine, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, Japan; [2]Department of Clinical Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, Kanda-Surugadai, Chiyoda-ku, Tokyo, Japan and [3]Department of Cardiovascular Diseases, Medical Research Institute, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, Japan

**1** Nicorandil is a hybrid compound of $K^+$ channel opener and nitrate. We investigated a possible interaction of acidosis and nitric oxide (NO)-donors on the nicorandil-activated ATP-sensitive $K^+$ channel ($K_{ATP}$) in guinea-pig ventricular myocytes using the patch-clamp technique.

**2** In whole-cell recordings, external application of 300 $\mu$M nicorandil activated $K_{ATP}$ in the presence of 2 mM intracellular ATP concentration ($[ATP]_i$) at external pH ($pH_o$) 7.4, but the activated current was decreased by reducing $pH_o$ to 6.5–6.0.

**3** Single-channel recordings of inside-out patches revealed decreased open-state probability ($P_o$) of $K_{ATP}$ activated by nicorandil with reducing internal pH ($pH_i$) from 7.2 to 6.0, whilst the channel activity increased at low $pH_i$ in the absence of nicorandil.

**4** Application of NO donors, 1 mM-sodium nitroprusside (SNP) or -NOR-3 to the membrane cytoplasmic side at $pH_i$ 7.2 increased the channel activity but decreased it at $pH_i$ 6.5–6.0. Neither removal of the drugs nor application of NO-scavengers reversed depression of channel activity induced by NO-donors.

**5** We conclude that an increase in $pH_o$ and $pH_i$ depresses rather than stimulates the nicorandil-activated $K_{ATP}$. Since NO-donors at low $pH_i$ exhibited a similar trend, involvement of $H^+$ and NO interaction can be considered as a mechanism of decreased $K_{ATP}$ activated by nicorandil.
*British Journal of Pharmacology* (2000) **131,** 1097–1104

**Keywords:** Nicorandil; $K^+$ channel opener; nitrates; NO-donors; patch-clamp

**Abbreviations:** $[ATP]_i$, intracellular ATP concentration; $[H^+]_o$, external proton-concentration; $[H^+]_i$, internal proton-concentration; $K_{ATP}$, ATP-sensitive $K^+$ channel; KCO; $K^+$ channel opener; $pH_o$, external pH; $pH_i$, internal pH; Po, open-state probability

## Introduction

Nicorandil is a potassium channel opener that targets the ATP-sensitive potassium channel ($K_{ATP}$) (Hiraoka & Fan, 1989; Nakayama *et al*., 1991; Takano & Noma, 1990). Based on its chemical structure nicorandil can be considered as a hybrid compound having nitrate and $K^+$ channel opening properties (Taira, 1987). The drug is clinically available by virtue of its ability to dilate the coronary vessels and increase the coronary flow reserve in patients with chronic ischaemic heart disease (Frampton *et al*., 1992; Goldschimidt *et al*., 1996; Krumenacker & Roland, 1992). Recently much attention has been paid to its effects mimicking ischaemic preconditioning (Matsubara *et al*., 2000; Patel *et al*., 1999). Whilst the potency for nicorandil to activate $K_{ATP}$ is not strong compared to other agents, the drug has a unique action requiring the presence of MgADP for the channel activation (Shen *et al*., 1991), whilst other studies demonstrated the channel activation by nicorandil in the absence of ADP at the single channel level (Takano & Noma, 1990). It has been further suggested that nicorandil could activate $K_{ATP}$ in the absence of ADP in external acidotic condition suggesting more effective action at low $pH_o$ than at normal $pH_o$ (Jahangir *et al*., 1994). Other studies indicated that $K_{ATP}$ activated by another KCO, pinacidil, was suppressed at low $pH_o$ and increased at high $pH_o$, suggesting an external site of interaction for the channel and $H^+$ (Kwok & Kass, 1994).

During myocardial ischaemia important changes in external and internal pH as well as changes in the ratio of intracellular ATP and ADP concentrations take place (Allen *et al*., 1985). The modulation of $K_{ATP}$ has been studied to demonstrate that moderate increase in $H^+$ concentrations, acidosis, itself has the ability to increase the activity of $K_{ATP}$ (Cuevas *et al*., 1991; Davies, 1990; Fan & Makielski, 1993; Koyano *et al*., 1993). Therefore, nicorandil may exert its $K^+$ channel opening potency more strongly during ischaemia or acidotic conditions than in normoxic state and normal pH. However, it is not known how the other aspect of nicorandil as nitrate interacts with the $K_{ATP}$ activation. The present study was undertaken to examine a possible interaction between low pH and NO on the nicorandil-activated $K_{ATP}$ activity.

## Methods

The investigation was conducted in accordance to the guidelines for the care and use of laboratory animals at Tokyo Medical and Dental University.

### *Cell isolation*

Single ventricular myocytes were isolated enzymatically from the ventricles of female guinea-pigs weighing 250–350 g. The

*Author for correspondence at: Department of Clinical Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10, Kanda-Surugadai, Chiyoda-Ku, Tokyo 101-0062, Japan; E-mail: sawa.cph@mri.tmd.ac.jp

technique for cell isolation used in our laboratory has been described previously (Hirano & Hiraoka, 1988). In brief, guinea-pigs were anaesthetized with Na-pentobarbital (30 mg $Kg^{-1}$, IP) and right after injected intravenously with heparin (300 units $Kg^{-1}$). The chest was opened under artificial respiration (Respirator Model 141, NEMI Corp, Medwey, MA, U.S.A.) and the aorta was canulated to perfuse retrogradely with the Tyrode solution before the heart was dissected out. After 6 min of perfusion on the Langendorff apparatus, the heart was perfused with a nominally $Ca^{2+}$ free Tyrode solution for an additional 6 min. The perfusate was switched to $Ca^{2+}$ free Tyrode solution containing collagenase (5 mg 50 $ml^{-1}$, Yakult, Tokyo, Japan). After 5–6 min perfusion, the heart was washed out with a high $K^+$, low $Cl^-$ solution. The temperature of the perfusates was maintained at 35–36°C. The isolated cells were obtained through a mesh (size 200 $\mu$M). Rod-shaped cells with clear margin and striation were used for the experiments.

### *Solutions*

In the whole-cell configuration the bath solution was Tyrode solution, and its composition was (mM): NaCl 144, $NaH_2PO_4$ 0.33, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, and HEPES 5.0; the pH was adjusted to 7.3–7.4 by addition of NaOH. The $Ca^{2+}$-free Tyrode solution was prepared by omitting $CaCl_2$ from the Tyrode solution. High $K^+$ low $Cl^-$ solution contained (mM): KOH 80, glutamic acid 70, taurin 15, $KH_2PO_4$ 10, HEPES 5, $MgCl_2$ 0.5, glucose 11, and $K_2$-ethyleneglycol-O-O′- bis(B- aminoethyl)-N,N,N′,N′- tetraacetic acid (EGTA) 0.5 (pH 7.4-KOH). The composition of the pipette solution was (mM): KCl 120, $K_2$ATP (Sigma Chemical Co., St. Louis, MO, U.S.A.) 2.0, HEPES 5.0 and $K_4$BAPTA (Dojin Co., Kumamoto, Japan) 5.0; the pH was adjusted to 7.2 with KOH. The final $K^+$ concentration was kept constant at 150 mM.

In the case of single-channel recordings (inside-out patch configuration), the bath solution (intracellular medium) contained (mM): KCl 140, glucose 5.5, EGTA 2 and HEPES (or PIPES) 5; the pH was adjusted to 7.3 with KOH. When experiments were conducted in acidotic conditions, bath solutions having different pH values (7.2, 6.5 or 6.0) were adjusted with HEPES or PIPES buffer systems, accordingly. Final pH adjustment was done with 0.1 N HCl. When nucleotides were present in the bath, $MgCl_2$ 0.6–0.8 mM was added to the bath. The drugs were dissolved in the bath solution at the concentration indicated in the text. The pipette solution (extracellular medium) contained (mM): KCl 140, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5 and HEPES 5; the pH was adjusted to 7.3 by adding KOH.

### *Electrophysiological measurements*

*Whole-cell current recordings* Membrane currents were recorded using the patch-clamp technique of whole cell configuration (Hamill *et al*., 1981), using a patch-clamp amplifier (Axopatch ID, Axon Instrument, Foster City, CA, U.S.A.). Glass patch electrodes were made from borosilicate capillary tubes with an outer diameter of 1.5 mm (Clark Electromedical Instruments, Pangbourne, England) using a microelectrode puller (Model PP-830, Narishige Co. Tokyo, Japan) and were heat-polished by a microforge (Model MF-830, Narishige Co., Tokyo, Japan). The electrode resistance was 2–4 MΩ when the pipettes were filled with an internal solution. The recording technique and the data acquisition systems have been described in previous reports (Hirano & Hiraoka, 1988). When the ramp voltage-clamp method was employed, an intelligent arbitrary function synthesizer (model 1731, NF Instruments, Yokohama, Japan) was used to supply the command pulse.

The temperature of the bath chamber was maintained at 34–35°C with a heating system (DTC-100TA, Dia Medical System, Tokyo, Japan). Before establishing contact between the electrode and the cell membrane, the junction potential was adjusted to zero at the level of the bath solution. At the end of each experiment the junction potential was verified again and, adjusted if a difference of more than $\pm 2$ mV existed between the first and second measurements. A time-interval of 2–3 min for sequential recording at different pH was established.

*Single-channel recordings* Single-channel current recordings were carried-out at room temperature with conventional inside-out patch configuration (Hamill *et al*., 1981) using the same patch-clamp amplifier as in the whole-cell experiments. The current signals were recorded and stored simultaneously on a videocassette recorder (HR-S7700, Victor, Tokyo, Japan) and a thermal recorder (Omnicorder 8M14-3, NEC-Sanei Instruments, Ltd., Tokyo, Japan) through a PCM data recorder system (RP-882, NF Instruments, Yokohama, Japan) at a conversion rate of 40 kHz. Recorded signals were filtered off-line through a programmable eight-pole Bessel low-pass filter (48 dB/octave, 3625, NF Instruments, Yokohama, Japan) and digitized at a sampling frequency of 10 kHz and stored into a MO disk of a computer (Physio PC-01; Physio-tech, Tokyo, Japan) using an analogue-to-digital converter (Digidata 1200 Interface, Axon Instruments, Foster City, CA, U.S.A.) for later analysis. pCLAMP software (version 6.0.4, Axon Instruments) was used to generate voltage pulse protocols, data acquisition and analysis.

*Single channel data analysis* Single channel records were analysed by pCLAMP 6.0 software program on a computer (Physio PC-01; Physio-tech, Tokyo, Japan). The unitary current amplitude of $K_{ATP}$ was measured from the all point histograms through Gaussian fitting, or by selecting well-defined long opening and close transitions and measuring the magnitude of the corresponding current steps with horizontal cursors.

Mean patch current ($I$) was obtained over 30 s as a time averaged currents of $K_{ATP}$, measured as the difference between the baseline (a current level where all channels are in a close state) and the current level where the channels were in the open state with a half height criteria. Data points over the signals were delimited by cursors. The number of active channels in the patch (N), while initially perfusing nucleotide-free solution was regarded as the maximum open state of activity for later comparisons to assess the effect of nucleotides and/or drugs upon the active channels in the membrane-patch. Since the unitary current ($i$) changes at different pH levels, the $I/i$ quotient was used as a proportional indicator of channel activity. Since $K_{ATP}$ could be subject to run-down phenomenon, during each experiment, maximum channel activity was always verified regularly and measurements and calculations were corrected accordingly assuming a linear decrease in the number of active channels.

### *Drugs*

All drugs were freshly prepared before every experiment and diluted into the test solution to obtain the final concentration

as indicated in the text. Nicorandil (2-nicotinamidoethyl nitrate; a gift from Chugai Pharmaceutical Co., Tokyo, Japan) was diluted into the test solution. Glibenclamide (a gift from Hoescht Japan, Tokyo) was dissolved in 2% dimethyl sulphoxide (DMSO) and diluted into the test solution. The final concentration of DMSO contained in the test solution was less than 0.01%. We used two NO-donors: Sodium nitroprusside (SNP, Sigma Chemical Co. St. Louis MO, U.S.A.) and (±)-(E)-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide (NOR-3, a gift from Fujisawa Pharmaceutical Co. Ltd. Osaka, Japan). They were directly dissolved into the perfusing solution at the concentration indicated in the text. Exposure to light and oxygen was minimized. NO-scavengers: 5–10 $\mu$M oxyhaemoglobin ($HbO_2$, Calzyme Laboratories, Inc. CA, U.S.A.) and 2–3 mM $C_{14}H_{16}N_2NaO_4$ (Carboxy-PTIO; Dojindo Kumamoto, Japan) were both directly dissolved into the perfusate to a final concentration as indicated in the text.

### *Statistical analysis*

Data are expressed as mean±s.d. The significance for pH-effect as the only changing factor when comparing more than two groups was assessed by analysis of variances (ANOVA). Comparisons between two groups of data were evaluated by paired or unpaired student *t*-test, accordingly. A value of $P<0.05$ was considered significant.

## Results

### *Reduction of external pH affects nicorandil-activated $K_{ATP}$ current*

Application of 300 $\mu$M nicorandil activated outward current at potentials positive to ~60 mV elicited by a ramp voltage clamp at $pH_o$ 7.4. The nicorandil-activated current was completely inhibited by 1 $\mu$M glibenclamide confirming the current as $K_{ATP}$ ($n=4$; not shown). When $pH_o$ was lowered from 7.4 to 6.8–6.0 in the presence of 2 mM $[ATP]_i$, the nicorandil-activated $K_{ATP}$ current was somewhat decreased at $pH_o$ 6.8 and further suppression was noted at $pH_o$ 6.0 (Figure 1a). Figure 1b presents a summary of the results obtained from 10 different myocytes. The current at 0 mV in the control was 1.22±0.6 nA and it was increased to 3.96±1.97 nA in the presence of 300 $\mu$M nicorandil at $pH_o$ 7.4 ($P<0.01$). The nicorandil-activated current was decreased to 1.96±0.95 nA at $pH_o$ 6.8 in the presence of the drug ($P<0.05$ versus nicorandil at $pH_o$ 7.4) and further decrease of the current was noted at $pH_o$ 6.0 (1.06±0.68 nA; $P<0.01$ versus nicorandil at $pH_o$ 7.4). As we could not detect any increase in the nicorandil-activated $K_{ATP}$ current by the whole-cell configuration, we proceeded to examine the modulatory action of $pH_i$ on the single $K_{ATP}$ currents with inside-out patch configuration.

### *Increased $K_{ATP}$ activity by internal acidosis*

First, we tested the low $pH_i$-induced modulation of the $K_{ATP}$ activity in the absence of nicorandil under our experimental conditions, since reducing $pH_i$ from ~7.4 to 6.5–6.0 was shown to increase the channel activity (Cuevas *et al*., 1991; Davies, 1990; Fan & Makielski, 1993; Koyano *et al*., 1993; Vivaudou & Forestier, 1995). Figure 2a presents the effect of lowering $pH_i$ on $K_{ATP}$ current. When the internal face of the patch-membrane was exposed to the ATP-free solution at

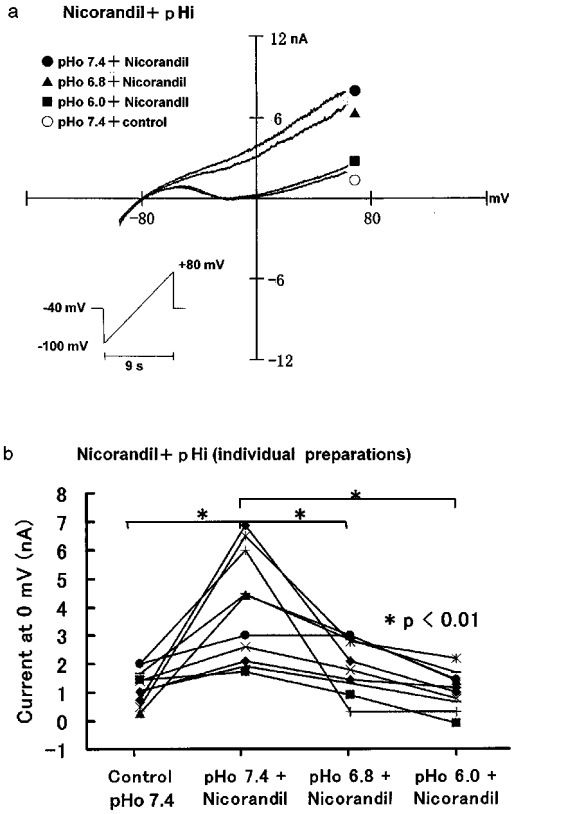


**Figure 1** Suppression of nicorandil-activated whole-cell current by lowering $pH_o$. (a) Superimposed traces of background I-V curves obtained from a typical case in a single myocyte, at 3 min intervals. ○: Control at $pH_o$ 7.4. ●: Addition of 300 $\mu$M nicorandil to the bath at $pH_o$ 7.4 (Nicorandil, $pH_o$ 7.4). ▲: Reducing $pH_o$ to 6.8 in the presence of nicorandil (Nicorandil, $pH_o$ 6.8). ■: Further reduction of $pH_o$ to 6.0 in the presence of nicorandil (Nicorandil, $pH_o$ 6.0). (b) Summarized data from 10 myocytes revealed a significant decrease of the nicorandil-activated $K^+$ current by reducing $pH_o$ from 7.2 to 6.8 and 6.0. Each symbol corresponds to the value obtained from individual preparations.

pH 7.2 many channels were kept in the open state, and the channels were quickly and largely inhibited by addition of 0.35 mM $ATP_i$. The open-state probability ($P_o$) of the channels was 89±10% in the ATP-free solution and it was 7±5% at 0.35 mM $[ATP]_i$ ($P<0.01$). A significant increase of channel activity became apparent by decreasing $pH_i$ from 7.2 to 6.0 ($P_o$; 7±5% versus 32±13%, respectively, $n=9$; $P<0.01$) in the presence of $Mg^{2+}$ as previously reported. Run-down was excluded by observing near complete recovery of the initial Po upon removal of ATP from the internal solution (Figure 2b). As reported previously reduction of $pH_i$ slightly decreased the unit amplitude of single-channel current (*i*) (data not shown).

### *Effect of acidosis on the nicorandil-activated $K_{ATP}$ current*

Application of 1 mM nicorandil to the internal solution in the presence of 0.25 mM ATP, 0.1 mM ADP and $Mg^{2+}$ at $pH_i$ 7.2, $K_{ATP}$ was activated as similar as the removal of ATP. The nicorandil-activated $K_{ATP}$ currents were decreased with reduction of $pH_i$ to 6.5 and 6.0 (Figure 3a). Return of $pH_i$ to 7.2 or removal of $[ATP]_i$ restored the channel activity. Figure 3b represents the amplitude histograms of the records shown in Figure 3a. Reduction of $pH_i$ decreased the channel activity. Figure 4 demonstrates the summary data from 10 similar experiments as shown in Figure 3. $P_o$ of nicorandil-

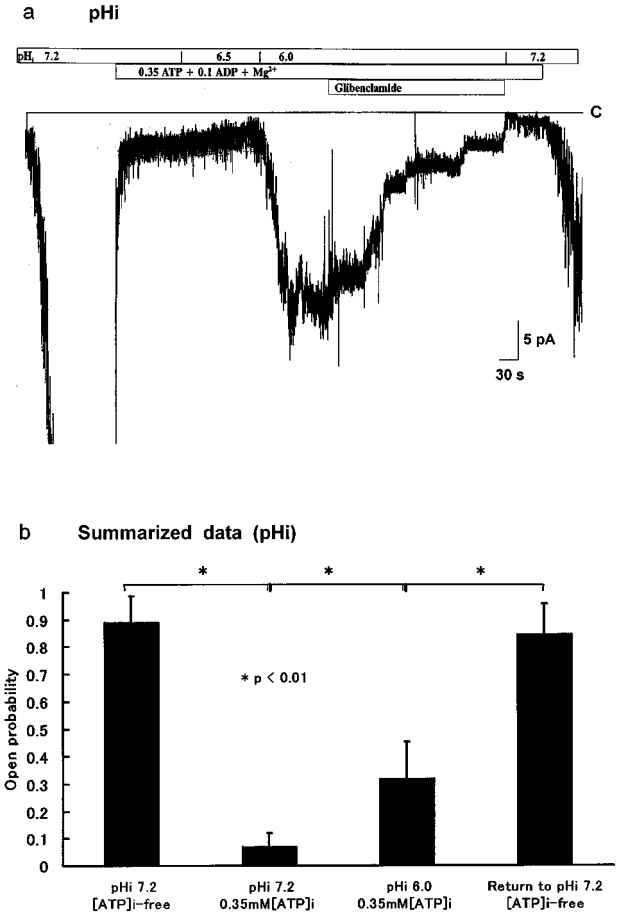


**Figure 2** Increased $K_{ATP}$ activity by lowering pH at the cytoplasmic side of the membrane ($pH_i$). (a) Single-channel record obtained with inside-out patch configuration. The membrane potential was held at $-40$ mV, while having a 140 mM symmetrical $K^+$ concentrations at both sides of the membrane. Control $K_{ATP}$ current was recorded in the absence of nucleotides at pH 7.2 in the bath ($pH_i$). The control current was of large amplitude and off-scaled. Addition of nucleotides, 0.35 mM $[Mg.ATP]_i + 0.12$ mM $[K.ADP]_i$ whilst keeping the same $pH_i$ markedly suppressed the current. $Mg^{2+}$ was also present at a 0.6–0.8 mM concentration. Whilst lowering $pH_i$ to 6.5 at this $[ATP]_i$ did not exhibit any change in channel activity, further reduction of $pH_i$ to 6.0 increased significantly the $P_o$ of the channels. Application of 4.0-$\mu$M glibenclamide suppressed the current. C represents the closed level of the channel. Downward deflection indicates inward currents in this and the following figures except Figure 3b. (b) The bar graph showing summarized data obtained from nine different patches under the same experimental condition as in (a).

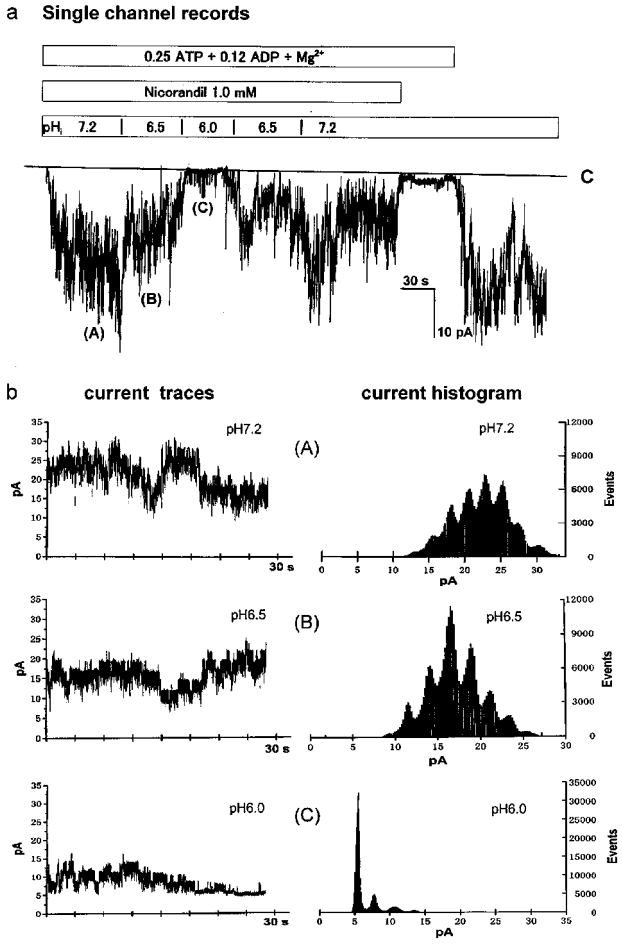


**Figure 3** Effects of low $pH_i$ on nicorandil-activated $K_{ATP}$ current. (a) Single channel current record with holding potential at $-40$ mV. The bath contained 0.25 mM Mg.ATP, 0.12 mM K.ADP, 0.8 mM $Mg^{2+}$ and 1.0 mM nicorandil. Lowering pHi from 7.2 to 6.5 and 6.0 in the presence of nicorandil reduced the nicorandil-activated $K_{ATP}$ current. These effects were reversible upon return of $pH_i$ to 7.2. (b) Current histograms of 30 s-analysed segments taken from (a). The letters in brackets indicate the corresponding segment in (a).

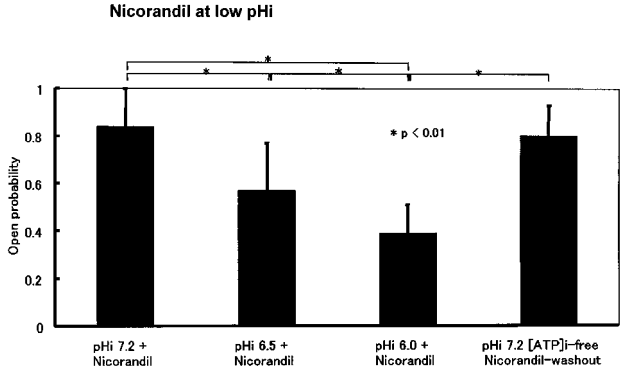


**Figure 4** Summarized data for effects of low $pH_i$ on nicorandil-activated $K_{ATP}$ current. The bar graphs summarize data from 10 different patches. Reducing $pH_i$ to 6.5 and 6.0 significantly decreased the channels-activity induced by 1.0 mM nicorandil compared at $pH_i$ 7.2. Upon washout of nicorandil and returning to ATP- and ADP-free $pH_i$ 7.2 solution, near complete recovery of the initial activity was achieved in all patches excluding the rundown of the channel. Experimental conditions same as those in Figure 3.

activated $K_{ATP}$ at $pH_i$ 7.2 was $84 \pm 16\%$. Decreasing $pH_i$ from 7.2 to 6.5 and 6.0, resulted in a significant reduction of $K_{ATP}$ activity ($P_o$; $57 \pm 20\%$ at $pH_i$ 6.5; $P < 0.01$ and $38 \pm 12\%$ at $pH_i$ 6.0; $P < 0.01$ when compared to $pH_i$ 7.2, respectively). Upon return to $pH_i$ 7.2, an almost complete recovery to the initial level of channel activity was restored ($P_o = 80 \pm 5\%$).

### *Effects of Nitric Oxide on $K_{ATP}$*

Since nicorandil is a hybrid compound including NO in its chemical structure, we investigated a possibility that the reduced activity of the nicorandil-activated $K_{ATP}$ at low $pH_i$ might be caused by an interaction between $H^+$ and NO. For this purpose, we examined effects of NO donors on $K_{ATP}$. We used two different NO-donors: SNP and NOR-3 (see Methods). Figure 5a shows the effect of 1 mM SNP in the presence of 0.25 mM $[ATP]_i$ at $pH_i$ 7.2. $K_{ATP}$ activity increased significantly in the presence of the NO-donor. Figure 5b summarizes data from 11 patches. $P_o$ was $17 \pm 11\%$

in the absence versus 32±12% in the presence of SNP 5 minutes ($P<0.05$). Upon removal of the NO-donor, an increase in $K_{ATP}$ current was kept ongoing to reach $P_o$ values up to 73±38% following 10 min of washout ($P<0.05$). On the contrary, lowering $pH_i$ from 7.2 to 6.0 at constant ATP and SNP concentration caused a significant reduction in $K_{ATP}$ activity ($P_o$; 54±34% at $pH_i$ 7.2 versus 27±19% at $pH_i$ 6.0; $P<0.01$; $n=11$) (Figure 6a, b). Using NOR-3, a different NO donor, we observed similar tendency as with SNP. In the presence of 0.25 mM $[ATP]_i$ at $pH_i$ 6.0, the $P_o$ was 28±15% in the absence, whilst in the presence of 1 mM NOR-3, $P_o$ decreased to 8±6% ($P<0.05$; $n=5$). Washout of the drug did not reverse the current depression, similarly as with the application of SNP. On the other hand, we also observed that a switch in $pH_i$ from 6.0–7.2 increased the activity of the channel ($P_o$; 20±16% at $pH_i$ 6.0 versus 46±19% at $pH_i$ 7.2; $P<0.05$; $n=4$) (Figure 6a). Contrary to what we observed at $pH_i$ 7.2, removal of NO-donor at $pH_i$ 6.0 did not get complete recovery of the channel activity to the previous level. This was not due to run-down of channel activity since the nucleotide-free solution at $pH_i$ 7.2 restored complete recovery of the channel activity to the initial level.

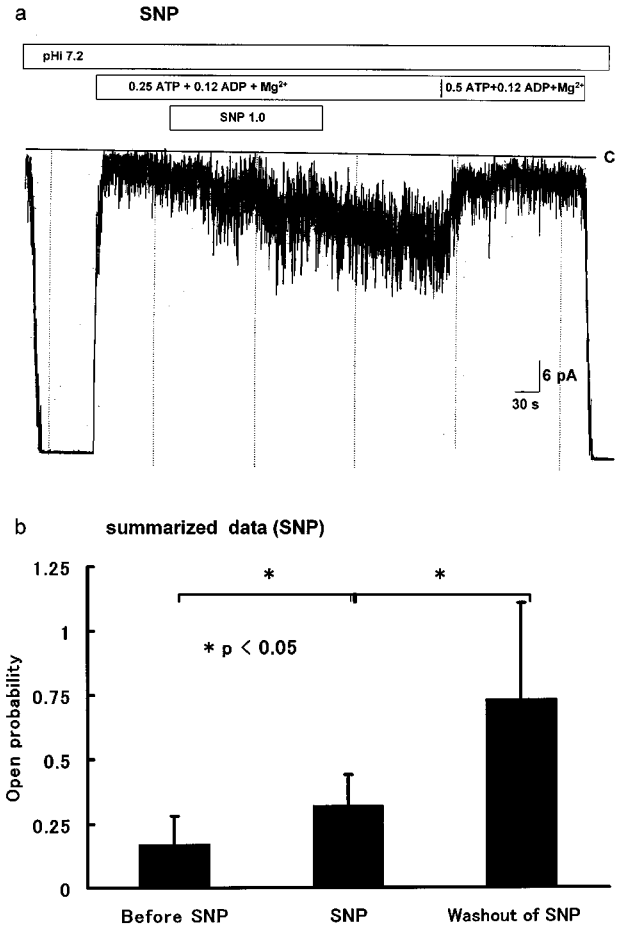


**Figure 5** Increased $K_{ATP}$ activity at normal $pH_i$ induced by NO-donor. (a) Single channel current record obtained from a typical patch. Application of nucleotides and NO-donor, 1.0 mM SNP in the bath is indicated at the top, $pH_i$ was kept constant at 7.2 throughout these experiments. In the absence of nucleotides a large current was observed and off-scaled in the record. Decreased channel activity upon addition of 0.25 mM Mg.ATP and 0.12 mM K.ADP was gradually increased by adding 1.0 mM SNP into the bath and continued after the removal of the NO-donor. Increasing $[Mg.ATP]_i$ to 0.5 mM caused a similar $P_o$ as the level observed at 0.25 mM $[Mg.ATP]_i$ before the exposure to SNP, suggesting a reduction to the inhibitory effect of ATP. (b) The bar graphs of data from 11 patches. The channel activity expressed by $P_o$ increased significantly by addition of SNP in the presence of nucleotides and washout of the drug increased $P_o$.

## *Effects of NO-donors and NO-scavengers on $K_{ATP}$*

In the presence of NO-donors at low $pH_i$, we attempted to reverse their depressing effects on $K_{ATP}$ by adding NO scavengers, either $HbO_2$ or carboxy-PTIO. However, 5–10 μM $HbO_2$ enhanced rather than reversed the depressing effects of NO donors (Figure 7). $P_o$ was 60±5% in the presence of 1 mM NOR-3 alone versus 20±2% in the presence of $HbO_2$ ($P<0.01$; $n=4$). At low $pH_i$ neither washout of $HbO_2$ nor removal of the NO donor altered the decreased $P_o$ of the channels, but an increase of the $pH_i$ to 7.2 could increase $P_o$. Run-down was excluded by observing complete recovery of channel activity upon removal of nucleotides at $pH_i$ 7.2. Either at normal or low $pH_i$, the compound itself at concentrations ranging 5–10 μM showed a direct suppressing effect on the channel activity. This effect was more prominent at low $pH_i$ revealing an additive action to that of the NO-donor. In two additional patches we tested the effect of $HbO_2$ on the nicorandil-activated $K_{ATP}$ at

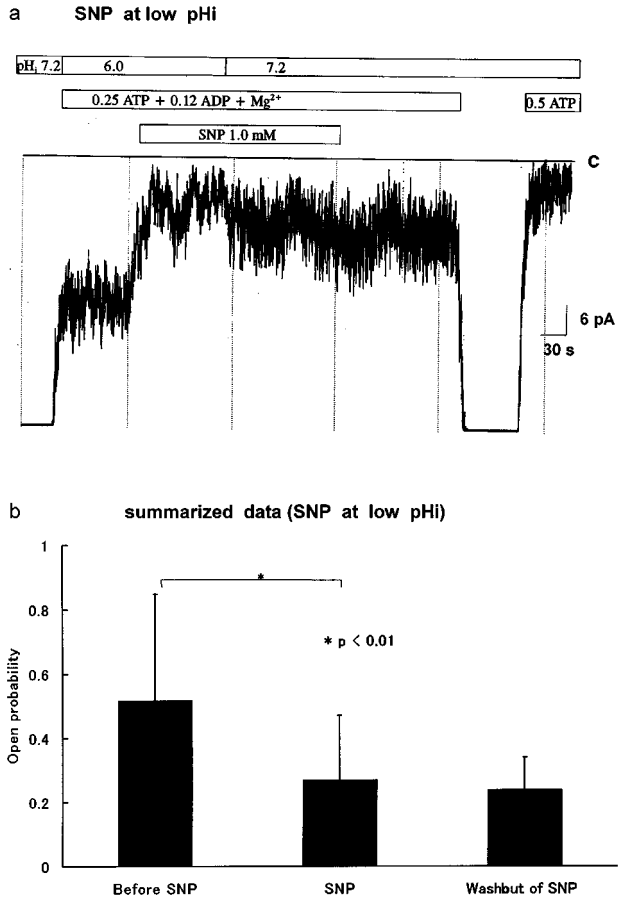


**Figure 6** Effects of SNP on $K_{ATP}$ at low $pH_i$ (a) Experimental conditions of $pH_i$, presence of nucleotides and SNP are indicated on the top. The channel activity at $pH_i$ 6.0 in the presence of nucleotides was significantly reduced by the presence of 1.0 mM SNP and return of $pH_i$ to 7.2 resulted in increase of channel $P_o$. (b) The bar graphs of summarized data from 11 patches. The $pH_i$ at 6.0 and nucleotides concentrations were kept constant with 0.25 mM $[Mg.ATP]_i$, and 0.12 mM $[K.ADP]_i$ throughout the experimental conditions. In all patches run-down was excluded by observing nearly complete recovery of activity when perfusing nucleotide-free, pH 7.2 solution after these treatments.

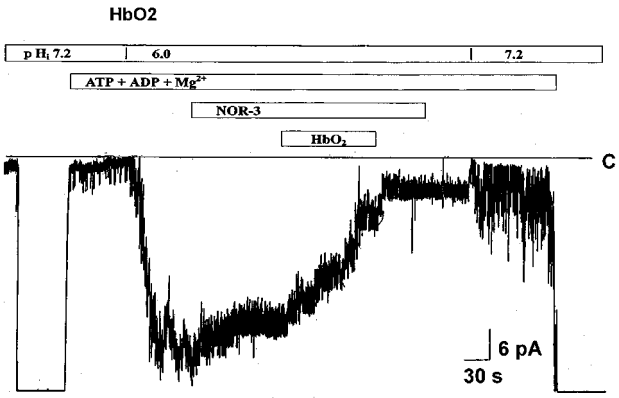


**Figure 7** Effects of NO scavenger, $HbO_2$ on $K_{ATP}$ activity in the presence of NO-donor, NOR-3. Single channel records show that lowering pHi from 7.2 to 6.0 in the presence of nucleotides and $Mg^{2+}$ increased $K_{ATP}$ activity. Increased channel activity was somewhat depressed by addition of 1.0 mM NOR-3 and further decrease was noted in the presence of 5 $\mu$M of $HbO_2$. Neither removal of $HbO_2$ nor NOR-3 reversed the depressed activity of the channel, but a change in $pH_i$ from 6.0–7.2 increased the $P_o$ of $K_{ATP}$. Upon removal of the nucleotides complete recovery of the initial activity took place ruling out the presence of run-down.

normal $pH_i$ and again we observed a clear reduction of activity, with its reversal upon washout of $HbO_2$. No significant changes in channel activity were observed by using carboxy-PTIO with a ratio 2:1 or 3:1 respect to the NO donor.

## Discussion

In the present study we examined two major issues: first, how an increase in external and internal $H^+$ concentrations modulates the activity of nicorandil-activated $K_{ATP}$ in guinea-pig ventricular myocytes. Second, the possibility that the modulation caused by acidosis in nicorandil-activated $K_{ATP}$ may be related to $H^+$ and NO interaction.

### *Effect of external pH on nicorandil-activated $K_{ATP}$ current*

Previous study demonstrated that lowering $pH_o$ could enhance the ability for nicorandil to activate whole-cell $K_{ATP}$ current in guinea-pig ventricular myocytes (Jahangir *et al*., 1994). The authors considered that the enhanced action of nicorandil during acidosis was not related to a different ionization state of the drug nor a change in the ATP/ADP ratio, but a direct effect of $H^+$ modulating the interaction between the channel and the drug. Effectiveness of another KCO, pinacidil decreased at low $pH_o$ and increased at high $pH_o$ (Kwok & Kass, 1994). The findings were interpreted as $H^+$ might modulate the binding site for pinacidil located at the extracellular side of the membrane. Thus, the interaction between $H^+$ and the different types of KCO might be different depending on their chemical structures.

We tested a slightly different view from that of Jahangir *et al*. (1994) as to the nicorandil action on $K_{ATP}$. That is how the nicorandil-activated $K_{ATP}$ could be modified by low $pH_o$, the condition prone to be associated with myocardial ischemia. It turned out that lowering $pH_o$ decreased rather than increased the nicorandil-activated $K_{ATP}$ in whole-cell currents. Since $pH_o$ might lead to change in $pH_i$, and $pH_i$ itself would have modifying action on $K_{ATP}$, we proceeded to explore the modulating mechanism by increased $H^+$ concentrations with inside-out patches, where involvement of the second messenger system produced by the drug could be largely excluded.

### *Effect of internal pH on nicorandil-activated $K_{ATP}$*

In the presence of MgATP and ADP in the bath (cytoplasmic face), lowering $pH_i$ remarkably increased the $P_o$ of the channels, the results consistent with previous reports (Cuevas *et al*., 1991; Davies, 1990; Fan & Makielski, 1993; Koyano *et al*., 1993; Vivaudou & Forestier, 1995). This action of low $pH_i$ can be interpreted that an intrinsic proton binding site regulates ATP sensitivity for the channel inhibition in $K_{ATP}$ (Fan & Makielski, 1993). Evidence of the importance of $Mg^{2+}$ as a cofactor necessary to keep the channel in operative state in the presence of either ATP or nucleotide diphosphates has been provided as well as its contribution in the enhancement of KCO effect (Findley, 1988; Lederer & Nichols, 1989; Shen *et al*., 1991; Terzic *et al*., 1994; Tung & Kurachi, 1991; Vivaudou & Forestier, 1995). We used MgATP combined with small doses of ADP in all our subsequent experiments in order to keep the channel in operative state and minimize run-down. At physiologic $pH_i$ 7.2 nicorandil markedly increased the $P_o$ of $K_{ATP}$, but when lowering $pH_i$, the $P_o$ of nicorandil-activated $K_{ATP}$ was significantly decreased. Therefore, it appears that variations in $pH_i$ directly affect the interaction between nicorandil and $K_{ATP}$ rather than *via* producing the second messengers such as the production of cyclic GMP (Kojda & Kottenberg, 1999; Kubo *et al*., 1994; Murphy & Brayden, 1995). This suppressive action on $K_{ATP}$ seems to overcome stimulating effect of low $pH_i$.

### *Modulation of $K_{ATP}$ by nitric oxide*

In the present study, we demonstrated at the single-channel level that NO donors of different chemical structures increased the $P_o$ of $K_{ATP}$ at $pH_i$ 7.2. Furthermore, removal of NO donors did not abolish the increased activity but the activity was kept increasing, and higher doses of ATP were needed to inhibit the channels than those before the drug treatment, suggesting a reduction of sensitivity to the nucleotide-inhibitory effect. Although the activation of the channels by NO donors was not as large as with nicorandil, the increase in $P_o$ was clear and significant. At low $pH_i$, however, the presence of NO donors decreased the $P_o$ of the channels and removal of the NO donors did not reverse the phenomenon. Also, with raising $pH_i$ from 6.0 to 7.2 in the presence of the NO donors, the increase of $P_o$ was evident. Therefore, the effects of the NO donors at either $pH_i$ 7.2 or $pH_i$ 6.0 were very similar to those observed with nicorandil.

Recently, NO was reported to enhance the KCO-activated $K_{ATP}$ current with undefined mechanism (Shinbo & Iijima, 1997). They demonstrated the enhancing action of NO with whole-cell and cell-attached patch recordings in guinea-pig ventricular myocytes at normal $pH_o$ and $pH_i$. In their experiments the cyclic GMP pathway and/or metabolic inhibition did not appear to be responsible for NO effects. Their findings were similar to the present results, except they failed to demonstrate the enhancing action of NOR-3 on $K_{ATP}$ in inside-out patches. Here, we were able to show a clear activation of the channels by application of two different NO donors to inside-out patches. At present, we could not find any proper reason to explain the discrepancy between the two studies, but a direct action of NO on the modulation of cardiac $K_{ATP}$ might be supported. In cultured

vascular smooth muscle cells, isosorbide dinitrate and atrial natriuretic factor were proven to modulate the gating of $K_{ATP}$ and the cyclic GMP pathway was involved (Kubo *et al*., 1994). There are, however, several lines of evidence that direct effects of NO on channel modulation; for example, expressed cardiac L-type $Ca^{2+}$ channels (Hu *et al*., 1997), calcium-regulated potassium channel in vascular smooth muscle cells (Bolotina *et al*., 1994) and potassium channels in colonic muscle cells (Koh *et al*., 1995). Therefore, it may be possible that NO could directly modulate $K_{ATP}$ and our findings add new evidence for its role on the modulation of cardiac $K_{ATP}$.

Because of these similarity of actions between the NO donors and nicorandil, the suppression of the nicorandil-activated $K_{ATP}$ at low $pH_i$ can be attributed to a direct interaction of NO with $H^+$ at ATP-binding inhibitory sites of the channels, where stimulating action of $pH_i$ is supposed to interact on this site. This action may be different from the enhancing action of NO and nicorandil on $K_{ATP}$ at normal $pH_i$, or some other mechanism to exhibit both stimulating and suppressive actions depending on pH may be involved. This issue is to be explored further. Dual modulations of cardiac $K_{ATP}$ have been demonstrated in the actions of pinacidil (Fan *et al*., 1990), nucleotides diphosphates and $Mg^{2+}$ (Findley, 1988; Lederer & Nichols, 1989; Tung & Kurachi, 1990; Terzic *et al*., 1994), and $pH_i$ (Cuevas *et at*., 1991; Fan & Makielski, 1993; Koyano *et al*., 1993; Fan *et al*., 1993). The dualistic modulations by these factors develop in variable ways and different mechanisms seem to be involved. For examples, pinacidil exerts voltage-independent activation of the channels and voltage dependent inhibition from inside of the membrane. Nucleotide diphosphates cause channel inhibition without $Mg^{2+}$ and stimulate the channels in the operative state in the presence of $Mg^{2+}$. $Mg^{2+}$ produces channel inactivation and run-down on the one hand, and on the other it promotes the channels into the operative states. Low $pH_i$ promotes increased channel open probability but induces decreased single channel current amplitude.

The fact that NO-scavengers were not able to prevent or reverse the actions of NO-donors on the $K_{ATP}$ is unclear and needs to be further investigated. In the case of $HbO_2$, the compound itself was shown to affect directly the channel at either normal or lower $pH_i$, and that explains the prominent enhancement of the depressive effect of NO-donors on the channel. Does the $K_{ATP}$ have the ability to sense and respond to $HbO_2$ levels? As for carboxy-PTIO, the dose required to abolish the NO effect is much higher than that for $HbO_2$, (Donor: Scavenger ratio = 3 : 1) and the release rate of NO is slower, perhaps that might explains why it did not affect NO-$H^+$ interaction respect to $K_{ATP}$.

### *Possible pathophysiological implications*

The openings of $K_{ATP}$ play an pivotal role for action potential shortening and are partly responsible for extracellular $K^+$ accumulation during early phase of myocardial ischemia and infarction, which may contribute to the development of arrhythmias (Venkatesh *et al*., 1992; Wilde & Janse, 1994). At the same time, $pH_i$ may go down quickly. On the other hand, the openings of $K_{ATP}$ may exert the protective action on the second severe ischemic insult after a brief period of ischemia (preconditioning) (Downey & Cohen, 1997; Gross, 1995; Grover, 1994). If the patients taking nicorandil as anti-anginal drug develop ischemic attack, a quick opening of $K_{ATP}$ can be anticipated during early ischemic episode and yet, the opening effects are subsequently suppressed due to decreased $pH_i$, which may give a self-limiting factor for the prevention of further shortening of action potential duration and extracellular $K^+$ accumulation. At the same time, $K_{ATP}$ as the end effector of cardioprotection is also modulated by NO production (Bolli *et al*., 1997; Imagawa *et al*., 1999; Yao and Gross, 1993). Therefore, these interplay and final results in the presence of nicorandil must be evaluated with caution. Acidosis is known to be pro-arrhythmogenic by affecting multiple ionic currents including $K_{ATP}$ (Orchard & Cingolani, 1994). Therefore, it might be necessary to further examine the pharmacological profile of these drugs in acidotic conditions.

### *Conclusions*

The present study provides information concerning the effect of $H^+$ at either side of the membrane in nicorandil-activated $K_{ATP}$. We present new evidence of a cyclic GMP-independent modulating effect of NO on $K_{ATP}$ during acidosis. These findings may be relevant to the therapeutic action of these drugs, especially during ischemic or hypoxic conditions.

We thank Miss K. Totsuka for secretarial services and Mrs K. Yamamoto for excellent technical assistant. We are also grateful for the generous supply of nicorandil by Chugai Pharmaceutical, Co. Ltd. (Tokyo, Japan). Thanks are due to Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan) for the generous supply of FK409 (NOR3).

## References

ALLEN, D.G., MORRIS, P.G., ORCHARD, C.H. & PIROLO, J.S. (1985). A nuclear magnetic resonance study of metabolism in the ferret heart during hypoxia and inhibition of glycolisis. *J. Physiol. (Lond.),* **361,** 185–204.

BOLLI, R., BHATTI, Z.A., TANG, X.-L., QIU, Y., ZHANG, Q., GUO, Y. & JADOON, A.K. (1997). Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. *Circ. Res.,* **81,** 42–52.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature,* **368,** 850–853.

CUEVAS, J., BASSET, A.L., CAMERON, J.S., FURUKAWA, T., MYERBURG, R.J. & KIMURA, S. (1991). Effect of $H^+$ on ATP-regulated $K^+$ channels in feline ventricular myocytes. *Am. J. Physiol.,* **261,** H755–H761.

DAVIES, N.W. (1990). Modulation of ATP-sensitive channels in skeletal muscle by intracellular protons. *Nature,* **343,** 375–377.

DOWNEY, J.M. & COHEN, M.V. (1997). Signal transduction in ischemic preconditioning. *Adv. Exp. Med. Biol.,* **430,** 39–55.

FAN, Z. & MAKIELSKI, J.C. (1993). Intracellular $H^+$ and $Ca^{2+}$ Modulation of Trypsin-Modified ATP-sensitive $K^+$ channels in Rabbit Ventricular Myocytes. *Circ. Res.,* **72,** 715–722.

FAN, Z., FURUKAWA, T., SAWANOBORI, T., MAKIELSKI, J.C. & HIRAOKA, M. (1993). Cytoplasmic acidosis induces multiple conductance states in ATP-sensitive potassium channels of cardiac myocytes. *J. Memb. Biol.,* **136,** 169–179.

FAN, Z., NAKAYAMA, K. & HIRAOKA, M. (1990). Multiple action of pinacidil on adenosine triphosphate-sensitive potassium channels in guinea-pig ventricular myocytes. *J. Physiol.,* **430,** 273–295.

FINDLAY, I. (1988). Effect of ADP upon the ATP-sensitive $K^+$ channel in rat ventricular myocytes. *J. Memb. Biol.,* **101,** 83–92.

FRAMPTON, J., BUCKLEY, M.M. & FILTON, A. (1992). Nicorandil. A review of its pharmacology and therapeutic efficacy in angina pectoris. *Drugs,* **44,** 625–655.

GOLDSCHIMIDT, M., LANDZBERG, B.R. & FRISHMAN, W.H. (1996). Nicorandil: a potassium channel opening drug for the treatment of ischemic heart disease (Review). *J. Clin Pharmacol.,* **36,** 559–572.

GROSS, G.J. (1995). ATP-sensitive potassium channels and myocardial preconditioning. *Basic Res. Cardiol.,* **90,** 85–88.

GROVER, G.J. (1994). Protective effects of ATP-sensitive potassium channel openers in experimental myocardial ischemia. *J. Cardiovasc. Pharmacol.,* **24** (suppl. 4)**,** S18–S27.

HAMILL, O.P., MARTY, A., NEHER, E., SACKMAN, B. & SIGWORTH, F. (1981). Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. *Pluggers Arch.,* **351,** 85–100.

HIRANO, Y. & HIRAOKA, M. (1988). Barium-induced automatic activity in isolated ventricular myocytes from guinea-pig hearts. *J. Physiol.,* **395,** 455–472.

HIRAOKA, M. & FAN, Z. (1989). Activation of ATP-sensitive outward $K^+$ current by nicorandil (2-nicotinamidoethyl nitrate) in isolated ventricular myocytes. *J. Pharmacol. Exp. Ther.,* **250,** 278–285.

HU, H., CHIAMVIMONVANT, N., YAMAGISHI, T. & MARBAN, E. (1997). Direct inhibition of expressed cardiac L-type $Ca^{2+}$ channels by S-nitrosothiol nitric oxide donors. *Circ. Res.,* **81,** 742–752.

IMAGAWA, J.-I., YELLON, D.M. & BAXTER, G.F. (1999). Pharmacological evidence that inducible nitric oxide synthase is a mediator of delayed preconditioning. *Br. J. Pharmacol.,* **126,** 701–708.

JAHANGIR, A., TERZIC, A. & KURACHI, Y. (1994). Intracellular acidification and ADP enhance nicorandil induction of ATP sensitive potassium channel current in cardiomyocytes. *Cardiovasc. Res.,* **28,** 831–835.

KOH, S.D., CAMPBELL, J.D., CARL, A. & SANDERS, K.M. (1995). Nitric oxide activates multiple potassium channels in canine colonic smooth muscle. *J. Physiol.,* **489,** 735–743.

KOJDA, G. & KOTTENBERG, K. (1999). Regulation of basal myocardial function by NO. *Cardiovasc. Res.,* **41,** 514–523.

KOYANO, T., KAKEI, M., NAKASHIMA, H., YOSHINAGA, M., MATSUOKA, T. & TANAKA, H. (1993). ATP-regulated $K^+$ channels are modulated by intracellular $H^+$ in guinea pig ventricular cells. *J. Physiol.,* **463,** 747–766.

KRUMENACKER, M. & ROLAND, E. (1992). Clinical profile of nicorandil: an overview of its hemodynamic properties and therapeutic efficacy. *J. Cardiovasc. Pharmacol.,* **20,** S93–S102.

KUBO, M., NAKAYA, Y., MATSUOKA, S., SAITO, K. & KURODA, Y. (1994). Atrial natriuretic factor and isosorbide dinitrate modulate the gating of ATP-sensitive $K^+$ channels in cultured vascular smooth muscle cells. *Circ. Res.,* **74,** 471–476.

KWOK, W.M. & KASS, R.S. (1994). Inhibition of pinacidil induced IK(ATP) in heart by changes in extracellular pH. *Cardiovasc. Res.,* **28,** 836–840.

LEDERER, W.J. & NICHOLS, C.G. (1989). Nucleotide modulation of the activity of rat heart ATP-sensitive $K^+$ channels in isolated membrane patches. *J. Physiol.,* **419,** 193–211.

MATSUBARA, T., MINATOGUCHI, S., MATSU, H., HAYAKAWA, K., SEGAWA, T., MATSUNO, Y., WATANABE, S., ARAI, M., UNO, Y., KAWASAKI, M., NODA, T., TAKEMURA, G., NISHIGAKI, K. & FUJIWARA, H. (2000). Three minute, but not one minute, ischemia and nicorandil have a preconditioning effect in patients with coronary artery disease. *J. Am. Coll. Cardiol.,* **35,** 345–351.

MURPHY, M.E. & BRAYDEN, J.E. (1995). Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. *J. Physiol. (Lond).,* **486,** 47–58.

NAKAYAMA, K., FAN, Z., MARUMO, F., SAWANOBORI, T. & HIRAOKA, M. (1991). Action of nicorandil on ATP-sensitive $K^+$ channel in guinea-pig ventricular myocytes. *Br. J. Pharmacol.,* **103,** 1641–1648.

ORCHARD, C.H. & CINGOLANI, H.E. (1994). Acidosis and arrhythmias in cardiac muscle (Review). *Cardiovasc. Res.,* **28,** 1312–1319.

PATEL, D.J., PURCELL, H.J. & FOX, K.M. (1999). Cardioprotection by opening of the K (ATP) channel in unstable angina. Is this a clinical manifestation of myocardial preconditioning? Results of a randomized study with nicorandil. CESAR 2 investigation. Clinical European studies in angina and revascularization. *Eur. Heart. J.,* **20,** 51–57.

SHEN, W.K., TUNG, R.T., MACHULDA, M.M. & KURACHI, Y. (1991). Essential role of nucleotide diphosphates in nicorandil-mediated activation of cardiac ATP-sensitive $K^+$ channels. *Circ. Res.,* **69,** 1152–1158.

SHINBO, A. & IIJIMA, T. (1997). Potentiation by nitric oxide of the ATP-sensitive $K^+$ current induced by $K^+$ channels openers in guinea-pig ventricular cells. *Br. J. Pharmacol.,* **120,** 1568–1574.

TAIRA, N. (1987). Similarity and dissimilarity in the mode and mechanism of action between nicorandil and classical nitrates: An overview. *J. Cardiovasc. Pharmacol.,* **10** (suppl. 8)**,** S1–S9.

TAKANO, M. & NOMA, A. (1990). Selective modulation of the ATP-sensitive $K^+$ channel by nicorandil in guinea-pig cardiac cell membrane. *Naunyn-Schmideberg's Arch. Pharmacol.,* **342,** 592–597.

TERZIC, A., FINDLAY, I., HOSOYA, Y. & KURACHI, Y. (1994). Dualistic behavior of ATP-sensitive $K^+$ channels toward intracellular nucleotide diphosphates. *Neurone,* **12,** 1049–1058.

TUNG, R.T. & KURACHI, Y. (1991). On the mechanism of nucleotide diphosphate activation of the cardiac ATP-sensitive $K^+$ channel in ventricular cell of guinea-pig. *J. Physiol. (Lond).,* **437,** 239–256.

VENKATESH, N., STUART, J.S., LAMP, S.T., ALEXANDER, L.D. & WEISS, J.N. (1992). Activation of ATP-sensitive $K^+$ channels by cromakalim. Effects on cellular $K^+$ loss and cardiac function in ischemic and reperfused mammalian ventricle. *Circ. Res.,* **71,** 1324–1333.

VIVAUDOU, M. & FORESTIER, C. (1995). Modification by protons of frog skeletal muscle $K_{ATP}$ channels: effects on ion conduction and nucleotide inhibition. *J. Physiol. (Lond.),* **486,** 629–645.

WILDE, A.A.M. & JANSE, M.J. (1994). Electrophysiological effects of ATP-sensitive poptassium channel modulation: implications for arrhythmogenesis. *Cardiovasc. Res.,* **28,** 16–24.

YAO, Z. & GROSS, G.J. (1993). Role of nitric oxide, muscarinic receptors, and the ATP-sensitive $K^+$ channel in mediating the effects of acetylcholine to mimic preconditioning dogs. *Circ. Res.,* **73,** 1193–1201.

*(Received June 28, 2000*
*Revised September 1, 2000*
*Accepted September 4, 2000)*