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# Effects of Adenosine on the Ionic Channel Activated by Metabolic Inhibition in Rabbit Ventricular Myocytes

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**=Abstract=**

**The objective of the present study was to characterize the role of adenosine in regulation of ATP-sensitive $K^+$ channel ($K_{ATP}$ channel) activity in isolated rabbit ventricular myocytes using the patch clamp technique. Internal adenosine had little effects on $K_{ATP}$ channel activity. In an outside-out patch with intrapipette GTP and ATP, external adenosine stimulated $K_{ATP}$ channel activity. In an inside-out patch with intrapipette adenosine, ATP reduced $K_{ATP}$ channel activity, and GTP stimulated $K_{ATP}$ channel activity. Adenosine receptor activation shifted the half-maximal inhibition of $K_{ATP}$ channel from 70 to 241 $\mu$M. These results suggest that activation of adenosine receptors stimulates $K_{ATP}$ channels in rabbit ventricular myocytes by reducing the apparent affinity of the channel for ATP. The effect may be important for activating $K_{ATP}$ channels during early phase of myocardial ischemia.**

**Key Words:** Adenosine, ATP-sensitive $K^+$ channel, Inside-out patch, Outside-out patch, Myocardial ischemia

## INTRODUCTION

It has been proposed that cardiac ATP-sensitive $K^+$ ($K_{ATP}$) channels, which open when cytosolic ATP concentration ($[ATP]_i$) falls below a critical level, are involved in the shortening of the action potential duration and the marked increase in $K^+$ efflux during myocardial ischemia or hypoxia (Faivre & Finfly, 1990; Venkatesch et al, 1991). However, the functional significance of $K_{ATP}$ channels remains controversial because of the discrepancy between the low levels (micromolar range) of $[ATP]_i$ at which the channels activates (Lederer & Nichols, 1989; Han et al, 1993) and the much higher levels (millimolar range) of $[ATP]_i$ maintained during ischemia (Elliot et al, 1989; Murry et al, 1990). To explain this discrepancy, it has been suggested that $K_{ATP}$ channels in the heart may be modulated by intracellular metabolites generated during ischemia other than ATP (Findly, 1988; Lederer & Nichols, 1988; Weiss & Lamp, 1989; Han et al, 1993). In the present study, we investigated the modulatory

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effects of adenosine on $K_{ATP}$ channel in rabbit ventricular myocytes. Many studies provide evidence that adenosine is released from cardiac myocytes during myocardial ischemia (Rubio et al, 1969; Olsson, 1970). Recent results obtained in rabbits suggest that activation of adenosine $A_1$ receptors may mediate ischemic preconditioning (Liu et al, 1991). This hypothesis is supported by the results that the adenosine $A_1$ receptor antagonists block the protective effect of ischemic preconditioning in the canine heart (Auchambach & Gross, 1993). However, the cellular mechanism by which activation of adenosine $A_1$ receptor provides protection to the myocardium remains to be determined. Recently, Kirsch et al (1990) showed in rat neonatal ventricular myocytes that activation of adenosine $A_1$ receptors opens the $K_{ATP}$ channel via a $G_i$ protein. The purpose of this study was to determine the role of adenosine on the modulation of the $K_{ATP}$ channel in rabbit ventricular myocytes.

## MATERIALS AND METHODS

### Cell preparation

Single ventricular myocytes were isolated from rabbit hearts by enzymatic dissociation, as discussed previously (Han et al, 1993). Initially, hearts were perfused retrogradely for 5 min with Tyrode solution. And then $Ca^{2+}$-free Tyrode solution was perfused for 5 min and $Ca^{2+}$-free Tyrode solution containing 0.01% collagenase (5 mg/50 cc, Yakult, Japan) was perfused for 15~25 min. After enzymatic treatment, Krafts Brühe (KB) solution (Isenberg & Klockner, 1982) was perfused. Langendorff column was kept at 37°C during all previous steps. Isolated ventricular cells were stored in a KB solution at 4°C and used within 12 hours.

### Solutions

Normal Tyrode solution contained (in mM): 143 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.5 glucose, 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); pH 7.4 with NaOH. The solutions facing the outside of the cell membrane in the excised patch recordings contained (in mM): 140 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES; pH 7.4 with KOH. The solutions facing the inside of the cell membrane in the excised patch recordings contained (in mM): 127 KCl, 13 KOH, 1 $MgCl_2$, 5 ethylene glycol-bis((β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 glucose, 10 HEPES; pH 7.4 with KOH. The modified KB solution had the following composition (in mM): 25 KCl, 10 $KH_2PO_4$, 16 KOH, 80 glutamic acid, 10 taurine, 14 oxalic acid, 10 HEPES, and 11 glucose at pH 7.4 adjusted by KOH (Isenberg & Klockner, 1982).

Adenosine (20 μM), ATP (100 μM or 1 mM), glibenclamide (50 μM), guanosine 5'-triphosphate (GTP, 100 μM) were added to either the extracellular or intracellular solutions according to the experimental protocols described in the text. After addition of drugs to the test solution, the pH was re-adjusted to 7.4 with KOH. Unless otherwise noted, these agents were obtained from Sigma (St. Louis, MO, U.S.A.). Experiments were done at a room temperature of 25±2°C.

### Electrophysiological methods and quantitation of channel activity

Single-channel currents were measured in inside-out and outside-out patch configurations of the gigaohm seal patch-clamp technique (Hamill et al, 1981). Channel activity was measured using a patch-clamp amplifier (EPC-7, LIST, Darmstadt, Germany; Axopatch-1D, Axon Instruments, Foster City, CA). Pipettes of 5-10 MΩ resistance were pulled from borosilicate glass capillaries (Clark Electrochemical, Pangbourne, England) using a vertical puller (Narishige PP-83, Japan). Their tips were coated with Sylgard and fire polished. Membrane currents were digitized at a sampling rate of 48 kHz and stored in digitized format on digital audio tapes using a Biologic DTR-1200 recorder (Grenoble, France). For the analysis of single channel activity,

the data were transferred to a computer (IBM-PC, 80486 DX2-66) with pClamp software (Axon Instruments, Foster City, CA, USA) through a 12-bit Labmaster analogue-to-digital converter interface. The threshold for judging the open state was set at half of the single-channel amplitude (Colquhoun & Sigworth, 1983). The open probability ($P_O$) was calculated using the formula:

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

where $t_j$ is the time spent at current levels corresponding to j=0,1,2, ... N channels in the open state, $T_d$ is the duration of the recording and N is the number of channels active in the patch. $P_O$ was calculated over 30 sec records.

## RESULTS

Experiments in the cell-attached configuration were performed to observe the metabolic inhibiton-induced currents. In these experiments, pipettes were filled with a 140 mM [KCl], the bath contained

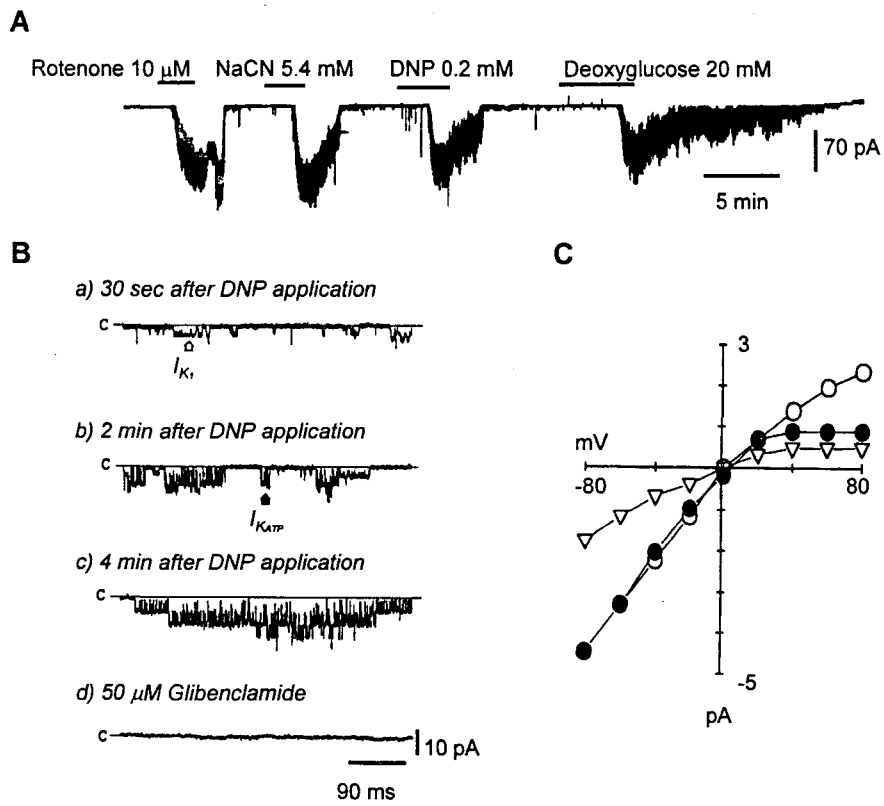


*Fig. 1. A: effect of various metabolic inhibitors on the membrane currents in rabbit ventricular myocytes. B: effect of 0.2 mM DNP on single-channel currents. (a), the inward rectifying $K^+$ channel ($I_{K1}$, about 25 pS, long lasting opening) observed in the control. (b) and (c), DNP induced the single-channel currents (about 71 pS, bursting kinetics) after a lag period. (d), application of glibenclamide reduced open state of channel. Single- channel currents were recorded in the cell-attached mode after perfusion with metabolic inhibitors at holding potential of 0 mV. Currents flowing from the external to internal side are displayed downwards. Low-pass filter, 1 kHz. C: Current-voltage relationship for $K_{ATP}$ channels of cell-attached patch and inside-out patch. Open circle, $K_{ATP}$ channel in inside-out patch; closed circle, $K_{ATP}$ channel in cell-attached patch; open triangle, $I_{K1}$ channel in cell-attached patch.*

Normal Tyrode solution. Application of each metabolic inhibitor (10 $\mu$M rotenone, 5.4 mM NaCN, 0.2 mM DNP, 20 mM Deoxyglucose) to the bath solution activated single-channel currents (Fig. 1A). The result suggests that inhibition of ATP synthesis results in the channel activation in the cell-attached patch. To test whether the metabolic inhibition-induced currents are $K_{ATP}$ currents, glibenclamide was added to the bath solution after activation of single-channel currents by the application of 0.2 mM DNP. The effect of DNP did not develop immediately upon application, but always showed a lag period of 1 to 2 min. Glibenclamide inhibited the DNP-induced single-channel currents completely (Fig. 1B). In these experiments, the membrane potential was expressed relative to the resting potential, which is assumed to be about $-70$ mV with 5.4 mM $K^+$ in the extracellular solution. As previously observed by others, $K_{ATP}$ channel activity was not observed from cell-attached patches and only openings of small inward rectifier $K^+$ ($I_{K1}$) channels were recorded. The addition of DNP (0.2 mM) in bath solution opened $K_{ATP}$ channels, which were easily distinguished from $I_{K1}$ channels because of their larger current amplitude. It can be therefore assumed that DNP activates the $K_{ATP}$ channel. The I-V relationship obtained from cell-attached patch configuration is shown in Fig. 1C.

The effects of adenosine on the $K_{ATP}$ channel were examined by the inside-out and outside-out patch configurations which were performed with symmetrical transmembrane $K^+$ concentration (140 mM). On formation of the inside-out patches in ATP-free internal solution, a population of $K^+$ channels with a unitary conductance of $71.6 \pm 0.8$ pS (mean $\pm$ S.E., n=15) appeared. The channel was inhibited by 1 mM ATP and 50 $\mu$M glibenclamide indicating that it was the $K_{ATP}$ channel (Fig. 2A). Upon wash-out of ATP, channel activity was restored. Figure 2B shows that adenosine, applied to

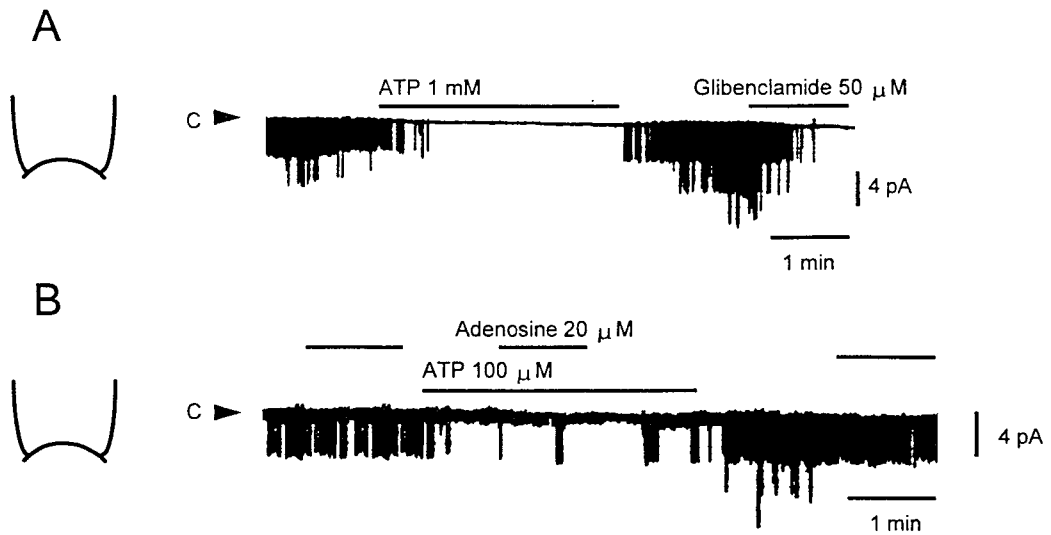


*Fig. 2. (A) Effect of intracellular ATP or glibenclamide on the $K_{ATP}$ channel in an inside-out membrane patch. Single $K_{ATP}$ channel currents recorded from chart recorder. The solution-exchange protocol for ATP (1 mM) and glibenclamide (50 $\mu$M) is shown above current traces. Patch membrane potential was held at $-50$ mV. Low-pass filter, 1 kHz. (B) Effects of intracellular adenosine on the $K_{ATP}$ channel activity in an inside-out patch. Adenosine (20 $\mu$M) was added to the bath solution for a period indicated by the bar. Patch membrane potential was held at -50 mV. Low-pass filter, 1 kHz.*

the internal side of patch membrane, could not induce opening or closing of the $K_{ATP}$ channel. The activity of most $K_{ATP}$ channels tended to decrease rapidly with time when the patch membrane was excised from an intact cell into ATP-free solution, the process known as 'run-down' of the channel activities.

Figure 3 shows a continuous record of activity in an outside-out patch exposed to 100 μM GTP plus 100 μM ATP at the intracellular surface. Few

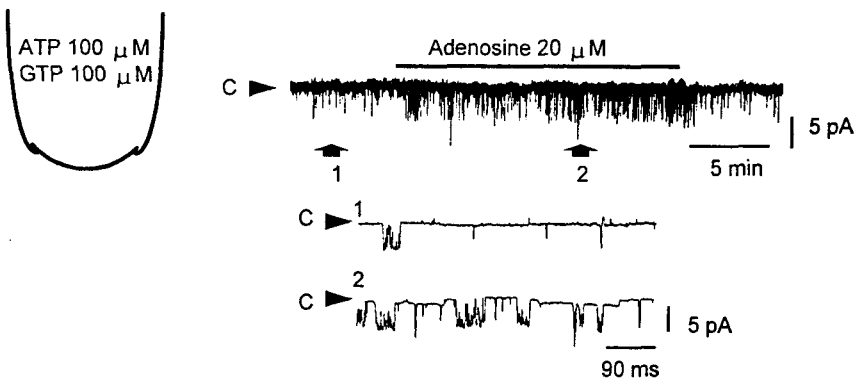


*Fig. 3. Effect of extracellular adenosine on the $K_{ATP}$ channel activity in an outside-out patch. Adenosine (20 μM) was added to the bath solution for a period indicated by the bar. Lower panels show short segments of the main traces (arrow 1 and 2) during experiments on an expanded scale. Patch membrane potential was held at -50 mV. Low-pass filter, 1 kHz.*

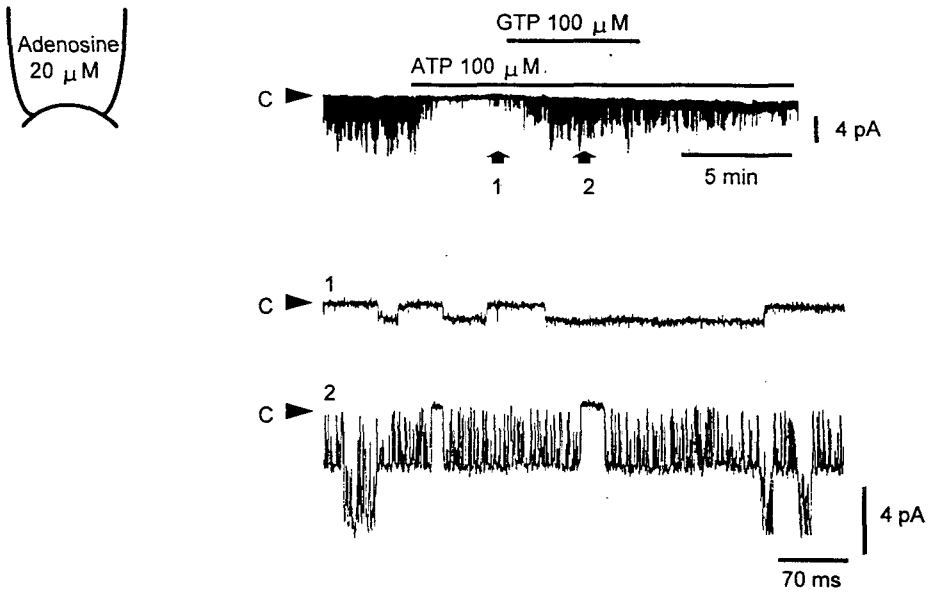


*Fig. 4. Effect of extracellular adenosine on the $K_{ATP}$ channel activity in an inside-out patch. Adenosine (20 μM) was added to the pipette solution continuously. Lower panels show short segments of the main traces (arrow 1 and 2) during experiments on an expanded scale. Patch membrane potential was held at -50 mV. Low-pass filter, 1 kHz.*

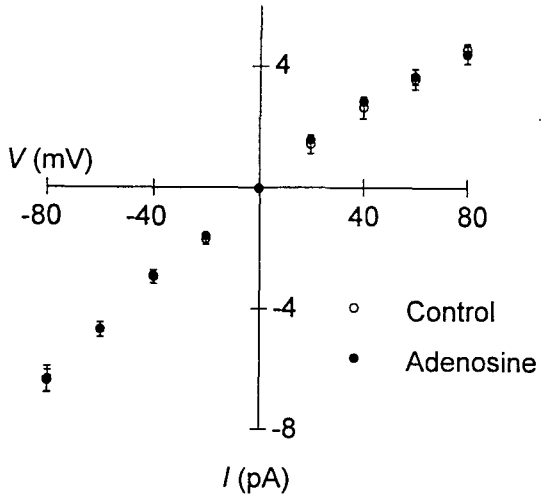


*Fig. 5. Current-voltage relationships of $K_{ATP}$ channel in inside-out patches in the absence (○) and the presence (●) of 20 μM extracellular adenosine. Every mean data point is associated with its standard error.*

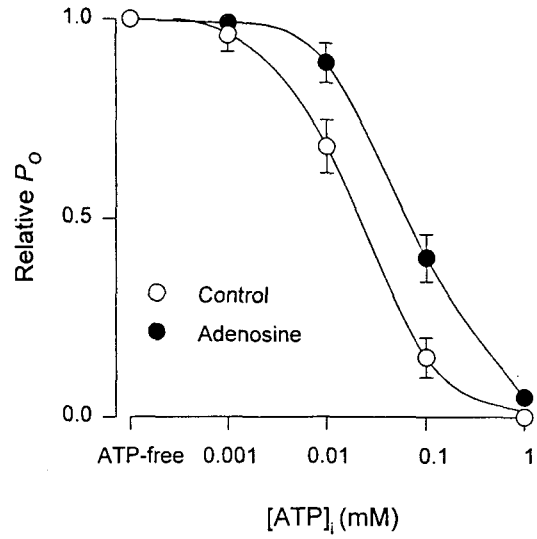


*Fig. 6. Effect of adenosine on the ATP dependence of $K_{ATP}$ channel activity. Dose-response relationship for the ATP-dependence of $K_{ATP}$ channel in the absence (open circle) and presence (filled circle) of adenosine. The open probability in each ATP concentration, Po, was normalized by referring to its value, Po max, in ATP-free solution. The points show mean ± S.E. for n values 7 (control) and 5 (adenosine), respectively. * Values are significantly different from control values ($P<0.05$). All data were filtered at 1 kHz.*

channel openings were observed until the extracellular bathing solution was changed to one containing 20 μM adenosine, where sustained activity was observed for several minutes until the adenosine was washed out. Similar results were obtained in two additional experiments.

The GTP-dependence of the effect was tested in an inside-out patch (Fig. 4) continuously exposed to 20 μM adenosine at the beginning of the experiment when the intracellular surface was first exposed to ATP-free solution. The activity was strongly reduced upon application of the ATP at 100 μM. Application of 100 μM GTP to the intracellular surface stimulated the channel activity in the continued presence of ATP. The effect was reversible upon washout with GTP-free solution.

In the presence of adenosine, a linear I-V relationship (Fig. 5) was again obtained at potentials negative to $E_K$ with a slope conductance 78.8 ± 1.9 pS (n=6). Extracellular adenosine did not change the conductance of $K_{ATP}$ channel.

In Figure 6, the graph shows the dose-response relationship for inhibition of $K_{ATP}$ channel activity by ATP in the absence and presence of adenosine. The continuous lines in the graph are fitted curve to the Hill equation using the least-squares method:

$$\text{Relative } P_O = 1/\{1+([ATP]/K_i)^n\}$$

where [ATP] = concentration of ATP, $K_i$ = the ATP concentration at the half-maximal inhibition, and n = Hill coefficient. In control conditions, $K_i$ and n were 70 μM and 1.1, respectively. In the presence of adenosine, the dose-response relation for ATP was shifted to higher ATP concentration (241 μM) and n was decreased slightly to 0.7.

## DISCUSSION

Activation of the $K_{ATP}$ channel has been thought to be a major component of the increased potassium conductance during myocardial ischemia or hypoxia (Favre & Findlay, 1990). The mechanism by which activation of the $K_{ATP}$ channel causes action potential shortening in metabolic inhibition despite of only modest reductions in tissue ATP is not completely understood at the present time. Several possibilities have been suggested to account for the observed discrepancy (Ascroft, 1988). First, because of the high density of cardiac $K_{ATP}$ channels, only a small increase in the open state probability is sufficient to play a role in the response to myocardial ischemia. Second, because the concentration of intracellular ATP in the immediate vicinity of $K_{ATP}$ channels is lower than the total cellular ATP content, the functional compartmentalization of ATP may contribute to the activation of $K_{ATP}$ channel during myocardial ischemia. Third, $K_{ATP}$ channels in the heart may be modulated by intracellular metabolites generated during ischemia other than ATP. One of them, our results support that some metabolic factors accumulated during ischemia and hypoxia may decrease the sensitivity of $K_{ATP}$ channel to ATP. Under the condition of myocardial ischemia or hypoxia, the intracellular concentrations of $H^+$, ADP, and GDP all rise and may potentially influence channel activity (Findlay, 1988; Lederer & Nichols, 1989; Weiss & Lamp, 1989). In the present study, we have observed that adenosine also play a significant role in activating $K_{ATP}$ channel. Extracellular adenosine shifted the half-maximal inhibition of $K_{ATP}$ channel from 70 to 241 $\mu$M.

The result suggests that activation of adenosine receptors stimulates $K_{ATP}$ channels by reducing the apparent affinity of the channel for ATP. Therefore, the effect may be important for activating $K_{ATP}$ channels even at moderate levels of ATP during early phase of myocardial ischemia.

Although the mechanism of ischemic preconditioning is unknown, recent results obtained in rabbits suggest that activation of adenosine $A_1$ receptors may trigger the protective effect. This hypothesis, first proposed by Liu et al (1991), is based on the observations that nonselective adenosine receptor antagonists block the protective effect of ischemic preconditioning in anesthetized rabbits and selective adenosine $A_1$ receptor agonist mimic preconditioning in perfused rabbit hearts. These studies provide strong evidence that adenosine, which is released from myocytes during ischemia, activates $A_1$ receptors, which subsequently mediates preconditioning. However, the cellular mechanism by which activation of this receptor provides protection to the myocardium remains to be determined. The objective of the present study was to test the hypothesis that adenosine provides protection to the myocardium during preconditioning by activating $K_{ATP}$ channels. We observed that in an outside-out patch exposed to GTP and ATP at the intracellular surface, $K_{ATP}$ channel openings were not observed, and addition of the extracellular adenosine (20 $\mu$M) restored the activity. In an inside-out patch exposed to adenosine (20 $\mu$M) at the extracellular surface, the channel activity was reduced upon ATP, and addition of GTP to the intracellular surface stimulated $K_{ATP}$ channel activity. Our work was similar to that of Carmeliet's group (1994). Our data confirm their results. There are, however, the novel aspects of our work in comparison with their work: (1) they measured currents in isolated guinea-pig ventricular myocytes, while we did in rabbit ventricular myocytes; (2) they measured currents only in inside-out patches, while we did both in inside-out and outside-out patches; (3) they mainly used GTPrS to determine the mechanism of G protein action, while we focused our interested on the modulation of $K_{ATP}$ channel by adenosine $A_1$ receptor activation itself. Considering the hypothesis that adenosine, which is formed and released during ischemia from breakdown of ATP, mediates pre-

conditioning by activating $K_{ATP}$ channel, it was really necessary to perform the experiments after adenosine application. We always performed the experiments after adenosine $A_1$ receptor activation. The first study to suggest that adenosine may act via opening the channels in the myocardium was performed in rat neonatal ventricular myocytes. Kirsch et al(1990) showed that $K_{ATP}$ channels are opened by adenosine and cyclohexylammonium, the selective adenosine receptor agonist, and that the $A_1$ receptor is coupled to the $K_{ATP}$ channel by a $G_i$ protein. The results suggested that the activation of $K_{ATP}$ channels by adenosine is a result of adenosine $A_1$ receptor activation. Therefore, considering these results whereby preconditioning was shown to be mediated via adenosine $A_1$ receptor activation, it seems reasonable to hypothesize that adenosine, which is formed during ischemia from the breakdown of ATP, acts on $A_1$ receptors, which serves to protect the myocardium from a subsequent ischemic insult by activating $K_{ATP}$ channels via a G protein (Kirsch et al, 1990; Ito et al, 1994). Theoretically, activation of $K_{ATP}$ channels may result in favorable metabolic effects. $K_{ATP}$ channel activation has been shown to shortening action potential duration and antagonize membrane depolarization (Faivre & Findlay, 1990; Venkatesch et al, 1991). These effects would be expected to reduce the open time of voltage-regulated calcium channels, which would be expected to ultimately lead to reduced free cytosolic calcium levels, a rapid loss of contractile activity, and preservation of ATP, which would be expected to delay cell death.

In conclusion, our results suggest that adenosine modulates $K_{ATP}$ channel activity in cardiac cell and may play an important role in activation of $K_{ATP}$ channel even at moderately high levels of cytosolic ATP concentrations during ischemia and hypoxia by reducing the sensitivity of $K_{ATP}$ channel to ATP.

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