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# The effect of glibenclamide on the production of interstitial adenosine by inhibiting ecto-5′-nucleotidase in rat hearts

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**1** Adenosine exerts cardioprotective effects on the ischaemic myocardium. The production of adenosine in the ischaemic myocardium is attributed primarily to the enzymatic dephosphorylation of adenosine 5′-monophosphate (AMP) by 5′-nucleotidase. We determined the activity of 5′-nucleotidase in rat hearts. The objective of the study was to determine the effects of ATP-sensitive $K^+$ ($K_{ATP}$) channel antagonists (glibenclamide and 5-hydroxydecanoate) on the production of adenosine, by use of a flexibly mounted microdialysis technique.

**2** Rats were anaesthetized and the microdialysis probe was implanted in the left ventricular myocardium, followed by perfusion with Tyrode solution. The baseline level of dialysate adenosine was $0.51 \pm 0.09$ $\mu$M ($n=16$). Introduction of AMP (100 $\mu$M) through the probe increased the dialysate adenosine markedly to $9.79 \pm 0.43$ $\mu$M ($n=12$, $P<0.001$ vs baseline), and this increase was inhibited by the ecto-5′-nucleotidase inhibitor, $\alpha,\beta$-methyleneadenosine 5′-diphosphate (100 $\mu$M), to $0.76 \pm 0.12$ $\mu$M ($n=8$). Thus, the dialysate adenosine noted during the perfusion of AMP originated from dephosphorylation of AMP by ecto-5′-nucleotidase, and the dialysate level of adenosine attained reflects the ecto-5′-nucleotidase activity in the tissue *in situ*.

**3** Glibenclamide (0.1–100 $\mu$M) decreased the adenosine concentration measured during the perfusion of AMP (100 $\mu$M) in a concentration-dependent manner ($IC_{50}=10.5$ $\mu$M). In contrast, 5-hydroxydecanoate (10–100 $\mu$M) did not affect the concentrations of dialysate adenosine, measured in the presence of AMP (100 $\mu$M). These results suggest that glibenclamide inhibits the activity of endogenous ecto-5′-nucleotidase and decreases the concentration of adenosine in the interstitial space of rat ventricular muscles *in situ*.

**Keywords:** Microdialysis; adenosine; ecto-5′-nucleotidase; glibenclamide; 5-hydroxydecanoate

## Introduction

Adenosine exerts multiple actions throughout the body and modifies various cardiovascular functions (Berne, 1980). Much attention has focused on the role of adenosine as an endogenous cardioprotective substance during myocardial ischaemia (Ely & Berne, 1992; Kitakaze *et al*., 1993). The formation and release of adenosine by the ischaemic myocardium is enhanced, and the major source of adenosine is the enzymatic dephosphorylation of adenosine 5′-monophosphate (AMP) by 5′-nucleotidase (Frick & Lowenstein, 1976; Schrader *et al*., 1991). Thus, the conversion of AMP to adenosine by 5′-nucleotidase may be a crucial step for cardioprotection during myocardial ischaemia.

In the present study, we sought to estimate the activity of 5′-nucleotidase in *in vivo* hearts. The activity of 5′-nucleotidase is estimated from the rate of adenosine production occurring in the presence of a certain concentration of AMP, a substrate for the enzyme to produce adenosine. Therefore, with introduction of flexibly mounted microdialysis technique (Obata *et al*., 1994), we measured the concentration of dialysate adenosine under a constant supply of AMP through the probe. Evidence is presented that the level of dialysate adenosine measured under the above conditions reflects the activity of tissue 5′-nucleotidase.

$A_1$-adenosine receptors are present in cardiomyocytes and appear to mediate the beneficial effects of adenosine during ischaemia and reperfusion (Lasley *et al*., 1990; Lasley & Mentzer, 1992; Thornton *et al*., 1992). Kirsch *et al*. (1990) showed that adenosine activates the ATP sensitive $K^+$ ($K_{ATP}$) channel in rat ventricular myocytes via the $A_1$-adenosine receptor-coupled to activation of a $G_i$ protein. Because activation of $K_{ATP}$ channels was proposed to mediate a protective effect on the ischaemic and reperfused myocardium (Cole *et al*., 1991; Hearse, 1995; Shigematsu *et al*., 1995), it is likely that $A_1$-adenosine receptor-mediated activation of $K_{ATP}$ channel plays, at least in part, a role to protect the heart during episodes of ischaemia. It has been shown that glibenclamide, a $K_{ATP}$ channel antagonist, abolished the cardioprotection afforded by the stimulation of $A_1$-adenosine receptors in the rabbit, dog and swine hearts (Auchampach & Gross, 1993; Yao & Gross, 1993; Schulz *et al*., 1994; Toombs *et al*., 1994; Van Winkle *et al*., 1994). However, Thornton *et al*. (1993) found that glibenclamide given intravenously into the rabbits hearts did not abolish preconditioning. In rat hearts, Liu and Downey (1992) showed that the ischaemic preconditioning could not be blocked by either an $A_1$-adenosine receptor antagonist or glibenclamide. Furthermore, Grover *et al*. (1993) failed to block ischaemic preconditioning with glibenclamide or 5-hydroxydecanoate in the rat isolated heart. However, more recently, Qian *et al*. (1996) demonstrated that glibenclamide blocked the cardioprotection conferred by ischaemic preconditioning in the rat. Thus, it appears that considerable controversy exists as to the role of $A_1$-adenosine receptors and $K_{ATP}$ channels in mediating ischaemic preconditioning.

In these previous studies, glibenclamide has been used as a pharmacological tool to block selectively the $K_{ATP}$ channels (Edwards & Weston, 1993). Whether or not glibenclamide mitigates the adenosine-dependent protection of myocardium against ischaemia by blocking $K_{ATP}$ channels or by decreasing adenosine concentration *per se* (via inhibition of the synthesis of adenosine or acceleration of its degradation) is unknown. This prompted us to examine the effect of glibenclamide on the production of interstitial adenosine (i.e., the activity of 5′-nucleotidase), by use of a flexibly mounted microdialysis technique. The results of our studies demonstrate that glibenclamide decreases the production of interstitial adenosine by inhibiting the activity of ecto-5′-nucleotidase.

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## Methods

### *Animal preparation*

Wistar rats of either sex weighing 250–300 g were anaesthetized by an intraperitoneal injection of chloral hydrate (400 mg $kg^{-1}$), and then were ventilated with room air and oxygen. The chest was opened at the left fifth intercostal space and the pericardium was removed to expose the left ventricle. All procedures in dealing with the experimental animals met the guideline principles stipulated by the Physiological Society of Japan and the Animal Ethics Committee of the Oita Medical University.

### *Microdialysis technique*

Details of the flexibly mounted microdialysis technique and its application to *in vivo* rat hearts to measure biological substances in the interstitial space have been described previously (Obata *et al*., 1994). In brief, the tip (cylinder-shape) of a microdialysis probe (3 mm length and 220 $\mu$m o.d. with the distal end closed) was made of dialysis membrane (cellulose membrane of 10 $\mu$m thick with 50,000 molecular weight cut-off). Two fine silica tubes (75 $\mu$m i.d.) were inserted from the open-end into the tip of the microdialysis tube consisting of a cylinder-shape dialysis membrane and which served as an inlet for the perfusate and an outlet for the dialysate, respectively. The inlet tube was connected to a micro-injection pump (Carnegie Medicine, CMA/100, Stockholm, Sweden), and the outlet tube was led to the dialysate reservoir. These tubes ($\sim$15 cm long) were supported loosely at the midpoint on a rotatable stainless steel wire (1 mm diameter), so that their movement was fully synchronized with a rapid up-and-down movement of the tip caused by the heart beats. The probe was implanted from the epicardial surface into the left ventricular myocardium to the depth of 3 mm and perfused through the inlet tube with Tyrode solution of the following composition (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, $NaH_2PO_4$ 0.16, $NaHCO_3$ 3.0, glucose 5.5 and HEPES 5.0 (pH = 7.4 adjusted with NaOH). The molecules that spilled out of the cut end of inlet tube entered the extracellular space across the dialysate membrane by diffusion, and various substances present or produced in the interstitial space then diffused back into the cavity of the probe; the dialysate eventually left the probe from the outlet tube.

To determine the recovery ratio of interstitial adenosine through the dialysis membrane the tip of the microdialysis probe was bathed in warm (37°C) Tyrode solution containing 10 $\mu$M adenosine, and the probe interior was perfused with an adenosine-free Tyrode solution (via inlet tube) at various rates of perfusion (0.5–5.0 $\mu$l $min^{-1}$). The recovery of adenosine was determined by measuring adenosine concentration in the dialysate, collected at different rates of perfusion. The relative recovery rate of adenosine ([dialysate adenosine concentration/bath adenosine concentration] × 100; in %) declined exponentially when the flow rate was increased stepwise from 0.5 to 5.0 $\mu$l $min^{-1}$. Conversely, when the flow rate was increased, the absolute amount of adenosine recovered for a certain period of time, i.e., [the volume of dialysate collected per minute] × [adenosine concentration of the particular dialysate] (in pmol $min^{-1}$) increased steeply at relatively low rates of perfusion (0.5–1.0 $\mu$l $min^{-1}$), and reached a steady state at rates over 1.0 $\mu$l $min^{-1}$. Thus, a low perfusion rate was needed in order to increase the relative recovery rate of adenosine while a high perfusion rate is recommended to increase the absolute recovery of adenosine. To handle these different conditions, a perfusion rate of 1.0 $\mu$ $min^{-1}$ was used in the present experiments. The relative recovery of adenosine attained with this perfusion rate was estimated to be 18.0 ± 1.6% ($n$ = 5), which was not affected by the drugs and vehicles used in the present study.

### *Measurements of adenosine in the dialysate*

The dialysate was collected continuously as a fraction recovered for a 15 min period (i.e. 15 $\mu$l of dialysate was collected in each reservoir), and immediate detection of adenosine was feasible, by use of a reversed phase high-performance liquid chromatography technique. Separation of the compounds was achieved on Eicompak MA-5 ODS columns (5 $\mu$m, 4.6 × 150 mm; Eicom, Kyoto, Japan), with the mobile phase consisting of 5% (vol) acetonitrile and 200 mM $KH_2PO_4$ (pH = 3.8 adjusted with phosphoric acid). The flow rate was set at 1.0 ml $min^{-1}$ by use of a pumping system (JASCO Corp., PU-980, Tokyo, Japan). The absorbance of the column eluate was monitored at 260 nm, by an ultraviolet detector (JASCO Corp., UV-970). Absorbance peak of adenosine or inosine was quantified by comparing the retention time and peak heights with a known standard of adenosine or inosine, at concentrations of 1 and 10 $\mu$M. Adenosine concentrations are presented as absolute values (actual concentrations of the dialysate in each reservoir), and not corrected for the recovery rate (18%) unless otherwise stated.

### *Assays of ecto-5′-nucleotidase*

Ventricular tissue from rat hearts was cut into $\sim$1 mm pieces and homogenized for 5 min in 3 ml of ice-cold 10 mM HEPES-KOH buffer (pH = 7.4) containing (in mM): sucrose 250, $MgCl_2$ 1 and mercaptoethanol 1 and 0°C. To prepare a membrane fraction, the homogenate was centrifuged at 1000 *g* for 10 min. The subsequent pellet was resuspended in HEPES-KOH buffer and divided into aliquots for measurement of ecto-5′-nucleotidase activity. The activity of 5′-nucleotidase was determined by an enzymatic assay technique (Smith *et al*., 1965), by use of a commercially available kit (Sigma Chemical Co., St. Louis, MO, U.S.A.). The protein concentration in each sample was measured by the method of Lowry *et al*. (1951).

### *Drugs*

Appropriate amounts of adenosine 5′-monophosphate (AMP, Wako Pure Chemical Co., Osaka, Japan) were dissolved in Tyrode solution immediately before use in order to yield various concentrations of AMP, as indicated in the text. $\alpha,\beta$-Methyleneadenosine 5′-diphosphate ($\alpha,\beta$-meADP, Sigma Chemical Co.), and sodium 5-hydroxydecanoate (5-HD, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) were dissolved in distilled water and kept as 10 mM stock solutions. Glibenclamide (Hoechst Japan Co., Tokyo, Japan) was prepared as a 5 mM stock solution in 5% dimethyl sulphoxide (DMSO). An appropriate volume of each stock solution was added to the Tyrode solution immediately before use to achieve the desired final concentrations, as described in the Results section. The final concentration of DMSO was less than 0.1% and this had no effect on the level of dialysate adenosine ($n$ = 4).

### *Statistical analysis*

All values are presented as mean ± s.e.mean. The significance of difference was determined by use of ANOVA followed by a Fisher's *post hoc* test. A $P$ value of less than 0.05 was considered to be statistically significant. Nonlinear curve fitting of concentration-response data was performed with commercially available software that uses a Marquardt procedure for parameter estimation (Sigmaplot, Jandel Corp., Corte Madera, CA, U.S.A.).

## Results

### In vivo *estimation of ecto-5′-nucleotidase activity*

We first determined the baseline level of adenosine concentration in the dialysate and examined the effect of added

AMP (a substrate of 5′-nucleotidase to produce adenosine) to the perfusate on the level of dialysate adenosine. Figure 1a shows sequential changes of dialysate adenosine concentration obtained from 6 rat hearts; the measurements were begun just after implantation of the probe. The dialysate adenosine concentration measured at the end of the initial 15 min (0–15 min) was $4.10 \pm 1.00$ $\mu$M, which declined to $1.67 \pm 0.79$ at 15–30 min and to $0.86 \pm 0.21$ at 30–45 min, and eventually reached a quasi steady state level of $0.60 \pm 0.13$ $\mu$M at 45–60 min. The perfusate was then switched to the Tyrode solution containing 100 $\mu$M AMP. The introduction of AMP through the probe caused a significant ($P < 0.001$) increase in the concentration of adenosine from $0.60 \pm 0.13$ μM (baseline) to $6.29 \pm 0.42$ $\mu$M at 30–45 min after the introduction (i.e., the dialysate fraction of 90–105 min in Figure 1a). The AMP-induced increase in dialysate adenosine concentration was attributed to the conversion of AMP to adenosine catalysed by 5′-nucleotidase present in the tissue. To gain support for this assumption, we determined whether α,β-methyleneadenosine 5′-diphosphate (α,β-meADP), an inhibitor of ecto-5′-nucleotidase (Headrick *et al*., 1992; Darvish & Metting, 1993), could prevent the increases in adenosine concentrations caused by AMP. After two dialysate fractions (0–15 min and 15–30 min) had been obtained, the perfusate was switched to the Tyrode solution containing α,β-meADP at a concentration of 100 $\mu$M for 30 min (cf the solid columns at 30–45 min and 45–60 min in Figure 1b). Additional application of AMP (100 $\mu$M) through the probe (in the continued presence of α,β-meADP) failed to increase the concentration of adenosine and the level of $0.56 \pm 0.14$ $\mu$M ($n = 5$) remained unchanged even at 45–60 min after the introduction of AMP (Figure 1b). These results suggest that the introduction of AMP through the probe did indeed produce adenosine via the ecto-5′-nucleotidase mediated conversion of AMP to adenosine.

Figure 2 shows a comparison of the steady state levels of adenosine in the dialysate, determined under various experimental conditions. As the equilibration period was extended up to 105–120 min in some experiments ($n = 5$), the mean levels of adenosine in the dialysate tended to decrease with time. However, because there was no significant difference between the adenosine concentrations in the dialysate fractions collected after 45 min of the probe implantation, the concentration of dialysate adenosine measured after 45–60 min of the probe implantation was considered to be the steady state level. When the probe was perfused with Tyrode solution, the steady state (baseline) level of dialysate adenosine measured after 45–60 min of probe implantation was $0.51 \pm 0.09$ $\mu$M (Figure 2). However, when the probe was perfused with the Tyrode solution containing 100 $\mu$M AMP from the outset of implantation, the level of dialysate adenosine was markedly elevated, in particular, immediately after probe implantation; this increased adenosine concentration was then followed by a slight decline in subsequent fractions of dialysate. The steady state level of adenosine (measured after 45–60 min of AMP introduction) was $9.79 \pm 0.43$ $\mu$M ($n = 12$, Figure 2), a value that is significantly ($P < 0.001$) higher than the baseline adenosine concentration. When α,β-meADP (100 $\mu$M) was included alone in the Tyrode solution, the concentration of dialysate adenosine measured at 45–60 min after its introduction was $0.42 \pm 0.05$ $\mu$M ($n = 7$, Figure 2), a value similar to the baseline level. In contrast, when α,β-meADP (100 $\mu$M) was included in combination with AMP, it significantly inhibited the AMP-induced increases in dialysate adenosine to $0.76 \pm 0.12$ $\mu$M ($n = 8$, Figure 2), a value significantly ($P < 0.001$) lower than that measured in the presence of AMP alone ($9.79 \pm 0.43$ $\mu$M), but not significantly different from the baseline level of adenosine ($0.51 \pm 0.009$ $\mu$M). These results taken together suggest that the dialysate adenosine measured during the constant supply of AMP originated from the dephosphorylation of AMP catalysed by endogenous ecto-5′-nucleotidase.

The effect of different concentrations of AMP on levels of dialysate adenosine was also determined. As shown in Figure 3, when various concentrations of AMP (ranging from 10 to 1000 $\mu$M) were applied through the probe, the steady state levels of dialysate adenosine (measured after 45–60 min of probe implantation) rose with increases in the concentration of AMP. Each experimental data point was fitted to the following equation:

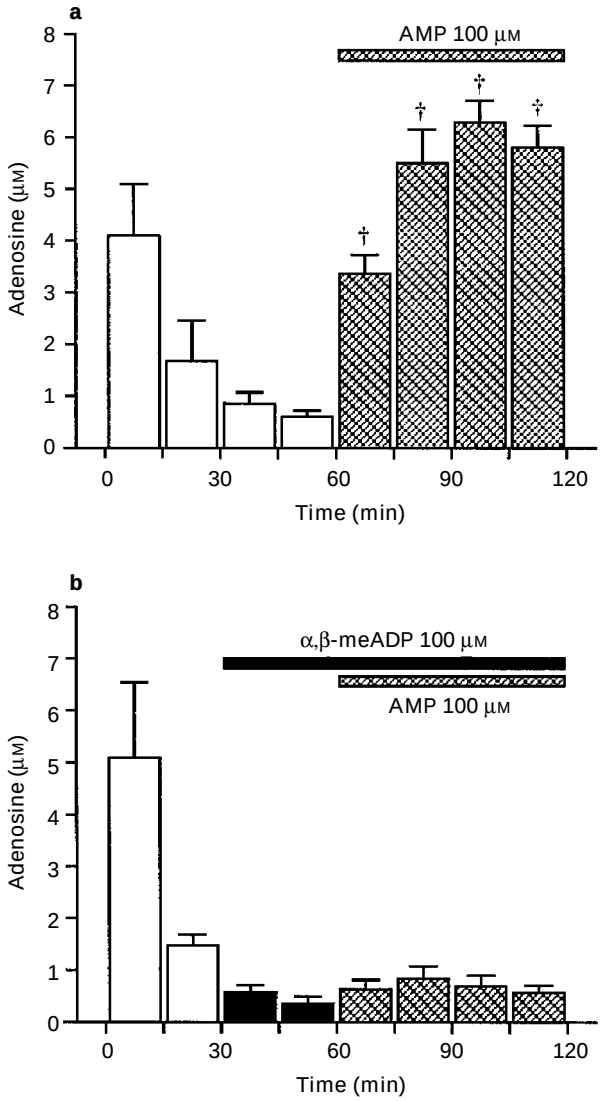


**Figure 1** Time course of changes in adenosine concentration and modification thereof by AMP and α,β-meADP, in the dialysate collected for every 15 min (as indicated) after implantation of the microdialysis probe. Time on abscissa scales denotes the time elapsed after probe implantation. (a) Effects of introduction of AMP (100 $\mu$M) on the level of dialysate adenosine ($n = 6$). After 60 min of probe implantation, AMP was introduced through the probe, as indicated by the horizontal bar. †$P < 0.001$, significant difference from the adenosine level immediately before AMP administration (corresponding to the dialysate collected 45–60 min after implantation). (b) Effect of introduction of AMP (100 $\mu$M) on dialysate adenosine examined in the presence of α,β-methyleneadenosine 5′-diphosphate (α,β-meADP, 100 $\mu$M) ($n = 5$). α,β-meADP was applied through the probe 30 min before AMP administration as indicated by the horizontal bars. Values are mean ± s.e.mean.

Dialysate adenosine concentration($\mu$M)

$$E_{max}[C/(EC_{50} + C)] \quad (1)$$

where $E_{max}$ is the maximum attainable concentration of dialysate adenosine; C, the AMP concentration perfused through the probe; and $EC_{50}$, the AMP concentration for the half-maximal effect. The $E_{max}$ and $EC_{50}$ values were 21.1$\mu$M and

107.2 μM, respectively. Based on these results, in the remaining experiments we used 100 μM AMP (a level close to the $EC_{50}$) to estimate the activity of ecto-5′-nucleotidase.

To determine the concentration-dependent inhibitory effect of α,β-meADP on the production of adenosine, experiments were performed in which the microdialysis probe was perfused with AMP (100 μM) containing Tyrode solution throughout an experiment. When the probe was perfused with Tyrode solution containing AMP from the beginning, the level of dialysate adenosine was fairly high and reached a steady state after 45–60 min of probe implantation (as already shown in Figure 2); there were no further significant changes over a period of at least 150 min after probe implantation. After two control fractions (dialysates of 30–45 min and 45–60 min in Figure 4a), had been obtained, the introduction of α,β-meADP at a concentration of 100 μM was begun in the continued presence of AMP. α,β-meADP significantly decreased the concentration of adenosine from 8.98±0.56 μM (a dialysate of 45–60 min in Figure 4a) to 0.79±0.11 μM within 45 min (a dialysate of 90–105 min in Figure 4a) ($n=5$, $P<0.001$). After removal of α,β-meADP from the perfusate, the concentration of dialysate adenosine was gradually restored and reached a level of 8.00±1.14 μM ( a dialysate of 135–150 min in Figure 4a). As shown in Figure 4b, these inhibitory effects of α,β-meADP on the AMP-primed production of adenosine were concentration-dependent. The % decrease in adenosine concentration caused by α,β-meADP was measured 30–45 min after its application and was plotted against a variety of α,β-meADP concentrations (ranged from 0.01 to 100 μM). The concentration-response relationship was fitted by the following equation:

$$\text{Decrease in adenosine concentration (\%)} = I_{max}[C/(IC_{50}+C)] \quad (2)$$

where $I_{max}$ is the maximum attainable decrease (%) in dialysate adenosine concentration; C, the concentration of α,β-meADP in the perfusate, and $IC_{50}$, the concentration of α,β-meADP to induce a half-maximal inhibitory effect. The values of $I_{max}$ and $IC_{50}$ were 87.7% and 0.41 μM, respectively. These findings taken together suggest that the level of dialysate adenosine observed during perfusion with AMP (100 μM) could serve as a meaningful measure of ecto-5′-nucleotidase activity in the tissue *in situ*.

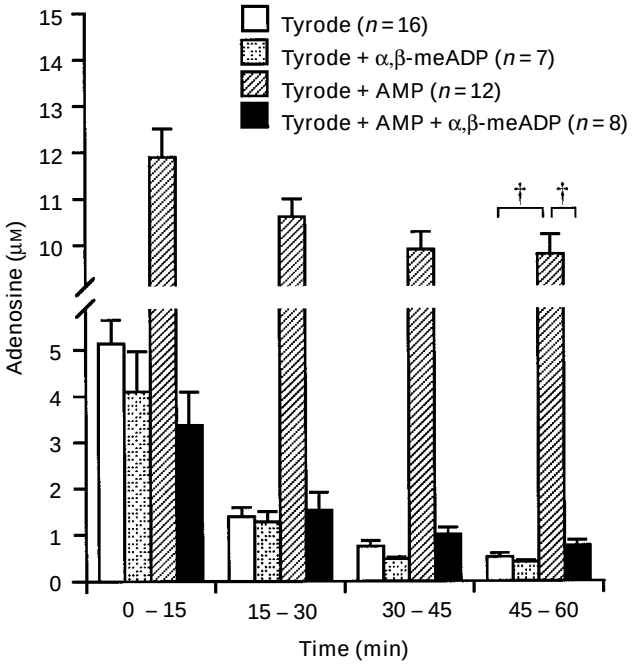


**Figure 2** Time-dependent changes of dialysate adenosine concentration measured in different perfusates, as indicated at the top. The microdialysis probe was perfused with Tyrode solution ($n=16$), Tyrode solution containing 100 μM α,β-meADP ($n=7$), Tyrode solution containing 100 μM AMP ($n=12$) and Tyrode solution containing both 100 μM AMP and 100 μM α,β-meADP ($n=8$), respectively. The dialysate was collected for every 15 min (as indicated) after implantation of the probe and the time on the abscissa scale indicates the time elapsed after probe implantation. †$P<0.001$, significant difference between the data connected by each bracket. Values are mean±s.e.mean.

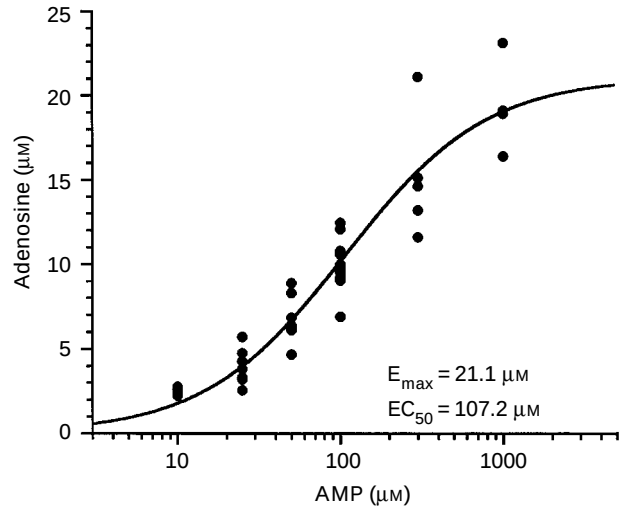


**Figure 3** Concentration-dependent effect of AMP on the level of dialysate adenosine. Ordinate scale: adenosine concentrations of the dialysate collected 45–60 min after introduction of AMP-containing Tyrode solution through the probe. Abscissa scale: concentrations of AMP added. Each point represents data from individual experiments. The solid curve was drawn as the best fit to equation (1) in the text.

## *Effects of glibenclamide on ecto-5′-nucleotidase*

We first assessed the effect of glibenclamide on the activity of ecto-5′-nucleotidase in the membrane fraction of the homogenate of rat ventricular tissue. Glibenclamide at a concentration of 30 μM significantly ($P<0.01$) decreased the activity of ecto-5′-nucleotidase from 245.6±5.3 nmol $mg^{-1}$ protein $min^{-1}$ to 199.6±6.3 nmol $mg^{-1}$ protein $min^{-1}$ ($n=4$). Therefore, in the next series of experiments, we determined whether glibenclamide could decrease the level of interstitial adenosine in rat hearts *in vivo*.

When glibenclamide (30 μM) was perfused with AMP-free Tyrode solution, the level of dialysate adenosine measured after 45–60 min of probe implantation was 0.51±0.08 μM ($n=7$), a value that was not significantly different from the baseline level of adenosine (0.51±0.09 μM). Figure 5a, shows the effect of glibenclamide on the level of dialysate adenosine, determined in the presence of 100 μM AMP. The experimental protocol was the same as for Figure 4, and α,β-meADP was replaced by glibenclamide. When glibenclamide (10 μM) was perfused through the probe for 45 min, the concentration of dialysate adenosine significantly decreased from 10.86±1.16 μM (a dialysate of 45–60 min in Figure 5a) to 6.79±1.02 μM after 30–45 min of its introduction (dialysate 90–105 min in Figure 5a) ($P<0.05$). After removal of glibenclamide from the perfusate, the level of adenosine was restored to 9.54±1.88 μM (dialysate 135–150 min in Figure 5a). Glibenclamide decreased the level of dialysate adenosine in a concentration-dependent manner (from 0.1 to 100 μM); the results are summarized in Figure 5b. The values for $I_{max}$ and $IC_{50}$ for glibenclamide inhibition, determined from equation (2) were 84.6% and 10.5 μM, respectively. These results suggest that glibenclamide decreased the production of interstitial adenosine by inhibiting the activity of ecto-5′-nucleotidase.

However, the reduction of AMP-primed dialysate adenosine by glibenclamide could be due to an enhanced breakdown of adenosine (which may lead to an increase in the production of inosine), via activation of adenosine deaminase. Thus, we determined if glibenclamide could affect the level of dialysate inosine in the presence of AMP (100 $\mu$M). Glibenclamide at a concentration of 10 $\mu$M did not increase but significantly decreased inosine concentrations by $29.9 \pm 4.3\%$ ($n=5$, $P<0.05$) and decreased the total sum of adenosine and inosine concentrations by $34.9 \pm 2.0\%$ ($n=5$, $P<0.05$). These findings suggest that the reduction of AMP-primed dialysate adenosine by glibenclamide was not due to enhanced activity of adenosine deaminase, rather it was due to inhibition of the activity of ecto-5′-nucleotidase.

The effects of 5-hydroxydecanoate (5-HD), another $K_{ATP}$ channel antagonist (McCullough *et al*., 1991; Notsu *et al*., 1992), were also examined. As shown in Figure 6a, 5-HD at a concentration of 100 $\mu$M did not decrease, but sometimes tended to increase the concentration of dialysate adenosine (primed by 100 μM AMP) from $7.85 \pm 1.77$ $\mu$M (dialysate 45–60 min in Figure 6a) to $8.94 \pm 2.37$ μM after 30–45 min application (dialysate 90–105 min in Figure 6a), albeit the changes were not statistically significant. The same experiments were repeated with various concentrations of 5-HD and the changes in dialysate adenosine concentrations measured 30–45 min after application of 5-HD are summarized in Figure 6b. 5-HD at concentrations of 10, 30 and 100 $\mu$M altered the dialysate adenosine to $99.3 \pm 10.6\%$ ($n=5$),

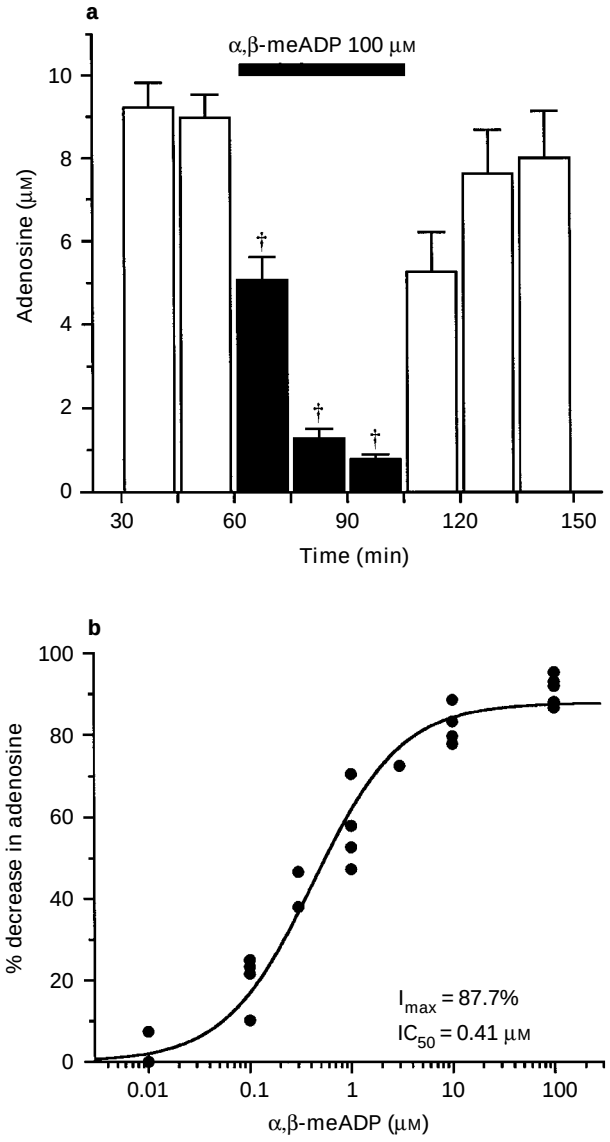


**Figure 4** Effect of α,β-meADP on the AMP-primed dialysate adenosine concentration. (a) Time-course of the changes of the dialysate adenosine concentration before, during and after application of α,β-meADP ($n=5$). Abscissa scale indicates the time after implantation of the probe. α,β-meADP (100 $\mu$M) was introduced in the probe in the presence of 100 $\mu$M AMP. †$P<0.001$, significant difference from the value immediately before introduction of α,β-meADP, i.e., dialysate 45–60 min. Values are mean ± s.e.mean. (b) Effects of different concentrations of α,β-meADP on the 100 $\mu$M AMP-primed dialysate adenosine concentration. Each point represents data from individual experiments, and are % inhibition of dialysate adenosine measured 30–45 min after the application of α,β-meADP plotted against various concentrations of α,β-meADP (0.01 to 100 $\mu$M). The solid curve was drawn as the best fit to equation (2) in the text.

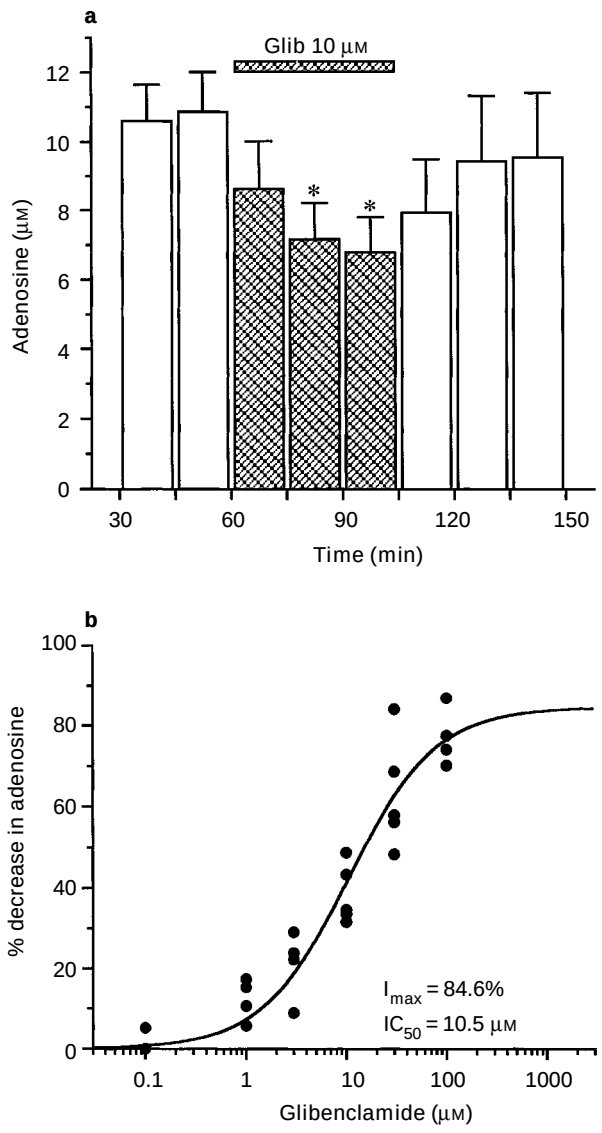


**Figure 5** Effects of glibenclamide (Glib) on the AMP-primed dialysate adenosine concentration. (a) Time-course of the changes of the dialysate adenosine concentration before, during and after application of glibenclamide ($n=5$). Abscissa scale indicates the time after implantation of the probe. Glibenclamide (10 $\mu$M) was applied for 45 min through the probe, as indicated by the horizontal bar (dialysates 60–105 min). *$P<0.05$ vs pre-drug value (dialysate 45–60 min). Values are mean ± s.e.mean. (b) Effects of different concentrations of glibenclamide on the 100 $\mu$M AMP-primed dialysate adenosine concentration. Each point represents data from individual experiments, and are % inhibition of dialysate adenosine measured after 30–45 min application of glibenclamide plotted against the various concentrations of glibenclamide (0.1–100 $\mu$M). The solid curve was drawn as the best fit to equation (2) in the text.

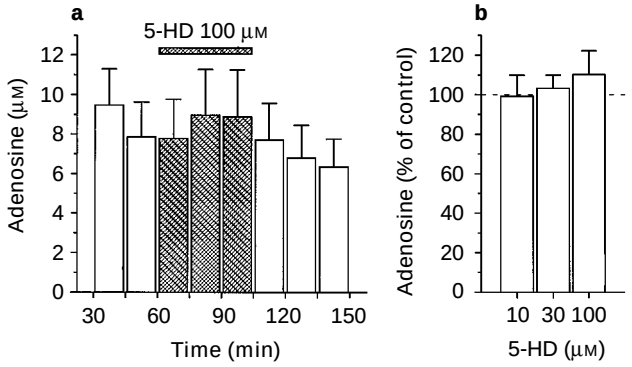


**Figure 6** Effect of 5-hydroxydecanoate (5-HD) on the 100 $\mu$M AMP-primed dialysate adenosine. (a) Time-course of the changes in the dialysate adenosine concentration caused by application of 5-HD ($n=5$). Abscissa scale indicates the time after implantation of the probe. 5-HD (100 $\mu$M) was added to the perfusate for 45 min, as indicated by the horizontal bar (dialysates 60–105 min). (b) Effects of various concentrations of 5-HD (abscissa scale) on the AMP-primed dialysate adenosine concentration (ordinate scale). Concentrations of dialysate adenosine measured 30–45 min after application of 5-HD (at 3 different concentrations) are given as a percentage relative to the value measured shortly before application of 5-HD (100%). Values are mean$\pm$s.e.mean ($n=5$).

103.3$\pm$6.7% ($n=5$) and 110.2$\pm$11.9% ($n=5$) of the control (in the absence of 5-HD). However, these changes were not significant when compared to control values.

## Discussion

Microdialysis techniques have been used to study neurotransmitter kinetics in the brain (Benveniste, 1989), and were recently introduced for *in vivo* heart experiments in order to measure interstitial biological substances, such as catecholamines, hydroxyl radical and purine metabolites (Van Wylen *et al*., 1990; 1992; Obata *et al*., 1994; Schulz *et al*., 1995). We measured interstitial adenosine in rat hearts by use of a flexibly mounted microdialysis technique, which involves the synchronized movement of the tip of the probe with the beating heart to reduce tissue injury (Obata *et al*, 1994). With this technique it is feasible to make stable and long-term measurements of interstitial adenosine. However, as evidenced by the elevated adenosine concentrations measured shortly after probe implantation, some tissue trauma might have occurred upon insertion of the probe. This is a limitation of the microdialysis technique (Benveniste, 1989) that should be kept in mind when interpreting the results of experiments in which this technique is used.

The baseline level of dialysate adenosine was 0.51$\pm$0.09 $\mu$M (Figure 2). Therefore, the concentration of adenosine in the interstitial space of rat ventricular muscles *in situ*, as calculated from the recovery rate of adenosine (18.0$\pm$1.6%), is estimated to be $\sim$2.8 $\mu$M. Estimates of myocardial interstitial adenosine concentrations by use of conventional microdialysis techniques have been found to be 0.9–1.3 $\mu$M in dog hearts (Van Wylen *et al*., 1990), 0.5 $\mu$M in rat isolated perfused rat hearts (Van Wylen *et al*., 1992) and 1.6 $\mu$M in swine hearts (Schulz *et al*., 1995). In guinea-pig isolated perfused hearts, by use of a porous nylon sampling discs technique the interstitial concentration of adenosine was estimated to be 0.3–3.6 $\mu$M (Zhu *et al*., 1991). Thus, the value of 2.8 $\mu$M obtained in the present study is within the range obtained in previous studies.

In the present study, an attempt was made to estimate the 5′-nucleotidase activity in hearts *in vivo* by measuring the dialysate adenosine concentration during perfusion with AMP. There are two known distinct forms of 5′-nucleotidase: one is the membrane-bound form (ecto-5′-nucleotidase) and the other is the free form in the cytoplasm (cytosolic 5′-nucleotidase). We demonstrated that the application of AMP through the microdialysis probe increased the concentration of dialysate adenosine in an AMP concentration-dependent manner (Figure 3), and that $\alpha,\beta$-meADP, an inhibitor of ecto-5′-nucleotidase (Headrick *et al*., 1992; Darvish & Metting, 1993), abolished the AMP-induced increase in the concentration of adenosine in the dialysate (Figures 2 and 4). These results can be explained as follows: AMP included in the perfusate, diffused out to the interstitial space through the dialysis membrane and was then converted to adenosine in the presence of endogenous ecto-5′-nucleotidase. The ecto-5′-nucleotidase has a fairly low $K_M$ (Michaelis constant) for degradation of AMP, as compared to cytosolic 5′-nucleotidase; the $K_M$ value for ecto-5′-nucleotidase was shown to be $\sim$20 $\mu$M (Sullivan & Alpers, 1971) and that for cytosolic 5′-nucleotidase to be $\sim$3 mM (Truong *et al*., 1988). Our results showed the AMP concentration for the half-maximal effect of adenosine production ($EC_{50}$) to be 107.2 $\mu$M (Figure 3). Although this $EC_{50}$ value was calculated for AMP in the perfusate and not for the AMP concentration that the ecto-5′-nucleotidase will be actually exposed to, the value is within the range of $K_M$ values allotted for ecto-5′-nucleotidase and not for cytosolic 5′-nucleotidase. $\alpha,\beta$-meADP did not affect the baseline level of adenosine (0.42$\pm$0.05 $\mu$M) (Figure 2), suggesting that the dialysate adenosine determined in the absence of AMP could not have originated from the enzymatic dephosphorylation of AMP by ecto-5′-nucleotidase. This finding is in agreement with data from a recent study by Kroll *et al*. (1993), in which they attributed the production of adenosine under normoxic conditions to the transmethylation of S-adenosylhomocysteine (SAH) by SAH hydrolase and/or hydrolysis of AMP mediated by cytosolic 5′-nucleotidase; the hydrolysis of extracellular AMP by ecto-5′-nucleotidase played only a minor role in the overall production of adenosine under normoxic conditions.

Under a constant supply of substrate (AMP), a limiting factor for the production of adenosine would be the activity of the catalytic enzyme for this reaction, namely ecto-5′-nucleotidase. Thus, it is reasonable to assume that the level of dialysate adenosine measured in the presence of a continuous supply of AMP reflects the activity of ecto-5′-nucleotidase in the tissue. In the present study, we used 100 $\mu$M AMP (a level close to the $IC_{50}$) to estimate the activity of ecto-5′-nucleotidase. The steady state level of dialysate adenosine seen in the presence of 100 $\mu$M AMP was 9.79$\pm$0.43 $\mu$M, which was $\sim$19 times higher than the baseline level of adenosine (0.51$\pm$0.09 $\mu$M) (Figure 2). based on the recovery rate of adenosine, the concentration of adenosine in interstitial fluid measured in the presence of 100 $\mu$M AMP was estimated to be $\sim$54 $\mu$M. Using a conventional microdialysis technique, Van Wylen *et al*. (1992) showed that the interstitial adenosine rat isolated in hearts increased to $\sim$46 $\mu$M after 60 min of ischaemia, a value comparable to that estimated in our study.

Another salient finding from the present study is that glibenclamide, $K_{ATP}$ channel antagonist, decreased the level of dialysate adenosine seen in the continued presence of AMP. We therefore believe that glibenclamide decreased the production of interstitial adenosine by inhibiting ecto-5′-nucleotidase activity. Moreover, the reduction of interstitial adenosine concentration by glibenclamide is unlikely to be due to increased activity of adenosine deaminase, because glibenclamide not only decreased the concentration of adenosine in the dialysate but also that of inosine. An alternative explanation is that glibenclamide increased the activity of adenosine kinase and/or facilitated the uptake of adenosine into cytoplasm. If this was the case, glibenclamide should have decreased not only the level of dialysate adenosine measured in the presence of AMP but also the level of adenosine measured in the absence of AMP (baseline adenosine concentration) that was not produced via dephosphorylation of AMP by ecto-5′-nucleotidase. Because glibenclamide (30 $\mu$M) did not decrease the baseline level of dialysate adenosine, it is unlikely that glibenclamide effects the scavenging or transporter system of adenosine.

Antidiabetic sulphonylureas such as glibenclamide and tolbutamide, inhibit $K_{ATP}$ channels by binding to a sulphonylurea receptor associated with the inwardly rectifying $K^+$ ($K_{ir}$) channel (Inagaki *et al*., 1995). Unlike glibenclamide, tolbutamide (1 mM) did not decrease the concentration of dialysate adenosine measured in the presence of AMP (100 $\mu$M) (data not shown). Moreover, 5-hydroxydecanoate (5-HD), a distinct type of $K_{ATP}$ channel blocker, did not affect the concentration of dialysate adenosine, when examined during a constant supply of AMP (Figure 6). Therefore, it is likely that the inhibitory effect of glibenclamide on ecto-5′-nucleotidase was not due to blockade of $K_{ATP}$ channels, thereby suggesting a direct interference of glibenclamide with the activity of ecto-5′-nucleotidase. Such an interpretation is further supported by our observation that glibenclamide (30 $\mu$M) decreased the activity of ecto-5′-nucleotidase, assessed by an enzymatic assay technique. However, the possible link between $K_{ATP}$ channel and ecto-5′-nucleotidase in ischaemic hearts needs to be further investigated. It has been shown that an increase in ecto-5′-nucleotidase activity derived from ischaemic preconditioning is blunted by glibenclamide and mimicked by the application of $K_{ATP}$ channel openers (Kitakaze *et al*., 1996).

Glibenclamide has been widely used to block selectively the $K_{ATP}$ channel. However, it should be emphasized that glibenclamide apparently decreases the production of adenosine by inhibiting ecto-5′-nucleotidase. The $IC_{50}$ of glibenclamide in the perfusates (10.5 $\mu$M) is similar to the concentration of glibenclamide required to produce half-maximal inhibition of $K_{ATP}$ channels ($\sim$6 $\mu$M) in rat ventricular myocytes (Ripoll *et al*., 1993). Therefore, it is likely that glibenclamide inhibits both ecto-5′-nucleotidase and $K_{ATP}$ channels in rat ventricular myocytes with comparable potency. Numerous mechanisms have been proposed to explain the cardioprotective action of adenosine (Ely & Berne, 1992), they include activation of $K_{ATP}$ channels, vasodilatation of coronary arteries and collateral vessels, inhibition of platelet aggregation, and inhibition of the release of oxygen free radicals from activated neutrophils. Cardioprotection mediated by these mechanisms may be mitigated or abolished in the presence of glibenclamide, because the latter drug decreases the tissue adenosine concentration *per se* in addition to blocking the $K_{ATP}$ channels.

In conclusion, we have found that glibenclamide reduces the accumulation of adenosine in the interstitial fluid by inhibiting the ecto-5′-nucleotidase. Consequently, the explanation as to why glibenclamide reduces the cardioprotective effects of adenosine and/or ischaemic preconditioning should now include the possibility that this drug, not only inhibits the $K_{ATP}$ channel, but also decreases the concentration of interstitial adenosine. Furthermore, it should be emphasized that glibenclamide is not a specific blocker of $K_{ATP}$ channels, and hence, should not be used as a pharmacological tool to implicate an effect on $K_{ATP}$ channels which might be mediated by adenosine.

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## References

AUCHAMPACH, J.A. & GROSS, G.J. (1993). Adenosine $A_1$ receptors, $K_{ATP}$ channels, and ischemic preconditioning in dogs. *Am. J. Physiol*., **264,** H1327–H1336.

BENVENISTE, H. (1989). Brain microdialysis. *J. Neurochem*., **52,** 1667–1679.

BERNE, R.M. (1980). The role of adenosine in the regulation of coronary blood flow. *Circ. Res*., **47,** 807–813.

COLE, W.C., MCPHERSON, C.D. & SONTAG, D. (1991). ATP-regulated $K^+$ channels protect the myocardium against ischemia/reperfusion damage. *Circ. Res*., **69,** 571–581.

DARVISH, A. & METTING, P.J. (1993). Purification and regulation of an AMP-specific cytosolic 5′-nucleotidase from dog heart. *Am. J. Physiol*., **264,** H1528–H1538.

EDWARDS, G., WESTON, A.H. (1993). The pharmacology of ATP-sensitive potassium channels. *Ann. Rev. Pharmacol. Toxicol*., **33,** 597–637.

ELY, S.W. & BERNE, R.M. (1992). Protective effects of adenosine in myocardial ischemia. *Circulation*, **85,** 893–904.

FRICK, G.P. & LOWENSTEIN, J.M. (1976). Studies of 5′-nucleotidase in the perfused rat heart, including measurements of the enzyme in perfused skeletal muscle and liver. *J. Biol. Chem*., **251,** 6372–6378.

GROVER, G.J., DZWONCZYK, S., SLEPH, P.G. & SARGENT, C.A. (1993). The ATP-sensitive potassium channel blocker glibenclamide (glyburide) does not abolish preconditioning in isolated ischemic rat hearts. *J. Pharmacol. Exp. Ther*., **265,** 559–564.

HEADRICK, J.P., MATHERNE, G.P. & BERNE, R.M. (1992). Myocardial adenosine formation during hypoxia: effects of ecto-5′-nucleotidase inhibition. *J. Mol. Cell. Cardiol*., **24,** 295–303.

HEARSE, D.J. (1995). Activation of ATP-sensitive potassium channels: a novel pharmacological approach to myocardial protection? *Cardiovasc. Res*., **30,** 1–17.

INAGAKI, N., GONOI, T., CLEMENT, J.P., NAMBA, N., INAZAWA, J., GONZALEZ, G., AGUILAR-BRYAN, L., SEINO, S. & BRYAN, J. (1995). Reconstitution of $I_{KATP}$: an inward rectifier subunit plus the sulfonylurea receptor. *Science*, **270,** 1166–1170.

KIRSCH, G.E., CODINA, J., BIRNBAUMER, L. & BROWN, A.M. (1990). Coupling of ATP-sensitive $K^+$ channels to $A_1$ receptors by G proteins in rat ventricular myocytes. *Am. J. Physiol*., **259,** H820–H826.

KITAKAZE, M., HORI, M. & KAMADA, T. (1993). Role of adenosine and its interaction with a adrenoceptor activity in ischemia and reperfusion injury of the myocardium. *Cardiovasc. Res*., **27,** 18–27.

KITAKAZE, M., MINAMINO, T., NODE, K., KOMAMURA, K., SHINOZAKI, Y., CHUJO, M., MORI, H., INOUE, M., HORI, M. & KAMADA, T. (1996). Role of activation of ectosolic 5′-nucleotidase in the cardioprotection mediated by opening of $K^+$ channels. *Am. J. Physiol*., **270,** H1744–H1756.

KROLL, K., DECKING, U.K.M., DREIKORN, K. & SCHRADER, J. (1993). Rapid turnover of the AMP-adenosine metabolic cycle in the guinea pig heart. *Circ. Res*., **73,** 846–856.

LASLEY, R.D. & MENTZER, R.M. (1992). Adenosine improves the recovery of postischemic myocardial function via an adenosine $A_1$ receptor mechanism. *Am. J. Physiol*., **263,** H1460–H1465.

LASLEY, R.D., RHEE, J.W., VAN WYLEN, D.G.L. & MENTZER, R.M. (1990). Adenosine $A_1$ receptor mediated protection of the globally ischemic rat heart. *J. Mol. Cell. Cardiol*., **22,** 39–47.

LIU, Y. & DOWNEY, J.M. (1992). Ischemic preconditioning protects against infarction in rat heart. *Am. J. Physiol*., **263,** H1107–H1112.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with folin phenol regent. *J. Biol. Chem*., **193,** 265–275.

MCCULLOUGH, J.R., NORMANDIN, D.E., CONDER, M.L., SLEPH, P.G., DZWONCZYK, S. & GROVER, G.J. (1991). Specific block of the anti-ischemic actions of cromakalim by sodium 5-hydroxydecanoate. *Circ. Res*., **69,** 949–958.

NOTSU, T., TANAKA, I., TAKANO, M. & NOMA, A. (1992). Blockade of ATP-sensitive $K^+$ channel by 5-hydroxydecanoate in guinea pig ventricular myocytes. *J. Pharmacol. Exp. Ther*., **260,** 702–708.

OBATA, T., HOSOKAWA, H. & YAMANAKA, Y. (1994). In vivo monitoring of norepinephrine and ·OH generation on myocardial ischemic injury by dialysis technique. *Am. J. Physiol*., **266,** H903–H908.

QIAN, Y., LEVASSEUR, J.E., YOSHIDA, K. & KUKREJA, R. (1996). $K_{ATP}$ channels in rat heart: blockade of ischemia and acetylcholine-mediated preconditioning by glibenclamide. *Am. J. Physiol*., **271,** H23–H28.

RIPOLL, C., LEDERER, W.J. & NICHOLS, C.G. (1993). On the mechanism of inhibition of $K_{ATP}$ channels by glibenclamide in rat ventricular myocytes. *J. Cardiovasc. Electrophysiol.*, **4,** 38–47.

SCHRADER, J., BORST, M., KELM, M., SMOLENSKI, T. & DEUSSEN, A. (1991). Intra- and extracellular formation of adenosine by cardiac tissue. In *Role of Adenosine and Adenine Nucleotides in the Biological System*. ed. Imai, S. & Nakazawa, M. pp. 261–270. Amsterdam: Elsevier.

SCHULZ, R., ROSE, J. & HEUSCH, G. (1994). Involvement of activation of ATP-dependent potassium channels in ischemic preconditioning in swine. *Am. J. Physiol.*, **267,** H1341–H1352.

SCHULZ, R., ROSE, J., POST, H. & HEUSCH, G. (1995). Involvement of endogenous adenosine in ischemic preconditioning in swine. *Pflügers. Arch.*, **430,** 273–382.

SHIGEMATSU, S., SATO, T., ABE, T., SAIKAWA, T., SAKATA, T. & ARITA, M. (1995). Pharmacological evidence for the persistent activation of ATP-sensitive $K^+$ channels in early phase of reperfusion and its protective role against myocardial stunning. *Circulation*, **92,** 2266–2275.

SMITH, K., VATON, H.H., RACE, G.J., PAULSON, D.L., URSHEL, H.C. & MALLAMS, J.T. (1965). Serum 5′-nucleotidase in patients with tumor in the liver. *Cancer*, **19,** 1281–1285.

SULLIVAN, J.M. & ALPERS, J.B. (1971). In vivo regulation of rat heart 5′-nucleotidase by adenine nucleotidase and magnesium. *J. Biol. Chem.*, **246,** 3057–3063.

THORNTON, J.D., LIU, G.S., OLSSON, R.A. & DOWNEY, J.M. (1992). Intravenous pre-treatment with $A_1$-selective adenosine analogues protects the heart against infarction. *Circulation*, **85,** 659–665.

THORNTON, J., THORNTON, C.S., STERLING, D.L. & DOWNEY, J.M. (1993). Blockade of ATP-sensitive potassium channels does not prevent preconditioning in the rabbit heart. *Circ. Res.*, **72,** 44–49.

TOOMBS, C.F., MOORE, T.L. & SHEBUSKI, R.J. (1994). Limitation of infarct size in the rabbit by ischaemic preconditioning is reversible with glibenclamide. *Cardiovasc. Res.*, **27,** 617–622.

TRUONG, V.L., COLLINSON, A.R. & LOWENSTEIN, J.M. (1988). 5′-Nucleotidase in rat heart: Evidence for the occurrence of two soluble enzymes with different substrate specificities. *Biochem. J.*, **253,** 117–121.

VAN WINKLE, D.M., CHIEN, G.L., WOLFF, R.A., SOIFER, B.E., KUZUME, K. & DAVIS, R.F. (1994). Cardioprotection provided by adenosine receptor activation is abolished by blockade of the $K_{ATP}$ channel. *Am. J. Physiol.*, **266,** H829–H839.

VAN WYLEN, D.G.L., SCHMIT, T.J., LASLEY, R.D., GINGELL, R.L. & MENTZER, Jr, R.M. (1992). Cardiac microdialysis in isolated rat hearts: interstitial adenosine purine metabolites during ischemia. *Am. J. Physiol.*, **262,** H1934–H1938.

VAN WYLEN, D.G.L., WILLIS, J., SODHI, J., WEISS, R.J., LASLEY, R.D. & MENTZER, Jr, R.M. (1990). Cardiac microdialysis to estimate interstitial adenosine and coronary blood flow. *Am. J. Physiol.*, **258,** H1642–H1649.

YAO, Z. & GROSS, G.J. (1993). Glibenclamide antagonizes adenosine $A_1$ receptor-mediated cardioprotection in stunned canine myocardium. *Circulation*, **88,** 235–244.

ZHU, Q., HEADRICK, J.P. & BERNE, R.M. (1991). Transmural distribution of extracellular purines in isolated guinea pig heart. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 657–660.

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