

Norepinephrine evoked by potassium depolarization increases interstitial adenosine concentration via activation of ecto-5' nucleotidase in rat hearts

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Abbreviations: AMP, adenosine 5'-monophosphate; AOPCP, α,β -methyleneadenosine 5'
diphosphate; PKC, protein kinase C; SAH, S-adenosylhomocysteine

Recommended section; cardiovascular

ABSTRACT

We examined whether the increase of the extracellular potassium-ion concentration, $[K^+]_o$, can increase the production of interstitial adenosine in the ventricular myocardium, with the use of microdialysis techniques in *in situ* rat hearts. A microdialysis probe was implanted in the left ventricular myocardium of anesthetized rat hearts and the tissue in the vicinity of the dialysis was perfused with Tyrode's solution containing adenosine 5'-monophosphate (AMP) through the dialysis probe at a rate of 1.0 μ l/min to assess the activity of ecto-5'-nucleotidase. When the K^+ concentration of the perfusate ($[K^+]_o$) was increased stepwise from 5.4 mM (control) to up to 140.4 mM, the level of dialysate adenosine significantly increased, in a $[K^+]_o$ -dependent manner. The presence of CsCl or $BaCl_2$ (20 mM), which markedly depolarized the resting potential, significantly increased the level of adenosine in the dialysate. Equivalent increases in the osmotic concentration of the perfusate, made by adding sucrose (270 mM), did not change the dialysate adenosine concentration. Introduction of high $[K^+]_o$ (140.4 mM) significantly increased the level of norepinephrine (NE) in the dialysate and this increase was abolished in the reserpinized rats hearts. In the presence of an antagonist of α_1 -adrenoceptor (prazosin, 50 μ M) or protein kinase C (PKC) (chelerythrine, 10 μ M) and in reserpinized rats, an introduction of high $[K^+]_o$ failed to increase the AMP-primed dialysate adenosine concentration. We conclude that high $[K^+]_o$ -induced NE release from sympathetic nerve terminals increases adenosine by stimulating the PKC-ecto-5'-nucleotidase cascade through α_1 -adrenoceptors.

Adenosine is considered to be an endogenous cardioprotective substance against cell damage caused by ischemia and reperfusion (Ely and Berne, 1992). In ischemia myocardium, adenosine derived from degradation of adenine nucleotides, via enzymatic dephosphorylation of AMP by 5'-nucleotidase (Berne, 1980). On the other hand, it is well known that in the case of acute myocardial ischemia, the extracellular potassium concentration, $[K^+]_o$, is markedly increased and the resting membrane potential of the ventricular muscle in the ischemia area seem to be depolarized (Hill and Gettes, 1980; Hirche et al., 1980). However, although adenosine and $[K^+]_o$ were increased in the heart during ischemia and reperfusion, the interaction between them has not yet been studied. It was suggested that the increase in external K^+ concentration, which decrease outward K current, is involved in the mechanism of norepinephrine (NE) release from sympathetic nerve terminals (Wakade and Kirpekar, 1974). Furthermore, NE activates protein kinase C (PKC)-ecto-5'-nucleotidase cascade through α_1 -adrenergic receptors. The present study was undertaken to clarify whether the increased $[K^+]_o$ affects the level of interstitial adenosine and if so, to further examine whether the change of adenosine concentration is due to the activation of PKC-ecto-5'-nucleotidase cascade by NE released from sympathetic nerve terminals. To achieve this goal, we measured the concentration of interstitial adenosine in *in vivo* rat hearts, with the use of a flexibly mounted microdialysis technique that we developed (Obata et al., 1994, 2001). The production of adenosine under normoxic conditions is attributed primarily to the transmethylation of S-adenosylhomocysteine (SAH) catalyzed by SAH hydrolase. In contrast, the hydrolysis of AMP by ecto-5'-nucleotidase is the main pathway for adenosine production under ischemic conditions (Lloyd and Schrader, 1987; Sparks and Bardenheuer, 1986). To mimic ischemic conditions, we measured the concentration of dialysate adenosine under continuous supply of AMP (the substrate for 5'-nucleotidase) through the microdialysis probe. With the use of this system, we have reported that the level of AMP-primed dialysate adenosine reflects the activity

of ecto-5'-nucleotidase in the particular site of the interstitial space of the myocardium (Sato et al., 1997a, 1997b). The results of our present studies claim that NE efflux induced by the membrane depolarization of sympathetic nerve terminals underlies the increased level of interstitial adenosine.

Materials and Methods

Animal preparation

The study was performed with Wistar rats of either sex weighing 300-400 g. The animals were anesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg). After intubation, the rat was artificially ventilated with room air supplemented with oxygen. The chest was opened at the left fifth intercostal space, and the pericardium was removed to expose the left ventricle. At the end of the experiments the rats were sacrificed using an overdose of chloral hydrate. In the case of reserpinized rats, reserpine (1 mg/kg) was injected intravenously into the rats 24 h before the experiments. All procedures treating the experimental animals conformed to 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 technique necessary for manipulation of the flexibly mounted microdialysis probe in in vivo rat hearts (to measure the interstitial adenosine) were described previously (Obata et al., 1994, 2001). In brief, the tip of the microdialysis probe (3 mm in length and 220 mm o.d. with the distal end closed) was made of dialysis membrane (cellulose membrane 10 mm thick, blocking components with molecular weights >50 kDa). Two fine silica tubes (75 mm i.d.) were inserted into the tip of the cylinder-shape dialysis probe and one of these served as the inlet for the perfusate and the other as the outlet for the dialysate. The inlet tube was connected to a microinjection pump (Carnegie Medicine, CMA/100, Stockholm, Sweden) and the outlet tube was led to the dialysate reservoir. These tubes were supported loosely at the mid-point on a semirotatable stainless steel wire, so that their movement fully synchronized with the rapid up-and-down motion of the tip caused by the heart beats. The probe was

implanted from the epicardial surface into the left ventricular myocardium and was perfused through the inlet tube with Tyrode's solution (when the interstitial NE concentration was measured, Ringer's solution was used instead of Tyrode's solution, see below) of the following composition (in mM): NaCl, 137; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 0.5; NaH₂PO₄, 0.16; NaHCO₃, 3.0; glucose, 5.5; and HEPES, 5.0 (pH = 7.4 adjusted with NaOH). Tyrode's solution (or Ringer's solution) that flowed out of the cut end of the inlet tube entered the extracellular space across the dialysate membrane by diffusion. The interstitial fluid diffused back into the cavity of the probe left the probe through the orifice of the outlet tube. We used a perfusion rate of 1.0 $\mu\text{l min}^{-1}$ and the relative recovery of adenosine measured using this flow rate was $18.0 \pm 1.6\%$ (Sato et al., 1997a).

Analytical procedure

i) Measurements of adenosine concentration in dialysate

The dialysate was collected (at the rate of 1.0 $\mu\text{l/min}$) into a series of wells for every 15 min consecutively (15 μl in each well). A 10-ml aliquot of the dialysate sample was used for the detection of adenosine, and we measured its concentration by using reversed-phase high performance liquid chromatography (HPLC). Separation of the compounds was achieved on Eicompak MA-5 ODS columns (5 mm, 4.6×150 mm; Eicom, Kyoto, Japan), with the mobile phase consisting of 200 mM KH₂P0₄ (pH = 3.8 adjusted with phosphoric acid) and 5% (v/v) acetonitrile. The flow rate was set at 1.0 ml/min using a pumping system (JASCO Corp., PU-980, Tokyo, Japan). The absorbance of the column eluate was monitored at 260 nm using an ultraviolet detector (JASCO Corp., UV-970). The absorbance peak of adenosine was quantified by comparing the retention time and peak height with a known adenosine standard concentration of 1 and 10 $\mu\text{mol/L}$. Concentrations of adenosine are presented as a raw value, without correction for recovery rate (18%), unless otherwise indicated.

ii) Measurements of NE concentration in dialysate

To determine the level of NE, the *in situ* heart appears to be blood-perfused. The dialysis fiber or the tissue in the vicinity of the dialysis fiber could be perfused Ringer's solution consisting 147 mM NaCl, 2.3 mM CaCl₂, and 4 mM KCl (pH 7.4) (Obata et al., 1994; Yamazaki et al., 1997). NE assay was performed using HPLC with an electrochemical (EC) procedure. To make the standard NE solution, NE was dissolved in the Ringer's solution. When the perfusion rate of 1.0 μ l/min was used, the relative recovery, which was tested using the standard NE solution (1.0 μ mol/L), was $17.0 \pm 0.7\%$ in our dialysis system. The dialysate samples (1.0 μ l min⁻¹) were collected for every 15 min consecutively for the adenosine measurements into a small collecting tube containing 15 μ l of 0.1 N HClO₄. The samples were immediately injected into an HPLC-EC system equipped with a glassy carbon working electrode (Eicom) and an analytic reverse-phase column on an Eicompak MA-5ODS column (5 μ m, 4.6 \times 150 mm; Eicom). The working electrode was set at a detector potential of 0.75 V. Each liter of mobile phase contained 1.5 g of 1-heptanesulfonic acid sodium salt, 0.1 g of Na₂EDTA, 3 ml of triethylamine and 125 ml of acetonitrile. The pH of the solution was adjusted to 2.8 with 3 ml of phosphoric acid. When dialysate NE levels reached a steady-state level at 105-120 min after probe implantation, KCl was directly infused in rat heart through a microdialysis probe.

Experimental protocol

We measured the time-dependent changes of the dialysate adenosine concentration in the presence of AMP (AMP-primed dialysate adenosine concentration) and evaluated the activity of ecto-5'-nucleotidase. Under a constant supply of AMP, the dialysate adenosine is considered to be produced via enzymatic dephosphorylation of AMP by endogenous ecto-5'-nucleotidase, since α,β -methyleneadenosine 5'-diphosphate (AOPCP; 100 μ M), an inhibitor of

ecto-5'-nucleotidase, completely inhibited the AMP-primed dialysate adenosine (Sato et al., 1997b). Therefore, the level of dialysate adenosine measured in the presence of AMP is an appropriate measure of the activity of ecto-5'-nucleotidase in rat hearts *in situ* (Sato et al., 1997b). In this series of experiments, AMP at a concentration of 100 μ M was perfused throughout the experiment via the probe, and the dialysate sampling was started 30 min (equilibration period) after implantation of the probe.

Assay of ecto-5'-nucleotidase

Ventricular tissue from rat hearts was cut into ~1 mm pieces and homogenized for 5 min in 3 ml of ice-cold 10 mM HEPES-KOH buffer (pH = 7.4) containing 250 mM sucrose, 1 mM $MgCl_2$, 1 mM mercaptoethanol and O • C. To prepare the 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 the activity of ecto-5'-nucleotidase. The activity was determined by an enzymatic assay technique (Smith et al., 1965), with the use of a commercially available kit (St. Louis, MO). The protein concentration in each sample was determined using the method of Lowry et al., (1951).

Drugs Used

AMP was purchased from Wako Pure Chemical (Osaka, Japan). Prazosin, AOPCP, 1-heptansulfonic acid sodium salt were purchased from Sigma Chemical. AOPCP was dissolved in distilled water and kept as a 10 mM stock solution and prazosin was dissolved in methanol and kept as a 10 mM stock solution. An appropriate volume of these stock solutions was added to Tyrode's or Ringer's solution immediately before use, as indicated in the Results.

Statistical analysis

All values are presented as means \pm S.E.M. The significance of differences was determined by using ANOVA with Fisher post hoc test. A P value of less than 0.05 was regarded as being statistically significant.

RESULTS

We first assessed the effect of increased KCl concentrations, i.e., increased $[K^+]_o$, on the activity of ecto-5'-nucleotidase in the membrane fraction of the homogenate of rat ventricular tissue. Changes in $[K^+]_o$ from control 5.4 mM to 140.4 mM did not significantly affect the activity of ecto-5'-nucleotidase from 239.6 ± 6.6 nmol/mg/protein to 243.6 ± 5.3 nmol/mg/protein ($n = 5$).

We then examined whether the increased $[K^+]_o$ depolarized the ventricular muscle and whether the depolarization increased the level of interstitial adenosine in rat hearts in vivo. The effects of high $[K^+]_o$ on the sequential changes of adenosine concentrations in the dialysate are shown in Figure 1. The microdialysis probe was perfused with Tyrode's solution containing 100 μ M AMP throughout the experiment. After obtaining two control fractions (30-45 and 45-60 min after implantation of the microdialysis probe, the introduction of high KCl (140.4 mM) Tyrode's solution was begun in the continued presence of AMP through the probe and continued for 45 min. During this perfusion the concentration of dialysate adenosine was significantly ($P < 0.05$) increased by $44.7 \pm 13.2\%$ (from 8.09 ± 1.23 μ M), immediately before the perfusion to 11.71 ± 1.07 μ M, after 30-45 min of perfusion. After returning the KCl concentration from 140.4 to 5.4 mM, the level of dialysate adenosine decreased to 7.12 ± 0.52 μ M in 15 min (the open bars at 105-120 min in Fig. 1A). In contrast, equivalent increases in the osmotic concentration of the Tyrode's solution, made by adding sucrose (270 mM), did not increase the dialysate adenosine concentration (Fig. 1B). In addition, when AOPCP (100 μ M) was present in the solution, we did not see any increase in the level of dialysate adenosine (0.75 ± 0.07 μ M before vs. 0.66 ± 0.08 μ M, 30-45 min after 140.4 mM KCl perfusion; $n = 6$, not shown).

As shown in Fig. 2., when the KCl concentration was increased stepwise from 5.4 mM (control) to up to 140.4 mM (26 times the control), the level of dialysate adenosine significantly

increased in a concentration-dependent manner. Application of a high concentration of CsCl (20 mM) that is known to depolarize the resting potential of cardiac muscles (Isenberg, 1976) increased the level of adenosine by $19.7 \pm 6.2\%$ (Fig. 3A). However, when CsCl (20 mM) was introduced in the presence of a high concentration of KCl (140.4 mM), the level of dialysate adenosine was not increased (Fig. 3B). Much lower CsCl concentration (10 mM) also increased the level of adenosine by $13.3 \pm 7.5\%$ ($n = 5$); however, this increase did not reach the statistical significance (not illustrated). When corresponding experiments were performed with BaCl₂ (20 mM), the BaCl₂-induced increase ($75.2 \pm 22.6\%$) in the adenosine concentration was significantly greater ($P < 0.05$) than that caused by CsCl ($19.7 \pm 6.2\%$) (Fig. 3C). However, when BaCl₂ (20 mM) was introduced in the presence of a high concentration of KCl (140.4 mM), the level of dialysate adenosine remained unchanged (Fig. 3D).

During acute regional ischemia, the interstitial concentration of NE in the ischemic region is reported to be increased (Schömig, 1989). We therefore examined whether an increase in interstitial KCl concentration during ischemia underlies this increase in NE concentration. Figure 4A shows that high concentrations of KCl (140.4 mM) significantly increased the level of NE in the dialysate. In contrast, an equivalent increase in the osmotic concentration produced by adding sucrose (270 mM), did not affect the level of NE (not illustrated). In rats treated with reserpine (see Methods) we did not see any increase in the dialysate NE concentration (Fig. 4B). Finally, we examined the effect of increased $[K^+]_o$ on the level of dialysate adenosine, in the presence of prazosin. In the presence of prazosin ($50 \mu\text{M}$), an introduction of high-KCl (140.4 mM) failed to increase the adenosine concentration (Fig. 5A). On the other hand, in the presence of chelerythrine ($10 \mu\text{M}$), an introduction of high KCl (140.0 mM) did not increase the dialysate adenosine (Fig. 5B). These results suggest that the high KCl-induced increases in adenosine concentrations in the dialysate were due to activation of PKC, mediated by stimulation of α_1 -adrenoceptors. In addition, in reserpine treated animals high KCl did not

JPET/2002/39917

increase the level of adenosine in the dialysate (Fig. 5C).

DISCUSSION

It is well known that myocardial ischemia increases interstitial K^+ concentrations of compromised ventricular muscles and decreases the resting membrane potential (RMP), leading to slow conduction, unidirectional block and re-entrant tachyarrhythmias (Hill and Gettes, 1980; Hirche et al., 1980). On the other hand, the concentration of adenosine in the coronary effluent was markedly increased after ischemia (Kitakaze et al., 1996); based on these reports we speculated that this increase in adenosine concentration seen after ischemia could have been due to high $[K^+]_o$ -mediated increases in the adenosine production. We initially hypothesized that the increased $[K^+]_o$ and the subsequent depolarization of the ventricular muscles alone might have activated ecto-5'-nucleotidase and increased adenosine production. Actually the increased KCl concentration (140.4 mM) of Tyrode's solution (introduced via the probe), which markedly depolarized the RMP of the rat, also significantly elevated the level of adenosine in the *in situ* heart of the same animals (Fig. 1A). CsCl and BaCl₂, which are well known to depolarize RMP by decreasing outward K^+ currents, also increased the level of adenosine, suggesting that these compounds may share the same mechanism of ecto-5'-nucleotidase activation as KCl: involvement of depolarization of RMP. However, additional increase of adenosine was not observed under conditions of increased $[K^+]_o$, perhaps because RMP had already been fully depolarized by high $[K^+]_o$, prior to the application of Cs⁺ or Ba²⁺. However, recent findings have (Obata, 2002) shown that mechanism of elevation of interstitial adenosine concentrations induced by high $[K^+]_o$ is more complicated and requires participation of several factors.

Several investigators have reported that myocardial ischemia increases the NE level in the interstitial space of the heart (Obata et al., 1994, 2001; Schömig et al., 1987, Schömig, 1989). This increase of NE during ischemia is result of increase of NE release from sympathetic nerve terminals and decrease in the reverse transport by the NE uptake carrier. However, the increase

in the level of myocardial interstitial NE seem to be mainly attributed to the former mechanism (Yamazaki et al., 1997), which may be secondary to the depolarization of the nerve terminals caused by ischemia-induced increases in the interstitial K^+ concentration (Schömig, 1989; Arita et al., 1983; Du et al., 1997; Snyder et al., 1995). We previously reported that diacylglycerol, a potent PKC activator (Nishizuka, 1995), increased the level of interstitial adenosine via stimulation of α_1 -adrenoceptors, followed by activation of ecto-5'-nucleotidase mediated by PKC (Sato et al., 1997a). It is known that NE stimulates α_1 -adrenoceptors and leads to activation of PKC (Fedida et al., 1993). These studies suggest the possibility that high $[K^+]_o$ -induced increases in adenosine is the results of increase in ecto-5'-nucleotidase activity via α_1 -adrenoceptor-PKC pathway by NE released from sympathetic nerve terminals. In the present study, we observed that dialysate NE concentration paralleled the perfusate K^+ concentration (Fig. 2) and in reserpinized heart, high $[K^+]_o$ failed to increase the NE level. Effects of high $[K^+]_o$ on adenosine production was mimicked by Cs^+ which is reported to induce NE release from the sympathetic nerve terminals (Takahashi et al., 1992). These present findings demonstrated that high $[K^+]_o$ promoted NE release from sympathetic nerve terminals. This released NE was involved in mechanism of increase in adenosine by high $[K^+]_o$, evidenced by the fact that high $[K^+]_o$ failed to increase adenosine production in reserpinized hearts where high $[K^+]_o$ -induced NE release was not found. In addition, this concept was supported by our previously study in which we examined the effects of NE on the adenosine production directly by administration of NE through the dialysis fiber and found the increase of adenosine (Sato et al., 1997a). Role of α_1 -adrenoceptor-PKC pathway in an increase of adenosine was confirmed here when we tested the effect of high $[K^+]_o$ on the dialysate adenosine concentration in the presence of prazosin, an α_1 -adrenoceptor blocker (Fig. 5A) or chelerythrine, an PKC inhibitor (Fig. 5B). In the presence of each of them, no increase of adenosine production was found. Thus present results demonstrated that high $[K^+]_o$ promotes

NE release from sympathetic nerve terminals and thereby increases the level of interstitial adenosine through α_1 -adrenoceptor stimulation followed by a PKC-mediated activation of ecto-5'-nucleotidase.

With all these findings taken together, we conclude that the increase in adenosine concentration reported to occur in the coronary effluent following ischemia/reperfusion is derived, at least in part, from the depolarization-induced release of NE from sympathetic nerve terminals, which is secondary to the ischemia-induced increases in the extracellular K^+ concentration in the compromised ventricular myocardium. We performed present study under simulated ischemia condition using a microdialysis technique. It is interesting to test if this pathway is really operative in actual ischemia and reperfusion.

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Footnotes

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Figure legends:

Figure 1.

Effects of increased KCl concentration (A) and an equivalent increase in osmotic concentration by adding sucrose (B) on the level of interstitial adenosine in *in situ* rat hearts. All microdialysis experiments in this and subsequent figures were conducted in the presence of 100 μ M AMP in the perfusate given through the probe. Ordinates show the concentrations of dialysate adenosine collected for every 15 min. In panel A, the KCl concentration of the perfusate was increased from 5.4 mM (control) to 140.4 mM (26 times the control) during the time indicated by the horizontal bar. In panel B, sucrose was added to the perfusate to make the final concentration of 270 mM as indicated. The abscissas denote the time after implantation of the dialysis probe in the heart. The level of adenosine reached a steady state 60 min after the implantation. Values are mean \pm S.E.M. * $P < 0.05$ versus the adenosine concentration immediately before introduction of 140.4 mM KCl (45-60 min) ($n = 5$ in both A and B, ANOVA and Fisher test)

Figure 2.

Effects of different concentrations of KCl in the perfusate on the level of dialysate adenosine. The ordinate indicates concentrations of dialysate adenosine, each measured 30-45 min after application of various concentrations of KCl (0, 2.7, 10.8, 21.6, 43.2, 86.4 and 140.4 mM; abscissa), and is shown as a percentage of the adenosine concentration measured at 5.4 mM KCl (100%). * $P < 0.05$ versus 5.4 mM KCl ($n = 6$ in each column).

Figure 3.

Effects of CsCl or BaCl₂ on the dialysate adenosine concentration under control and increased

KCl concentrations in the perfusate. The abscissas indicate the time after implantation of the probe. CsCl (A and B; 20 mM) and BaCl₂ (C and D; 20 mM) were applied for 45 min through the probe, as indicated by each horizontal shaded bar in the presence of 5.4 mM KCl (A or C) and 140.4 mM KCl (B or D). *P < 0.05 versus the value at 45-60 min (n = 6 in A; n = 6 in B, n = 6 in C; n = 6 in D)."

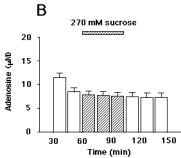
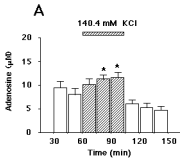
Figure 4.

Effects of introduction of high KCl concentration in the perfusate on the norepinephrine (NE) concentration in the dialysate. The KCl concentration in the perfusate was increased from control (5.4 mM) to 140.4 mM in intact (A) and reserpinized rat hearts (B), as indicated by horizontal bars. Then, the NE concentration was determined. The abscissas show the time after implantation of the dialysis probe. *P < 0.05 versus the value at 135-150 min (n = 6 in A; n = 6 in B).

Figure 5.

Inhibitory effect of prazosin on the KCl-induced increases in the dialysate adenosine concentration. An increase of KCl concentration in the perfusate from 5.4 mM to 140.4 mM (indicated by the shaded bar) failed to increase the adenosine concentration in the presence of 50 μ M prazosin (n = 5; A) or 10 μ M chelerythrine (n = 5; B) in intact rat hearts or in reserpinized rat hearts (C).

Fig.1



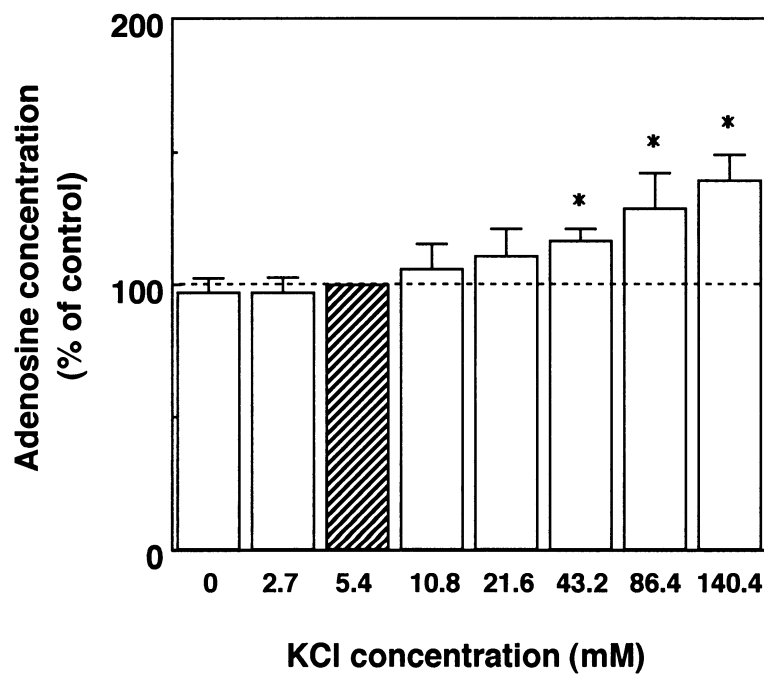


Fig.3

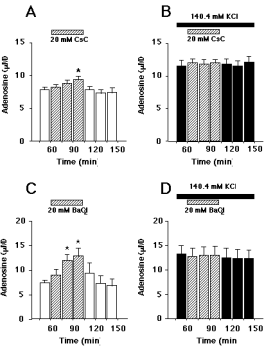


Fig.4

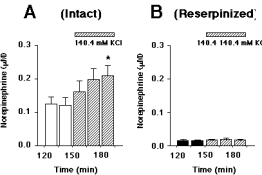


Fig.5

