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Differential Modulation of Exogenous and Endogenous Adenosine-induced Coronary Vasodilation by Dipyridamole

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Some recent investigations revealed that vasodilatory action of adenosine is mainly not mediated by surface A2 receptor and suggested the existence of an intracellular action site. In the present study, we tried to differentiate intracellular from extracellular site of adenosine action in the regulation of coronary flow. In perfused rabbit hearts, concentration-response curve of coronary flow to exogenous adenosine was constructed in the presence or absence of dipyridamole, an inhibitor of transmembrane purine transport. Inhibition of cellular adenosine uptake by dipyridamole suppressed the increase of flow rate while enhancing the decrease in heart rate induced by exogenous adenosine. In another series of experiments, perfused rabbit hearts were subjected to energy deprivation in order to increase the production of endogenous adenosine. Energy deprivation along with dipyridamole administration resulted in higher coronary flow rate. Lower perfusate adenosine concentration was observed along with higher tissue adenosine content in this group. These results implied that coronary flow rate is determined not by interstitial adenosine concentration but by intracellular activity of adenosine. To confirm the effects of dypiridamole in vivo, direct measurement of interstitial adenosine concentration by mycrodialysis along with the assay of intracellular adenosine content was performed after intranenous dipyridamole administration. After dipyridamole infusion, intracellular adenosine content was markedly increased while interstitial adenosine concentration was not altered. In another series of experiments, the right shift of concentration-response curve of adenosine-induced vasodilation by 8-phenyltheophilline, a representative adenosine receptor antagonist, was mostly abolished by prior administration of prazosin, indicating that the influence of 8-PT on the adenosine action is not attributed to the inhibition of A2 receptor but related to the suppression of α-adrenoceptor activation. From these results, we concluded that adenosine acts intracellularly to regulate the coronary blood flow.

Key Words: Adenosine, Dipyridamole, Vasodilation, A2 receptor, 8-phenyltheophylline

INTRODUCTION

Myocardial oxygen demand is relatively high and varies widely according to physiologic or pathophysiologic changes. Therefore, metabolic regulation of coronary blood flow is critical for the maintenance of cardiac function. Adenosine, a main degradation product of adenosine triphosphate, has been reported to possess a variety of pharmacological effects (Drury & Szent-Gyorgyi, 1929), and it was proposed that this substance plays an important role in the regulation of blood flow in many organs including the heart (Berne et al, 1979). The vasodilator action of adenosine has been attributed to the effects on the P1 receptor, based on the observation that it was blocked by theophylline, a non-specific P1 receptor blocker (Afonso, 1970; Bunger et al, 1975). It was further reported that the P1 receptor involved in adenosine-induced vasodilation was presumably A2 subtype (Kusachi et al, 1989; Olsson & Pearson, 1990).

However, several studies revealed that only a part of vasodilatory action of adenosine was mediated by

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A2 receptor activation. Collis et al (1983) showed that the vasodilation of vascular preparation induced by the higher doses of exogenous adenosine was insensitive to 8-phenyltheophylline, a P1 receptor antagonist, while abolished by dipyridamole, an inhibitor of purine transport across the cell membrane. With these results, they concluded that exogenous adenosine acts on vascular tissues via both A2 receptor and an unknown intracellular site. Chinellato et al (1992) also reported that the blockade of adenosine-induced aortic vasodilation by methylxanthine derivatives was negligible, and concluded that any P1-purinoceptor-mediated component of adenosine-induced relaxation is small or nil. An experiment conducted in the coronary vasculature of the arrested perfused heart also showed that the blockade of adenosine action by 8-phenyltheophylline was incompatible with a typical competitive inhibition at a receptor site (Harden et al, 1996). Moreover, similar results were obtained from a clinical study, in which an A1-receptor mediated action of exogenous adenosine (atria-to-His bundle interval prolongation) was abolished by the pretreatment of theophylline while the increase in coronary blood flow was minimally affected (Bertolet et al, 1996). These reports suggest the possible existence of an intracellular action site. However, most studies failed to differentiate the effects of adenosine to the putative intracellular action site unequivocally from those to purinergic surface receptors. Relative contribution of this putative intracellular action site to the vasodilatory action induced by exogenously administered adenosine or endogenously produced adenosine was left unclear.

Moreover, as theophylline derivatives are presumed to increase the release of norepinephrine in the sympathetic nerve endings, this will influence the coronary blood flow (Rutherford et al, 1981; Vestal et al, 1983; Richardt et al, 1987; Minamino et al, 1995). However, in most previous studies, it has not been considered that adenosine agonists or its antagonists influence the adrenergic system as well as the vascular tissues. In this regard, effects of theophylline or related drugs on endogenous catecholamine system might be quite confounding when interpreting the inhibition of adenosine-induced vasodilation by these compounds.

In the present study, we tried to clarify the contribution of the putative intracellular action site in adenosine-induced coronary vasodilation using perfused rabbit hearts, with the following specific aims: 1) to quantitate the response of coronary vasculature to exogenous adenosine and investigate the effects of dipyridamole on this response; 2) to evalauate the changes in coronary flow under augmentation of intracellular adenosine production, in the presence or absence of dipyridamole, and 3) to characterize the nature of 8-phenyltheophylline sensitive portion in the coronary vascular response to exogenous adenosine and determine the contribution of catecholamine in it.

METHODS

Experimental Protocol

Experiment I: Responses to exogenous adenosine with or without dipyridamole pretreatment. In control group, adenosine (Sigma, USA), freshly dissolved in normal saline in 1000-fold of expected concentration, was directly added to the perfusate after the initial equilibration. In dipyridamole group, 1 μM dipyridamole (Boeringer Ingelheim Korea, Korea) was included in perfusate to block the transmembrane transport of adenosine, and after 15 minutes equilibration with dipyridamole, concentration-response curve was constructed in the same manner as the control group.

Experiment II: Responses to energy deprivation with or without dipyridamole pretreatment. To augment the coronary vasodilation by endogenous adenosine, oxygen and glucose were omitted from the perfusate. The perfusate was bubbled with 95% N_2-5% CO_2 mixture, and glucose was substituted by mannitol of the same molarity. This hypoxic, glucose-deprived perfusion was started after the initial equilibration and continued for 30 minutes (control group). In dipyridamole group, 1 μM dipyridamole was added to the hypoxic, glucose-deprived perfusate to inhibit the release of intracellular adenosine. Perfusate was collected before and at 5, 15, 25 minutes of hypoxic perfusion to measure the adenosine concentration. After the experiment, hearts were removed from perfusion apparatus, blotted and weighed. Left ventricle was immediately frozen with metal tongue precooled in liquid nitrogen and stored at $-70^{\circ}C$ for tissue adenosine content measurement.

Experiment III: Effects of dypiridamole in vivo. To confirm the intracellular action of endogenous adenosine *in vivo*, interstitial adenosine concentration in heart was measured before and after intavenous dipyridamole administration. And, at the same time, cell-

ular adenosine content after dypiridamole injection was also measured to compare to the control level.

Experiment IV: Effects of 8-phenyltheophylline. To examine the effects of 8-phenyltheophylline on the response to exogenous adenosine, another series of isolated perfused heart preparations were subjected to 5 μM 8-phenyltheophylline (RBI, USA) pretreatment, and concentration-response curve was constructed in the same manner as that of experiment I after 15 minutes equilibration with 8-phenyltheophylline (8-phenyltheophylling group). 8-phenyltheophylline was dissolved in 0.2 M NaOH solution (80% methanol in water) to give a stock concentration of 10 mM. In prazosin + 8-phenyltheophylline group, 1 μM prazosin-HCl (RBI, USA) was coadministered with 8-phenyltheophylline to rule out the indirect effect of 8-phenyltheophylline on vascular α-adrenergic system. Prazosin-HCl was dissolved in distilled water with heating to give a stock concentration of 1 mM.

Perfusion of isolated rabbit heart

New Zealand White rabbits (1.5~2.5 kg, male) were anesthetized with intravenous administration of 30 mg/kg pentobarbital sodium. Hearts were excised, immediately connected to aortic cannulae, and perfused in a constant pressure, non-circulating Langendorff mode with Krebs-Henseleit buffer containing: NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 1.25 mM, $MgSO_4$ 1.2 mM, glucose 10 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM. The physiologic salt solution was bubbled with 95% O_2~5% CO_2 mixture at 37°C, and perfusion pressure was maintained at 80 cm H_2O. Left ventricular pressure and heart rate were continuously monitored via a pressure transducer attached to a water-filled latex balloon inserted into the left ventricle. The balloon was initially inflated until the left ventricular end diastolic pressure reached 5 mmHg. Coronary flow was determined continually by measuring the weight of perfusate. Each preparation was equilibrated for 20 minutes before experimental manupulations.

Operative Procedures for in vivo Experiments

After tracheotomy, rabbits were intubated and ventilated with room air, and the heart was exposed by a left thoracotomy through 4th intercostal space. Microdialysis probe (0.5 × 10 mm; CMA/Microdialysis AB, Sweden) was inserted into the anterior wall of left ventricle to measure the interstitial adenosine concentration. Following stabilization for 1 hour, dialysate samples were collected for every 10-minute period before and after intravenous dipyridamole (0.5 mg/kg) injection. Thirty minutes after dipyridamole administration, hearts were removed and washed by perfusion with cold saline, and then frozen quickly in liquid nitrogen. Control myocardial samples were taken from anesthetized rabbits after the same procedures except dipyridamole treatment.

Biochemical assay of adenosine

Perfusate, dialysate or tissue samples were stored at −70°C until assayed. For determination of perfusate and dialysate adenosine concentration, a high performance liquid chromatography (HPLC) method (Gruber et al, 1989) was adopted. Samples (20 μL) were loaded onto a C-18 reverse phase column (Rainin, USA). Mobile phase was composed of 10 mM phosphate buffer, pH 2.9, 1% (v/v) acetonitrile in water. Adenosine and inosine were eluted at 16~18 and 18~20 minutes, and its identity was confirmed by spiking samples with authentic standard and reanalysis on HPLC.

In order to estimate the intracellular adenosine level, total tissue content of adenosine was measured. As contribution of interstitial and vascular adenosine to total tissue content in perfused heart is minimal (Deussen et al, 1988), changes in total tissue content of adenosine is presumed to represent the changes in intracellular adenosine content. Frozen tissues were pulverized and homogenised in 4 volumes of 1 M perchloric acid using a Polytron homogenizer (Brinkman Instruments, USA). The homogenate was centrifuged at 10,000 g for 5 minutes and the supernatant was neutralized with 4 M K_2CO_3. After another centrifugation at 10,000 g for 2 minutes, the supernatant was used for enzymatic assay of adenosine concentration. Measurement of tissue adenosine content was performed by a method modified from the original method of Heinz and Reckel (Heinz and Rechel, 19 85). 0.2 ml of tissue sample was added to 1 ml of 100 mM phosphate buffer, pH 7.5. In this reaction mixture, 5 μL of xanthine oxidase (20 kU/l), 5 μL of nucleoside phosphorylase (6.5 kU/l), and 2 μL of adenosine deaminase (400 kU/l) were successively added with monitoring the changes of absorbance at 293 nm. Standard curve was constructed with authentic adenosine solutions. All the enzymes were pur-

chased from Sigma, USA and freshly prepared by dilution in distilled water. Identity of adenosine was confirmed by addition of known amount of adenosine before polytron homogenation and reassay.

Data analysis

Coronary flow and heart rate changes are expressed as a percentage of the increase or decrease compared to the values before administration of drug or hypoxic perfusate. The results represent the mean±standard error of at least five individual determinations.

Statistical analyses of results were performed using unpaired student's t tests. A P value less than 0.05 was considered significant.

RESULTS

Responses to exogenous adenosine

Under perfusion conditions described above, coronary flow and contractile function were stabilized within 15 min, and the mean coronary flow rate was 9.8 ml/min/g wet weight after the stabilization period.

Administration of adenosine evoked an increase in the coronary flow immediately. At the same time, a decrease in heart rate was manifested. Although the coronary flow response reached a plateau at 10 μM adenosine, this concentration of adenosine failed to evoke the maximal response of heart rate decrease (Fig. 1).

In dipyridamole-pretreated preparations, increase of the flow rate by exogenous adenosine was markedly suppressed. 30 μM adenosine, the highest concentration tested, increased the flow rate by only 11.1%, while the same concentration evoked an increase of 68.6% in control group. However, the decrease of heart rate by exogenous adenosine was enhanced by dipyridamole pretreatment, to show the left shift of concentration-response curve. Comparing the responses to 3 μM adenosine, dipyridamole-pretreated hearts showed 28.6 % decrease in heart rate while control hearts showed 12.7% decrease (Fig. 1).

Responses to endogenous adenosine

Energy deprivation by hypoxia and glucose deprivation induced an immediate decrease in contractility and a gradual increase of left ventricular end diastolic pressure. Coronary flow rate was initially increased by 30 to 40% at 5 min of energy deprivation, but decreased thereafter, presumably due to the contracture. In dipyridamole-treated hearts, coronary flow rate was higher than that of control hearts, and the difference of flow rate between two groups was most prominent at 15 and 25 min of energy deprivation (Fig. 2).

Perfusate adenosine concentration during normal perfusion was 0.3 to 0.4 μM and increased enormously after the onset of energy deprivation. The

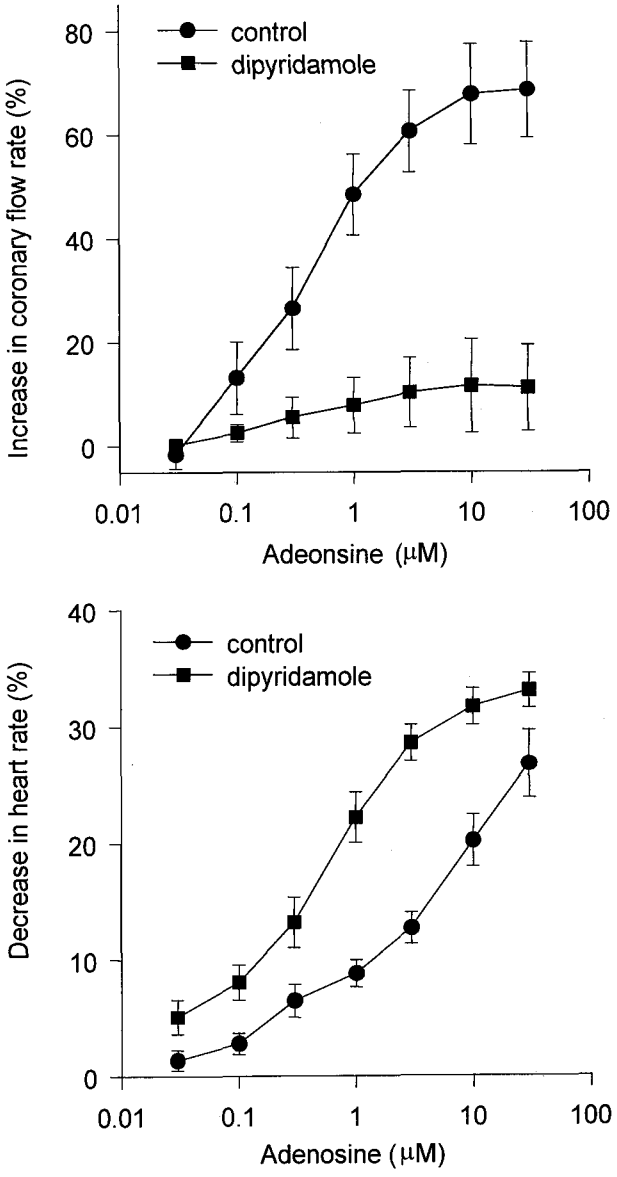

Fig. 1. Effects of exogenous adenosine on the coronary flow rate and heart rate of perfused rabbit hearts. Control: control hearts, dipyridamole: hearts treated with adenosine in the presence of 1 μM dipyridamole.

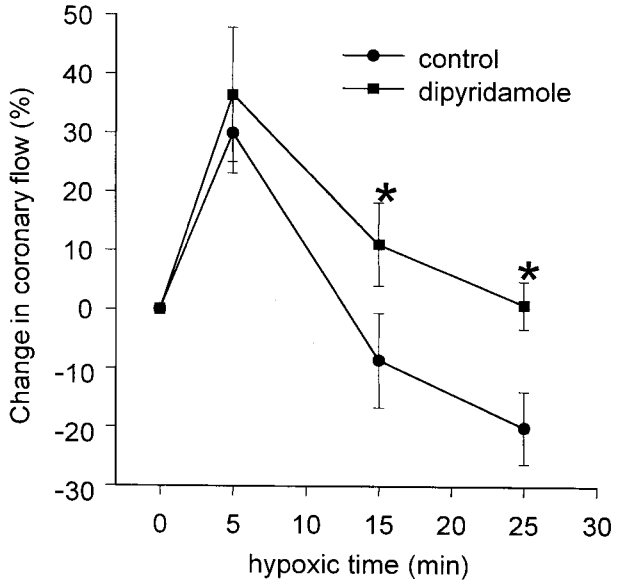

Fig. 2. Effect of energy deprivation on the coronary flow rate of perfused rabbit hearts. Control: control hearts, dipyridamole: hearts subjected to energy deprivation in the presence of 1 μM dipyridamole. *: $P<0.05$ vs con.

increase in perfusate adenosine concentration showed a peak at 15 min of energy deprivation. In dipyridamole-treated hearts, perfusate adenosine concentration was much lower throughout the experiment (Fig. 3).

Tissue adenosine content was markedly increased after 30 minutes of energy deprivation (227.0±57.5 nmole/g wet weight) as compared to the control level (less than 5 nmole/g wet weight, data not shown). Dipyridamole augmented this increase by about 5 fold (1128.0±93.0 nmole/g wet weight) (Fig. 3).

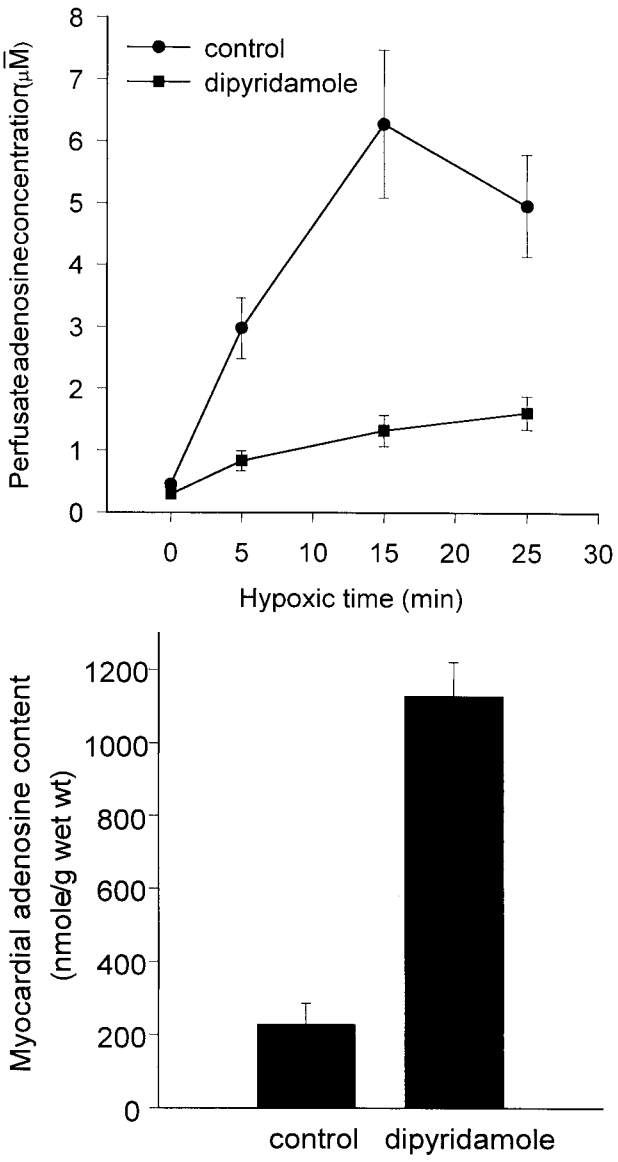

Fig. 3. Changes in perfusate adenosine concentration and myocardial adenosine content in energy-deprived perfused rabbit hearts. Control: control hearts, dipyridamole: hearts subjected to energy deprivation in the presence of 1 μM dipyridamole. Myocardial adenosine content was measured at the end of energy-deprived perfusion for 30 minutes.

Effects of dypiridamole in vivo

Dialysate adenosine concentration was transiently increased by the insertion of dialysis probe, but stabilized within 1 hour and showed no fluctuation thereafter (data not shown). Measured 25 min after intravenous dipyridamole administration, dialysate adenosine concentration was not increased, while tissue adenosine content was markedly increased compared to the basal level measured in untreated hearts (Fig. 4).

Effects of 8-phenyltheophylline

8-phenyltheophylline (8-PT) significantly decreased the coronary flow rate by 20.9%, but did not induce any change in heart rate. As shown in Fig. 6, effects of 8-PT on the response of coronary vasculature to exogenous adenosine were different according to the concentration of adenosine. While the vascular responses to lower concentrations of adenosine were suppressed by the pretreatment of 8-PT, the responses to highest concentrations of adenosine were slightly

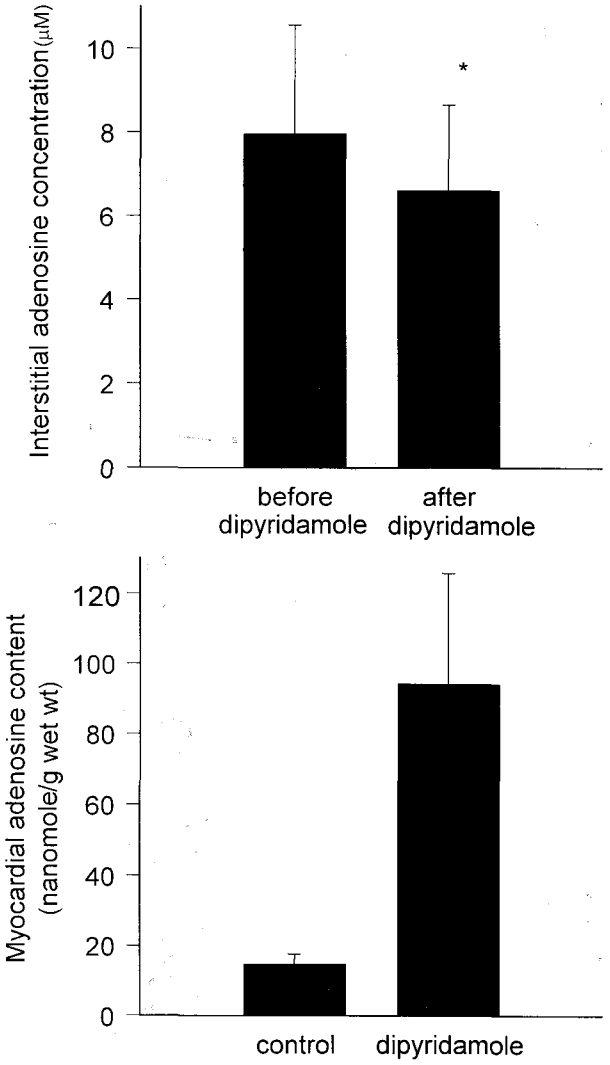

Fig. 4. Effect of intravenous dipyridamole administration on the interstitial concentration and myocardial content of adenosine in anesthesized rabbits. Interstitial adenosine concentration was measured before and after dipyridamole injection. Myocardial content of adenosine in dip group hearts was measured in 30 minutes after intravenous dipyridamole injection. Control: control animals, dipyridamole: animals treated with dipyridamole. *: $P<0.05$ vs con.

augmented by 8-PT. However, prior adeministration of prazosin abolished the suppression of vascular response by 8-PT (Fig. 5). Percent increases in flow rate by 1 μM adenosine in control, 8-PT pretreated, and 8-PT+prazosin pretreated hearts were 48.4, 6.2 and 46.2%, respectively.

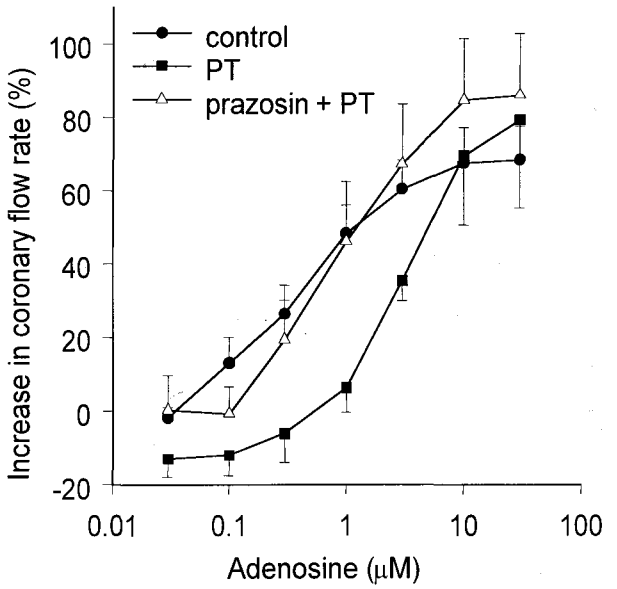

Fig. 5. Effect of exogenous adenosine on the coronary flow rate of perfused rabbit hearts. Control: control, PT: 8-phenyltheophylline pretreated, prazosin+PT: prazosin and 8-phenyltheophylline pretreated.

DISCUSSION

It is generally accepted that the vasodilator action of adenosine is attributed to the effects on the surface A2 receptor, but there has recently emerged a confusion about this dogma. Some investigators reported existance of other action sites such as an unknown intracellular site (Collis & Brown, 1983), or a novel surface recepter (Chinellato et al, 1992). In the present study, we observed an almost complete suppression of the adenosine-induced vasodilation by blocking the transport of adenosine into the intracelluar compartment. Although dipyridamole, the transport inhibitor used in the experiments, itself has vasodilatory action, the degree of increased coronary flow rate by dipyridamole pretreatment was relatively low when compared to the maximal response by adenosine (data not shown). Therefore, the supression of adenosine-induced vasodilation by dipyridamole was not attributed to the exhaustion of vasodilator response. This is supported by the observation that dipyridamole, while reducing the maximal responses of adenosine, has no effects on those evoked by other vasodilators (Collis & Brown, 1983).

On the other hand, this transport inhibition rather enhanced the decrease in heart rate by adenosine ad-

ministration. These results imply that the local interstitial concentration of adenosine and the occupation of cell surface receptor, which mediates the effects on heart rate, was increased by the transport inhibition, but vasodilation is not augmented in the same condition. In other words, it was presumed that the two biological actions of adenosine, the increase in coronary flow and the decrease in heart rate, are mediated by two distinct action sites differentiated by cellular uptake inhibition. As vasodilatory action required cellular uptake of exogenous adenosine, it is speculated that the intracellular adenosine itself or a vasoactive metabolite derived from intracellular adenosine is responsible for the vasodilation.

There are some opposing reports reguarding the effect of dipyridamole on vasodilation by adenosine. Headrick and colleagues reported that dipyridamole does not affect the maximal vascular response by adenosine (Headrick et al, 1992). In another report, Collis and Brown observed a reduction of the amplitude of adenosine-induced vasodilation in the presence of dipyridamole, but in this experiment, submaximal responses were potentiated by dipyridamole (Collis & Brown, 1983). We speculate that this inconsistancy in dipyridamole effects might be attributable to the difference of vascular preparations, as these authors used aorta or ear artery preparation contracted with noradrenaline.

More recently, Headrick et al reported that, in the presence of dipyridamole, adenosine induced an increase in coronary flow of perfused rabbit heart reaching up to 120% of baseline at 10 μM adenosine (Headrick et al, 1996). This result is unexpectable in the view of the present study, and we speculate that there might have been some unpredicted actions of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an adenosine deaminase inhibitor which was co-administered in their experiments to reduce the degradation of exogenous adenosine, since this drug might also inhibit the degradation of endogenously-produced adenosine to augment the intracellular action of it.

The results of the present study strongly support the existence of an intracellular action site of adenosine. However, we still couldn't exclude the role of vascular surface A2 receptor because vasodilation through a surface receptor with low potency might be masked by the dipyridamole-induced vasodilation.

Therefore, to clarify the dominance of the intracellular action in a physiological condition, we tried to reveal the effects of enhanced intracellular adenosine action combined with reduced interstitial adenosine. For augmentation of the endogenous adenosine production, several researchers used a catecholamine-stimulated hearts (Knabb et al, 1984; Headrick & Willis, 1988), but this model was thought incompatible because in these conditions, the production of adenosine in vascular cells may not be stimulated as much as in myocardial cells. Therefore, we adopted an energy-deprived condition which stimulates adenosine production in both vascular and myocardial cells (Deussen et al, 1986; Pekka Raatikainen et al, 1994). Perfusate adenosine concentration was markedly increased by this intervention, and the increase was suppressed by dipyridamole, as was expected. Tissue adenosine content, which represents the activity of intracellular adenosine, was about 5 times higher in dipyridamole-treated hypoxic hearts than untreated hypoxic hearts. Although tissue content of adenosine may not directly reflect the intracellular adenosine concentration, it would be possible to compare the relative intracellular activity of adenosine in dipyridamole-treated and dipyridamole-untreated hearts. For interstitial level of adenosine is presumed to be much lower and the contribution of this compartment to total tissue content of adenosine may be even lower in dipyridamole-treated hearts. Therefore, higher coronary flow rate observed in dipyridamole-treated hearts indicated that the augmentation of intracellular rather than interstitial adenosine plays the major role in coronary vasoregulation in face of metabolic stimulation.

Collis et al have suggested that the intracellular effects of adenosine in aorta are mediated by inosine with the results showing decreased flow response to adenosine in the presence of EHNA, which blocks the conversion of adenosine to inosine (Collis et al, 1986). However, our results indicated no involvement of inosine, as tissue inosine level and presumed intracellular inosine concentration was not increased by dipyridamole-treated hypoxia (data not shown). This conclusion is further supported by reports from other laboratories showing increased flow response by EH NA (Foker et al, 1980; Zughaib et al, 1993).

Another important implication of the present study lies on the mechanism of vasodilatory action of dipyridamole. Dipyridamole is known as a coronary vasodilator (Kuebler et al, 1970). The ability of dipyridamole to increase the coronary flow was attributed to the augmentation of the actions of endogenous adenosine by adenosine uptake inhibition (K-

nabb et al, 1984). However, further studies revealed that this agent inhibit the transport of adenosine symmetrically to reduce both the uptake and the release of adenosine (Meghji et al, 1985). Because inhibition of the release was supposed to result in a decreased interstitial adenosine concentration, dipyridamole might rather reduce the ocupation of surface receptors by adenosine. Therefore, the mechanism of coronray vasodilation by dipyridamole is subject to controversy. The involvement of intracellular adenosine in vasodilation as proved in the present study, would give a satisfactory solution to this dilemma. As might be predicted, dipyridamole, in a concentration in which the receptor mediated decrease of heart rate was not apparent, significantly increased the coronary flow rate. In these dipyridamole-treated cardiac preparations, perfusate adenosine concentration was decreased while tissue content was increased (data not shown). These results indicated that dipyridamole reduced the adenosine release and augmented the intracellular adenosine action without an increase of the interstitial concentration. This conclusion was further confirmed by in situ experiments showing that the interstitial adenosine concentration was not increased by intravenous dipyridamole while tissue adenosine content was markedly increased.

The most strong argument against the dominance of intracellular action site in adenosine-induced vasodilation is the observation that adenosine-induced vasodilation is suppressed by the surface receptor antagonists such as methylxanthine derivatives (Afonso, 1970; Bunger et al, 1975; Headrick and Willis, 1988; Headrick et al, 1992). However, some investigators recently reported that the methylxanthine-sensitive component of adenosine-induced vasodilation is small or nil (Chinellato et al, 1992), or only partial (Collis & Brown, 1983; Harden et al, 1996). In the present study, we also observed an inhibitory effect of 8-PT on adenosine-induced vasodilation. Considering that methylxanthines have multiple actions (Olsson & Pearson, 1990; Rall, 1990), it is suggested that the opposing action of these agents to adenosine-induced vasodilation might be attributed to some other action mechanisms than vascular adenosine receptor antagonism. We thought that the increased release of endogenous catecholamine by methylxanthines (Richardt et al, 1987; Minamino et al, 1995) might operate to reduce the adenosine-induced vasodilation and observed whether the α-adrenoceptor activation is involved in the 8-PT-induced alterations in concentration-response curve to adenosine. As shown in the results, previous α-adrenoceptor blockade by prazosin recovered most of the typical adenosine-induced vasodilation. From these results, we speculated that at least a part of theophylline induced suppression of adenosine action was attributable to the α-adrenoceptor occupation and subsequent vasoconstiction.

In conclusion, an intracellular action site of unknown character was revealed to mediate coronary vasodilation induced by exogenous and endogenous adenosine. Roles of surface receptors were presumed minimal. Conventional viewpoint accepting the surface A2 receptor as the primary action site of adenosine-induced vasodilation should be reconsidered at least in the coronary vasculature. With the same rationale, vasodilatory action of dipyridamole is supposed to be mediated by increased intracellular adenosine activity rather than increased interstitial concentration. In addition, vasoconstrictive action of 8-phenyltheophylline opposing the action of adenosine was presumed to be attributable to α-adrenoceptor activation.

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