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Tyramine produces interstitial adenosine-mediated activation of ecto-5′-nucleotidase in rat heart in vivo

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Abstract

We examined the effect of tyramine on the production of adenosine in rat heart. A flexibly mounted microdialysis setup was used to measure the concentration of interstitial adenosine and to assess the activity of ecto-5′-nucleotidase in in vivo rat hearts. The microdialysis probe was implanted in the left ventricular myocardium of anesthetized rats and perfused with Tyrode solution containing adenosine 5′-monophosphate (AMP) at a rate of 1.0 μl/min. The concentration of adenosine in the effluent (dialysate) was measured by high-performance liquid chromatography (HPLC). Dialysate adenosine obtained during perfusion with the AMP-containing solution through the probe originated from the hydrolysis of AMP by endogenous ecto-5′-nucleotidase, and the level of adenosine reflected the activity of ecto-5′-nucleotidase in the tissue. Tyramine (0–4 mM) increased the adenosine concentration measured during the perfusion of AMP (100 μM) in a concentration-dependent manner. α,β-Methyleneadenosine 5′-diphosphate (α,β-meADP, 100 μM), an inhibitor of ecto-5′-nucleotidase, abolished the AMP-induced increase in dialysate adenosine. Tyramine (1 mM) increased the adenosine concentration measured in the presence of 100 μM AMP (i.e., the activity of ecto-5′-nucleotidase) by 65.8 ± 19.9% ($n = 6$, $P < 0.05$), an increase which was inhibited by an antagonist of the α_1-adrenoceptor (prazosin, 50 μM) or of protein kinase C (chelerythrine, 10 μM). These data provide the first evidence that α_1-adrenoceptor stimulation and the subsequent activation of protein kinase C can increase adenosine concentrations in the interstitial space of ventricular muscle in vivo, through activation of endogenous ecto-5′-nucleotidase. To examine the effect of tyramine on the production of adenosine by ischemia–reperfusion of the rat myocardium, the heart was subjected to myocardial ischemia for 15 min by occlusion of the left anterior descending coronary artery. When the heart was reperfused, elevation of the level of adenosine in the ischemic zone was observed, but this change was not significant. However, when corresponding experiments were performed with a subsequent systemic administration of tyramine (1 mM), a marked elevation in the level of adenosine was observed. The results suggest that tyramine elevates adenosine via stimulation of α_1-adrenoceptors and protein kinase C-mediated activation of ecto-5′-nucleotidase in rat heart.

Keywords: Microdialysis; Tyramine; Adenosine; Ecto-5′-nucleotidase; Protein kinase C

1. Introduction

Adenosine is considered to be an endogenous cardioprotective substance which protects the heart from cell damage caused by ischemia and reperfusion (Ely and Berne, 1992). Catecholamine stimulation was induced by tyramine, which triggers the release of endogenous catecholamine (Downing and Chen, 1985). Numerous investigators have reported that myocardial ischemia also increases catecholamine levels (Dart et al., 1984; Schömig et al., 1984; Rona, 1985; Schömig et al., 1987). Ischaemia activates protein kinase C via α_1-adrenoceptor-dependent and -independent mechanisms (Strasser et al., 1992). Adenosine, an endogenous nucleoside, is an important biochemical intermediate in cellular metabolism and has cardioprotective effects in myocardial ischaemia (Lasley et al., 1990; Ely and Berne, 1992; Lasley and Mentzer, 1992; Thornton et al., 1992). Kitakaze et al. (1994) have recently argued that α_1-adrenoceptor stimulation contributes to the infarct size-limiting effect of ischemic preconditioning in dog hearts by augmenting 5′-nucleotidase activity. They also showed that enhanced activation of protein kinase C increased 5′-nucleotidase activity; thereby leading to an increase of adenosine release, in isolated rat cardiomy-

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ocytes (Kitakaze et al., 1995; Sato et al., 1997a). 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 and Berne, 1992; Kitakaze et al., 1993). The formation and release of adenosine by the ischemic myocardium is enhanced, and the major source of adenosine is the enzymatic dephosphorylation of adenosine 5′-monophosphate (AMP) by 5′-nucleotidase (Frick and Lowenstein, 1976; Schrader et al., 1991). The production of adenosine under normoxic conditions is primarily attributed to the transmethylation of S-adenosylhomocysteine catalyzed by *S*-adenosylhomocysteine hydrolase; the hydrolysis of AMP by ecto-5′-nucleotidase, the main stream for adenosine production under ischemic conditions, is considered to be minimal (Sparks and Bardenheuer, 1986; Headrick et al., 1992). Thus, to mimic ischemic conditions, we measured the concentration of dialysate adenosine under a continuous supply of AMP (a substrate for 5′-nucleotidase) through the microdialysis probe. With this system, the level of dialysate adenosine reflects the activity of ecto-5′-nucleotidase in a particular site of the interstitial space of the myocardium (Sato et al., 1997a,b). The present study was undertaken to clarify further the mechanism of tyramine production of interstitial adenosine in rat heart.

2. Materials and methods

2.1. Animal preparation

Adult male Wistar rats weighing 300–400 g were kept in an environmentally controlled room (20–23°C, 50–60% humidity, illuminated from 0700 to 1900 h) and allowed food and water ad libitum. The rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and the level of anaesthesia was maintained by intraperitoneal injection of chloral hydrate (20 mg/kg). Artificial ventilation was maintained by constant-volume respiration with room air mixed with oxygen. The heart rate, arterial blood pressure, and electrocardiogram (ECG) were monitored and recorded continuously. At the end of the experiments the rats were killed by an overdose of anesthetic. All procedures for dealing with the experimental animals met the guideline principles stipulated by the Physiological Society of Japan and the Animal Ethics Committee of Oita Medical University.

2.2. Microdialysis technique

Details of the flexibly mounted microdialysis setup and its application to the measurement of biological substances in the interstitial space have been described previously (Obata et al., 1994). We created a suitable microdialysis probe. The tubes of the dialysis probe (~15 cm long) were supported loosely at the mid-point on a rotatable stainless-steel wire, so that their movement was totally 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 perfusate was introduced through the inlet tube. The synchronized movement of the tip of the microdialysis probe with the beating ventricle minimized the tissue injury that would otherwise be caused by friction between probe and muscle tissue. The tip of the microdialysis probe (3 mm length and 220 μm o.d. with the distal end closed) was made of dialysis membrane (cellulose hollow fiber 10 μm thick with 50,000 molecular weight cut-off). Two fine silica tubes (150 μm o.d.) were inserted from the open end into the tip of the microdialysis tube consisting of a cylinder-shaped dialysis membrane and serving 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 high-performance liquid chromatography (HPLC) pump (Fig. 1).

2.3. Measurement of adenosine concentration in dialysate

The probe was perfused through the inlet tube with Tyrode solution of the following composition (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 relative recovery of adenosine measured using this flow rate (1.0 μl/min) was 18.0 ± 1.6% ($n = 5$). The dialysate (1.0 μl/min) was collected into a series of reservoirs for each consecutive 15 min (15 μl in each reservoir), throughout the observation period (usually ~3 h). A 10-μl aliquot of the dialysate sample was used for the detection of adenosine and we measured its concentration by reversed phase HPLC. Separation of the compounds was achieved on Eicompak MA-5 ODS columns (5 μm, 4.6 mm × 150 mm; Eicom, Kyoto, Japan), with the mobile phase consisting of 200 mM KH_2PO_4 (pH 3.8, adjusted with phosphoric acid) and 5% (v/v) acetonitrile. The flow rate was set at 1.0 ml/min with a pumping system (PU-980; JASCO, Tokyo, Japan). The absorbance of the column eluate was continuously monitored at 260 nm, using an ultraviolet detector (UV-970; JASCO). The absorbance peak of adenosine was quantified by comparing the retention time and peak magnitudes of samples containing known concentrations (1 or 10 μM) of adenosine and inosine. The limit of detection of adenosine concentration was 10 nM. Adenosine concentrations are presented as the raw data (actual concentrations of the dialysate in each reservoir), and are not corrected for the recovery rate (18%) unless otherwise stated.

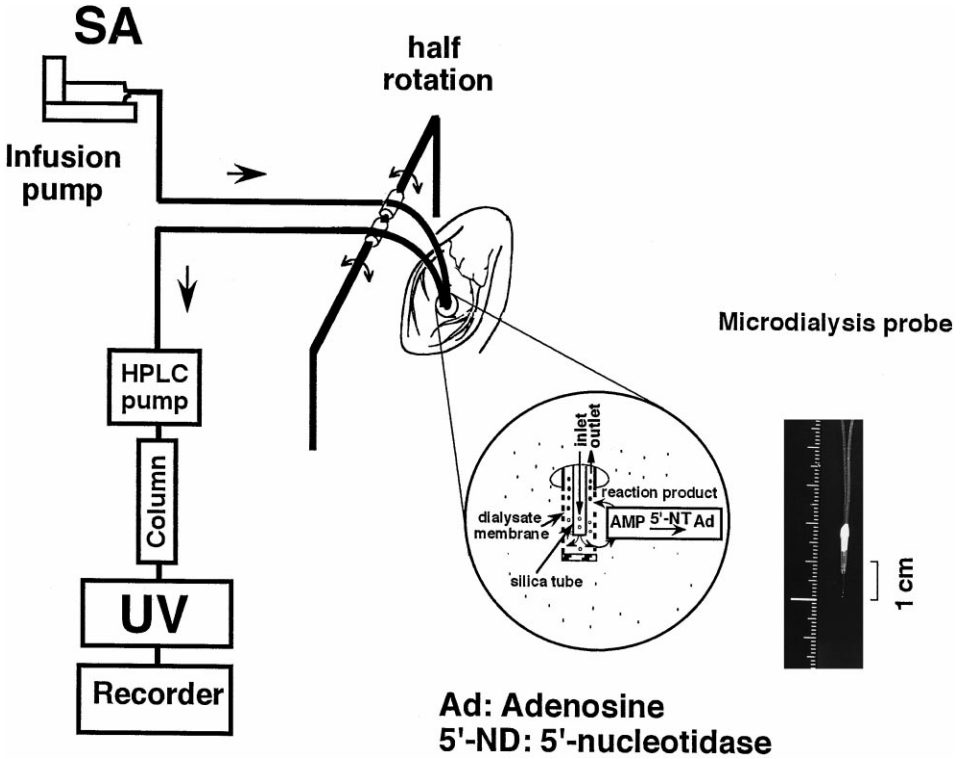

Fig. 1. Microdialysis technique with rat heart. The microdialysis probe was perfused with Tyrode solution containing 100 μM AMP throughout the experiment. The probe (3-nm exposure) was implanted from the epicardial surface into the left ventricular myocardium to the depth of 3 mm. The sequential change in dialysate adenosine measured under a constant supply of AMP reflects the activity of ecto-5′-nucleotidase. Adenosine assayed by HPLC-UV.

2.4. Preparation of ischemic rats

After microdialysis probe implantation in the ischemic zone, the left anterior descending artery branch was clamped by a thread through a tube surrounding the coronary artery. The heart was subjected to regional ischemia for 15 min by the occlusion of the left anterior descending artery coronary artery followed by reperfusion for 75 min.

2.5. Drugs used

AMP (Wako, Osaka, Japan) was prepared immediately before the start of experiments by dissolving an appropriate amount of each agent in the Tyrode solution to give the desired final concentrations, as detailed in the text. Tyramine · HCl, α,β-methyleneadenosine 5′-diphosphate (α,β-meADP) and chelerythrine (Sigma, St. Louis, MO, USA) were dissolved in distilled water and kept as 10 mM stock solutions. Prazosin and diacylglycerol; 1,2-dioctadec-9′-enoyl-*sn*-glycerol (Sigma) were dissolved in methanol as 10-mM stock solutions. An appropriate volume of these stock solutions was added to Tyrode solution immediately before use, as indicated in Section 3.

2.6. Statistical analysis

All values are presented as means ± S.E.M. The significance of differences was determined by using an analysis of variance (ANOVA) with Fisher's post-hoc test. A *P*-value of less than 0.05 was regarded as being statistically significant.

3. Results

3.1. Effect of tyramine on the production of adenosine

We determined the activity of 5′-nucleotidase in rat heart. The rats were anesthetized and the microdialysis probe was implanted in the left ventricular myocardium, followed by perfusion with Tyrode solution. We previously reported (Sato et al., 1997a,b) that the level of adenosine measured during AMP perfusion gives an index of the activity of ecto-5′-nucleotidase in the tissue. The microdialysis probe was perfused with Tyrode solution containing 100 μM AMP throughout the experiment. The effects of tyramine (1 mM) on the sequential change of adenosine concentrations in the dialysate obtained from six rats are demonstrated in Fig. 2. The microdialysis probe was perfused with Tyrode solution containing 100 μM AMP throughout the experiment. After obtaining two control fractions (30–45 and 45–60 min after probe implantation), the introduction of tyramine (1 mM) was begun in the continued presence of AMP. Tyramine significantly increased the concentration of dialysate adenosine, by 61.4 ± 19.4% (from 7.57 ± 0.66 to 12.21 ± 1.47 μM, *n* =

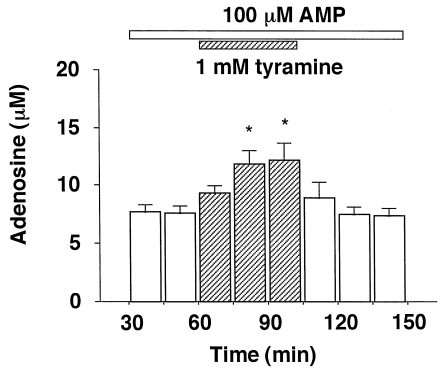

Fig. 2. Effects of tyramine on the production of interstitial adenosine during perfusion with AMP. Tyramine (1 mM) was added to the perfusate in the presence of 100 μM AMP at 60 min after probe implantation. Values are means ± S.E.M. for six animals. $^{*}P < 0.05$ vs. pre-drug value (ANOVA and Fisher's test).

6, $P < 0.05$), at 30–45 min after the beginning of tyramine application (diagonally shaded column at 90–105 min in Fig. 2). After removal of tyramine from the perfusate, the level of dialysate adenosine decreased significantly to 8.86 ± 1.48 μM in 15 min (open column at 105–120 min in Fig. 2). In contrast, in experiments in which α,β-meADP (100 μM) was perfused concomitantly with AMP (100 μM) via the probe, the addition of tyramine (1 mM) failed to increase the concentration of dialysate adenosine (0.85 ± 0.25 μM before vs. 0.73 ± 0.20 μM after tyramine; $n = 5$, not illustrated). When the concentration of tyramine was increased stepwise from 0 to 4 mM, the level of dialysate tyramine significantly increased in a concentration-dependent manner (Fig. 3). The tyramine concentration for a half-maximal effect on adenosine release (EC_{50}) was 0.81 mM. The maximum attainable concentration of dialysate adenosine (E_{max}) obtained with tyramine was 21.48 μM. However, when corresponding experiments were performed with prazosin (50 μM; α_1-adrenoceptor antagonist) pretreated animals, tyramine slightly increased the level of dialysate adenosine.

To determine if the tyramine-induced increases in dialysate adenosine were the result of increases in protein kinase C activity achieved via α_1-adrenoceptor stimulation, the effect of tyramine was examined in the presence of prazosin, an α_1-adrenoceptor antagonist, or chelerythrine, a potent and selective protein kinase C inhibitor that interacts with the catalytic domain of this enzyme (Herbert et al., 1990). In the presence of prazosin (50 μM), tyramine (1 mM) failed to increase dialysate adenosine. In contrast, atenolol, a β_1-adrenoceptor antagonist, did not prevent the tyramine-induced increase in dialysate adenosine: even in the presence of a high concentration of atenolol (50 μM), the application of tyramine (1 mM) significantly increased the dialysate adenosine concentration, by 58.6 ± 18.3% ($n = 5$, $P < 0.05$, not illustrated). On the other hand, in the presence of chelerythrine (10 μM), tyramine did not increase the dialysate adenosine (data not shown). These results suggest that tyramine-induced increases in adenosine concentrations in the dialysate resulted from activation of protein kinase C, mediated by stimulation of α_1-adrenoceptor.

3.2. Effect of tyramine in ischemic–reperfused rat heart

The heart was subjected to myocardiac ischemia for 15 min (45–60 min after probe implantation) by occlusion of the left anterior descending artery. After the dialysate probe was implanted in the left ventricular myocardium, an elevation of the level of adenosine was observed in ischemic–reperfused rat heart. However, when the heart was

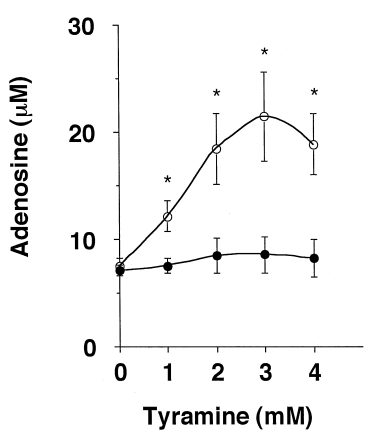

Fig. 3. Concentration-dependent effect of tyramine. The concentration of tyramine was increased gradually from 0 to 4 mM. The level of adenosine in the prazosin (50 μM)-pretreated group (●) was compared with that in the tyramine only-treated group (○). Ordinate scale: adenosine concentration after introduction of tyramine (0, 1, 2, 3 and 4 mM) containing Tyrode solution through the probe. Abscissa scale: concentration of tyramine added. Values are means ± S.E.M. for six animals. $^{*}P < 0.05$ vs. pre-drug values (ANOVA and Fisher's test).

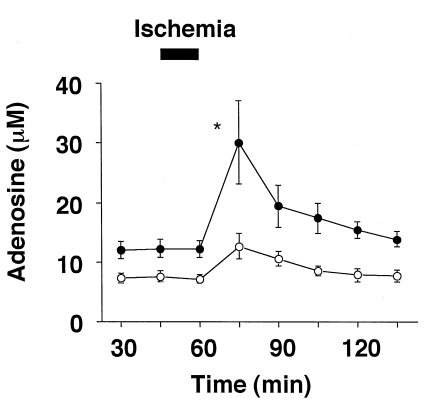

Fig. 4. Effect of tyramine on the production of interstitial adenosine during perfusion with AMP in ischemic–reperfused rat heart. Tyramine (1 mM, ●)-treated group of perfusion–ischemia (closed column)-reperfusion was compared with control group (○). Dialysate samples were collected and immediately assayed for adenosine using HPLC-UV. Values are mean ± S.E.M. for six animals. $^{*}P < 0.05$ vs. level at 135–150 min after probe implantation (steady state level) (ANOVA and Fisher's test).

reperfused, the elevation of adenosine was still observed, but was not significant. This elevation of adenosine was not observed outside of the ischemic area. To define the ischemic zone, after reperfusion, the heart was removed and cannulated into the aorta. The ligature remaining around the left anterior descending artery was retightened, and ethanol was injected into the heart. Then the heart was sliced. Discolored areas in the sliced sections of the heart show non-ischemic regions (not illustrated). However, when corresponding experiments were performed in the presence of tyramine (1 mM), a marked increase in adenosine was observed in the ischemic–reperfused heart (Fig. 4).

4. Discussion

In the present study, we have demonstrated that tyramine produced interstitial adenosine on stimulation of α_1-adrenoceptors and protein kinase C-mediated activation of ecto-5′-nucleotidase in rat heart, using a microdialysis technique. 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 free radical and purine metabolites (Van Wylen et al., 1990, 1992; Schulz et al., 1995). Details of the microdialysis technique (O System) (Obata et al., 1994) for using a flexibly mounted microdialysis probe in vivo in rat hearts to measure biological substances in the interstitial space have already been described. With this technique, it is feasible to make stable and long-term measurements of interstitial adenosine. In the present experiments, drugs were administered through the microdialysis probe. Accordingly, the concentration profile of the administered compounds in the surrounding interstitial space is unknown; in general, the extracellular concentration of a compound given through the probe would never reach the same concentration as in the dialysis probe (Benveniste, 1989). This is an unavoidable limitation of the microdialysis technique that should be kept in mind when interpreting the experimental data.

We previously reported that the sequential changes in dialysate adenosine measured under a constant supply of AMP reflected the activity of endogenous ecto-5′-nucleotidase, using the same experimental design as in the present study (Sato et al., 1997a,b). The baseline level of dialysate adenosine measured in the absence of exogenous AMP was ~ 0.5 μM, which was ~ 14 times lower than the level of dialysate adenosine seen in the presence of 100 μM AMP (~ 7 μM). This report was confirmed by findings as follows: the application of AMP through the probe increased the concentration of dialysate adenosine in a concentration-dependent manner, and the EC_{50} of AMP was ~ 100 μM. This value is much closer to the K_m (Michaelis constant) for 'ecto' than that for 'cytosolic' 5′-nucleotidase (Sparks et al., 1986). The ecto-5′-nucleotidase has a fairly low K_m for degradation of AMP, as compared to cytosolic-5′-nucleotidase; the K_m value for ecto-5′-nucleotidase was shown to be ~ 20 μM (Sullivan and Alpers, 1971) and that for cytosolic 5′-nucleotidase to be ~ 3 mM (Truong et al., 1988). When the selective inhibitor of ecto-5′-nucleotidase, α,β-meADP (Hori and Kitakaze, 1991), at a concentration of 100 μM was present in the perfusate, the AMP (100 μM)-induced increases in the dialysate adenosine concentration were completely inhibited and remained at the level of ~ 0.5 μM, close to the baseline. Therefore, it is reasonable to assume that the level of dialysate adenosine is proportional to the adenosine concentration in the interstitial space of the myocardium, and reflects the ecto-5′-nucleotidase activity in this tissue. This finding is 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 by *S*-adenosylhomocysteine 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.

Although the mechanism of tyramine-induced adenosine production is unclear, tyramine clearly produced an increase in the level of adenosine in the rat heart. Further, an α_1-adrenoceptor antagonist (prazosin) prevented the tyramine-induced noradrenaline increase in dialysate adenosine. This finding suggests that noradrenaline stimulation was induced by tyramine. Our results showed that the tyramine concentration for a half-maximal effect on adenosine release (EC_{50}) was 0.81 mM. Therefore, in the present study, we used 1 mM tyramine. AMP supplied from an inlet tube diffused out into the interstitial fluid through the dialysis membrane, and was converted to adenosine by endogenous 5′-nucleotidase. It is well known that catecholamine stimulation is induced by tyramine, which triggers the release of endogenous catecholamine (Downing and Chen, 1985). Tyramine induced an increase in the adenosine concentration measured in the presence of 100 μM AMP, an increase which was prevented by an antagonist of the α_1-adrenoceptor (prazosin, 50 μM) or of protein kinase C (chelerythrine, 10 μM). Stimulation of the α_1-adrenoceptor is known to activate protein kinase C by increasing the level of cytosolic diacylglycerol (Fedida et al., 1993). In the present study, application of tyramine increased the level of dialysate adenosine in the presence of a continuous supply of AMP and this response was abolished by α,β-meADP. Assuming, as argued above, that the levels of adenosine measured during the continuous availability of AMP reflect ecto-5′-nucleotidase activity, we can conclude that tyramine-induced adenosine, by stimulating α_1-adrenoceptors, increased the level of interstitial adenosine via a protein kinase C-mediated activation of ecto-5′-nucleotidase. It is unlikely that the increase in

adenosine concentration resulted from the inhibition of adenosine deaminase, because tyramine increased not only the adenosine concentration, but also the inosine concentration in the dialysate. The present study showed a clear and important link between the α_1-adrenoceptor stimulation, activation of protein kinase C and the production of adenosine, through enhanced activity of ecto-5′-nucleotidase in the rat heart in vivo.

Recently, protein kinase C has received much attention as an intracellular signal transducer involved in the evolution of ischemic preconditioning (Kitakaze et al., 1994; Liu et al., 1994; Speechly-Dick et al., 1994). Furthermore, both adenosine and α_1-adrenoceptors have been implicated in preconditioning in experiments with hearts from rat (Banerjee et al., 1993), rabbit (Liu et al., 1991; Toombs et al., 1992), dog (Kitakaze et al., 1993) and swine (Schulz et al., 1995). We examined the effect of tyramine on the production of adenosine by ischemia–reperfusion of rat myocardium. In the presence of tyramine, ischemia–reperfusion significantly increased the level of AMP-primed dialysate adenosine. It is difficult to know whether ischemia affected the baseline (no AMP) adenosine levels. However, when corresponding experiments were performed in the absence of tyramine, there was a small increase in the level of AMP-primed dialysate adenosine in ischemic–reperfused rat heart (Fig. 4). To confirm the suggested involvement of an α_1-adrenoceptor/protein kinase C-linked mechanism, we examined the effect of prazosin on the production of interstitial adenosine via the action of tyramine in ischemic–reperfused rat heart. Prazosin completely abolished the increase of dialysate adenosine by tyramine (data not shown). We previously reported that diacylglycerol, a potent protein kinase C activator (Nishizuka, 1995), increased the AMP-primed dialysate adenosine (Sato et al., 1998). It is known that noradrenaline stimulates α_1-adrenoceptors and leads to activation of protein kinase C (Fedida et al., 1993). These results suggest that tyramine-induced increases in adenosine were the results of increases in protein kinase C activity via α_1-adrenoceptor stimulation by tyramine-released noradrenaline. Our results seem to indicate that tyramine, a well known trigger of this catecholamine-stimulated pathway, increases adenosine by 5′-nucleotidase activation. Specifically, it has been shown that α_1-adrenoceptor stimulation and subsequent protein kinase C activation is apparently one of the pathways which causes an adenosine rise through 5′-nucleotidase activation (Sato et al., 1997a). Alternatively, increased NO production with consecutive cGMP formation has been identified as another pathway to trigger 5′-nucleotidase activation and adenosine increase (Obata et al., 1998). Based on this pathway, it is possible to speculate that NO mediates ischemic preconditioning via an adenosine-dependent mechanism. It is also possible to speculate on the same basis, that NO mediates ischemic preconditioning via an adenosine-dependent mechanism. However, further investigation is necessary to confirm the latter speculation. Ischaemia activates protein kinase C via α_1-adrenoceptor-dependent and -independent mechanisms (Strasser et al., 1992). The latter mechanism of activation was secondary to translocation of protein kinase C from the cytosol to the sarcolemma of cardiac muscles; the translocated protein kinase C may then activate ecto-5′-nucleotidase, perhaps via modification of some part of the latter enzyme from inside the membrane, and as a consequence, interstitial adenosine would increase. The adenosine thus produced may then stimulate adenosine receptors of the A_1 subtype located in the surface membrane of the ischemic myocardium (Liu et al., 1991; Thornton et al., 1992).

In conclusion, the present study provided in vivo evidence that tyramine elevates adenosine via stimulation of α_1-adrenoceptors and protein kinase C-mediated activation of ecto-5′-nucleotidase in rat heart. Estimation of 5′-nucleotidase activity by using flexibly mounted microdialysis probes perfused with AMP may be useful in future studies to elucidate the actual mechanism for the action of endogenous adenosine in rat heart.

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