Stimulation of α_1-adrenoceptors and protein kinase C-mediated activation of ecto-5′-nucleotidase in rat hearts *in vivo*

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1. To determine whether protein kinase C (PKC)-mediated activation of ecto-5′-nucleotidase would increase interstitial adenosine concentrations in the rat heart *in vivo*, we made use of the microdialysis technique and a flexibly mounted probe, which was implanted in the left ventricular myocardium and perfused with Tyrode solution.

2. The baseline level of dialysate adenosine was $0{\cdot}51 \pm 0{\cdot}09$ μM ($n = 16$). Perfusion of adenosine 5′-monophosphate (AMP, 100 μM) through the probe increased the dialysate adenosine concentration markedly to $9{\cdot}25 \pm 0{\cdot}46$ μM ($n = 15$). α,β-Methyleneadenosine 5′-diphosphate (AOPCP, 100 μM), an inhibitor of ecto-5′-nucleotidase, abolished the AMP-induced increase in dialysate adenosine, but did not affect the baseline level of adenosine. These observations suggest that the dialysate adenosine obtained during the perfusion with AMP, but not the baseline levels of adenosine, originated from the dephosphorylation of AMP by ecto-5′-nucleotidase. Thus, the level of adenosine measured during AMP perfusion gives an index of the activity of ecto-5′-nucleotidase in the tissue.

3. Noradrenaline (10 μM) increased the adenosine concentration measured in the presence of 100 μM AMP (i.e. the activity of ecto-5′-nucleotidase) by $38{\cdot}7 \pm 9{\cdot}6\%$ ($n = 5$, $P < 0{\cdot}05$), an increase which was inhibited by an antagonist of the α_1-adrenoceptor (prazosin, 50 μM) or of PKC (chelerythrine, 10 μM). Further application of either the α_1-adrenoceptor agonist methoxamine (100 μM) or the diacylglycerol analogue 1,2-dioctanoyl-*sn*-glycerol (DOG, 100 μM) also increased the adenosine concentration by $35{\cdot}1 \pm 10{\cdot}0\%$ ($n = 6$, $P < 0{\cdot}05$) or $40{\cdot}6 \pm 8{\cdot}3\%$ ($n = 5$, $P < 0{\cdot}05$), respectively.

4. The presence of okadaic acid (50 μM), an inhibitor of protein phosphatase, enhanced the noradrenaline-induced increase in adenosine concentration by $112{\cdot}4 \pm 35{\cdot}9\%$ ($n = 4$, $P < 0{\cdot}05$), to a level significantly ($P < 0{\cdot}05$) greater than the increase caused by noradrenaline alone ($38{\cdot}7 \pm 9{\cdot}6\%$).

5. These data provide the first evidence that α_1-adrenoceptor stimulation and the subsequent activation of PKC can increase adenosine concentrations in interstitial spaces of ventricular muscle *in vivo*, through activation of endogenous ecto-5′-nucleotidase.

Adenosine is considered to be an endogenous cardioprotective substance which protects the heart from cell damage caused by ischaemia and reperfusion (Ely & Berne, 1992). During ischaemia, the release of adenosine is markedly increased and most of this increase has been attributed to enzymatic dephosphorylation of adenosine 5′-monophosphate (AMP) by 5′-nucleotidase (Frick & Lowenstein, 1976; Schrader, Borst, Kelm, Smolenski & Deussen, 1991; Kitakaze, Hori & Kamada, 1993). It has been suggested that, in dog hearts, α_1-adrenoceptor stimulation augments adenosine production during ischaemia by enhancing 5′-nucleotidase activity and thus can limit the size of the infarct (Kitakaze *et al.* 1994). Furthermore, in isolated rat cardiomyocytes, the activation of 5′-nucleotidase was shown to be mediated by the activation of protein kinase C (PKC) (Kitakaze *et al.* 1995); for these measurements inhibitors of adenosine kinase and deaminase were used to avoid phosphorylation or degradation of adenosine. However, as far as we know, there has been no convincing demonstration that α_1-adrenoceptor stimulation can increase PKC activity and activate 5′-nucleotidase in the heart *in vivo* and so increase the adenosine concentration in the extracellular space or interstitial fluid of intact cardiac muscle.

Thus, the aims of the present experiments were to test this hypothesis. For this purpose, we used the flexibly mounted microdialysis probes developed by Obata, Hosokawa & Yamanaka (1994) in our laboratories. This technique has made it possible not only to collect dialysate samples from interstitial fluid, but also to apply desired substrates into the interstitial space, with minimum injury to the cardiac

muscles located around the probe of the microdialysis system.

In the non-ischaemic myocardium, the production of adenosine is mainly attributed to the hydrolysis of *S*-adenosylhomocysteine (SAH) mediated by SAH hydrolase, and the hydrolysis of AMP by 5′-nucleotidase is considered to be a minor pathway for overall adenosine production (Lloyd & Schrader, 1987), whereas the latter pathway predominates under ischaemic conditions (Sparks & Bardenheuer, 1986; Hori & Kitakaze, 1991). We considered that evaluation of the activity of ecto-5′-nucleotidase *in vivo* may be difficult, if not impossible, in normally perfused hearts because under physiological conditions, the extracellular level of AMP, the substrate of ecto-5′-nucleotidase, may be quite low. Therefore, we elected to estimate the concentration of dialysate adenosine by applying a constant supply of AMP through the probe, a condition mimicking ischaemia. The activity of 5′-nucleotidase was then estimated from the rate of adenosine production in the presence of a certain concentration of AMP. Using this system, we obtained evidence that the level of dialysate adenosine measured in the presence of AMP does indeed reflect the activity of ecto-5′-nucleotidase in a particular region of the myocardial interstitial space.

METHODS

Animal preparation

Wistar rats of either sex ($n = 115$; body weight, 267·1 ± 5·7 g) were anaesthetized with chloral hydrate (400 mg kg^{-1}, I.P.), and were ventilated (after intubation) with room air mixed 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 killed by an overdose of anaesthetic. 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 technique required for the use of flexibly mounted microdialysis probes *in vivo* in rat hearts to measure biological substances in the interstitial space have already been described (Obata *et al.* 1994). In brief, the tip of the microdialysis probe (3 mm long and 220 μm o.d., with the distal end closed) was made from dialysis membrane (cellulose membrane 10 μm thick with 50 000 molecular weight cut-off). Two very fine silica tubes (150 μm o.d.) were inserted into the tip from the open end and served as an inlet for the perfusate and an outlet for the dialysate, respectively. The inlet tube was connected to a microinjection pump (CMA/100; Carnegie Medicine, Stockholm, Sweden), and the outlet tube was led to the dialysate reservoir. These tubes (~15 cm long) were supported loosely at the mid-point on a semi-rotating 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 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 the probe and the muscle tissue. The probe was implanted from the epicardial surface into the left ventricular myocardium and 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 Tyrode solution that flowed out of the cut end of inlet tube entered the extracellular space across the dialysate membrane by diffusion. The interstitial fluid diffused back into the cavity of the probe and the dialysate left the probe through the orifice of the outlet tube.

To determine the recovery ratio of interstitial adenosine through the dialysis membrane, the tip of the microdialysis probe was bathed in Tyrode solution containing 10 μM adenosine (37 °C), and the probe was perfused with the adenosine-free Tyrode solution (via the inlet tube) at various rates of perfusion (0·5–5·0 μl min^{-1}). The recovery of adenosine was assessed from the adenosine concentration in the dialysate, collected with different rates of perfusion. The relative recovery rate of adenosine ((dialysate concentration/bath concentration) × 100; in %) declined exponentially when the flow rate was increased stepwise (in steps of 0·5 μl min^{-1}) from 0·5 to 5·0 μl min^{-1}. Conversely, the absolute amount of adenosine recovered over a certain period of time, i.e. the volume of perfusate collected for a minute × the adenosine concentration of the perfusate (in pmol min^{-1}), increased steeply when the flow rate was increased over the range of 0·5–1·0 μl min^{-1}, and reached a steady state at rates over 1·0 μl min^{-1}. Thus, a low rate of perfusion increased the relative recovery of adenosine, whereas a high rate of perfusion increased the absolute recovery of adenosine. As a compromise, we used a perfusion rate of 1·0 μl min^{-1}, in the present experiments. The relative recovery of adenosine measured using this flow rate (1·0 μl min^{-1}) was 18·0 ± 1·6% ($n = 5$).

Measurements of adenosine concentration in dialysate

The dialysate (1·0 μl min^{-1}) was collected into a series of reservoirs for every 15 min consecutively (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 using the technique of reversed phase high performance liquid chromatography (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^{-1} by using a pumping system (PU-980; JASCO Corp., Tokyo, Japan). The absorbance of the column eluate was continuously monitored at 260 nm, using an ultraviolet detector (UV-970; JASCO Corp.). 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 raw data (actual concentrations of the dialysate in each reservoir), and are not corrected for the recovery rate (18%) unless otherwise stated.

Experimental protocols

Two protocols were used. In the first protocol, we measured the time-dependent changes of the dialysate adenosine concentration after implantation of the probe, in the absence and presence of AMP and/or α,β-methyleneadenosine 5′-diphosphate (AOPCP) (Figs 1 and 2). In each experiment, the sampling of dialysate was started just after probe implantation and repeated at intervals of 15 min: the concentrations of dialysate adenosine collected at 45–60 min (duration, 15 min) after probe implantation were considered to be the steady-state level of adenosine. In the second protocol, we examined the effect of various compounds (noradrenaline, prazosin, chelerythrine, methoxamine, 1,2-dioctanoyl-

sn-glycerol (DOG), and okadaic acid; see below for the supply source) on the dialysate adenosine concentration, in the presence of AMP (AMP-primed adenosine concentration) and so evaluated the activity of 5′-nucleotidase (Figs 3–6). In the latter series of experiments, AMP at a concentration of 100 μM was perfused throughout the experiment via the probe, and the dialysate sampling was started after a 30 min equilibration period. The number of experiments in each protocol is noted in the respective figure legends.

Chemicals

AMP or noradrenaline (Wako Pure Chemical Co., Osaka, Japan) was prepared immediately before the start of experiments by directly dissolving an appropriate amount of each agent in the Tyrode solution to acquire the desired final concentrations, as given in the text. AOPCP, methoxamine, and chelerythrine (Sigma) were dissolved in distilled water and kept as 10 mM stock solutions. Prazosin (Sigma) was dissolved in methanol as a 10 mM stock solution. Okadaic acid (a kind gift from Fujisawa Pharmaceutical Co., Osaka, Japan) and DOG (Sigma) were dissolved in dimethyl sulphoxide, as 10 mM stock solutions. An appropriate volume of these stock solutions was added to Tyrode solution immediately before use, as indicated in the Results.

Statistical analysis

All values are presented as means ± S.E.M. The significance of difference was determined by using ANOVA with Fisher's *post hoc* test. A P value of less than 0·05 was regarded as being statistically significant. Non-linear curve fitting of the concentration–response relationship (Fig. 2) was performed by using commercially available software (SigmaPlot) that accommodates a Marquardt procedure for parameter estimation (Jandel Corp., Corte Madera, CA, USA).

RESULTS

Time-dependent changes in the dialysate adenosine concentrations harvested in the absence and presence of AMP in the perfusate are demonstrated in Fig. 1*A*. In the absence of AMP (control, filled bars), the concentration of adenosine in the dialysate within the first 15 min after implantation of the microdialysis probe was ~5 μM (Fig. 1*A*, filled bar on the extreme left). There was then a gradual decline in adenosine concentration so that it reached a virtually steady-state level of 0·51 ± 0·09 μM ($n = 16$) at 45–60 min (Fig. 1*B*, Control). When the probe was perfused with Tyrode solution containing AMP at a concentration of 100 μM, the level of dialysate adenosine was markedly elevated to ~12 μM, immediately after implantation of the probe (Fig. 1*A*, hatched bar on the extreme left), then there was a slight decline in the subsequent fractions of dialysate (Fig. 1*A*). The steady-state level of dialysate adenosine measured in the presence of 100 μM AMP and at 45–60 min after implantation of the probe was 9·25 ± 0·46 μM ($n = 15$), and was significantly ($P < 0·001$) higher than that measured in the absence of AMP (Fig. 1*B*).

It seemed likely that the marked increase in adenosine concentrations in the dialysate seen in the presence of AMP reflects the production of adenosine from AMP, catalysed by 5′-nucleotidase in the tissue. Thus, we recorded the level of dialysate adenosine in the presence of AOPCP, an inhibitor of ecto-5′-nucleotidase (Sparks & Bardenheuer, 1986). As can be seen from Fig. 1*B*, when AOPCP alone was

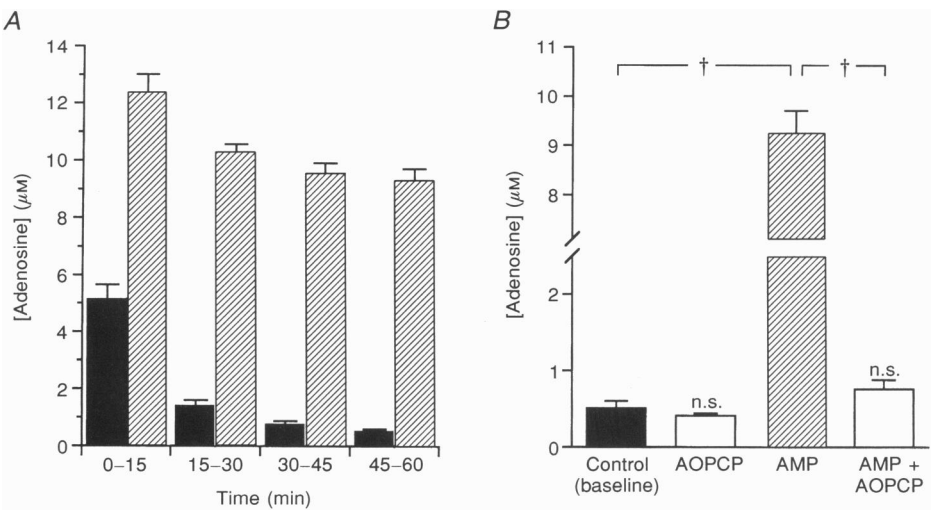

Figure 1. Time-dependent changes and steady-state level of dialysate adenosine measured under various conditions

A, time course of changes of adenosine concentration in the dialysate collected during perfusion with Tyrode solution ($n = 16$, ■) or with Tyrode solution containing 100 μM AMP ($n = 15$, ▨). Abscissa indicates the time elapsed after implantation of the probe. *B*, effects of α,β-methyleneadenosine 5′-diphosphate (AOPCP) on the steady-state level of dialysate adenosine in baseline (left 2 columns) and AMP-primed (right 2 columns) conditions. AOPCP, AOPCP (100 μM) was included in the Tyrode solution ($n = 8$); AMP + AOPCP, AOPCP (100 μM) and AMP (100 μM) were included in the Tyrode solution ($n = 8$). † $P < 0·001$, significant difference between the data indicated by the bracket; n.s., no significant difference *vs.* Control (baseline).

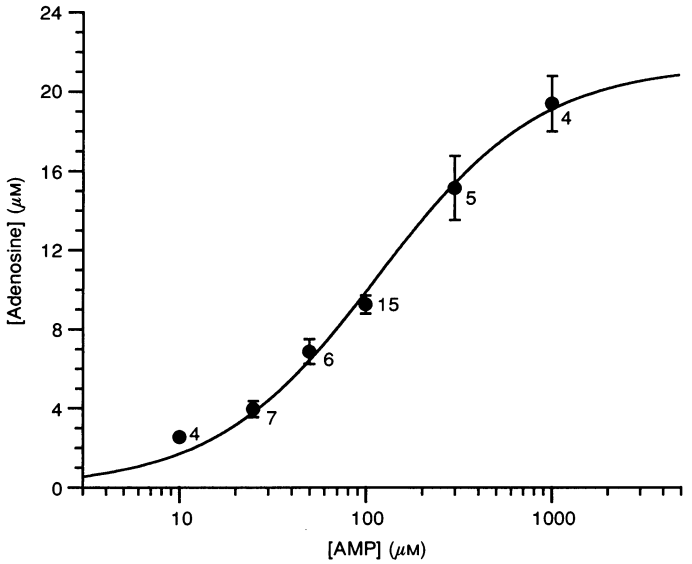

Figure 2. Concentration-dependent effect of AMP on the steady-state level of dialysate adenosine

The steady-state level of dialysate adenosine concentration was plotted against AMP concentration injected through the microdialysis probe. The numbers close to data points indicate the number of experiments. The continuous curve is the best fit to the data points calculated using the following equation:

$$[\text{Adenosine}] = E_{\max}[C/(EC_{50} + C)],$$

where $E_{\max}$ is the maximum attainable concentration of adenosine; EC_{50}, AMP concentration for the half-maximal effect, and C, AMP concentration in the perfusate.

added to the perfusate (Tyrode solution in the absence of AMP) at a concentration of 100 μM, the steady-state level of dialysate adenosine (measured at 45–60 min after the probe implantation) was 0·41 ± 0·03 μM ($n = 8$), a value not significantly different from the baseline adenosine level. In contrast, the augmentation of the adenosine concentration induced by 100 μM AMP was completely inhibited in the presence of 100 μM AOPCP (on extreme right of Fig. 1*B*), and the steady-state level of adenosine remained low, at 0·76 ± 0·12 μM ($n = 8$). The latter value was significantly ($P < 0·001$) lower than that measured in the presence of AMP alone (9·25 ± 0·46 μM) and was not significantly different from the baseline level of adenosine (0·51 ± 0·09 μM), measured without addition of AMP or AOPCP.

The steady-state levels of dialysate adenosine determined by applying various concentrations (10–1000 μM) of AMP through the probe are summarized in Fig. 2, which shows that the concentration of dialysate adenosine measured at 45–60 min after each probe implantation increased in an AMP concentration-dependent manner. The maximum attainable concentration of dialysate adenosine ($E_{\max}$) and the AMP concentration for the half-maximal effect (EC_{50}) were 21·3 and 116·1 μM, respectively. If it is assumed that the AMP-related adenosine fraction, i.e. the adenosine produced after introduction of AMP, was derived from dephosphorylation of the exogenously applied AMP by endogenous ecto-5′-nucleotidase then the level of adenosine in the dialysate measured in the presence of a certain concentration of AMP can serve as an appropriate measure of ecto-5′-nucleotidase activity in the tissue. In subsequent experiments, we therefore used 100 μM AMP, a level close to the EC_{50} (116·1 μM), in order to estimate the activity of ecto-5′-nucleotidase in the tissue.

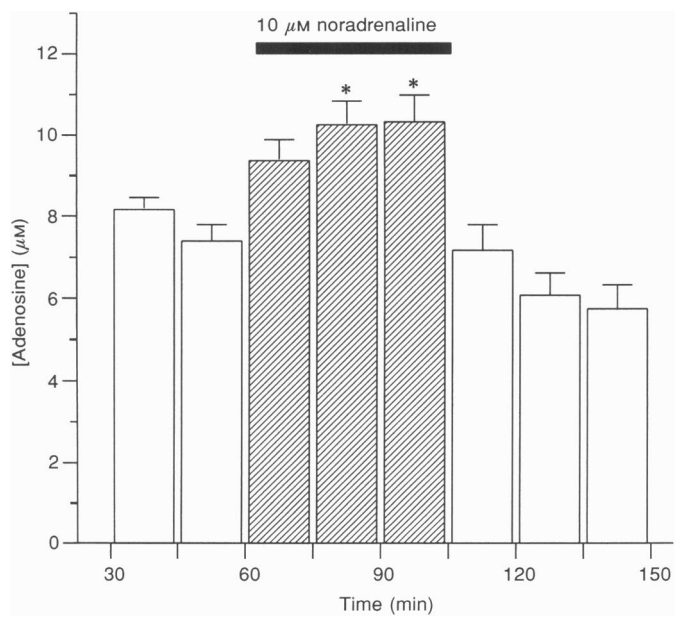

Figure 3. Effects of noradrenaline on the production of interstitial adenosine during perfusion with AMP

Noradrenaline (10 μM) was added to the perfusate in the presence of 100 μM AMP after 60 min implantation of the dialysis probe ($n = 5$). * $P < 0·05$ *vs.* pre-drug value.

Noradrenaline stimulates α_1-adrenoceptors and leads to activation of PKC through accumulation of diacylglycerol (Fedida, Braun & Giles, 1993). We therefore asked whether noradrenaline would increase the level of dialysate adenosine, i.e. the activity of ecto-5′-nucleotidase. The effects of noradrenaline (10 μM) on the sequential change of adenosine concentrations in the dialysate obtained from five rats are demonstrated in Fig. 3. 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), the introduction of noradrenaline (10 μM) was begun in the continued presence of AMP. Noradrenaline significantly increased the concentration of dialysate adenosine by 38·7 ± 9·6% (from 7·36 ± 0·38 to 10·19 ± 0·70 μM, $n = 5$, $P < 0·05$) at 30–45 min after beginning noradrenaline application (hatched bar at 90–105 min in Fig. 3). After removal of noradrenaline from the perfusate, the level of dialysate adenosine significantly ($P < 0·05$) decreased to 7·10 ± 0·64 μM in 15 min (open bar at 105–120 min in Fig. 3). By contrast, in experiments in which AOPCP (100 μM) was perfused concomitantly with AMP (100 μM) via the probe, the addition of noradrenaline (10 μM) failed to increase the concentration of dialysate adenosine (0·75 ± 0·15 μM, before *vs.* 0·69 ± 0·20 μM, after noradrenaline; $n = 4$, not illustrated).

To determine if the noradrenaline-induced increases in dialysate adenosine were the result of increases in PKC activity afforded via α_1-adrenoceptor stimulation, the effect of noradrenaline was examined in the presence of prazosin, an α_1-adrenoceptor antagonist, or chelerythrine, a potent and selective PKC inhibitor that interacts with the catalytic domain of this enzyme (Herbert, Augereau & Maffrand, 1990). In the presence of prazosin (50 μM), noradrenaline (10 μM) failed to increase the dialysate adenosine (Fig. 4*A*). By contrast, atenolol, a β_1-adrenoceptor antagonist, did not prevent the noradrenaline-induced increase in dialysate adenosine: even in the presence of a high concentration of atenolol (50 μM), the application of noradrenaline (10 μM) significantly increased the dialysate adenosine concentration, by 35·8 ± 11·4% ($n = 4$, $P < 0·05$, not illustrated). On the other hand, in the presence of chelerythrine (10 μM), noradrenaline did not increase the dialysate adenosine (Fig. 4*B*). These results suggest that the noradrenaline-induced increases in adenosine concentrations in the dialysate resulted from activation of PKC, mediated by stimulation of α_1-adrenoceptors.

For the experiments shown in Fig. 5, we examined the effects of methoxamine, an α_1-adrenoceptor agonist, and DOG, a diacylglycerol analogue, on the AMP (100 μM)-primed adenosine concentration in the dialysate. Methoxamine (100 μM) significantly increased the level of adenosine by 35·1 ± 10·0% (from 8·33 ± 0·82 to 11·01 ± 0·70 μM, $n = 6$, $P < 0·05$) at 30–45 min after beginning the application. In addition, DOG (100 μM) also significantly elevated the level of adenosine by 40·6 ± 8·3% (from 7·52 ± 0·53 to 10·44 ± 0·26 μM, $n = 5$, $P < 0·05$) at 30–45 min after its introduction.

If, as suggested above, the activity of ecto-5′-nucleotidase is increased by PKC, it may be that this increase is achieved by phosphorylation of the enzyme. We therefore examined the effect of okadaic acid, a protein phosphatase inhibitor (Bialojan & Takai, 1988). Okadaic acid (50 μM) *per se* did not affect the dialysate adenosine measured in the presence of AMP (100 μM, $n = 4$, not illustrated). However, when okadaic acid (50 μM) was introduced concomitantly with

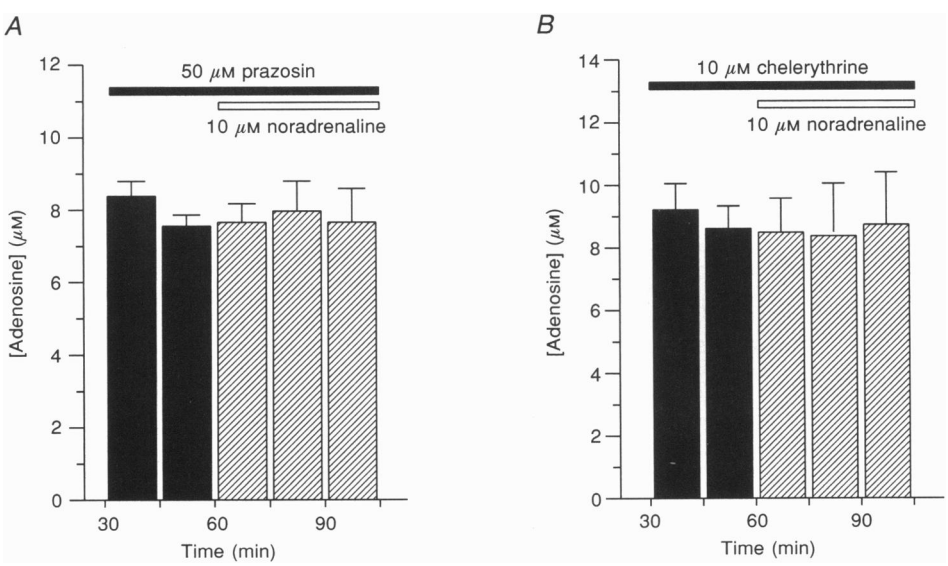

Figure 4. Inhibitory effects of prazosin and chelerythrine on the noradrenaline-induced increases in adenosine concentration

Noradrenaline (10 μM) was added to the perfusate containing 100 μM AMP in the presence of 50 μM prazosin ($n = 5$; *A*), and in the presence of 10 μM chelerythrine ($n = 5$; *B*).

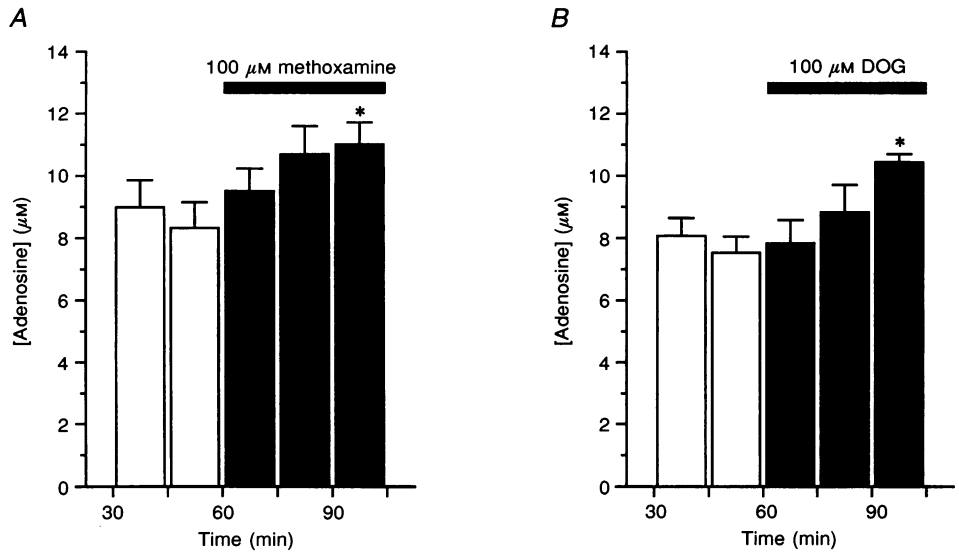

Figure 5. Effects of methoxamine and DOG on the AMP-primed dialysate adenosine concentration
Note that methoxamine (100 μM, $n = 6$; *A*) and DOG (100 μM, $n = 5$; *B*) significantly increased dialysate adenosine concentrations at 30–45 min after the application. * $P < 0{\cdot}05$ *vs.* pre-drug values.

noradrenaline (10 μM) after obtaining two control fractions of dialysate (30–45 and 45–60 min), the AMP (100 μM)-primed dialysate adenosine concentration increased significantly, by as much as 112·4 ± 35·9% at 30–45 min after beginning the application (from 8·30 ± 0·70 to 17·37 ± 2·80 μM, $n = 4$, $P < 0{\cdot}05$) (Fig. 6*A*). This increase (by 112·4 ± 35·9%) in adenosine concentration was significantly ($P < 0{\cdot}05$) greater than that seen with application of noradrenaline alone (by 38·7 ± 9·6%), when compared at 30–45 min after the drug application (Fig. 6*B*). These findings lend support to the notion that phosphorylation of ecto-5′-nucleotidase by PKC augmented the activity of this enzyme.

Adenosine is deaminated by adenosine deaminase and degrades to inosine. Therefore, if the activation of PKC inhibited adenosine deaminase, the result would also be an increase in the adenosine concentration, even without any

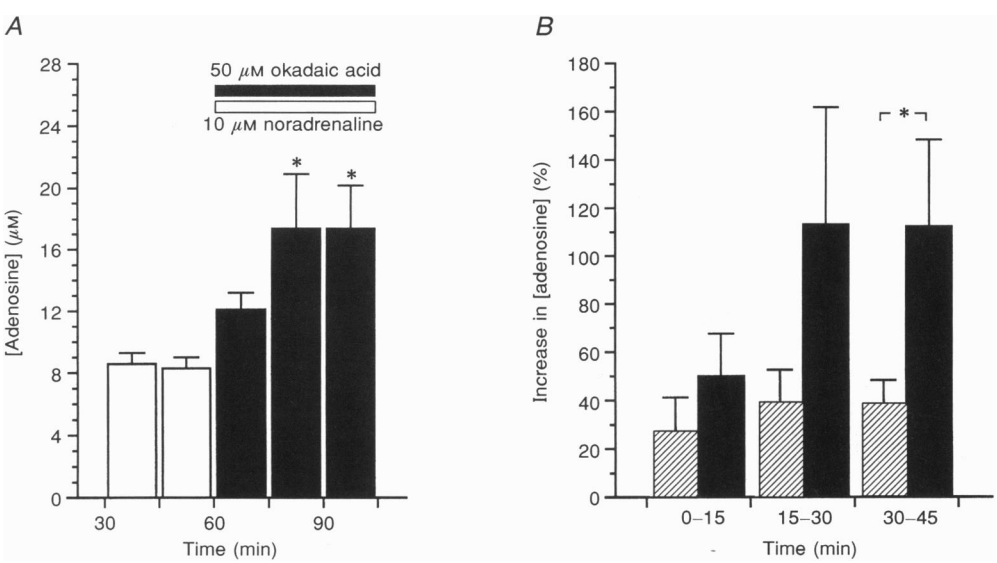

Figure 6. Effects of okadaic acid on noradrenaline-induced increases in AMP-primed adenosine concentration
A, effects of simultaneous application of noradrenaline (10 μM) and okadaic acid (50 μM) on dialysate adenosine concentrations ($n = 4$). * $P < 0{\cdot}05$ *vs.* pre-drug value. *B*, comparison of the effect of noradrenaline on the per cent increase of adenosine concentration in the absence (▨) or presence (■) of 50 μM okadaic acid. Abscissa indicates the time intervals elapsed after application of noradrenaline. * $P < 0{\cdot}05$: significant difference between the data connected by the bracket.

effect on the activity of ecto-5′-nucleotidase, but in this case, the increase in adenosine levels would be accompanied by a reduction in interstitial inosine. However, this was not the case judging from our observations that the interstitial inosine level was significantly elevated (not lowered) by 45·5 ± 2·9% ($n = 5$, $P < 0·05$) after application of noradrenaline (10 μM; not illustrated), thereby indicating that the inhibition of adenosine deaminase by PKC cannot account for the observed increase in adenosine concentrations.

DISCUSSION

Adenosine, an endogenous nucleoside, is an important biochemical intermediate in cellular metabolism and has cardioprotective effects in myocardial ischaemia (Lasley, Rhee, Van Wylen & Mentzer, 1990; Ely & Berne, 1992; Lasley & Mentzer, 1992; Thornton, Liu, Olsson & Downey, 1992). Kitakaze *et al.* (1994) have recently argued that α_1-adrenoceptor stimulation contributes to the infarct size-limiting effect of ischaemic preconditioning in dog hearts by augmenting 5′-nucleotidase activity. They also showed that enhanced activation of PKC increased 5′-nucleotidase activity, thereby leading to an increase of adenosine release, in isolated rat cardiomyocytes (Kitakaze *et al.* 1995). The present study was performed to see if α_1-adrenoceptor stimulation and subsequent activation of PKC increases the interstitial adenosine in the heart *in vivo*, by augmenting 5′-nucleotidase activity. The flexibly mounted microdialysis probe we used for this purpose was capable of measuring adenosine concentration in the interstitial space of the ventricular muscle for more than 6 h without tissue damage (Obata *et al.* 1994).

As shown in Fig. 1, the baseline level of dialysate adenosine was 0·51 ± 0·09 μM. Based on our estimate of the recovery rate of tissue adenosine of 18·0%, the concentration of adenosine in the interstitial fluid of the ventricular muscle located adjacent to the dialysis membrane was therefore ~2·8 μM, a value comparable to that reported in other studies, i.e. 0·3–3·6 μM, using conventional microdialysis techniques (Van Wylen, Willis, Sodhi, Weiss, Lasley & Menztzer, 1990; Van Wylen, Schmit, Lasley, Gingell & Mentzer, 1992) or porous nylon sampling disc techniques (Zhu, Headrick & Berne, 1991). In contrast, the level of dialysate adenosine measured during perfusion of 100 μM AMP was 9·25 ± 0·46 μM, i.e. ~18 times higher than the baseline concentration. This finding suggests that 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. We therefore examined the effect of AOPCP, a selective inhibitor of ecto-5′-nucleotidase, which has no access to the cytosolic 5′-nucleotidase because the drug cannot penetrate the sarcolemma of heart muscle cells (Headrick, Matherne & Berne, 1992). Since AOPCP completely inhibited AMP-induced increases in dialysate adenosine concentrations, without affecting the baseline level of adenosine, the AMP-induced increases in the adenosine concentration most probably originated from enzymatic dephosphorylation of AMP by ecto-5′-nucleotidase, and the baseline production of adenosine probably derived from hydrolysis of SAH.

There are in fact two known forms of 5′-nucleotidase: one is membrane bound (ecto-5′-nucleotidase) and the other is a free or soluble form found in the cytoplasm (cytosolic 5′-nucleotidase). Ecto-5′-nucleotidase has a lower K_m for AMP (~20 μM) than does cytosolic 5′-nucleotidase (~3 mM) (Sullivan & Alpers, 1971; Truong, Collinson & Lowenstein, 1988). In the present study the AMP-induced increases in the dialysate adenosine concentration depended on the AMP concentration, and the AMP concentration for the half-maximal effect of adenosine production (EC_{50}) was 116·1 μM (Fig. 2). This value is much closer to the K_m for 'ecto-' than that for 'cytosolic' 5′-nucleotidase. We therefore used 100 μM AMP (a value close to EC_{50}) in our subsequent experiments. Since the activity of 5′-nucleotidase was measured from the rate of adenosine production under a constant supply of the substrate for this reaction, we believe it is reasonable to consider that the level of dialysate adenosine we measured reflects the activity of ecto-5′-nucleotidase, under these conditions.

Stimulation of the α_1-adrenoceptor is known to activate PKC by increasing the level of cytosolic diacylglycerol (Fedida *et al.* 1993). In the present study, application of noradrenaline increased the level of dialysate adenosine in the presence of a continued supply of AMP and this response was abolished by AOPCP. Furthermore, an α_1-antagonist (prazosin) or a PKC inhibitor (chelerythrine) prevented the noradrenaline-induced increase in dialysate adenosine (Figs 3 and 4). Assuming, as argued above, that the levels of adenosine measured during the continuous availability of AMP reflects the ecto-5′-nucleotidase activity, we can conclude that noradrenaline, by stimulating α_1-adrenoceptors, increased the level of interstitial adenosine via a PKC-mediated activation of ecto-5′-nucleotidase. It is unlikely that the increase in adenosine concentration induced by noradrenaline resulted from the inhibition of adenosine deaminase, because noradrenaline increased not only the adenosine concentration, but also the inosine concentration in the dialysate. Our conclusion is also supported by the finding that either stimulation of the α_1-adrenoceptor by methoxamine, or activation of PKC by the diacylglycerol analogue, DOG, increased the level of dialysate adenosine in the presence of AMP (Fig. 5).

However, other possibilities must be considered: (i) noradrenaline may have attenuated the uptake of adenosine into cytoplasm or enhanced the release of adenosine itself from the intracellular to the extracellular space, and (ii) α_1-adrenoceptor stimulation may have caused a local vasoconstriction, resulting in localized ischaemia which in turn increased the adenosine concentration. If either of these were the case, noradrenaline should have increased the level of dialysate adenosine measured in the 'absence' of exogenous supply of AMP (i.e. the baseline level of adenosine). However, we found that noradrenaline (10 μM) did not affect the

baseline level of adenosine ($n = 3$, unpublished observation). Moreover, noradrenaline should still have been able to increase the adenosine concentration in the presence of AOPCP and yet, it could not (see above).

Noradrenaline, methoxamine and 1,2-dioctanoyl-*sn*-glycerol increased the level of dialysate adenosine by 38·7, 35·1 and 40·6%, respectively. These values were in agreement with data obtained using biochemical assay techniques and isolated rat cardiomyocytes (Kitakaze *et al.* 1995), in that noradrenaline and methoxamine increased ecto-5′-nucleotidase activity by ~55 and ~53%, respectively. Now, the steady-state production of adenosine, i.e. the steady-state concentration of dialysate adenosine may depend on the equilibrium between phosphorylation and dephosphorylation of ecto-5′-nucleotidase. Okadaic acid enhances phosphorylation by inhibiting protein phosphatase (Bialojan & Takai, 1988). Thus, our observation that okadaic acid enhanced the effect of noradrenaline on the production of adenosine is consistent with the idea that it is phosphorylation of ecto-5′-nucleotidase by PKC that increases the enzyme activity.

Recently, PKC has received much attention as an intracellular signal transducer involved in the evolution of ischaemic preconditioning (Kitakaze *et al.* 1994; Liu, Ytrehus & Downey, 1994; Speechly-Dick, Mocanu & Yelon, 1994). Furthermore, both adenosine and α_1-adrenoceptors have been implicated in preconditioning in studies using rat (Banerjee *et al.* 1993), rabbit (Liu, Thornton, Van Winkle, Stanley, Olsson & Downey, 1991; Toombs, Mcgee, Johnstone & Johansen, 1992), dog (Kitakaze *et al.* 1993) and swine (Schulz, Rose, Post & Heusch, 1995) hearts. The present study shows a clear and important link between the α_1-adrenoceptor stimulation, activation of PKC and the production of adenosine, by an enhanced activity of ecto-5′-nucleotidase in the rat heart *in vivo*. Ischaemia activates PKC via α_1-adrenoceptor-dependent and -independent mechanisms (Strasser, Braun-Dullaeus & Marquetant, 1992). The latter mechanism of activation was secondary to translocation of PKC from the cytosol to the sarcolemma of cardiac muscles; the translocated PKC may then activate ecto-5′-nucleotidase, perhaps via modification of some part of the latter enzyme from inside of 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 ischaemic myocardium (Liu *et al.* 1991; Thornton *et al.* 1992).

In conclusion, we have obtained *in vivo* evidence that α_1-adrenoceptor stimulation can activate PKC, which, in turn, activates ecto-5′-nucleotidase via phosphorylation thereby enhancing the production of interstitial adenosine. Estimation of 5′-nucleotidase activity by using flexibly mounted microdialysis probes perfused with AMP may be useful in future studies to elucidate the regulatory influences of 5′-nucleotidase on the production of adenosine.

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