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Activation of ecto-5′-nucleotidase by protein kinase C and its role in ischaemic tolerance in the canine heart

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1 Ischaemic preconditioning (IP) protects the myocardium against irreversible ischaemic injury by activating protein kinase C (PKC). The mechanism by which PKC protects the myocardium is unknown. We have shown that PKC increases the activity of ecto-5′-nucleotidase (ecto-5′-N) and thereby the production of adenosine in cardiomyocytes which may protect the myocardium against ischaemia-reperfusion injury *in vivo*.

2 The objective of this study was to elucidate the possible role of PKC-induced activation of ecto-5′-N in the cardioprotection associated with IP in the canine heart.

3 IP increased the activities of both ecto-5′-N and PKC, and minimized ischaemic damage (infarct size: 7.5 ± 1.8 vs. $42.3 \pm 2.8\%$, $P < 0.01$ vs. the control group). Treatment with the PKC activator (4β-phorbol 12-myristate-13-acetate) also reduced infarct size ($13.5 \pm 2.9\%$, $P < 0.01$ vs. the control group). 8-Sulfophenyltheophylline (an antagonist of adenosine receptors) or α,β-methyleneadenosine 5′-diphosphate (an inhibitor of ecto-5′-N) eliminated the cardioprotective effect of the PKC activator (infarct size: 36.6 ± 3.9 and $34.7 \pm 4.2\%$, respectively), suggesting that PMA limits infarct size by increasing the activity of ecto-5′-N and the adenosine level.

4 The PMA-induced cardioprotection was blunted by GF109203X (an inhibitor of PKC, infarct size: $36.2 \pm 3.1\%$), but not by pretreatment with dexamethasone (infarct size, $14.2 \pm 2.6\%$).

5 We conclude that the PMA- and IP-induced cardioprotection is attributable to phosphorylation and activation of ecto-5′-N.

Keywords: Protein kinase C; ecto-5′-nucleotidase; adenosine; ischaemic preconditioning; 4β-phorbol 12-myristate 13-acetate

Introduction

Brief periods of myocardial ischaemia and reperfusion decrease the extent of cellular injury following subsequent prolonged ischaemia. Although this phenomenon, termed ischaemic preconditioning (IP), has been demonstrated in virtually every animal model, the underlying mechanism of the protection has been unclear. Several cellular mechanisms have been implicated in IP. Adenosine, which is synthesized from adenosine 5′-monophosphate (AMP) by the action of ecto-5′-nucleotidase (ecto-5′-N), protects the heart against the injury induced by ischaemia-reperfusion (Olafsson *et al*., 1987), and Liu *et al*. (1991) were the first to show that activation of adenosine receptors triggers IP. Indeed, adenosine receptor antagonists effectively inhibit cardioprotection due to IP, and adenosine receptor agonists mimic the infarct size-limiting effect of preconditioning in rabbits (Thornton *et al*., 1992), dogs (Kitakaze *et al*., 1993) and man (Walker *et al*., 1995). Ytrehus *et al*. (1994) have shown that ischaemic preconditioning (IP) protects the rabbit heart against ischaemic injury by activating protein kinase C (PKC), and the activation of PKC is attributable to either adenosine (Liu *et al*., 1994) or catecholamines (Tsuchida *et al*., 1994). Since we have shown that activation of PKC increases ecto-5′-N activity in rat cardiomyocytes (Kitakaze *et al*., 1995a) and mediates cardioprotection, it is possible that activation of PKC protects the myocardium against irreversible injury by activation of ecto-5′-N. Recently, we have further demonstrated that IP increases ecto-5′-N activity through activation of PKC (Kitakaze *et al*., 1996b). However, we have not proved that activation of PKC can mediate cardioprotection through increases in ecto-5′-N activity and adenosine release during reperfusion following sustained ischaemia.

To investigate whether the cardioprotective effect of PKC activation is mediated by increased ecto-5′-N activity, we examined the effect of the PKC activator (4β-phorbol 12-myristate 13-acetate, PMA) on the activities of PKC and ecto-5′-N, as well as the relationship between enhanced ecto-5′-N activity and amelioration of irreversible myocardial injury induced by ischaemia-reperfusion in the canine heart. To clarify the role of activation of adenosine receptors, ecto-5′-N and PKC in the infarct size-limitation by PMA, we investigated whether administration of either an antagonist of an adenosine receptor (8-sulphophenyltheophylline, 8-SPT) or an inhibitor of ecto-5′-N (α, β-methyleneadenosine 5′-diphosphate, AMP-CP) inhibits the infarct size-limiting effect due to these procedures. Dexamethasone has been shown to inhibit the induction of ecto-5′-N by lipopolysaccharide in canine myocardium (Node *et al*., 1995b). Hence, to clarify whether increased 5′-N activity is due to an increased protein level of the enzyme or increased phosphorylation of the enzyme, we investigated the effect of intravenous dexamethasone, which inhibits the induction of ecto-5′-N, and the effect of an inhibitor of PKC (GF109203X) on the PMA-induced cardioprotection.

Methods

Experimental system

Mongrel dogs, (body weight, 16 to 24 kg) of either sex, were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} body weight, i.v.), intubated and ventilated with room air mixed with oxygen (100% O_2 at a flow rate of 1.0 to 1.5 l min^{-1}). The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. After admin-

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istration of heparin (500 u kg^{-1}, i.v.), we cannulated the left anterior descending (LAD) coronary artery and perfused it with blood from the left carotid artery through an extracorporeal bypass tube. Coronary blood flow (CBF) in the perfused area was measured with an electromagnetic flow probe attached to the bypass tube, and coronary perfusion pressure (CPP) was monitored at the tip of the coronary artery cannula. A small-calibre (1 mm), short (7 cm) collecting tube was introduced into a small coronary vein near the centre of the perfused area to sample coronary venous blood for the measurements of plasma adenosine, catecholamine and lactate concentrations. Coronary venous blood was sampled 1 min before 90 min of coronary occlusion and 5 min after the onset of reperfusion.

Drained coronary venous blood was collected in a reservoir placed at the level of the left atrium and was returned to the jugular vein. The left atrium was catheterized for microsphere injection. Hydration was maintained by slow infusion of normal saline with an infusion rate of 0.0167 ml kg^{-1} min^{-1}. High fidelity left ventricular (LV) pressure was measured by a micromanometer (Konigsberg P-5, Pasadena, CA, U.S.A.) placed in the left ventricular cavity through the apex. A pair of ultrasonic crystals was placed in the inner one-third of the myocardium about 1 cm apart to measure myocardial segment length with an ultrasonic dimension gauge (5 MHz, 2-mm in diam, Schuessler, Cardiff-by-the-Sea, CA, U.S.A.). Haemodynamic parameters were recorded on a multichannel recorder (Rm-6000; Nihon Kohden, Tokyo, Japan). We calculated fractional shortening (FS) from the equation, $FS = [(EDL - ESL)/EDL] \times 100\%$, where EDL and ESL are end-diastolic and end-systolic segment lengths, respectively.

Heart rate averaged 141 ± 2 beats min^{-1} during control conditions, and did not change during the study. The pH, partial pressure of O_2, and partial pressure of CO_2 in the systemic arterial blood before the start of the experimental protocols were 7.40 ± 0.03, 104 ± 4 mmHg, and 37.5 ± 1.9 mmHg, respectively.

Experimental protocols

Four cycles of coronary occlusion and subsequent 5 min of reperfusion were performed to precondition the myocardium against 90 min of ischaemia followed by 6 h of reperfusion ($n = 8$, the IP group). In the control group, we infused saline at the rate of 0.0167 ml kg^{-1} min^{-1} into the coronary artery 45 min before the sustained occlusion and continued until the first 60 min of reperfusion except during the 90 min occlusion period: the coronary artery was occluded for 90 min by clamping with an occluder attached to the bypass tube, after 40 min of haemodynamic stabilization, and then reperfused for 6 h ($n = 8$). Coronary arterial and venous blood were sampled for blood gas analysis and plasma adenosine and noradrenaline (NA) concentrations measured. In all groups, coronary venous blood was sampled 1 min before 90 min of coronary occlusion. Coronary arterial blood was also sampled with a 60–90 min interval to check the condition of the dogs. Five minutes after the onset of reperfusion, coronary venous blood was sampled.

To evaluate whether transient exposures to PMA can mimic the infarct size-limiting effect of IP, we administered PMA (0.5 pmol kg^{-1} min^{-1}, i.c.) for four cycles of 5 min exposures, each separated by a 5 min interval, and the dogs were subjected to 90 min of coronary occlusion followed by 6 h of reperfusion ($n = 8$, the PMA group). In a preliminary study, PMA (5.0 pmol kg^{-1} min^{-1}, i.c.) increased PKC activity the same as 0.5 pmol kg^{-1} min^{-1} of PMA (i.c.). Therefore we employed a single dose of PMA (0.5 pmol kg^{-1} min^{-1}, i.c.) in the present study.

In a separate group of animals, an intracoronary infusion of either the inhibitor of PKC, GF109203X (40 μg kg^{-1} min^{-1} with an infusion rate of 0.0167 ml kg^{-1} min^{-1}, $n = 7$, the PMA + GF109203X group), the inhibitor of ecto-5′-N, AMP-CP (80 μg kg^{-1} min^{-1}, 4.8 mg ml^{-1} at an infusion rate of 0.0167 ml kg^{-1} min^{-1}, $n = 7$, the PMA + AMP-CP group), or the adenosine receptor antagonist, 8-SPT (25 μg kg^{-1} min^{-1}; 1.5 mg kg^{-1} min^{-1} at an infusion rate of 0.0167 ml kg^{-1} min^{-1}, $n = 7$, the PMA + 8-SPT group) was initiated 10 min before the administration of PMA and continued until the first 60 min of reperfusion except the 90-min occlusion period. We also examined the effects of GF109203X ($n = 8$, the GF109203X group), AMP-CP ($n = 7$, the AMP-CP group) and 8-SPT ($n = 7$, the 8-SPT group) on infarct size caused by 90 min of coronary occlusion followed by 6 h of reperfusion in the absence of PMA treatment. To clarify whether increased 5′-N activity is due to an increased protein level of the enzyme or phosphorylation levels of the enzyme, we investigated the effect of intravenous injection of dexamethasone (4 mg kg^{-1}, 60 min before the injection of PMA) on infarct size ($n = 7$, the PMA + dexamethasone group); the effect of dexamethasone on infarct size in animals not given PMA was also determined ($n = 8$, the dexamethasone group). In a preliminary study, we confirmed that the dose of 8-SPT used completely blocked the coronary vasodilatation induced by exogenous adenosine, that the dose of AMP-CP used inhibited the production of adenosine during ischaemia, and that of dexamethasone inhibited the induction of the protein level of ecto-5′-N by endotoxin (lipopolysaccharide) in canine myocardium (Node *et al.*, 1995b). In the present study, we administered AMP-CP, 8-SPT, or GF109203X into the coronary artery via the bypass tube. Since intracoronary infusions of these agents did not cause systemic haemodynamic changes, we were able to assess the primary effects of these agents on infarct size.

In addition, PKC activity was determined in cytosolic and membrane fractions of the endocardial myocardium in the control ($n = 5$), IP ($n = 5$), and PMA ($n = 5$) groups, and ecto-5′-N activity in membrane fractions of the endocardial and epicardial myocardium from the area of the LAD coronary artery in control ($n = 7$), IP ($n = 8$), PMA ($n = 7$), PMA + GF109203X ($n = 6$), GF109203X ($n = 7$), PMA + 8-SPT ($n = 6$), 8-SPT ($n = 8$), PMA + dexamethasone ($n = 7$), and dexamethasone ($n = 8$) groups.

Criteria for exclusion

To ensure that all of the animals included in the analysis of infarct size data were healthy and exposed to a similar degree of ischaemia, we excluded dogs that fulfilled any of the following criteria: (i) subendocardial collateral flow of > 15 ml 100 g^{-1} min^{-1}, (ii) heart rate of > 170 beats min^{-1}, or (iii) more than two consecutive attempts required to revert ventricular fibrillation with low-energy d.c. pulses applied directly to the heart.

Measurement of infarct size

After 6 h of reperfusion, the LAD coronary artery was reoccluded and perfused with autologous blood, and Evans blue dye was injected into a systemic vein to determine the anatomic risk area and the nonischaemic area in the heart. The heart was then removed immediately and sliced into serial transverse sections 6 to 7 mm in thickness. The nonischaemic area was identified by blue stain, and the ischaemic region was incubated at 37°C for 20 to 30 min in sodium phosphate buffer (pH 7.4) containing 1% triphenyltetrazolium chloride (TTC) (Sigma). TTC stained the noninfarcted myocardium brick-red, indicating the presence of a formazan precipitate formed by reduction of TTC by dehydrogenase enzymes present in viable tissue. Infarct size was expressed as a percentage of the area at risk.

Measurement of regional myocaridal blood flow

The regional myocardial blood flow was determined by the microsphere technique as previously described (Mori *et al.*, 1992). The nonradioactive microspheres (Sekisui Plastic, To-

kyo, Japan) were made of inert plastic labelled with different types of stable heavy elements. In the present study, microspheres labelled with bromine or zirconium, with a mean diameter of 15 μm and a specific gravity of 1.34 or 1.36, respectively, were used. The microspheres were suspended in isotonic saline containing 0.01% Tween 80 to prevent aggregation, sonicated for 5 min and then agitated for 5 min with a vortex mixer immediately before injection. Approximately 1 ml of the microsphere suspension (2×10^6 to 4×10^6 microspheres) was injected into the left atrium, followed by several warm (37°C) saline flushes. Microspheres were administered 80 min after the onset of coronary occlusion. A reference blood flow sample was withdrawn from the femoral artery at a constant rate of 8 ml min^{-1} for 2 min immediately before microsphere administration.

The X-ray fluorescence activity of microsphere heavy elements was measured with a wavelength dispersive spectrometer (Phillips model PW 1480) the specifications of which have been described previously (Mori *et al*., 1992). Myocardial blood flow was calculated according to the formula times reference flow divided by reference counts, and was expressed in ml min^{-1} 100 g^{-1} wet mass. We measured endomyocardial blood flow of the left ventricular wall.

Chemical analysis

Myocardial oxygen consumption (MVO_2) (ml 100 g^{-1} min^{-1}) was calculated by CBF (ml 100 g^{-1} min) $\times$ coronary arterial and venous blood oxygen difference (ml dl^{-1}). Lactate was assessed by enzymatic assay (Bergmeyer, 1963), and lactate extraction ratio (LER) was obtained by coronary arteriovenous difference in lactate concentration multiplied by 100 and divided by arterial lactate concentration.

Measurement of adenosine concentration

The measurement of plasma adenosine concentration has been described previously (Sato *et al*., 1982; Yamane *et al*., 1992). Briefly, 1 ml of blood was drawn into a syringe containing 0.5 ml of 0.02% dipyridamole and 100 μl of 2′-deoxycofromycin (0.1 mg ml^{-1}), and 500 mM EDTA, to block both the uptake of adenosine by red blood cells and its degradation. The samples were centrifuged, and the adenosine concentration in the supernatant determined by radioimmunoassay. Adenosine in the plasma (100 μl) was succinylated by incubation for 20 min with 100 μl of dioxane containing succinic acid anhydride and triethylamine. After addition of 100 μl of 5 nM adenosine 2′,3′-O-disuccinyl-3-[^{125}I]-iodotyrosine methyl ester and 100 μl of diluted rabbit antiserum to adenosine, the mixture was maintained at 4°C for 18 h, antiserum to goat rabbit (500 μl) was then added and the mixture incubated 4°C for 20 min. Radioactivity remaining in the tube was determined with a gamma counter. Adenosine degradation during this blood sampling procedure has been shown to be negligible (Sato *et al*., 1982; Yamane *et al*., 1992). With this radioimmunoassay adenosine concentrations as low as 5 pmol ml^{-1} were able to be detected, with intra-assay and interassay coefficients of variance of 1.3 to 3.1 and 4.1 to 9.4%, respectively. Unlike measurement of adenosine by high-performance liquid chromatography, the radioimmunoassay does not require the removal of protein from samples. The difference in adenosine concentration between coronary venous and arterial blood [ΔVA(Ado)] was used to quantify the amount of adenosine released from the myocardium.

Measurement of noradrenaline concentration

The measurement of plasma NA concentration has been described previously (Sato *et al*., 1989). Five milliliters of coronary arterial or venous blood were collected into a tube containing EDTA, immediately placed on ice, and subsequently centrifuged for 20 min. The plasma supernatant was stored at -80°C. Within 2 weeks, plasma noradrenaline was adsorbed on alumina, separated by high-performance liquid chromatography (LC-3A;Zpax-SCX column; Shimazu Seisakusho, Kyoto, Japan), and assayed spectrofluorometrically by the trihydoxyindole method (Shimazu spectrofluorophotometer RF-500LCA). Noradrenaline 10 pg ml^{-1} can be detected with this assay and the intra-assay coefficient of variation was 6.8% (Sato *et al*., 1989).

Measurement of PKC and ecto-5′-N activity

A biopsy specimen (1 to 2 g) of the LAD-perfused subendocardial myocardium was obtained without sustained coronary occlusion in the various treatment groups. In these dogs, hearts were excised and endo- and epicardial tissue quickly removed. Tissue samples were frozen and stored under liquid nitrogen until further analysis. Myocardial tissue was subsequently homogenized with a Potter-Elvehjem homogenizer (30 strokes) for 5 min in 10 volumes of an ice-cold solution containing 10 mM HEPES-KOH (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, and 1 mM mercaptoethanol. The homogenate was strained through a double-layer nylon sieve and homogenized again for 1 min. To prepare a crude membrane fraction, a portion of part of the homogenate was centrifuged at 1000 *g* for 10 min. The resulting pellet was washed three times and resuspended in the HEPES-KOH buffer. To prepare the cytosolic fraction, the remaining portion of the homogenate was centrifuged at 3000 *g* for 10 min, and the resulting supernatant further centrifuged at 200,000 *g* for 1 h. The membrane and cytosolic fractions were dialysed at 4°C for 4 h against a solution containing 10 mM HEPES-KOH (pH 7.4), 1 mM $MgCl_2$ and 1 mM mercaptoethanol, and then stored at -80°C. In a preliminary study, it was demonstrated that the recovery of ecto-5′-N activity in the membrane fraction was $97 \pm 2\%$ ($n = 5$).

The activity of ecto-5′-N was measured by an enzymatic assay (Smith *et al*., 1965) and is expressed as nanomol mg^{-1} protein min^{-1}. Protein concentration was measured by the method of Lowry *et al*. (1951) with bovine serum albumin as standard.

The activity of PKC was measured with an enzyme assay kit (Amersham, Arlington Heights, IL, U.S.A.), which provides a simple and reliable method of estimating PKC activity without extensive purification of the enzyme (Kitakaze *et al*., 1995b). PKC activity was expressed as nanomol mg^{-1} protein min^{-1}. The dependence of PKC activity on Ca^{2+} and phospholipid was examined by adding 0.5 mM excess EGTA or eliminating phosphatidylserine from the assay system (Kitakaze *et al*., 1995b).

Drugs

PMA and 8-SPT were obtained from Research Biochemicals (Natick, MA, U.S.A.). GF109203X (bisindolylmaleimide) was obtained from Calbiochem (La Jolla, CA, U.S.A.), and AMP-CP and dexamethasone from Sigma (St. Louis, MO, U.S.A.).

Statistical analysis

Data are expressed as mean $\pm$ s.e.mean. Statistical significance was assessed by analysis of variance (ANOVA) and Bonferroni's test (Snedecor & Cochran, 1972; Winer, 1982). The effect of endocardial collateral blood flow on infarct size was analysed by analysis of covariance (ANCOVA), with regional collateral flow in the inner half of the left ventricle wall as the covariant. A P value of < 0.05 was considered statistically significant.

Results

Mortality and exclusions

One-hundred and seven dogs were randomly assigned to 11 groups for assessment of infarct size: The control group, the IP group, PMA group, PMA + GF109203X group, GF109203X

group, PMA + AMP-CP group, AMP-CP group, PMA + 8-SPT group, 8-SPT group, PMA + dexamethasone group, and dexamethasone group (Table 1). Seven dogs were excluded from data analysis because subendocardial collateral blood flow was >15 ml 100 g^{-1} min^{-1}. No dogs were excluded because of >170 beats min^{-1}. Ventricular fibrillation developed in 19 of the remaining 98 animals at least once, matching the exclusion criterion in eight dogs during the 90 min of ischaemia and in ten dogs during subsequent reperfusion; these animals were also excluded from the assessment of infarct size.

Haemodynamic and metabolic parameters

No significant differences in systolic and diastolic blood pressures and heart rate were detected between the 11 groups before, during, and after 90 min of myocardial ischaemia (Table 2). FS did not significantly increase (24.5 ± 2.1 to $28.3 \pm 1.7\%$) and CBF slightly decreased during administration of PMA in the PMA group (90.5 ± 2.1 to 82.4 ± 1.1 ml $^{-1}$ 100 g^{-1} min, $P<0.05$), and returned to the control level 5 min after the fourth exposure to PMA (Table 3). CPP did not significantly

Table 1 The number of dogs assigned to and excluded from each group for measurement of infarct size

Group	*Number of dogs originally assigned*	*Number of dogs used for data analysis*	*Reason for exclusion* *VF during 90 min of ischaemia*	*VF during 6 h of reperfusion*	*High collateral flow (>15 ml 100 g^{-1} min^{-1})*
Control	11	8	1	2	0
IP	11	8	1	1	1
PMA	10	8	1	0	1
PMA + GF109203X	9	7	0	1	1
GF109203X	10	8	1	0	1
PMA + AMP-CP	9	7	1	1	0
AMP-CP	8	7	1	0	0
PMA + 8-SPT	9	7	0	2	0
8-SPT	9	7	0	1	1
PMA + dexamethasone	10	7	1	1	1
Dexamethasone	11	8	1	1	1

Abbreviations: VF, ventricular fibrillation; IP, ischaemic preconditioning; PMA, 4β-phorbol 12-myristate13-acetate; AMP-CP, α,β-methyleneadenosine 5′-diphosphate; 8-SPT, 8-sulphophenyltheophylline.

Table 2 Sequential measurements of systolic and diastolic blood pressure and heart rate before ischaemia, after 80 min of ischaemia, and after 10 min or 3 h of reperfusion

Group	*Before ischaemia*	*Ischaemia 80 min*	*Reperfusion 10 min*	*3 h*
		Systolic blood pressure (mmHg)		
Control	143 ± 4	141 ± 2	138 ± 2	137 ± 2
IP	140 ± 4	141 ± 4	140 ± 4	138 ± 3
PMA	143 ± 2	143 ± 3	139 ± 3	138 ± 3
PMA + GF109203X	143 ± 2	142 ± 3	139 ± 3	138 ± 3
GF109203X	141 ± 3	140 ± 3	141 ± 3	138 ± 4
PMA + AMP-CP	142 ± 4	140 ± 4	141 ± 4	139 ± 4
AMP-CP	140 ± 3	139 ± 3	138 ± 4	137 ± 5
PMA + 8-SPT	141 ± 3	140 ± 3	139 ± 5	139 ± 3
8-SPT	142 ± 4	144 ± 2	137 ± 4	137 ± 4
PMA + dexamethasone	140 ± 2	140 ± 3	142 ± 4	138 ± 3
Dexamethasone	141 ± 4	140 ± 2	141 ± 3	139 ± 4
		Diastolic blood pressure (mmHg)		
Control	83 ± 6	82 ± 4	84 ± 3	81 ± 6
IP	86 ± 3	84 ± 4	83 ± 4	82 ± 4
PMA	84 ± 4	88 ± 4	86 ± 3	82 ± 3
PMA + GF109203X	82 ± 3	83 ± 3	83 ± 4	82 ± 4
GF109203X	84 ± 3	83 ± 2	82 ± 3	82 ± 4
PMA + AMP-CP	85 ± 5	83 ± 5	81 ± 3	80 ± 4
AMP-CP	84 ± 2	83 ± 2	80 ± 4	79 ± 2
PMA + 8-SPT	83 ± 4	81 ± 5	82 ± 5	82 ± 6
8-SPT	87 ± 6	89 ± 7	84 ± 7	78 ± 5
PMA + dexamethasone	85 ± 3	83 ± 2	82 ± 3	82 ± 3
Dexamethasone	83 ± 4	82 ± 5	83 ± 3	81 ± 2
		Heart rate (beats min^{-1})		
Control	144 ± 2	146 ± 3	145 ± 4	140 ± 3
IP	143 ± 2	142 ± 2	141 ± 5	140 ± 3
PMA	144 ± 5	138 ± 4	141 ± 6	140 ± 5
PMA + GF109203X	141 ± 3	139 ± 2	138 ± 3	139 ± 2
GF109203X	142 ± 3	141 ± 2	141 ± 3	140 ± 4
PMA + AMP-CP	138 ± 5	136 ± 4	137 ± 4	135 ± 5
AMP-CP	142 ± 6	141 ± 3	141 ± 4	139 ± 4
PMA + 8-SPT	142 ± 3	139 ± 2	140 ± 5	138 ± 4
8-SPT	141 ± 5	137 ± 6	138 ± 6	137 ± 6
PMA + dexamethasone	143 ± 3	141 ± 2	140 ± 4	141 ± 2
Dexamethasone	141 ± 3	140 ± 4	140 ± 3	138 ± 3

Values are mean $\pm$ s.e.mean. No statistically significant differences were detected by ANOVA. Abbreviations as in Table 1.

increase during PMA administration (100±2 to 102±2 mmHg). CPP, CBF, FS, MVO_2, pH in coronary arterial and venous blood, NA concentration in coronary arterial and venous blood, and LER did not differ significantly betwen the eleven groups before the onset of 90 min of ischaemia (Table 3). ΔVA(Ado) did not increase significantly 5 min after the fourth exposure to PMA (62±7 vs. 68±6 pmol ml^{-1}, respectively) compared with the baseline condition. Five minutes after the onset of coronary reperfusion, ΔVA(Ado) was significantly higher in the IP and PMA groups than in the control group (478±22 and 461±29 vs. 156±8 pmol ml^{-1}, respectively; $P<0.01$); in the PMA+AMP-CP group, ΔVA(Ado) was similar to that in the AMP-CP group (173±12 vs. 156±8 pmol ml^{-1}, respectively) at this time.

PKC and ecto-5′-N activity

PKC activity in the membrane fraction of the myocardium was increased in the IP and PMA groups relative to the control group (Figure 1a). In contrast, PKC activity in the cytosolic fraction did not differ between the three groups (Figure 1b). Removal of Ca^{2+} or phospholipid from the PKC assay reduced PKC activity in the cytosolic fraction, but not in the membrane fraction of the control group. The increase in PKC activity in the membrane fraction induced by either PMA or IP was also apparent only when the assay was performed in the presence of both Ca^{2+} and phospholipid.

Exposure of dogs to PMA increased ecto-5′-N activity in the epicardium (Figure 1c) and endocardium (Figure 1d) to values

Table 3 Baseline coronary haemodynamic and metabolic parameters before sustained ischaemia

Group	*CPP* (mmHg)	*CBF* (ml 100 g^{-1} min^{-1})	*FS* (%)	*MVO_2* (ml dl^{-1})	*LER* (%)	*pH(A)*	*pH(V)*	*NA(A)* (pg ml^{-1})	*NA(V)* (pg ml^{-1})
Control	101±3	91.2±0.9	25.9±1.4	6.45±0.24	24.1±1.5	7.42±0.01	7.42±0.02	382±23	389±28
IP	102±4	91.5±0.4	24.6±1.3	6.76±0.25	25.2±2.1	7.41±0.02	7.40±0.02	376±29	409±21
PMA	103±5	89.2±1.5	25.0±2.3	6.62±0.30	24.1±1.2	7.42±0.02	7.41±0.02	362±18	388±18
PMA+GF109203X	102±3	88.1±2.2	23.4±2.1	5.59±0.31	25.7±0.2	7.40±0.05	7.41±0.02	357±34	351±22
GF109203X	101±3	90.2±1.6	25.3±2.1	6.33±0.20	25.9±1.2	7.41±0.02	7.41±0.01	380±38	391±22
PMA+AMP-CP	99±2	88.2±1.4	22.6±2.9	5.98±0.22	22.2±3.1	7.40±0.02	7.40±0.03	378±21	401±23
AMP-CP	103±4	90.1±1.6	24.2±1.8	6.89±0.18	23.7±2.1	7.43±0.03	7.38±0.02	384±15	408±13
PMA+8-SPT	101±2	90.0±1.9	25.0±2.1	6.13±0.20	27.9±1.2	7.43±0.01	7.42±0.01	388±28	399±12
8-SPT	102±5	89.1±3.3	24.5±2.3	5.56±0.21	25.0±0.2	7.41±0.08	7.42±0.03	467±24	451±32
PMA+dexamethasone	100±2	88.0±1.4	23.0±2.9	6.46±0.20	23.1±1.8	7.40±0.01	7.41±0.01	369±22	392±11
Dexamethasone	101±3	89.3±1.3	23.5±1.6	6.73±0.14	23.2±1.7	7.42±0.02	7.39±0.02	405±18	422±18

Values are means±s.e.mean. No statistically significant differences were detected by Bonferroni's test. Abbreviations: CPP, coronary perfusion pressure; CBF, coronary blood flow; FS, fractional shortening; MVO_2, myocardial oxygen consumption; LER, lactate extraction ratio; pH(A) and pH(V), pH in coronary arterial and venous blood, respectively; NA(A) and NA(V), noradrenaline concentrations in coronary arterial and venous blood, respectively.

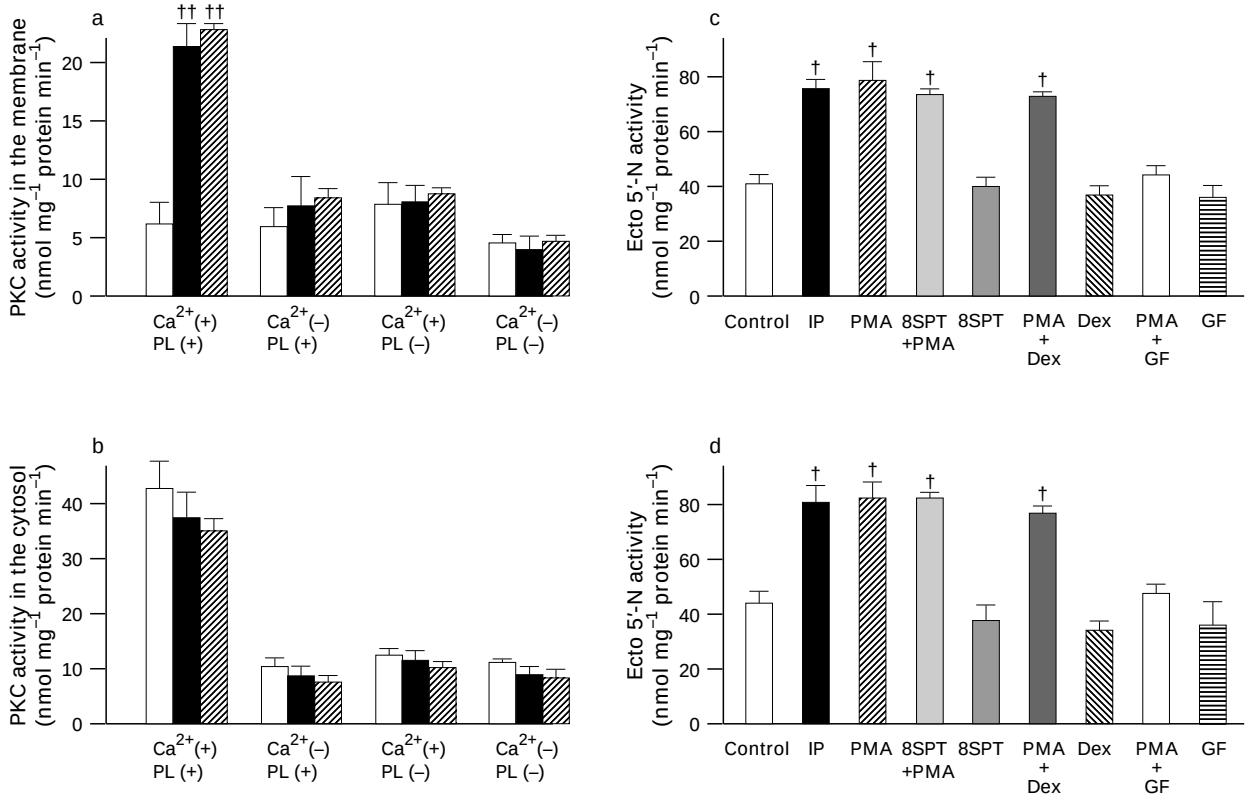

Figure 1 Activity of protein kinase C (PKC) in myocardial membrane (a) and cytosolic (b) fractions, and ecto-5′-nucleotidase (ecto-5′-N) activity in the epicardium (c) and endocardium (d), in various experimental groups. (a and b) PKC activity was assayed in the absence or presence of Ca^{2+} and phospholipid (PL) as indicated for the control (open columns), IP (solid columns) and PMA (hatched columns) groups. Dex, dexamethasone; GF, GF109203X. All data are mean±s.e.mean. †$P<0.01$, ††$P<0.001$ vs. control group (Bonferroni's test).

similar to those obtained with IP. The PMA-induced increase in ecto-5′-N activity was blunted by GF109203X but not by pretreatment with dexamethasone.

Infarct size

The area at risk and collateral blood flow during ischaemia was comparable between all the groups (Table 4). Transient exposures to PMA mimicked the infarct size-limiting effect of IP (Figure 2). This effect of PMA was not observed in the presence of either GF109203X, AMP-CP or 8-SPT, suggesting that PMA limits infarct size by increasing the activity of PKC, ecto-5′-N and release of adenosine. The relationship between infarct size normalized by the risk area and the collateral blood flow to the inner half of the LAD-dependent myocardium during the sustained ischaemic period clearly indicated that (1) both PMA and IP limited infarct size to the same extent; and (2) administration of either GF109203X, AMP-CP or 8-SPT during the infusion of PMA and the reperfusion period following 90 min of ischaemia blunted the infarct size-limiting effect (Figures 3 and 4). Pretreatment with dexamethasone did not blunt the infarct-size limiting effect of PMA. These results indicate that increased ecto-5′-N activity due to the activation of PKC contributes to the infarct size-limiting effect of either IP or PMA, and that these effects of IP and PMA do not depend on the synthesis of ecto-5′-N.

Table 4 Area at risk and collateral blood flow during ischaemia

	Area at risk (% of left ventricle)	*Collateral flow during ischaemia* (ml 100 g^{-1} min^{n-1})
Control	34±4.7	8.2±2.3
IP	36±3.2	9.4±2.8
PMA	38±5.2	9.1±3.0
PMA+GF109203X	37±3.4	8.4±2.0
GF109203X	35±3.2	8.5±2.1
PMA+AMP-CP	35±2.2	8.8±2.7
AMP-CP	39±4.1	8.9±2.1
PMA+8-SPT	40±3.8	8.1±2.2
8-SPT	33±4.8	7.8±2.6
PMA+dexamethasone	35±4.2	8.1±2.3
Dexamethasone	36±3.2	8.7±2.9

Values are means±s.e.mean. No statistically significant differences were detected by Bonferroni's test. Abbreviations as shown in Table 1.

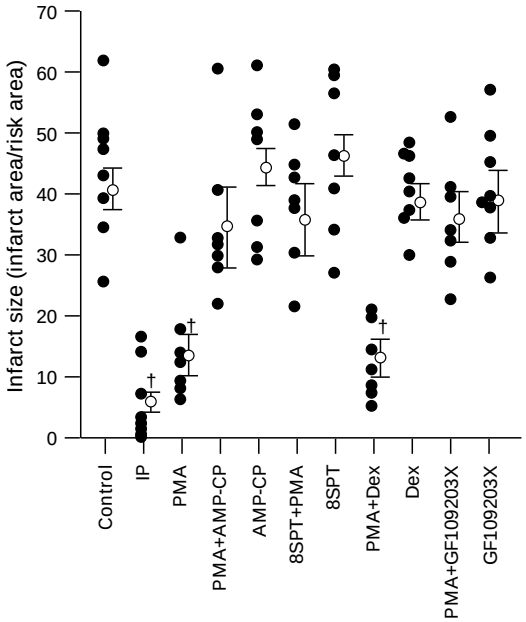

Figure 2 Infarct size (%) in the various experimental groups. (●) Represent individual animals, (○) represent mean±s.e.mean (vertical lines). †$P<0.01$ vs. control group (Bonferroni's test). Dex, dexamethasone.

Discussion

Murray *et al*. (1986) proposed that reduced ATP depletion during ischaemia is an important component of the mechanism of IP. Liu *et al*. (1991) and Thornton *et al*. (1992) showed that an exposure to adenosine before and during sustained ischaemia limits the infarct size, and that activation of A_1 adenosine receptors mediates IP. We have shown that increased ecto-5′-N activity, presumably via activation of PKC, renders the myocardium resistant to sustained ischaemia (Kitakaze *et al*., 1993;

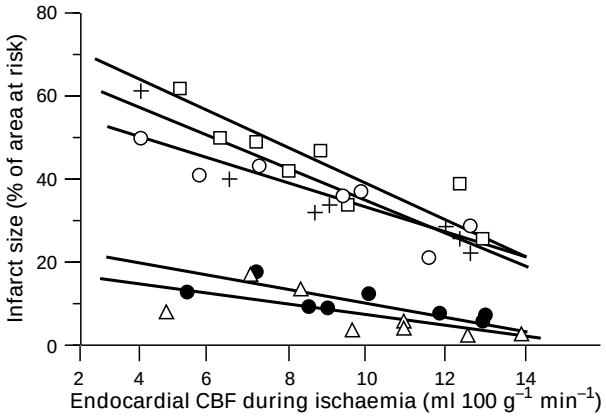

Figure 3 Plots of infarct size expressed as a percentage of risk area versus regional collateral blood flow (CBF) during ischaemia. Infarct size in hearts subjected to IP (△) or PMA-stimulated preconditioning (●) was significantly smaller than in the control group (□) at any given value of collateral blood flow. The infarct size-limiting effect of PMA was completely abolished by the administration of either AMP-CP (+) or 8-SPT (○) ($P<0.01$, ANCOVA).

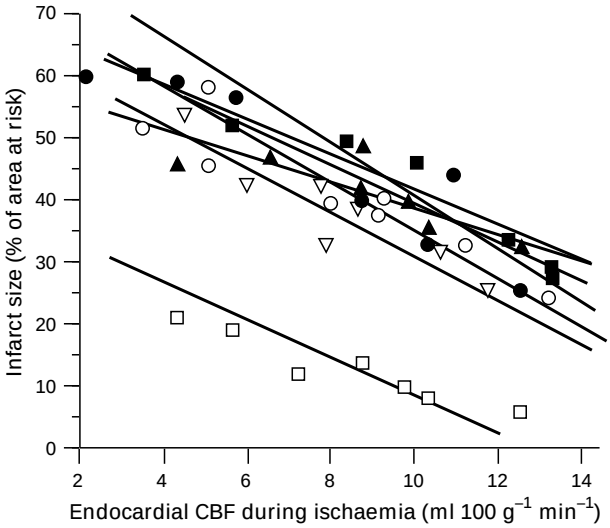

Figure 4 Plot of infarct size expressed as a percentage of risk area versus regional collateral blood flow (CBF) during ischaemia. The infarct size-limiting effect of PMA was completely abolished by the administration of GF109203X (▽) but not by dexamethasone (□). AMP-CP (■), 8-SPT (●), GF109203X (○) or dexamethasone (▲) did not increase the infarct size compared with the control group ($P<0.01$, ANCOVA).

1994a; 1996a). PKC is generally a critical component of intracellular signal transduction pathways, it plays an important role in cellular regulation, tumour promotion and oncogenesis (Nishizuka, 1988). With regard to the central role of PKC in IP, endogenous adenosine in the rabbit (Downey *et al*., 1994; Liu *et al*., 1994) or in the dog (Strasser *et al*., 1994) binds to A_1 adenosine receptors, while catecholamines bind to α_1-adrenoceptors in the dog (Kitakaze *et al*., 1995a), which may result in G protein-mediated activation of phospholipase C. The enzyme catalyzes the hydrolysis of phospatidylinositol 4,5-bisphosphate to produce diacylglycerol and inositol 1,4,5-trisphosphate. Diacylglycerol activates PKC present in the membrane, induces translocation to the membrane and activation of cytosolic PKC (Otani *et al*., 1988). Activated PKC then phosphorylates protein substrates and thereby is thought to mediate the protective effects of IP (DeJonge *et al*., 1995). Indeed, a PKC inhibitor, chelerythrine, has been shown to reduce the infarct size-limiting effect of IP in a rat model of ischaemia (Speechly-Dick *et al*., 1994). Both staurosporine and chelerythrine also blunt the functional recovery attributable to IP in isolated hearts of the rat (Mitchell *et al*., 1995). The precise mechanism by which activation of PKC protects the ischaemic heart has not been elucidated. We have shown that PKC activation induced by either IP or PMA appears to confer cardioprotection through activation of ecto-5′-N. A role for other substrates phosphorylated by PKC such as ATP-sensitive K^+ channels (Speechly-Dick *et al*., 1995) cannot be excluded, although the infarct size-limiting effect due to transient ligand-gated opening of these channels has also been shown to be blocked by AMP-CP (Kitakaze *et al*., 1995b). These observations are thus consistent with the hypothesis that activation of ecto-5′-N secondary to activation of PKC contributes to the cardioprotective effect of IP. Although the mechanism by which PKC increases ecto-5′-N activity has not been elucidated, it is likely that phosphorylation of ecto-5′-N by PKC changes either the characteristics of the active site or the conformation of the enzyme (Kitakaze *et al*., 1995c).

In the present study, we observed that IP translocates PKC from the cytosolic fractions to the membrane fraction, indicating that IP activates PKC in the canine myocardium. This observation is consistent with the observations of Strasser *et al*. (1994). However, PKC has also been shown to be inactive 10 min after the IP procedure (Simkhovich *et al*., 1995). Since we measured PKC activity 5 min after the IP procedure, PKC translocated to the membrane fraction returned to the cytosolic fraction in several minutes. This indicates that activation and translocation of PKC caused by IP is transient. Przyklenk *et al*. (1995) found that H-7 and polymyxin B did not attenuate the reduction in infarct size achieved with IP in dogs. The reason for the disparity between these and our results is unknown; however the different inhibitors of PKC used and their route of administration may provide a possible explanation. We administered GF109203X, a highly specific inhibitor of PKC, via a continuous intracoronary route.

In the present study, PKC activity in the cytosolic fraction was higher than that in the membrane fraction in both basal and postischaemic states. However, PKC in the cytosolic fraction is believed to be inactive for the signal transduction and activation of PKC during ischaemia is believed to be due to the translocation of PKC from the cytosolic to the membrane fraction. The present finding of PKC activity in the cytosolic fraction is compatible with the results of Przyklenk *et al*. (1995).

PMA may mimic the effect of IP by inducing coronary vasoconstriction (Ito *et al*., 1994), and in the present study CBF was found to be slightly decreased during administration of PMA. However, PMA did not affect the LER, an indicator of the severity of ischaemia. PKC increases the sensitivity of contractile proteins to Ca^{2+} in cardiomyocytes, and thus exerts a positive inotropic effect (Dixon & Haynes, 1989). Such an effect is not likely to contribute to the IP-like action of PMA because an increase in myocardial oxygen demand can expand infarct size. PMA also increases the release of NA (Shuntoh & Tanaka, 1986; Imamura *et al*., 1995; Tomsig & Suzuki, 1995), which may precondition the myocardium. Indeed, transient stimulation of α_1-adrenoceptors mimics IP in the rabbit (Tsuchida *et al*., 1994) and the dog (Kitakaze *et al*., 1994b). In the present study, PMA treatment did not increase plasma NA concentration.

In the present study, PMA at the dose used did not increase FS significantly. Although PKC increases the sensitivity of contractile proteins to Ca^{2+} (Watson & Karmazyn, 1991), other factors influencing myocardial contractility may decrease myocardial contraction. For example, CBF slightly decreased following the administration of PMA, which can decrease myocardial contractility via Gregg's phenomenon. Indeed, this dose of PMA has been found to have little effect on myocardial contractility in the rat heart (Watson & Karmazyn, 1991).

Although transient exposures to PMA did not increase ΔVA(Ado) before sustained ischaemia, both IP and PMA increased the production of adenosine during the early reperfusion period relative to the control group. This suggests that an increase in adenosine release during reperfusion may contribute to the cardioprotection induced by IP or PMA in the canine heart; the protective effect of PKC activation was blunted when either an inhibitor of ecto-5′-N or an antagonist of adenosine receptors was present.

Myocardial ischaemia increases the levels of NA (Schomig *et al*., 1984) and adenosine in coronary venous blood (Hori *et al*., 1986). However, since both NA and adenosine are rapidly washed out from the myocardium and especially adenosine is quickly degraded, the topical levels in coronary venous blood of these substances returned to the base-line 5 min after the 4th coronary occlusion or PMA exposure.

We have shown that adenosine release is increased 5 min after the onset of reperfusion in the IP and PMA groups. However, IP has been shown to cause a reduction in the degradation of adenine nucleotides, and less production of adenine nucleosides including adenosine, in the ischaemic myocardium (Jennings *et al*., 1991). Therefore, we needed to show that the level of myocardial adenosine and adenine nucleotides can be independent of the extracellular adenine nucleotides and adenosine levels. Extracellular adenosine levels depend on the activity of ecto-5′-N, concentration of AMP, activity of adenosine deaminase, and adenosine transport and washout, and intracellular adenine nucleotide levels depend on the energy state of cardiomyocytes. AMP, a substrate for ecto-5′-N, has been shown to exist in the extracellular space at levels sufficient to produce adenosine (Imai *et al*., 1987; Bunger *et al*., 1991). Therefore, even if levels of adenosine and adenine nucleotides in myocardial tissue are low during sustained ischaemia, extracellular adenosine levels near the plasma membrane may be higher in the IP and PMA group. When the adenosine level in the cellular surface is high, adenosine A_1-receptor-mediated-energy sparing effects may preserve high energy phosphates, and cause less degradation of high energy phosphates and adenine nucleotides in the cardiomyocytes. On the other hand, evidence has been presented in the cardiomyocytes. On the other hand, evidence has been presented indicating that increases in adenosine levels in the interstitial space are not augmented during sustained ischaemia after IP (Van Wylen, 1994). We have no explanation for this apparent difference.

Although basal adenosine release was detectable in the non-ischaemic condition, neither 8-SPT nor AMP-CP affected CBF. While the coronary vasodilator effects of adenosine are abolished, other coronary vasodilator substances, e.g., nitric oxide and prostacyclin, may compensate for the lack of the adenosine-induced vasodilatation and maintain CBF. Indeed, we have preliminary data showing that inhibition of nitric oxide increases the release of adenosine, which minimally affects CBF (Minamino & Hasegawa, 1995). However, in the ischaemic condition, both 8-SPT and AMP-CP reduce CBF probably because other vasodilator mechanisms are fully activated. Furthermore, AMP-CP only reduced the PMA-induced increase in adenosine levels to control values (493 ± 24 to 205 ± 21 pmol ml^{-1} after 5 min of reperfusion). This in-

dicates that other factors apart from ecto-5′-N may be involved in IP. Indeed, the adenosine concentration is mainly determined by (1) synthesis enzymes for adenosine, i.e., 5′-N and S-adenosylhomocysteine-hydrolase, (2) degradation or salvage enzymes for adenosine, i.e., adenosine deaminase and adenosine kinase, and (3) substrates, AMP and S-adenosylhomocysteine. These many pathways to increase the adenosine levels suggest that AMP-CP cannot completely attenuate the increased adenosine levels.

The present results have potential implications for the treatment of acute myocardial infarction: pretreatment with activators of PKC or ecto-5′-N may limit infarct size. Another possibility is to enhance adenosine release. Administration of adenosine (Olafsson *et al*., 1987) or adenosine derivatives such as ATP and diadenosine tetraphosphate (Node *et al*., 1995b) may limit infarct size. Although both strategies merit clinical investigation, the basic mechanism of IP requires further elucidation.

Limitation of the present study

In this study, we used a single dose of PMA, and did not use an inactive phorbol ester as a control. To exclude the possibility of nonspecific effects of PMA, the inhibitory effect of a specific inhibitor of PKC on the infarct size-limiting effect of PMA was examined. As GF109203X abolished the cardioprotective effect of PMA, PMA may mediate cardioprotection by activation of PKC. Hence, in a preliminary study it was shown that the dose of PMA used was the lowest dose needed to exert maximal cardioprotective effects.

Although AMP-CP was infused into the coronary artery during the experimental protocol, except during the 90 min ischaemic period, the arterial blood concentration of AMP-CP was not measured during sustained ischaemia. Therefore, it was not confirmed that this dose of AMP-CP was high enough to inhibit ecto-5′-N. The estimated AMP-CP level was 58.5 ± 4.9 μg ml^{-1} in the coronary artery, a level shown previously to inhibit ecto-5′-N (Kitakaze *et al*., 1994b). Also, in a previous study, we showed that the dose of AMP-CP used in the present experiments inhibits ecto-5′-N by 80–90% *in vivo* (Kitakaze *et al*., 1994a). Therefore, the dose of AMP-CP used in the present study may reach the level effective for reduction of production of adenosine.

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