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Effects of lysophosphatidylcholine on the production of interstitial adenosine *via* protein kinase C-mediated activation of ecto-5′-nucleotidase

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1 Adenosine plays a crucial role in the evolution of ischemic preconditioning. With the use of microdialysis techniques in *in situ* rat hearts, we assessed the activity of ecto-5′-nucleotidase (a key enzyme responsible for adenosine production), and examined the effects of lysophosphatidylcholine (LPC) on the production of interstitial adenosine.

2 The microdialysis probe was implanted in the left ventricular myocardium of anesthetized rat hearts and perfused with Tyrode solution containing adenosine 5′-monophosphate (AMP, 100 μM). With this system, the dialysate adenosine originates from the dephosphorylation of AMP, catalyzed by endogenous ecto-5′-nucleotidase. The level of dialysate adenosine is a measure of the ecto-5′-nucleotidase activity *in vivo*.

3 LPC at concentrations of 25 and 50 μM significantly increased the level of dialysate adenosine to $122.7 \pm 4.3\%$ ($n=4$, $P<0.05$) and $158.6 \pm 7.2\%$ ($n=5$, $P<0.05$) of the control, respectively. Chelerythrine (200 μM), a protein kinase C (PKC) inhibitor, completely abolished the increase of dialysate adenosine afforded by LPC (50 μM) ($n=5$).

4 These data provide the first evidence that LPC does increase the concentration of interstitial adenosine in rat hearts *in situ*, through the PKC-mediated activation of endogenous ecto-5′-nucleotidase.

Keywords: Lysophosphatidylcholine; palmitoylcarnitine; adenosine; ecto-5′-nucleotidase; protein kinase C; microdialysis

Introduction

Brief periods of ischemia attenuate the extent of cellular injury afforded by a subsequent prolonged ischemia, a phenomenon defined as ischemic preconditioning (Murry *et al*., 1987). Although the precise mechanism of ischemic preconditioning remains to be elucidated, the activation of protein kinase C (PKC) appears to be a prime and common factor, because several endogenous agonists thought to be coupled to PKC activation (e.g., adenosine, norepinephrine, bradykinin, or endothelin-1) mimic the ischemic preconditioning (cf. Cohen & Downey, 1996 for review). Adenosine, which is mainly produced by the dephosphorylation of adenosine 5′-monophosphate (AMP), catalyzed by 5′-nucleotidase, under ischemic conditions in the heart (Hori & Kitakaze, 1991; Schrader *et al*., 1991), is believed to be a central figure in ischemic preconditioning (Liu *et al*., 1991). It has been reported that the activity of ecto-5′-nucleotidase is markedly increased in preconditioned canine hearts (Kitakaze *et al*., 1993; Kitakaze *et al*., 1994). More recently, we have presented evidence that α_1-adrenoceptor stimulation enhances the production of interstitial adenosine *via* PKC-mediated activation of ecto-5′-nucleotidase in *in vivo* rat hearts, using microdialysis techniques as a tool to assess the activity of ecto-5′-nucleotidase *in situ* (Sato *et al*., 1997a).

Lysophosphatidylcholine (LPC), an endogenous amphiphilic lipid metabolite, accumulates within a few minutes after ischemia and contributes to the development of arrhythmias in ischemic hearts (Corr *et al*., 1995). Although LPC is known to exert detergent-like cytotoxic effects, it has been reported that low concentrations of LPC activate PKC, purified from pig brains (Oishi *et al*., 1988) and that LPC is also able to activate phospholipase D (PLD) in coronary endothelial cells (Cox & Cohen, 1996). The latter enzyme is reported to be involved in the activation cascade of PKC in ishcemia-preconditioning of the heart (Cohen *et al*., 1996). Accordingly, it is reasonable to speculate that the activation of PKC by LPC contributes to the evolution of ischemic preconditioning. The present study was designed to investigate whether LPC does increase the production of interstitial adenosine in *in situ* hearts and to determine the possible role of LPC-mediated PKC activation in the ischemic preconditioning. To achieve this goal, we used a flexibly mounted microdialysis technique (Obata *et al*., 1994) and measured the concentration of interstitial adenosine under a constant supply of AMP through the microdialysis probe. With this system, the level of dialysate adenosine gave an appropriate index of the activity of ecto-5′-nucleotidase, in a particular region of the myocardial interstitial space (Sato *et al*., 1997a, b). Our results indicate that LPC increases the production of interstitial adenosine via the PKC-mediated activation of ecto-5′-nucleotidase. The implications of this event for ischemic preconditioning are discussed.

Methods

Animal preparation

Wistar rats of either sex (250–300 g) were anesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and were ventilated (after intubation) with room air supplemented with oxygen. The chest was opened at the left fifth intercostal space, and the pericardium was removed to expose the left ventricle. At the end of the experiments the rats were killed by an overdose of

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the same anesthetic. All procedures followed in dealing with the experimental animals met the guidelines stipulated by the Physiological Society of Japan.

Microdialysis technique

Details of the technique required for manipulation of the flexibly mounted microdialysis probe in *in vivo* rat hearts (to measure the interstitial adenosine) have been described (Obata *et al*., 1994; Sato *et al*., 1997a, b). In brief, the tip of the microdialysis probe (3 mm in length and 220 μm o.d. with the distal end closed) was made of dialysis membrane (cellulose membrane 10 μm thick with a 50,000 molecular weight cut-off). Two fine silica tubes (75 μm i.d.) were inserted into the tip of a cylinder-shape dialysis probe and served 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 dialysate reservoir. These tubes were supported loosely at the mid-point on a semi-rotatable stainless-steel wire, so that their movement fully synchronized with the rapid up-and-down motion of the tip caused by the heart beats. The probe was implanted from the epicardial surface into the left ventricular myocardium and was perfused through the inlet tube with Tyrode solution of the following composition (in 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 the 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. 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 the adenosine concentration in the dialysate

The dialysate (flowing at the rate of 1.0 μl min^{-1}) was collected into a series of reservoirs for every 15 min, consecutively (15 μl in each reservoir). 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 × 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} using a pumping system (JASCO Corp., PU-980, Tokyo, Japan). The absorbance of the column eluate was monitored at 260 nm using a ultraviolet detector (JASCO Corp., UV-970). The absorbance peak of adenosine was quantified by comparing the retention time and peak height with a known adenosine standard concentration of 1 or 10 μM. The adenosine concentrations are presented here as raw data, and are not corrected for recovery rate (18%) unless otherwise mentioned.

Chemicals

Adenosine 5′-monophosphate (AMP, Wako Pure Chemical Co., Osaka, Japan), L-α-lysophosphatidylcholine palmitoyl (LPC, Sigma Chemical Co., St. Louis, MO, U.S.A.) and L-palmitoylcarnitine hydrochloride (PALC, Sanwa Chemicals, Nagoya, Japan), were prepared immediately before the start of the 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. Chelerythrine and α,β-methyleneadenosine 5′-diphosphate (Sigma Chemical Co.) were dissolved in distilled water and kept at 10 mM stock solutions. Diacylglycerol (DAG); 1,2-dioctadec-9′-enoyl-*sn*-glycerol (Sigma Chemical Co.) was dissolved in methanol as a 10 mM stock solution. An appropriate volume of each stock solution was added to the Tyrode solution immediately before use, as indicated in the Results. In the final concentrations used, the solvent had no effect on the level of dialysate adenosine.

Statistical analysis

All values are indicated as means ± s.e.mean. ANOVA combined with a Fisher's *post hoc* test or Student *t*-test was used to determine the significant difference. A *P*-value of less than 0.05 was regarded as statistically significant.

Results

We first examined the effect of LPC on the dialysate adenosine concentration in the presence of AMP and evaluated the activity of ecto-5′-nucleotidase *in vivo*. In this series of experiments, an AMP concentration of 100 μM was perfused throughout the experiments *via* a microdialysis probe; and the dialysate sampling was started after a 45 min equilibration period, the time defined previously (Sato *et al*., 1997a, b). The application of LPC (50 μM) through the probe increased the adenosine concentrtion in the dialysate significantly from 7.44 ± 1.23 μM to 11.88 ± 2.05 μM at 30–45 min after the beginning of the LPC application ($n = 5$, $P < 0.05$) (Figure 1a). After the removal of LPC from the perfusate, the adenosine concentration reverted to 6.58 ± 0.94 μM in 30 min. By contrast, when α,β-methyleneadenosine 5′-diphosphate (100 μM) was perfused concomitantly with AMP (100 μM), LPC (50 μM) did not increase the level of dialysate adenosine (0.75 ± 0.06 μM, before *vs* 0.68 ± 0.09 μM, after 30–45 min application of 50 μM LPC; $n = 5$).

To determine whether the LPC-induced increases in dialysate adenosine concentration were the result of an increase in PKC activity, the effect of LPC on the level of AMP-primed dialysate adenosine was examined in the presence of cherlerythrine, a potent and selective PKC inhibitor that interacts with the catalytic domain of this enzyme (Herbert *et al*., 1990). As shown in Figure 1b, when chelerythrine at 200 μM was present in the perfusate concomitantly with AMP, the introduction of LPC (50 μM) could not increase the concentrationof dialysate adenosine at all (7.61 ± 0.23 μM, baseline *vs* 7.28 ± 0.70 μM, 30–45 min after LPC; $n = 5$). This effect of chelerythrine was concentration-dependent and the level of adenosine was increased ~10% by 50 μM LPC (after 30–45 min) in the presence of much lower concentration of chelerythrine (100 μM, not illustrated). On the other hand, the application of chelerythrine (at a concentration of 200 μM) *per se* did not affect the dialysate adenosine measured in the presence of 100 μM AMP ($n = 5$, cf. Figure 2).

The change of LPC concentrations in the perfusate (25–200 μM) altered the levels of dialysate adenosine as summarized in Figure 2. LPC at concentrations of 25 and 50 μM significantly increased the level of dialysate adenosine (measured at 30–45 min after application) to 122.7 ± 4.3% ($n = 4$, $P < 0.05$) and 158.6 ± 7.2% ($n = 5$, $P < 0.05$) of the

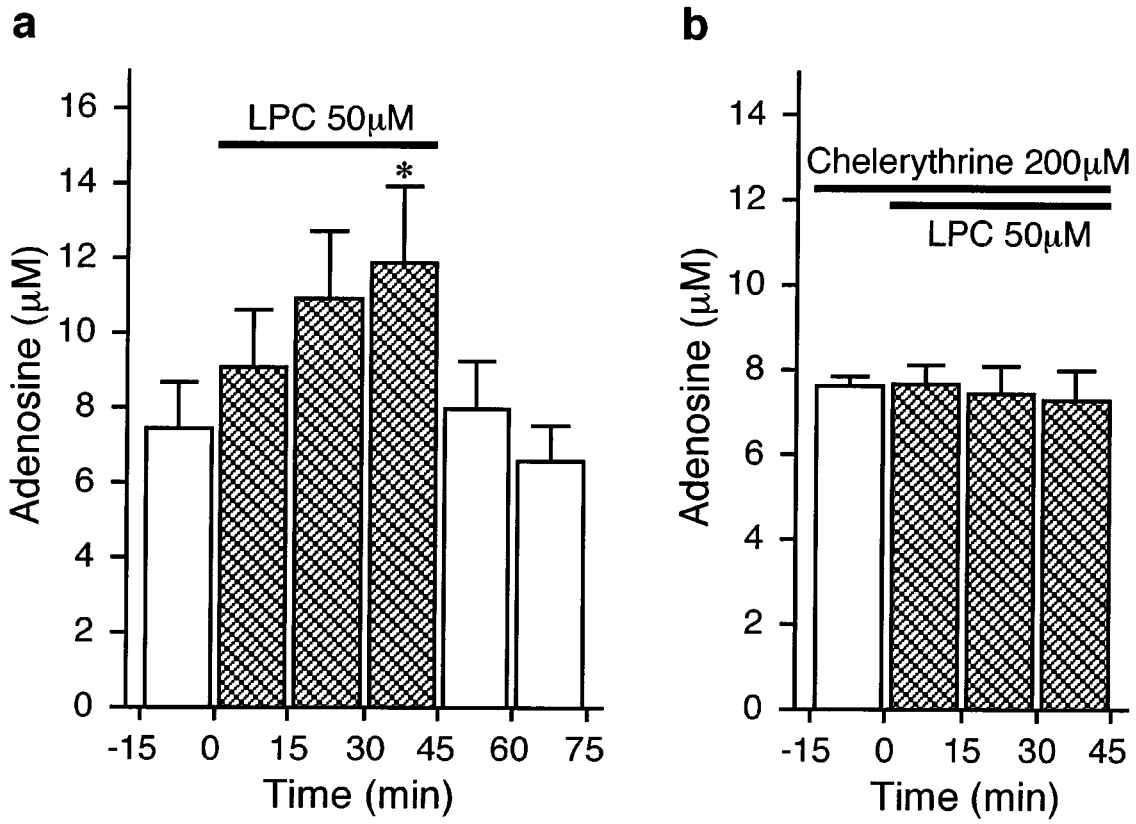

Figure 1 Effects of L-α-lysophosphatidylcholine palmitoyl (LPC) on the production of intersitial adenosine in rat ventricular myocardium. (a) Sequential changes of the dialysate adenosine concentration measured in the presence of 100 μM AMP throughout. LPC (50 μM) was added to the perfusate for 45 min, as indicated by a horizontal bar ($n=5$). (b) Antagonizing effects of chelerythrine on LPC-induced increases in dialysate adenosine ($n=5$). The abscissa denotes the time in min before and after the introduction of LPC. Values are means±s.e.mean. *$P<0.05$ *vs* pre-LPC value.

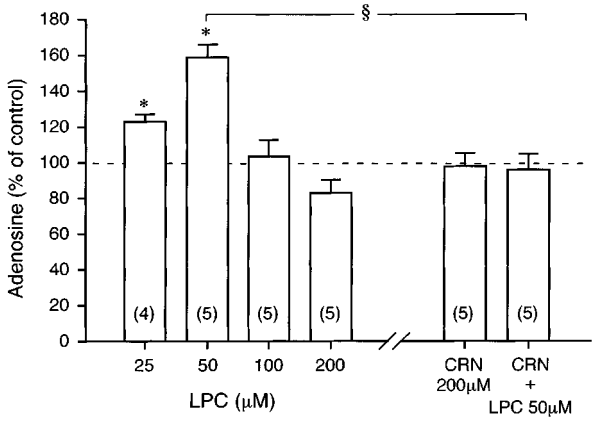

Figure 2 Effects of LPC and chelerythrine (CRN) on the AMP-primed dialysate adenosine concentration. Concentrations of adenosine, measured at 30–45 min after application of LPC and/or CRN, are given as a percentage of the control (pre-drug) value. Each column and vertical bar indicates mean±s.e.mean with the number of experiments in parenthesis. *$P<0.05$ *vs* control (pre-LPC). §$P<0.01$: significant difference between the data connected by the bracket.

control, respectively. In contrast, LPC at concentrations of 100 μM did not increase and 200 μM rather decreased the level of dialysate adenosine. In the presence of chelerythrine, the LPC (50 μM)-induced increases in dialysate adenosine concentration were significantly inhibited, and the levels of dialysate adenosine remained at the level of 95.7±8.8% of the control ($n=5$, $P<0.01$ *vs* LPC alone). These results suggest that LPC increased the AMP-primed dialysate adenosine concentration (i.e., the activity of ecto-5′-nucleotidase) *via* activation of PKC.

To further support this notion, we examined the effects of diacylglycerol (DAG), a potent PKC activator (Nishizuka, 1995), and L-palmitoylcarnitine (PALC), a long-chain acylcarnitine or a reported PKC inhibitor (Katoh *et al*., 1981), on the production of interstitial adenosine. The experimental protocol was the same as used for Figure 1a, except that LPC was replaced by DAG or PALC. As shown in Figure 3, DAG at a concentration of 50 μM increased the concentration of dialysate adenosine from 8.56±1.42 μM to 11.70±1.57 μM within 45 min ($n=5$, $P<0.05$). After the removal of DAG from the perfusate, the level of adenosine significantly decreased to 6.60±1.28 μM in 30 min. By contrast, as shown in Figure 4a, PALC at a concentration of 50 μM did not increase, but rather decreased the level of dialysate adenosine (primed by 100 μM AMP) from 8.51±0.85 μM to 7.13±0.41 μM after a 30–45 min application ($n=5$), albeit the changes were not statistically significant. The same experiments were repeated with different concentrations of PALC and the changes in dialysate adenosine concentrations measured after a 30–45 min application of PALC are summarized in Figure 4b. In all the concentrations tested (25–200 μM), the changes were not significant when compared with the value measured without this compound.

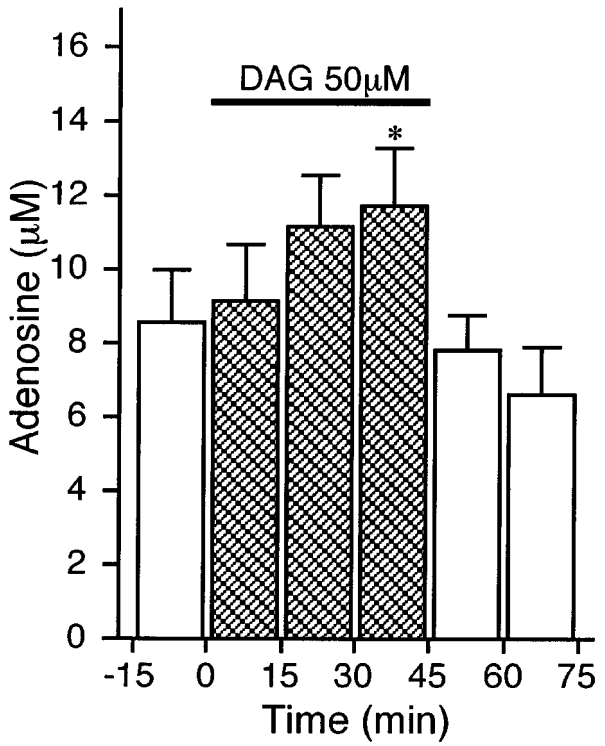

Figure 3 Effects of diacylglycerol (DAG) on the interstitial adenosine concentration. DAG (50 μM) was added to the perfusate in the continued presence of 100 μM AMP, as indicated by a horizontal bar ($n=5$). Values are means ± s.e.mean. *$P<0.05$ *vs* pre-DAG value.

Discussion

It has been reported that adenosine behaves as an endogenous cardioprotectant (Ely & Berne, 1992). In the present study, we evaluated the effects of LPC on the production of interstitial adenosine in *in situ* rat hearts, with the use of flexibility mounted microdialysis techniques (Obata *et al*., 1994). More recently, with this technique, we provided the evidence that the level of dialysate adenosine measured in the presence of AMP reflects the activity of *ecto*-5′-nucleotidase in the particular tissue (Sato *et al*., 1997a, b). This notion was supported by the findings as follows: (i) AMP-induced increases in the dialysate adenosine concentration depended on the AMP concentrations used, and the EC_{50} of AMP was ~100 μM, a value close to the K_m estimated for *ecto-* (rather than for *cytosolic*) 5′-nucleotidase in rat hearts (Sullivan & Alpers, 1971; Truong *et al*., 1988); (ii) When a selective ecto-5′-nucleotidase inhibitor, α,β-methyleneadenosine 5′-diphosphate (Headrick *et al*., 1992; Darvish & Metting, 1993) was present in the perfusate, AMP-induced increases in the dialysate adenosine concentration could not be detected.

With the use of the same experimental design, we demonstrated that LPC at concentrations less than 50 μM increased the level of AMP-primed dialysate adenosine in *in situ* rat hearts (Figures 1 and 2). In addition, in the presence of α,β-methyleneadenosine 5′-diphosphate, LPC failed to increase the level of AMP-primed dialysate adenosine. Therefore, it is likely that the LPC-induced increases in adenosine concentration originated from the activation of ecto-5′-nucleotidase. However, other possibilities should be considered: (i) LPC attenuated the break-down of adenosine (e.g., by inhibiting

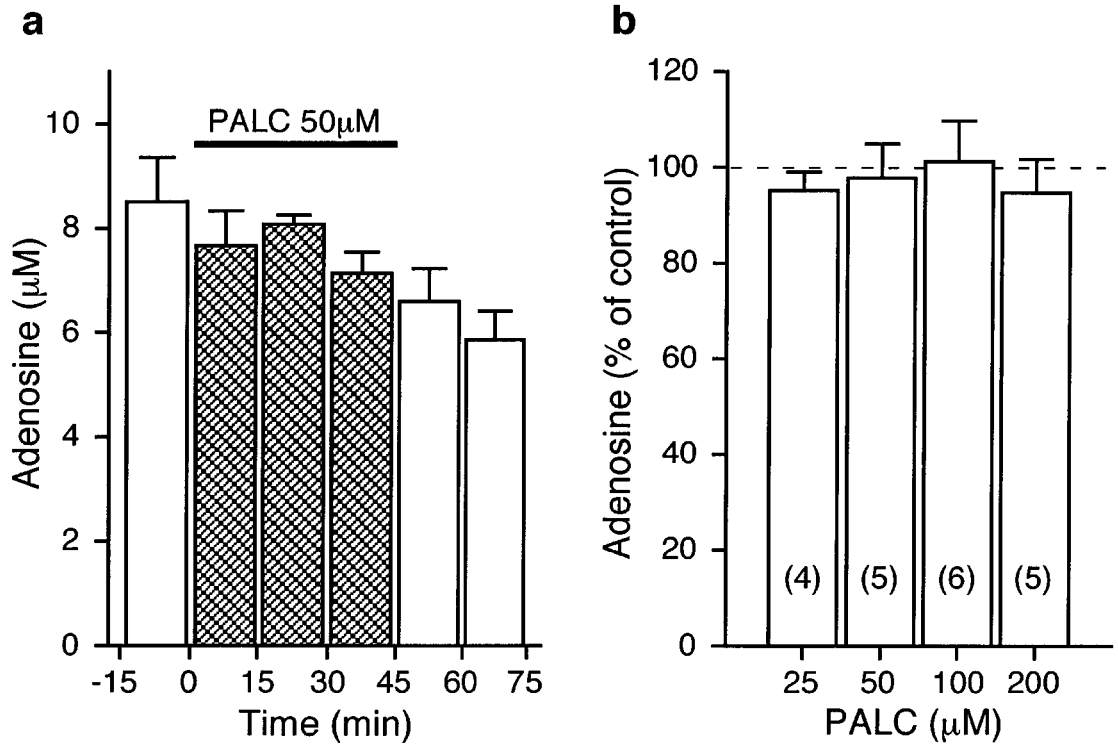

Figure 4 Effects of L-Palmitoylcarnitine hydrochloride (PALC) on the level of AMP (100 μM)-primed dialysate adenosine. (a) Sequential changes of the dialysate adenosine concentration seen after application of PALC. Abscissa denotes the time in min before and after the introduction of PALC for 45 min, as indicated by a horizontal bar ($n=5$). (b) Effects of various concentrations of PALC on the AMP-primed dialysate adenosine concentration. Concentrations of adenosine measured after 30–45 min after application of PALC at each concentration are given as a percentage of the value measured before PALC application (100%). Each column and vertical bar indicates mean ± s.e.mean with the number of experiments in parenthesis.

adenosine deaminase) or the uptake of adenosine into cytoplasm and increased dialysate adenosine; and/or (ii) the non-specific detergent effect of LPC led to the increase in dialysate adenosine. However, in our preliminary observation, LPC increased not only the level of adenosine but also the level of inosine measured in the presence of AMP (from 7.76 ± 0.72 μM to 11.11 ± 1.14 μM, $n=4$). Moreover, LPC (50 μM) did not affect the level of dialysate adenosine when measured in the absence of AMP (our unpublished observation). Therefore, the possibility (i) listed above is not likely. Both LPC and PALC incorporate into the sarcolemmal phospholipid bilayer of the myocardium and exert deleterious effects on the membrane function *via* the amphipathic activity, a character shared by both compounds (Corr *et al*., 1995). Unlike LPC, PALC which has a structural similarity to LPC (i.e., a single and long aliphatic hydrocarbon chain with 16 carbon atoms esterified to the hydrophilic polar headgroup) did not increase the AMP-primed dialysate adenosine concentration (Figure 4). Furthermore, propionylcarnitine, which was reported to be a potent antagonist to the detergent action of LPC (Sato *et al*., 1996), could not inhibit the LPC-induced increases in the dialysate adenosine (our unpublished observation). These results suggest that the increase in the adenosine concentration induced by LPC did not result from a non-specific or detergent-like effect of this compound.

We have shown that α_1-adrenoceptor stimulation and subsequent activation of PKC increased the interstitial adenosine in the rat heart, *via* the activation of endogenous ecto-5′-nucleotidase (Sato *et al*., 1997a). LPC at low concentrations increased the AMP-primed dialysate adenosine, whereas high concentrations of LPC did not (Figure 2). In agreement with our present observation, Oishi *et al*. (1988) reported that LPC modified PKC activity (purified from pig brains) in a biphasic manner, i.e., LPC at low concentrations (<20 μM) stimulated, while at high concentrations (>30 μM), inhibited PKC. Moreover, LPC failed to increase the AMP-primed dialysate adenosine in the presence of chelerythrine (an inhibitor of PKC). In addition, DAG, a potent activator of PKC (Nishizuka, 1995), increased the AMP-primed dialysate adenosine concentration (Figure 3). Taken in concert, these findings lend support to the notion that LPC increases the interstitial adenosine concentration via the PKC-mediated activation of ecto-5′-nucleotidase.

Several lines of experimental evidence suggest that stimulation of a variety of G protein-coupled receptors (e.g. adenosine A_1, α_1-adrenergic, muscarinic, bradykinin, and endothelin-1 receptors) leads to the activation of PKC and which is involved in the evolution of ischemic preconditioning (cf. Cohen & Downey, 1996 for review). Moreover, phospholipase D (PLD) plays a crucial role in the activation cascade of PKC in preconditioned hearts (Cohen *et al*., 1996). In the myocardium subjected to ischemia, LPC is rapidly produced and accumulates in both the extra- and intra-cellular side of the membrane, within a few minutes (Corr *et al*., 1995). Recently, it has been reported that LPC stimulates PLC in human endothelial cells (Cox & Cohen, 1996). Thus, we speculate that LPC accumulated in the ischemic heart activates PKC, perhaps *via* stimulation of PLD, and enhanced the ecto-5′-nucleotidase activity, leading to increased interstitial adenosine. Although the increase in dialysate adenosine became significant only after a 30 min application of LPC, the time may depend on the rate of diffusion of the compound into the interstial space. Our present observation may provide an attractive explanation that LPC (at relatively low concentrations) is, at least in part, responsible for the alleged protection mechanism against ischemic insult, i.e. ischemic preconditioning.

The metabolism of phospholipids is significantly altered in an ischemic myocardium, and numerous end-products, such as lysophospholipids and plasmalogenic diglycerides, seem to be potential activators of PKC (Oishi *et al*., 1988; Ford & Gross, 1990; Sasaki *et al*., 1993; Liscovitch & Cantley, 1994; Nishizuka, 1995; Sando & Chertihin, 1996). Therefore, the role of ischemia-derived phospholipid metabolites, for the evolution and maintenance of ischemic preconditioning, needs to be further investigated.

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