Lysophosphatidylcholine induces arachidonic acid release and calcium overload in cardiac myoblastic H9c2 cells

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Abstract **Lysophosphatidylcholine (lyso-PC) and arachidonate are products of phosphatidylcholine hydrolysis by phospholipase A_2. In this study, the modulation of arachidonate release by exogenous lyso-PC in rat heart myoblastic H9c2 cells was examined. Incubation of H9c2 cells with lyso-PC resulted in an enhanced release of arachidonate in both a time- and dose-dependent fashion. Lyso-PC species containing palmitoyl ($C_{16:0}$) or stearoyl ($C_{18:0}$) groups evoked the highest amount of arachidonate release, while other lysophospholipid species were relatively ineffective. Cells treated with phospholipase A_2 inhibitors resulted in the attenuation of the enhanced arachidonate release in the presence of lyso-PC. Lyso-PC caused the translocation of phospholipase A_2 from the cytosol to the membrane fraction and induced an increase in Ca^{2+} flux from the medium into the cells. Nimodipine, a specific Ca^{2+}-channel blocker, partially attenuated the lyso-PC-induced rise in intracellular Ca^{2+}. Concurrent with Ca^{2+} influx, lyso-PC caused an enhancement of protein kinase C activity. The lyso-PC-induced arachidonate release was attenuated when cells were preincubated with specific protein kinase C and mitogen activated protein kinase kinase inhibitors. Taken together, these results strongly indicate that the lyso-PC-induced increases in levels of intracellular calcium and stimulation of protein kinase C lead to the activation of cytosolic phospholipase A_2 which results in the enhancement of arachidonate release in H9c2 cells.**—Golfman, L. S., N. J. Haughey, J. T. Wong, J. Y. Jiang, D. Lee, J. D. Geiger, and P. C. Choy. **Lysophosphatidylcholine induces arachidonic acid release and calcium overload in cardiac myoblastic H9c2 cells.** ***J. Lipid Res.*** **1999.** 40: **1818–1826.**

Supplementary key words lysophospholipids • calcium transport • myoblasts • MAPK • PKC • sarcolemma • Phospholipase A_2 • myocardial ischemia

The principal pathway for the hydrolysis of phosphatidylcholine is via the action of phospholipase A_2 (1). In mammalian tissues, several major isoforms of phospholipase A_2 have been identified. Each form appears to differ from the others in the primary structure, subcellular localization, calcium requirement for activation, and substrate specificity (reviewed in 2–6). Two forms of calcium-dependent phospholipase A_2 are prominent in mammalian cells; the type II 14-kDa secretory form (s-form) (7) and the type IV, 85–110 kDa cytosolic form (c-form) (3, 6, 8, 9). In addition to both isoforms of phospholipase A_2, a calcium-insensitive 40-kDa phospholipase A_2 isoform is present in cardiovascular tissues (9). The c-phospholipase A_2 requires that Ca^{2+} increase to the micromolar range for translocation of the enzyme to the phospholipid-containing membrane, and possesses a high specificity for the arachidonoyl group at the *sn*-2 position of the phospholipid molecule (9). The s-phospholipase A_2 requires that Ca^{2+} increase to millimolar levels for maximum activity and shows little or no selectivity towards the chain length, number of unsaturated bonds of the acyl group at the *sn*-2 position (9). The c-phospholipase A_2 activity is regulated directly by phosphorylation at serine[505] via the mitogen-activated protein kinase (MAPK) (6, 9), and indirectly by protein kinase C, but the role of protein kinase C in direct phosphorylation of c-phospholipase A_2 in vivo is unclear at present (4, 6).

The hydrolysis of phosphatidylcholine by the action of phospholipase A_2 results in the production of lysophosphatidylcholine (lyso-PC) and a fatty acid. Due to its amphipathic property, lyso-PC is cytolytic at high concentrations and its intracellular levels are therefore under rigid control (10). The majority of lyso-PC formed in the tissue is rapidly metabolized or reacylated under normal physiological conditions (11–13). In ischemic myocardium, lyso-PC accumulates (14–16) and leads to electrophysiological and mechanical dysfunction of the heart (17, 18). A relationship between high lyso-PC concentrations and calcium overloading in cardiac tissues has been postulated (19–21), but the mechanism for this phenomenon has been subjected to much debate (20–23). In plasma, the concentration of lyso-PC is normally low (24) but high amounts of lyso-PC are found in atherogenic lipoproteins

Abbreviations: lyso-PC, lysophosphatidylcholine; MAPK, mitogen-acitivated protein kinase; $[Ca^{2+}]_i$ intracellular calcium concentration.

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such as the oxidatively modified low density lipoprotein and β-very low density lipoprotein (25, 26). In the last decade, the accumulation of lyso-PC in atherosclerotic and inflammatory lesions of vascular vessels has been reported (14, 27). Furthermore, lyso-PC induces the expression of mononuclear leukocyte adhesion molecules (28), gene expression of potent smooth muscle growth factors in monocytes and in cultured human endothelial cells (29), modulates smooth muscle contractility (30), and acts as a chemotactic factor for human T lymphocytes (31) and monocytes (32).

It is clear that lyso-PC produced in the plasma may be an important signal molecule that impairs endothelium-dependent relaxation of blood vessels (27). In addition to lyso-PC, free fatty acids are produced from the hydrolysis of phosphatidylcholine by phospholipase A_2. The release of arachidonic acid from phospholipids is regarded as an important step for the biosynthesis of eicosanoids (33). In most mammalian tissues including the cardiac tissue, arachidonate is converted to prostacyclin, a potent vasodilator which would attenuate the impairment of endothelium-dependent relaxation of blood vessels produced by lyso-PC.

In view of the antagonistic effect of lyso-PC and arachidonic acid on endothelium-dependent relaxation of blood vessels, the primary objective of this study was to determine the effects of lyso-PC on arachidonic acid release in non-vascular cells. The H9c2 cell line was selected as a model for this study because it was derived from the rat heart but retained many of the properties of the skeletal muscle (34). Secondary objectives of this study were to determine the involvement of Ca^{2+}, protein kinase C, and mitogen-activated protein kinase in the modulation of c-phospholipase A_2 activity.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium and Dulbecco's phosphate-buffered saline were obtained from Sigma Chemical Company (St. Louis, MO). Heat-inactivated newborn calf serum and trypsin were obtained from Life Technologies, Inc. Staurosporine, H-89, arachidonyl trifluoromethyl ketone ($AACOCF_3$), PD098059, and bisindolylmaleimide I were products of Biomol Inc. (Plymouth Meeting, PA). Ro31-8220 was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA) and para-bromophenacyl bromide and all other chemicals were reagent grade and were purchased from Sigma. [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (210 Ci/mmol), adenosine 5′-[γ-^{32}P]triphosphate (3,000 Ci/mmol), 1-stearoyl-2-[1-^{14}C]arachidonyl-l-3-phosphatidylcholine (55 mCi/mmol) and 1-[1-^{14}C]palmitoyl-l-lyso-3-phosphatidylcholine (55 mCi/mmol) were all obtained from Amersham Corp. Lysophospholipids and all lipid standards were purchased from Serdary Research Laboratory (London, Ontario, Canada). Thin-layer chromatography plates (silica gel G) were the product of Fisher Scientific.

Cell culture, radiolabeling, and challenge of cells

Rat heart myoblast (H9c2) cells were obtained from ATCC and cultured according to the company's guidelines. The cells were grown in flasks or culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum albumin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1.25 μg/ml fungizone. Cells 85–90% confluent were used for all subsequent experiments.

Cells were radiolabeled (35) in 35-mm culture dishes and were incubated for 16–20 h with 1 μCi/ml [^{3}H]arachidonate in Dulbecco's modified Eagle's medium containing 1% bovine serum albumin. The cells were washed 3 times with HEPES-buffered saline containing 140 mm NaCl, 4 mm KCl, 5.5 mm glucose, 10 mm HEPES, 1.5 mm $CaCl_2$, and 1.0 mm $MgCl_2$, pH 7.4, and 0.1% (w/v) essentially fatty acid-free bovine serum albumin. Aliquots of lysophospholipids were dissolved in chloroform–methanol 2:1 (v/v), evaporated under N_2, and the lysophospholipid samples were resuspended in HEPES-buffered saline containing bovine serum albumin.

The binding of lysophospholipid to H9c2 cells was studied in the following manner. Cells were cultured on 60-mm plates with Dulbecco's modified Eagle's medium containing 100 nm [^{14}C]lyso-PC (55 nCi/nmol) for 15 min. Subsequently, the medium was removed and cells were incubated for another 15 min with the same medium containing 10 μm non-radioactive lysophospholipid (100 times excess) or 0.1% bovine serum albumin. After the second incubation, the cells were dislodged from the culture dish in HEPES-buffered saline. The labeled lysophospholipid content in each dish was determined by scintillation counting.

Measurement of arachidonate release

The arachidonate released from the cells was determined as described previously (36). Briefly, the lysophospholipid was added to the cell culture and incubated for the prescribed period. The buffer was then removed and acidified with 50 μl of glacial acetic acid. A 0.8-ml aliquot was used for lipid extraction in a solvent mixture consisting of chloroform–methanol–water 4:3:2 (by volume). Oleic acid was added as an internal fatty acid standard. The free fatty acid fraction in the organic phase was resolved by thin-layer chromatography in a solvent system consisting of hexane–diethyl ether–acetic acid 70:30:1. The fatty acid fraction was visualized by iodine vapor, and its radioactivity was determined by liquid scintillation counting.

Determination of phospholipase A_2 activity

Cells were lysed by sonication in a buffer containing 50 mm Tris-HCl (pH 8.0), 1 mm EDTA, 10 μm leupeptin, 10 μm aprotinin, 20 mm NaF, and 10 mm Na_2HPO_4. Cell lysates were centrifuged at 100,000 *g* for 1 h. The supernatant was designated as the cytosolic fraction, while the pellet was designated as the membrane fraction and was resuspended in the buffer described above. Phospholipase A_2 activity in the subcellular fractions was determined by the hydrolysis of 1-stearoyl-2-[1-^{14}C] arachidonyl-*sn*-glycero-3-phosphocholine to yield free radiolabeled arachidonate (35). The assay mixture contained 50 mm Tris-HCl (pH 8.0), 1.5 mm $CaCl_2$, 0.9 nmol of 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (100,000 dpm/assay), and approximately 10 μg of protein in a final volume of 100 μl. The reaction mixtures were incubated at 37°C for 30 min and the reactions were terminated by the addition of 1.5 ml of chloroform–methanol 2:1. Total lipid was extracted and the radioactivity of arachidonate released was determined as described above. The amounts of protein in samples were determined by the bicinchoninic acid method.

Intracellular Ca^{2+} determinations

H9c2 cells were plated on poly-d-lysine-coated 35-mm glass cover slips 3–4 days prior to experimentation. $[Ca^{2+}]_i$ levels were determined using the Ca^{2+}-specific fluorescent probe Fura-2/AM. Cells were incubated for 40 min at 25°C followed by 10 min at 37°C in HEPES-buffered saline buffer containing 1.2

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mm Ca^{2+}, 0.1% BSA, and 2 μm Fura-2/AM. The cover slips containing Fura-2-loaded cells were placed in a PDMI-2 open perfusion micro-incubator (Medical Microsystems Corp., Greenvale, NY) set at 37°C. Cells were superfused at a rate of 2 ml/min with HEPES-buffered saline during baseline measurements. After 5 min, flow was stopped and buffer was removed and replaced with HEPES-buffered saline containing lyso-PC (25–150 μm). Cells were alternately excited at 340 and 380 nm, and emission was recorded at 510 nm with a video-based Universal Imaging System (EMPIX, Mississauga, ON). R_{max}/R_{min} ratios were converted to nanomolar $[Ca^{2+}]_i$ according to the method of Grynkiewicz, Poenie, and Tsien (37). Images were acquired every 15 sec during baseline measurements and every 5 sec after lyso-PC additions by real-time averaging of 16 frames of each wavelength that included a background reference subtraction from each of the acquired images. Increases in $[Ca^{2+}]_i$ represent average cytosolic concentrations determined by subtracting $[Ca^{2+}]_i$ levels at 5, 10, and 15 min after lyso-PC applications from baseline $[Ca^{2+}]_i$. All cells in the visual field were monitored for time periods up to 30 min.

Determination of PKC activity

Cells were sonicated in buffer B (50 mm Tris-HCl, pH 7.5, 5 mm EDTA, 10 mm EGTA, 0.25 m sucrose, 0.3% β-mercaptoethanol, 10 μm benzamidine, 1 mm PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and were centrifuged at 1,500 *g* for 10 min. The supernatants were subjected to ultracentrifugation at 100,000 *g* for 1 h to obtain soluble and membrane fractions. Approximately 15–30 μg of protein from these fractions was used to determine PKC activity using a PKC assay kit (Amersham), which is based on the incorporation of ^{32}P from [γ-^{32}P]ATP into a PKC-specific substrate peptide.

Statistical analyses

The data were analyzed with a two-tailed independent Student's *t* test or, where appropriate, a one-way analysis of variance (ANOVA) followed by Duncan's new multiple range post hoc test to detect individual differences. The level of significance was determined at $P < 0.05$. All values represent mean ± standard error of the mean.

RESULTS

The effect of lyso-PC on arachidonate release in H9c2 cells was examined. Cells were prelabeled with [^{3}H]arachidonate and then incubated with HEPES-buffered saline containing 0.1% bovine serum albumin and 0 or 150 μm lyso-PC for various time periods (**Fig. 1A**). Lyso-PC elicited a time-dependent enhancement of arachidonate release, which reached a maximum at 15 min of incubation. The enhancement of arachidonate release was slightly diminished after the first 15 min. The presence of 100 μm lyso-PC resulted in optimal arachidonate release (Fig. 1B) irrespective of the bovine serum albumin concentration (0.025–0.1% w/v) (4–16 μm). Hence, 100 μm lyso-PC in a medium containing 0.1% bovine serum albumin was used in all subsequent studies. Cell viability was confirmed by trypan blue exclusion that showed minimal dye infiltration under the conditions described above.

Lyso-PC is an amphiphilic molecule which can be incorporated into lipid membranes. Thus, we performed binding studies to determine the nature of the association of lyso-PC with the H9c2 cells. These cells were labeled with 1-[1-^{14}C]palmitoyl-lyso-PC (100 nm, 55 nCi/nmol) for 15 min, and a considerable amount (45 ± 7%) of radioactivity was found to be associated with cells after the incubation. When lipids were extracted from these cells and analyzed by thin-layer chromatography, the majority of the radioactivity (>90%) in the lipid extract was found in the lyso-PC fraction. Subsequently, these cells were incu-

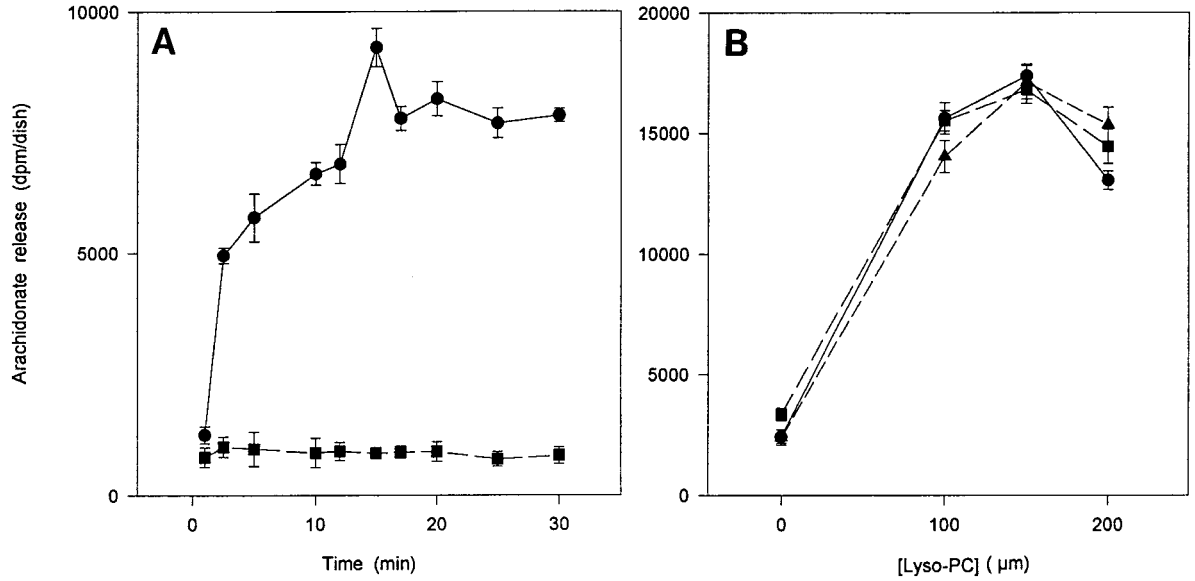

Fig. 1. Effect of lyso-PC on arachidonate release in H9c2 cells. Cells were prelabeled with 1 μCi/ml of [^{3}H]arachidonate in Dulbecco's modified Eagle's medium containing 1% newborn calf serum for 16–20 h. Cells were washed 3× with HEPES-buffered saline containing 0.1% bovine serum albumin (w/v) prior to challenge. A: cells were challenged with 0 μm (■) or 100 μm (●) lyso-PC in HEPES-buffered saline containing 0.1% bovine serum albumin for the time period as indicated. B: cells were challenged for 15 min with lyso-PC in HEPES-buffered saline containing 0.025% (▲), 0.05% (■), or 0.10% (●) bovine serum albumin. Arachidonate release into the medium was determined. Values represent means ± standard error of the mean from 4 separate experiments.

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bated for another 15 min in the control medium (without lyso-PC) or medium containing 10 μm non-radioactive lyso-PC. The majority of the radioactivity (70–76%) associated with the cells was not removed by either treatment. However, more than two-thirds (67–74%) of the radioactivity was removed from these cells by incubation with medium containing 0.1% albumin. Taken together, our data indicate that the binding of lyso-PC to cells was non-specific and was not significantly metabolized within 15 min of incubation.

As egg lysolecithin contains mainly saturated species of lyso-PC, the ability of myristoyl-($C_{14:0}$), palmitoyl ($C_{16:0}$)-, stearoyl ($C_{18:0}$)- lyso-PC as well as several unsaturated lyso-PC species (i.e., $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$) to stimulate arachidonate release were investigated. As depicted in **Fig. 2**, lyso-PC containing palmitoyl and stearoyl chains caused the highest amount of arachidonate release by these cells. The specificity of other lysophospholipids for the stimulation of arachidonate release in H9c2 cells was also examined. Cells were incubated with 100 μm of lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, or lysophosphatidate under identical conditions and the arachidonic acid released was determined. As indicated in Fig. 2, lysophospholipids with head groups other than choline were minimally effective in the stimulation of arachidonate release. Based on these results, lyso-PC containing palmitoyl ($C_{16:0}$) chain was used in subsequent experiments.

In order to determine whether the enhanced release of arachidonate is mediated via phospholipase A_2, the effect of lyso-PC on arachidonate release in H9c2 cells was studied in the presence of arachidonoyl trifluoromethyl ketone ($AACOCF_3$), a specific inhibitor of c-phospholipase A_2 (6, 9). As shown in **Table 1**, cells pre-incubated with $AACOCF_3$ at concentrations of 0.5, 5.0, and 25 μm significantly reduced arachidonate release when compared to control cells. Our results indicate that c-phospholipase A_2 is a major phospholipase involved in lyso-PC-induced arachidonate release in the H9c2 cells.

To determine the mechanism of enzyme activation by lyso-PC, the enzyme activities in the cytosolic and membrane fractions were assayed. Direct addition of lyso-PC to the in vitro assay of phospholipase A_2 activity did not cause any significant changes in enzyme activity (data not shown). When enzyme activity was assayed in subcellular fractions prepared from cells incubated with lyso-PC, enzyme activity was decreased by 40% in cytosolic fractions and increased by 47% in membrane fractions (**Table 2**). These results are consistent with lyso-PC-induced translocation of the enzyme from the cytosol to the membrane where it is in its most active form (6).

Enzyme phosphorylation has been shown to play a major role in the regulation of phospholipase A_2 activity in a number of cell types (6, 38). We therefore examined the involvement of protein kinase A and/or C as potential modulators of phospholipase A_2 activity. Cells were pre-

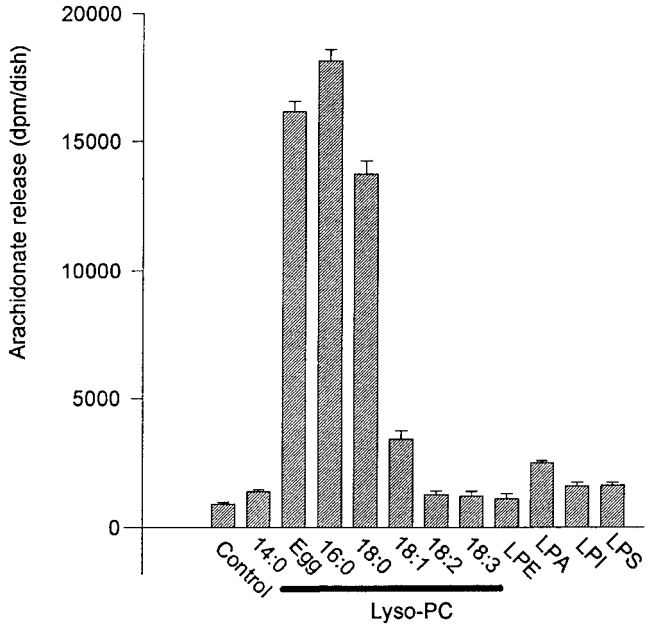

Fig. 2. Effect of various lysolipids on arachidonate release in H9c2 cells. Cells were prelabeled with [^{3}H]arachidonate and challenged for 15 min with 0 μm (Control) or 100 μm of the indicated lysolipid. Myristoyl-lyso-PC (14:0); lysolecithin from egg (egg); palmitoyl-lyso-PC (16:0); stearoyl-lyso-PC (18:0); oleoyl-lyso-PC (18:1); linoleoyl-lyso-PC (18:2); linolenoyl-lyso-PC (18:3); lysophosphatidylethanolamine (LPE); lysophosphatidic acid (LPA); lysophosphatidylinositol (LPI); lysophosphatidylserine (LPS). Arachidonate release into the medium was determined. Values represent means ± standard error of the mean from 3 experiments.

TABLE 1. Effect of arachidonoyl trifluoromethyl ketone ($AACOCF_3$) on arachidonate release in H9c2 cells

Treatment	Arachidonate Release	Inhibition
	dpm/dish ($\times 10^{-2}$)	*%*
Control	10 ± 1.1	
Lyso-PC	132 ± 2.3	
$AACOCF_3$ + Lyso-PC		
0.5 μm	75 ± 2.2[a]	43
5 μm	69 ± 1.9[a]	48
25 μm	54 ± 2.4[a]	59

Cells were prelabeled with [^{3}H]arachidonate and incubated with the indicated concentrations of $AACOCF_3$ for 2 min prior to challenge with 120 μm lyso-PC for 15 min. The release of arachidonate into the medium was determined. Results are expressed as mean ± standard error of the mean from 5 separate experiments.

[a] $P < 0.05$.

TABLE 2. Effect of lyso-PC on c-phospholipase A_2 activity in H9c2 cells

Treatment	c-Phospholipase A_2 Activity	
	Cytosol	Membrane
	pmol/min/mg	
Control (without lyso-PC)	8.2 ± 0.1	1.2 ± 0.1
Lyso-PC (100 μm)	4.9 ± 0.2[a]	1.8 ± 0.1[a]

Cells were treated with or without 100 μm lyso-PC in HEPES-buffered saline containing 0.1% bovine serum albumin. Cells were lysed and phospholipase A_2 activity was assayed in the cytosolic and membrane fractions. Results are expressed as mean ± standard error of the mean from 4 separate experiments.

[a] $P < 0.05$.

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TABLE 3. Effect of protein kinase C and protein kinase A inhibitors on lyso-PC-induced arachidonate release in H9c2 cells

Treatment	Arachidonate Release	Inhibition
	dpm/dish (×10⁻²)	%
Control	7.3 ± 0.6	
Lyso-PC	130 ± 2.2	
Staurosporine + lyso-PC		
0.1 μm	49 ± 1.9[a]	62
1.0 μm	36 ± 2.0[a]	72
Ro31-8220 + lyso-PC		
5 μm	51 ± 2.0[a]	61
10 μm	18 ± 2.0[a]	86
BIS-I + lyso-PC		
1.0 μm	69 ± 2.5[a]	47
10.0 μm	30 ± 2.2[a]	77
H-89 + lyso-PC		
0.5 μm	112 ± 3.0	14
1.0 μm	109 ± 2.0	17

Cells were prelabeled with [^{3}H]arachidonate and then treated with indicated concentrations of staurosporine, Ro31-8220, or H-89 for 15 min or BIS-I (bisindolylmaleimide I) for 30 min prior to challenge with 100 μm lyso-PC for an additional 15 min. Arachidonate release into the medium was determined. Results are expressed as mean ± standard error of the mean from 5 separate experiments.

[a] $P < 0.05$.

treated with the protein kinase inhibitors, staurosporine (39), Ro31-8220 (40), bisindolylmaleimide-I (BIS-I) (41) and H89 (42) prior to the lyso-PC challenge. Ro31-8220, BIS-I, and staurosporine significantly inhibited lyso-PC-induced arachidonate release from 47 to 86% (**Table 3**). In contrast, the arachidonate release was not significantly attenuated by H89 even at concentrations 20-times its K_i value. Thus, on the basis of pharmacological inhibition, it appeared that protein kinase C was involved in the activation of phospholipase A_2 activity that resulted in the enhancement of arachidonate release. Furthermore, cells incubated with lyso-PC for 5 min resulted in a 84% increase in protein kinase C activity in the membrane fraction (**Table 4**). It is clear, however, that lyso-PC was a less potent modulator of protein kinase C activity than was phorbol 12-myristate 13-acetate.

It has been shown that the phosphorylation of c-phospholipase A_2 via the activation of protein kinase C is mediated downstream by the mitogen-activated protein kinase

TABLE 4. Effects of lyso-PC and phorbol 12-myristate 13-acetate (PMA) on protein kinase C activity in H9c2 cells

Treatment	Protein Kinase C Activity	Increase
	pmol/min/mg	%
Control	130 ± 10	
Lyso-PC	239 ± 8[a]	84
PMA	412 ± 17[a]	214

Cells were challenged with 100 μm lyso-PC or 200 nm phorbol 12-myristate 13-acetate for 5 min. Protein kinase C activity was determined in the cell lysate. Results are expressed as mean ± standard error of the mean from 4 separate experiments.

[a] $P < 0.05$.

TABLE 5. Effect of PD098059 on lyso-PC-induced arachidonate release in H9c2 cells

Treatment	Arachidonate Release	Inhibition
	dpm/dish (×10⁻²)	%
Control	7.4 ± 0.8	
Lyso-PC	131 ± 1.9	
PD098059		
10 μm PD098059 + lyso-PC	64 ± 3.2[a]	51
30 μm PD098059 + lyso-PC	59 ± 2.8[a]	55
50 μm PD098059 + lyso-PC	41 ± 2.3[a]	69

Cells were prelabeled with [^{3}H]arachidonate and then treated with the indicated concentrations of PD098059 for 30 min prior to challenge with 100 μm lyso-PC for 15 min. Arachidonate release into the medium was then determined. Results are expressed as mean ± standard error of the mean from 5 experiments.

[a] $P < 0.05$.

(MAPK) (36, 38). Hence, the involvement of the MAPK pathway in lyso-PC-induced arachidonate release was tested using PD098059, an inhibitor of the MAPK/extracellular regulated kinase kinase (43). Cells were preincubated with 10, 30, and 50 μm concentrations of PD098059 for 30 min prior to challenge with lyso-PC (**Table 5**). PD098059 significantly inhibited lyso-PC-induced increases in the arachidonate release by 51% at 10 μm, 55% at 30 μm, and 69% at 50 μm (Table 5).

Another mode of phospholipase A_2 activation occurs by increased levels of intracellular calcium $[Ca^{2+}]_i$. An elevation of $[Ca^{2+}]_i$ induces enzyme translocation and increases enzyme activation (44). Hence, cells were chal-

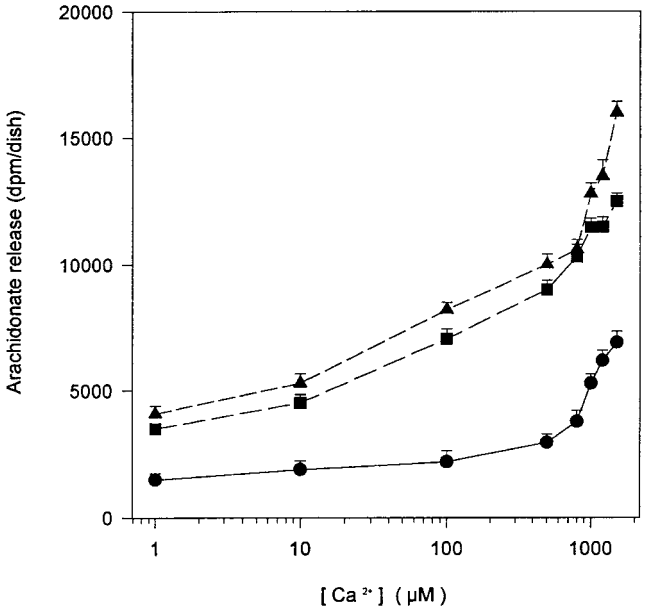

Fig. 3. Effect of extracellular Ca^{2+} on lyso-PC-induced arachidonate release in H9c2 cells. Cells were prelabeled with [^{3}H]arachidonate and challenged for 15 min with 50 μm (●), 100 μm (■), and 150 μm (▲) LPC at various Ca^{2+} concentrations. Arachidonate release into the medium was determined. Values represent means ± standard error of the mean from 5 separate experiments.

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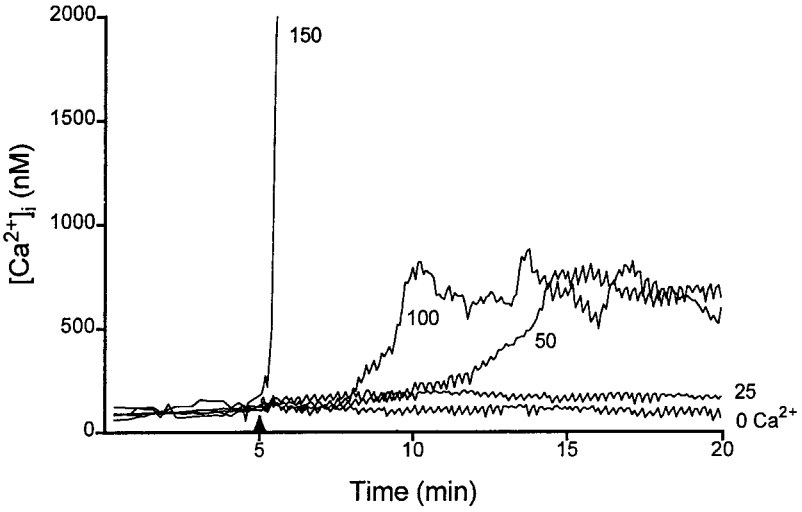

Fig. 4. Effect of lyso-PC on intracellular calcium concentration $[Ca^{2+}]_i$ in H9c2 cells. Numbers next to traces indicate micromolar (μm) concentrations of lyso-PC in HEPES-buffered saline containing 0.1% bovine serum albumin. 0 Ca^{2+} represents the absence of extracellular calcium. Arrow indicates the time of lyso-PC application. $[Ca^{2+}]_i$ was monitored using Fura-2 as described under Materials and Methods.

lenged with varying levels of lyso-PC (50 to 150 μm) in the presence of 0–1.5 mm Ca^{2+}. As shown in **Fig. 3**, arachidonate release induced by each level of lyso-PC was progressively suppressed at the lower Ca^{2+} concentrations. When extracellular Ca^{2+} was removed from the challenge buffer (calcium-free buffer also contained 1.0 mm EDTA and 1.0 mm EGTA), arachidonate release was completely abolished (data not shown). Thus, the lyso-PC-induced arachidonate release was dependent on Ca^{2+} from the extracellular media. In determining the mechanism(s) by which lyso-PC increased $[Ca^{2+}]_i$ cells were treated with varying concentrations of lyso-PC (0–150 μm) in the presence of 1.2 mm Ca^{2+} in HEPES buffer. As shown in **Fig. 4**, the absence of lyso-PC did not cause any change in $[Ca^{2+}]_i$. A dose-dependent increase in $[Ca^{2+}]_i$ was observed at 25, 50, and 100 μm lyso-PC. A progressive decrease in the time to achieve maximum $[Ca^{2+}]_i$ was also dose related. At 150 μm lyso-PC, a dramatic increase in $[Ca^{2+}]_i$ was followed by quenching of Fura 2-fluorescene, and a significant number of these cells stained trypan blue positive, suggesting that some of the cells became leaky. The increased levels of $[Ca^{2+}]_i$ achieved at various doses of lyso-PC and at different time points subsequent to lyso-PC challenge are depicted in **Table 6**.

The involvement of L-type Ca^{2+} channel in the lyso-PC-induced elevation of $[Ca^{2+}]_i$ was investigated. Cells were preincubated with nimodipine for 5 min prior to incubation with lyso-PC. As shown in **Fig. 5**, nimodipine in the presence of lyso-PC did not significantly influence the time required to achieve peak $[Ca^{2+}]_i$, however, the amplitude of peak $[Ca^{2+}]_i$ was significantly decreased. In order to investigate the potential effect of nimodipine on attenuation of lyso-PC-induced $[Ca^{2+}]_i$ levels and lyso-PC-induced arachidonate release, cells were stimulated with 50, 100, and 150 μm lyso-PC in the presence and absence of nimodipine (10 μm), and arachidonate release was determined. **Table 7** indicates that at all three lyso-PC concentrations, nimodipine caused significant declines in arachidonate release 2.5 and 10 min subsequent to lyso-PC challenge.

DISCUSSION

The present study was designed to investigate the effects of lyso-PC on arachidonate release in H9c2 cells. This cell line is derived from quiescent cardiac myoblastic cells and it retains many morphological and structural properties similar to the cardiac muscle myoblast (34). Alternatively, the biochemical and electrophysiological properties of these cells resemble those found in skeletal muscle cells. Using the H9c2 cells as a model, we observed that lyso-PC induced both time- and dose-dependent increases in the release of arachidonates. The concentration of lyso-PC used in this study is similar to its plasma concentrations (0.13–0.15 mm) (24, 45), but the presence of serum proteins may attenuate the ability of lyso-PC to en-

TABLE 6. Effect of lyso-PC concentration on intracellular calcium concentration $[Ca^{2+}]_i$ in H9c2 cells

Lyso-PC Incubation	Increases of $[Ca^{2+}]_i$ above Baseline Lyso-PC Concentration			
	25 μm	50 μm	100 μm	150 μm
min		*n*m		
5	100 ± 5 (46)	216 ± 20 (28)	990 ± 7 (26)	1781 ± 93 (34)
10	109 ± 5 (46)	496 ± 47 (27)	1174 ± 111 (18)	L.O.F.
15	64 ± 5 (46)	575 ± 41 (27)	877 ± 216 (10)	L.O.F.

Cells were incubated with different concentrations of lyso-PC for the time indicated and $[Ca^{2+}]_i$ was determined. Numbers in parentheses indicate cells that maintained fluorescent signal during the time periods indicated. L.O.F. indicates loss of fluorescence.

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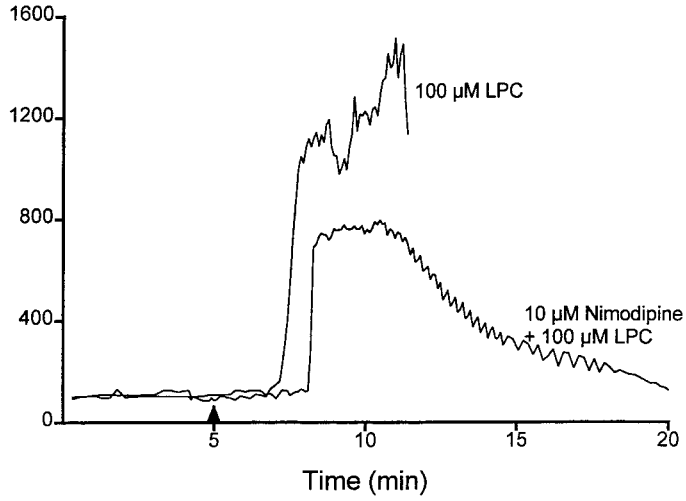

Fig. 5. Effect of nimodipine (NIM) on the lyso-PC induced elevation of $[Ca^{2+}]_i$ in H9c2 cells. Cells were incubated with lyso-PC (100 μm) in the presence or absence of (10 μm) NIM. The presence of NIM attenuated $[Ca^{2+}]_i$ elevation induced by lyso-PC. NIM caused a delay in obtaining maximum $[Ca^{2+}]_i$ (from 3.3 ± 0.3 min to 4.09 ± 0.5 min; $P > 0.05$), and the maximum value of $[Ca^{2+}]_i$ was also reduced. Arrow indicates the point of lyso-PC application.

hance arachidonate release. The effect of lyso-PC was most pronounced in lyso-PC species containing long and saturated acyl chains. Although the rationale for the effectiveness of lyso-PC with acyl chains containing $C_{16:0}$ or $C_{18:0}$ remains unclear, the ability of these lyso-PC species containing aliphatic chains of 16–18 carbon atoms to produce optimal lytic activity has been documented (10). Lyso-PC appears to induce arachidonate release by the elevation of $[Ca^{2+}]_i$ and activation of protein kinase C. We postulate that both regulatory factors acted in concert to fully activate the cytosolic phosholipase A_2, leading to the enhanced release of arachidonate in H9c2 cells.

In cardiac and other mammalian cells, the content of unesterified arachidonate is very low, and the majority of the acyl group is incorporated into the membrane phospholipid pool (33). The esterified and free arachidonate levels are controlled by a fine-tuned deacylation–reacylation cycle of phopholipids in which phospholipase A_2 plays a dominant role (33). Although there are several types of phospholipase A_2, the cytosolic form is the predominant form of the enzyme for the intracellular release of arachidonate. It has a molecular mass of 85–110 kDa, requires Ca^{2+} in the micromolar range for translocation of the enzyme to its target (phospholipid-containing membrane), and in contrast to the s-form of the enzyme, possesses a high specificity for the arachidonoyl residues at the *sn*-2 position of the phospholipid molecule (6, 8, 9). This form is activated by phosphorylation followed by the translocation of the phosphorylated enzyme to the membrane in a calcium-dependent manner (6, 36). Using $AACOCF_3$, a specific inhibitor of the c-form of phospholipase A_2, we have shown that the increase in arachidonate release was mediated by the enhancement of c-phospholipase A_2. In agreement with our earlier study (35), we were unable to demonstrate that the loss of enzyme activity in the soluble fraction was quantitatively recovered in the membrane fraction. Based on previous findings from our group and by others, the failure to recover all the enzyme activity after translocation was probably caused by interference of the enzyme assay with the membrane fraction (35, 46). This phenomenon also occurs when other enzymes are translocated to the membrane fraction (46). Factors attributing to this loss of activity include the reduced accessibility of exogenous radioactive substrate to the enzyme and the dilution effect on the exogenous radioactive substrate.

TABLE 7. Influence of nimodipine (NIM) on lyso-PC-induced arachidonate release in H9c2 cells

	Arachidonate Release	
Lyso-PC	2.5 min	10 min
μm	*dpm/dish ×10⁻²*	
50 (−NIM)	11 ± 1.0	91 ± 2.5
50 (+NIM)	6.9 ± 0.7[a]	67 ± 3.1[a]
100 (−NIM)	41 ± 1.9	143 ± 1.3
100 (+NIM)	22 ± 3.5[a]	121 ± 6.9[a]
150 (−NIM)	85 ± 4.2	168 ± 5.0
150 (+NIM)	51 ± 3.7[a]	132 ± 5.1[a]

Cells were prelabeled with [^{3}H]arachidonate and then treated with lyso-PC in the presence (+) or absence (−) of nimodipine (NIM) for the indicated period of time. Arachidonate release into the medium was determined. Results are expressed as mean ± standard error of the mean from 6 separate experiments.

[a] $P < 0.05$, +NIM vs. −NIM under the same incubation conditions.

The resting $[Ca^{2+}]_i$ in quiescent cardiomyocytes is about 100 nm (47) and values obtained for resting H9c2 cells (85–110 nm) are in agreement with this value. Lyso-PC caused a 2- to 17-fold rise in $[Ca^{2+}]_i$ levels (~200 nm to >1.7 μm), consistent with the elevation level of $[Ca^{2+}]_i$ shown to cause the association of c-phospholipase A_2 with membranes and also correlates with the level of calcium required to stimulate the catalytic activity of the enzyme in vitro using phospholipid vesicles as substrates (6, 44, 46). Inhibition of lyso-PC-induced increases of $[Ca^{2+}]_i$ by nimodipine suggests the involvement of L-type Ca^{2+} channels.

Although c-phospholipase A_2 is a substrate for protein kinase C in vitro, the direct phosphorylation of c-phospholipase A_2 by protein kinase C does not result in enhanced phospholipase activity, nor is there any evidence that the enzyme is phosphorylated in vivo by protein kinase C (3, 36). However, protein kinase C has been shown to phosphorylate and activate Raf-1 (48), which in turn activates MAP-kinase through a protein kinase cascade. In-

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deed, the direct phosphorylation and activation of c-phospholipase A_2 by MAP-kinase has been demonstrated (36, 49). Our study indicates that the MAP kinase kinase is involved in the lyso-PC-induced activation of c-phospholipase A_2 for the enhancement of arachidonate release, and suggests the involvement of MAP-kinase in this process.

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