Lysophosphatidylcholine Inhibits 
Bradykinin-Induced Phosphoinositide Hydrolysis and Calcium Transients in Cultured Bovine Aortic Endothelial Cells

Nobutaka Inoue, Ken-ichi Hirata, Mitsuhiko Yamada, Yasuo Hamamori, Yuichi Matsuda, Hozuka Akita, and Mitsuhiro Yokoyama

Vascular endothelium, which produces endothelium-derived relaxing and constricting factors, plays an important role in regulating the vascular tone. We recently demonstrated that oxidized low density lipoprotein inhibited endothelium-dependent relaxation and that lysophosphatidylcholine accumulated during the oxidative modification of low density lipoprotein was the essential substance for the inhibition of endothelium-dependent relaxation. To clarify the mechanisms of the inhibitory effect of lysophosphatidylcholine, we used a bioassay system to investigate the effect of lysophosphatidylcholine on the production and/or release of endothelium-derived relaxing factor and its effect on the cytosolic Ca\(^{2+}\) level ([Ca\(^{2+}\)]\(_i\)) and phosphoinositide hydrolysis in cultured bovine aortic endothelial cells. [Ca\(^{2+}\)]\(_i\) was monitored by the fura 2 method, and the accumulation of inositol phosphates in cells labeled with myo-[2-\(^3\)H]inositol was measured. Bioassay experiments showed that lysophosphatidylcholine inhibited the production and/or release of endothelium-derived relaxing factor from cultured endothelial cells. Lysophosphatidylcholine (5–20 \(\mu\)g/ml) induced a biphasic increase in [Ca\(^{2+}\)]\(_i\), which consisted of a rapid increase followed by a sustained increase, and the initial component was a result of mobilization from intracellular Ca\(^{2+}\) stores without detectable synthesis of inositol 1,4,5-trisphosphates. Furthermore, lysophosphatidylcholine (5–20 \(\mu\)g/ml) dose-dependently inhibited both phosphoinositide hydrolysis and the increases in [Ca\(^{2+}\)]\(_i\) evoked by bradykinin. These results indicate that the impairment of endothelium-dependent relaxation induced by lysophosphatidylcholine is due to the inhibition of phosphoinositide hydrolysis and the subsequent increases in [Ca\(^{2+}\)]\(_i\), in endothelial cells. Lysophosphatidylcholine that accumulates in oxidized low density lipoprotein and atherosclerotic arteries may play an important role in the modification of endothelial function. (Circulation Research 1992;71:1410–1421)

**Key Words** • lysophosphatidylcholine • vascular endothelium • intracellular calcium concentration • phosphoinositide hydrolysis • endothelium-derived relaxing factor

Coronary artery spasm is well recognized as playing an important role in the pathogenesis of ischemic heart disease, including variant angina, unstable angina, and acute myocardial infarction.\(^1\)–\(^3\) However, the mechanisms of coronary artery spasm are still unknown. Vascular endothelial cells in response to a variety of neurohumoral and physical stimuli produce prostacyclin, endothelium-derived relaxing factor (EDRF),\(^4\) and endothelium-derived constricting factor\(^5\) and regulate the vascular tone. Atherosclerosis has been implicated in the pathogenesis of coronary artery spasm, and local hyperreactivity or supersensitivity of the coronary artery may be involved.\(^6\)–\(^9\) In atherosclerotic arteries, endothelium-dependent relaxation is markedly reduced, and its impairment may play an important role in the pathogenesis of coronary artery spasm.\(^10\)

Modification of low density lipoprotein (LDL) is speculated to be an important factor in the atherosclerotic process.\(^11\) Oxidized LDL can cause foam cell formation via the scavenger receptors in macrophages, and oxidized LDL is detected in atherosclerotic lesions of the rabbit and human.\(^12\) Recently, we\(^13\) and other investigators\(^14\) demonstrated that modified LDL with copper oxidation, phospholipase A\(_2\) treatment, or the incubation with endothelial cells inhibited endothelium-dependent relaxation of the rabbit aorta evoked by acetylcholine and that lysophosphatidylcholine (LPC) generated during these modifications was the main factor in the impairment of endothelium-dependent relaxation. It has been proposed that the synthesis and the release of EDRF are mediated by intracellular Ca\(^{2+}\) signal transduction through a receptor-mediated phospholipase C–coupled system that hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol, the specific activator of protein kinase C, and inositol
1,4,5-trisphosphate (IP₃), a compound that mobilizes Ca²⁺ from intracellular Ca²⁺ stores. To clarify the cellular mechanism of the inhibitory effect of LPC on endothelium-dependent relaxation, we used a bioassay system to investigate the effect of LPC on the production and release of EDRF from cultured bovine aortic endothelial cells (BAECs), on the cytosolic Ca²⁺ level ([Ca²⁺]), and on phosphoinositide hydrolysis in cultured BAECs.

Materials and Methods

Culture of Endothelial Cells

BAECs were obtained by scraping the internal surface of the aorta excised from a freshly slaughtered cow with a knife, as described previously. They were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with fetal calf serum (FCS, 15% [vol/vol]). The cells were seeded on 25-cm² flasks and incubated at 37°C under an atmosphere of 5% CO₂–95% air. The medium was changed on the following day and every 3 days thereafter. After 4 or 5 days, the primary cultures formed a confluent monolayer and could be subcultured. The cells were separated for subculture with 0.25% trypsin solution containing 0.02% EDTA. Cultures used in the present study were from the third to 12th passage. The endothelial cells were identified by their typical cobblestone appearance, positive immunofluorescence for anti-factor VIII antibody, and positive uptake of acetylated LDL (Biomedical Technologies Inc., Stoughton, Mass.). Cell viability used in this study was greater than 98% by trypan blue exclusion. Neither cell passages 1–18 nor trypsin treatment affected the response of Ca²⁺ transients and phosphoinositide hydrolysis to bradykinin (BK) or ATP.

Bioassay of Endothelium-Derived Relaxing Factor

EDRF was released from BAECs cultured on microcarrier beads, and it was detected by bioassay on helical strips of rabbit aorta superfused in a cascade. BAECs (6 × 10⁴ cells) were seeded onto 3 × 10² Cytodex 3 microcarrier beads (Pharmacia LKB Biotechnology, Sweden). Microcarrier beads were maintained in suspension by continuous stirring. Microcarrier beads were coated with a confluent cellular monolayer within 3 days and were used for bioassay experiments in the next 1 or 2 days. A column packed with BAECs (3–5 × 10⁶) cultured on microcarrier beads was perfused with oxygenated Krebs’ buffer composed of (mM) NaCl 118, KCl 4.0, CaCl₂ 1.5, MgSO₄ 1.2, NaHCO₃ 25, and glucose 5.0, containing 10 µM indomethacin. The helical strips of rabbit thoracic aortas without endothelium were used as the detector tissue for EDRF. Japanese white rabbits (2.5–3.5 kg) were anesthetized with pentobarbital sodium (30 mg/kg), and descending aortas were isolated, cleaned of surrounding tissues, and cut into helical strips approximately 2 mm wide and 15 mm long. Endothelium of the aorta was removed mechanically by rubbing the internal surface with filter paper. For recording isometric force, one end of the strip was attached to the bottom of the 30-ml muscle chamber, and the other was attached to a force transducer, which was connected to an amplifier/recorder system. The strips were precontracted with 0.3 µM phenylephrine and then relaxed by the addition of the effluent from the column packed with BAECs. The transit time from a cell column to a detector tissue was 3 seconds. The sensitivity of the bioassay tissues was standardized by administration of nitroglycerin before and after each experiment.

Fura 2 Fluorescence Measurement of Intracellular Calcium Concentration

The [Ca²⁺], level was monitored by measuring the fluorescence of fura 2. The cells were incubated for fura 2 fluorescence, grown on glass coverslips, where they exhibited normal growth and appearance. The FCS in culture medium was depleted for 3 hours, and then the cells attached to glass coverslips were loaded with fura 2 by incubating them with 5 µM fura 2 acetylomethyl ester for 60 minutes at 25°C in HEPES-buffered Eagles’ minimum essential medium (MEM). Loaded cells were washed with HEPES-buffered MEM containing 0.1% bovine serum albumin (BSA) and physiological salt solution (PSS) composed of (mM) NaCl 135, KCl 5, CaCl₂ 1, MgCl₂ 1, glucose 5.5, and HEPES 10 (pH 7.4). The cells of a confluent monolayer on a glass coverslip were placed on the stage of an inverted microscope (Diaphot, Nikon, Japan) equipped for fluorescence measurements in the ultraviolet range. Fluorescence excitation was performed with a 100-W xenon lamp (Nikon). The cells were excited alternately at 340 and 380 nm every 250 msec. Light from the xenon lamp was reflected through 10-nm interference filters (340 nm/380 nm) mounted on a computer-controlled motorized wheel that was programmed to shuttle between the two wavelengths every 250 msec. Fura 2 fluorescence from the cells was imaged with a Nikon UV-Fluor objective lens. The dichroic mirror was used as a beam splitter to transmit emitted fluorescence (510 nm) into the photomultiplier (P1, Nikon) that was coupled to an RS232C converter (NEC, Japan) interfaced to a personal computer (PC9801, NEC) that also controlled filter alternation. A pinhole diaphragm was placed in the emission beam in front of the photomultiplier to regulate the area of recording fields. Analyzed fields were approximately 50 µm in diameter, and signals from two or three cells were recorded. During the experiments, the cells of a monolayer were perfused with thermostatically controlled PSS at 37°C. The cells were stimulated by perfusing PSS containing adequate concentrations of various agents. In some experiments, before the measurements of fluorescence, fura 2-loaded cells were incubated at 25°C for 10 minutes with PSS containing adequate concentrations of various lipids.

In this study, the changes in [Ca²⁺], were expressed qualitatively by using the ratio of two fluorescence values (340 nm/380 nm) after subtracting the autofluorescence and determined quantitatively by using the following equation:

\[
[Ca^{2+}] = K_d \frac{(R - R_{min})/(R_{max} - R)}{(S_f/S_b)}
\]

where \(K_d\) is 224 nM, \(R\) is the fluorescence ratio within the cells, \(R_{max}\) is the maximal fluorescence ratio after addition of 10 µM ionomycin in the presence of 1 mM CaCl₂, \(R_{min}\) is the minimal ratio determined by the subsequent addition of 4 mM EGTA, and \(S_f/S_b\) is the ratio of fluorescence values at 380-nm excitation determined at \(R_{min}\) and \(R_{max}\), respectively. Figure 1 is a representative tracing of calibration. We confirmed that
the ratio values are comparable to those determined by using ionomycin and EGTA in the independent experiments and that they did not vary from day to day.

Assay Condition for Phospholipase C Reaction

The cells were plated into 35-mm dishes. After 2 days the cultured cells reached confluence. The final cell density on the day of the assay was 1 x 10^6 cells per dish. For assay of phospholipase C reaction, the cells were rinsed three times with 2 ml phosphate-buffered saline with 0.9 mM Ca^{2+} and 0.8 mM Mg^{2+} and labeled with myo-[2-^3H]inositol (10 μCi/ml) at 37°C for 24 hours in inositol-free DMEM without FCS. After the labeling, the cells were washed three times with 2 ml DMEM to remove unincorporated [^3H]inositol. The cells were preincubated with 0.9 ml HEPES-buffered solution composed of (mM) NaCl 130, KCl 5, MgCl_2 1, CaCl_2 1.5, glucose 10, and HEPES 20 (pH 7.4) containing 10 mM LiCl for a tray in a water bath at 37°C for 15 minutes. Then, the cells were stimulated with 0.1 ml of various agents. The reaction was stopped by rapid aspiration of the medium and addition of 1 ml ice-cold 15% trichloroacetic acid. The cells were chilled on ice for 1 hour to extract water-soluble inositol phosphates. To investigate the effects of phospholipids on BK-induced phosphoinositide hydrolysis, BAECs were preincubated with LPC and phosphatidylcholine (PC) at 37°C for 10 minutes and then stimulated with 100 nM BK for 30 seconds.

Separation of [^3H]Inositol Phosphates

The extract was transferred to a test tube, and the cells were washed twice with 1 ml distilled water. The washings were combined with the original acid extract. Trichloroacetic acid was removed with 3 ml diethyl ether four times. The final extract was neutralized, applied to columns containing 1 ml Dowex AG 1 x 8 formate-form resins (Bio-Rad Laboratories, Richmond, Calif.), and eluted to separate the water-soluble inositol phosphates by the method described previously, which was based on that described by Berridge et al. After application of the sample, the column was washed with 8 ml distilled water. Glycerophosphoinositol was eluted with 16 ml of 5 mM disodium tetraborate/60 mM sodium formate. Inositol 1-monophosphate (IP_1) was eluted with 8 ml of 0.1 M formic acid/0.2 M ammonium formate, followed by an 8-ml wash with the same buffer. Inositol 1,4-bisphosphate (IP_2) was eluted with 8 ml of 0.1 M formic acid/0.4 M ammonium formate, followed by an 8-ml wash with the same buffer. IP_3 was eluted with 8 ml of 0.1 M formic acid/1.0 M ammonium formate.

Drugs

1-Palmitoyl-2-oleoyl-phosphatidylcholine was purchased from Avanti Polar Lipids Inc., Alabaster, Ala. LPC (1-palmitoyl, 1-stearoyl, or 1-caproyl), lysophosphatidylethanolamine (LPE), palmitoyl-L-carnitine, BK, BSA, and ATP were purchased from Sigma Chemical Co., St. Louis, Mo. DMEM was from Flow Laboratories, Inc., McLean, Va. MEM was purchased from Nissui Pharmaceuticals, Tokyo. FCS was purchased from Boehringer Mannheim Yamanouchi Co., Tokyo. Fura 2 acetoxymethyl ester was obtained from Doyingo Laboratories, Kumamoto, Japan. Myo-[2-^3H]inositol (20 Ci/mmol) was obtained from Amersham, Arlington Heights, Ill. Sodium pentobarbital was from Abbott Laboratories, Chicago. Other materials and chemicals were obtained from commercial sources. Phospholipids were dissolved in a mixture (1:1 [vol/vol]) of methanol and chloroform. Appropriate aliquots of the solutions were dried with a stream of N_2 gas, followed by sonication for 3 minutes in distilled water or phosphate-buffered saline without Ca^{2+} and Mg^{2+} before use, and then they were diluted in each solution. The purity of the phospholipid preparation was surveyed by standard thin-layer chromatography on precoated, activated silica gel plates using the solvent system, chloroform/methanol/water (65:35:6 [vol/vol]). Lipid bands were detected by lightly staining with I_2 vapor. The contaminated compounds were not detected. Palmitic acid, which was first dissolved in absolute ethanol, was combined with K_2CO_3 dissolved in distilled water. It was dried with the stream of N_2 gas and then redissolved as potassium palmitate. Concentrations were expressed as final concentrations.

Statistical Analysis

All values were expressed as mean±SEM. Statistical analysis was performed using Student’s t test for unpaired observations.

Results

LPC Inhibited the Production and/or Release of EDRF in Endothelial Cells

Using a bioassay system, we examined the effect of LPC on the production and release of EDRF. The strips of rabbit aortas without endothelium were relaxed by 100 nM nitroglycerin or EDRF released from BAECs on microcarrier beads stimulated with BK (10–100 nM) (Figure 2). Treatment of BAECs with 1-palmitoyl LPC (10 μg/ml) for 30 minutes almost abolished EDRF-induced relaxation of the detector arteries (Figure 2A). On the other hand, after the detector arteries were treated with 1-palmitoyl LPC (10 μg/ml), EDRF-in-
duced relaxation was not altered (Figure 2B). These results suggest that LPC inhibited the production and/or release of EDRF in endothelial cells, whereas LPC did not act on vascular smooth muscles and had little effect on the degradation of EDRF.

**LPC Induced an Increase in \([\text{Ca}^{2+}]_i\) in Endothelial Cells**

Figure 3A shows the effect of BK on \([\text{Ca}^{2+}]_i\) in cultured BAECs. BK (0.1 nM-10 \(\mu\)M) induced a biphasic rise of \([\text{Ca}^{2+}]_i\), i.e., a rapid increase followed by a sustained increase in a dose-dependent manner. The threshold concentration of BK was 0.1 nM. 1-Palmitoyl LPC (10 \(\mu\)g/ml) also elicited a biphasic increase in \([\text{Ca}^{2+}]_i\) (Figure 3B). Figure 3D is a fluorescence tracing of the experiment of Figure 3B. LPC caused an increase of the 510-nm fluorescence emission at 340-nm excitation and a simultaneous decrease of the 510-nm fluorescence emission at 380-nm excitation. The ratio of the fluorescence emission at two wavelengths was therefore substantially increased, consistent with an increase in \([\text{Ca}^{2+}]_i\). Figure 4 shows the dose dependence of 1-palmitoyl LPC on \([\text{Ca}^{2+}]_i\) in BAECs. The effect was observed in concentrations of 1–20 \(\mu\)g/ml 1-palmitoyl LPC. The threshold concentration was 5 \(\mu\)g/ml. At high doses of 1-palmitoyl LPC (20 \(\mu\)g/ml), the second phase was more pronounced. By stimulation with more than 20 \(\mu\)g/ml 1-palmitoyl LPC, morphological changes such as cytoplasmic vacuolation and the abrupt leakage of fluorescence dye were observed (data not shown). These changes were not observed at the lower concentration of LPC. Verapamil (100 nM) had no effects on the \(\text{Ca}^{2+}\) transients evoked by both BK and LPC (data not shown).

**Structural Specificity of Lysophospholipids**

As shown in Table 1, LPC with a long aliphatic chain (i.e., 18-carbon stearyl, 16-carbon palmitoyl) could elicit a prominent increase in \([\text{Ca}^{2+}]_i\), in BAECs. However, neither LPC with a short aliphatic chain (i.e., 6-carbon caproyl) nor LPE, at an equimolar concentration of 10 \(\mu\)g/ml of 1-palmitoyl LPC, could induce an increase in \([\text{Ca}^{2+}]_i\) in BAECs. Neither PC nor palmitic acid, even at high doses, could induce an increase in \([\text{Ca}^{2+}]_i\), (Figure 3C).

Palmitoyl-L-carnitine, another amphiphilic substance, showed a biphasic increase in \([\text{Ca}^{2+}]_i\). The time course and pattern of \(\text{Ca}^{2+}\) transients induced by palmitoyl-L-carnitine were very similar to those induced by 1-palmitoyl LPC (data not shown).

**LPC Induced \(\text{Ca}^{2+}\) Mobilization From Intracellular Calcium Stores**

To determine the sources from which the mobilized \(\text{Ca}^{2+}\) originated, BAECs were exposed to LPC in \(\text{Ca}^{2+}\)-free PSS with 1 mM EGTA. The result in Figure 5A shows that the absence of extracellular \(\text{Ca}^{2+}\) abolished the second sustained phase of an increase in \([\text{Ca}^{2+}]_i\). However, the initial rapid increase was preserved in the absence of extracellular \(\text{Ca}^{2+}\). This result indicates that the initial rise in \([\text{Ca}^{2+}]_i\) did not depend on extracellular \(\text{Ca}^{2+}\) and that it originated from intracellular \(\text{Ca}^{2+}\) stores. We examined whether the \(\text{Ca}^{2+}\) stores mobilized by LPC were the same ones evoked by receptor agonists, i.e., whether they were IP\(_3\)_sensitive. In \(\text{Ca}^{2+}\)-free PSS with 1 mM EGTA, BAECs were first stimulated with 10 nM BK, and after a transient increase in \([\text{Ca}^{2+}]_i\), subsided to the basal level, 10 nM BK was repeatedly added to the cells. Figure 5B shows that the second stimulation with BK failed to increase \([\text{Ca}^{2+}]_i\), probably because of depletion of the IP\(_3\)_sensitive \(\text{Ca}^{2+}\) stores. The third stimulation with BK, after BAECs were perfused with PSS with 1 mM \(\text{Ca}^{2+}\), caused a rise in \([\text{Ca}^{2+}]_i\), similar to that of the first stimulation (data not shown). These results were consistent with the previous report indicating that in endothelial cells, after \(\text{Ca}^{2+}\) was released from intracellular store sites to cytosol, the stores required refilling from extracellular \(\text{Ca}^{2+}\). Similarly, the cells were first stimulated with 10 nM BK, and then 1-palmitoyl LPC (10 \(\mu\)g/ml) was added. Figure 5C shows that the sequential stimulation with LPC had no effects on \([\text{Ca}^{2+}]_i\). In Figure 5D, the order of addition of these two agents was reversed. When BAECs were first
stimulated with 1-palmitoyl LPC (10 μg/ml), subsequently followed by 10 nM BK, there was no increase in [Ca\(^{2+}\)], by BK. Ionomycin (1 μM) could elicit further increases in [Ca\(^{2+}\)] in the cells prestimulated with BK or LPC (data not shown). These results indicate that intracellular Ca\(^{2+}\) stores from which LPC mobilized Ca\(^{2+}\) during the initial transient overlapped with the IP\(_3\)-sensitive Ca\(^{2+}\) stores probably present in the endoplasmic reticulum.

**LPC Inhibited BK-Induced Ca\(^{2+}\) Transients**

The effects of LPC on BK-induced Ca\(^{2+}\) transients are demonstrated in Figure 6A. BAECs were preincubated with 1-palmitoyl LPC (10 μg/ml) for 10 minutes in PSS containing 1 mM CaCl\(_2\), and then 10 nM BK was added. The Ca\(^{2+}\) transients evoked by BK were suppressed with the small increase in the resting level. In contrast, after BAECs were prestimulated with 10 nM BK for 10 minutes in the presence of extracellular 1 mM CaCl\(_2\), 1-palmitoyl LPC (10 μg/ml) could induce an increase in [Ca\(^{2+}\)] (data not shown).

As shown in Figure 3A, the Ca\(^{2+}\) transient evoked by BK was composed of two components. The initial component reflects a release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores, and the second component results from Ca\(^{2+}\) influx across the plasma membrane from the extracellular space. We investigated the effects of LPC on each component of the Ca\(^{2+}\) transients evoked by BK. In the absence of extracellular Ca\(^{2+}\), the addition of 1 mM CaCl\(_2\) to BAECs without any stimulation caused only a slight increase in [Ca\(^{2+}\)]. (Figure 6B). As shown in Figure 6C, in the absence of extracellular Ca\(^{2+}\), BK induced a transient increase in [Ca\(^{2+}\)], and then the subsequent readdition of 1 mM CaCl\(_2\) to the buffer produced a large increase in [Ca\(^{2+}\)], indicative of BK-stimulated Ca\(^{2+}\) influx. This procedure allowed temporal separation of the second component from the initial one. BAECs were preincubated with LPC or PC for 10 minutes in PSS containing 1 mM CaCl\(_2\), the perfusate was replaced by Ca\(^{2+}\)-free PSS with 1 mM EGTA, and then the BAECs were stimulated with 10 nM BK. When [Ca\(^{2+}\)] \(_{i}\) returned to the basal level, 1 mM CaCl\(_2\) was added. Figures 6D and 6E show that, after the preincubation of BAECs with 1-palmitoyl LPC (5–10 μg/ml), both the initial and the sustained components evoked by 10 nM BK were suppressed.

ATP (100 nM–10 μM), another endothelium-dependent relaxant, induced Ca\(^{2+}\) transients in BAECs. 1-Palmitoyl LPC also inhibited ATP (10 μM)–induced Ca\(^{2+}\) transients (data not shown). PC, even at a high

---

**Figure 3.** Tracings showing the effects of bradykinin (BK), lysophosphatidylcholine (LPC), and phosphatidylcholine (PC) on 
[Ca\(^{2+}\)], of bovine aortic endothelial cells (BAECs) in the presence of extracellular Ca\(^{2+}\). Panel A: In physiological salt solution (PSS) containing 1 mM CaCl\(_2\), 10 nM BK was added to the monolayer of fura 2–loaded BAECs at the time indicated by the arrow. Panel B: In PSS containing 1 mM CaCl\(_2\), 10 μg/ml 1-palmitoyl LPC was added to the monolayer of fura 2–loaded BAECs. Panel C: In PSS containing 1 mM CaCl\(_2\), 20 μg/ml PC was added to the cells. Panel D: An outline of a tracing of 510-nm fluorescence emission (F) is shown at two wavelengths (340 and 380 nm) from the experiment in panel B. Similar tracings were repeatedly observed using these concentrations of the agents. Each tracing is a representative of 10 independent experiments.
Inoue et al. Inhibition of EDRF by Lysophospholipids

FIGURE 4. Tracings showing the dose dependence of lysophosphatidylcholine (LPC)-induced rise of [Ca^{2+}] in bovine aortic endothelial cells (BAECs). F indicates fluorescence. In physiological salt solution containing 1 mM CaCl_2, 1-palmitoyl LPC (1–20 μg/ml) was added to the monolayer of fura 2–loaded BAECs. Each tracing is a representative tracing of five independent experiments.

dose (20 μg/ml), had no inhibitory effects on Ca^{2+} transients induced by BK (Figure 6F) and ATP. The results are summarized in Table 2.

After the preincubation of BAECs with 1-palmitoyl LPC (10 μg/ml), they were washed three times with 2 ml PSS containing 0.1% BSA, and then they were stimulated with 10 nM BK. The inhibitory effect of LPC was abolished by washing cells with PSS containing 0.1% BSA, and BK-induced Ca^{2+} transients were similar to those of BAECs without LPC (data not shown). This result indicates that the inhibitory effect of LPC is reversible.

With regard to the inhibitory effect, the structural specificity of LPC was examined. The specificity for inhibition was similar to that for the effect on Ca^{2+} transients (Table 1). 1-Stearoyl LPC similarly inhibited BK-induced Ca^{2+} transients. But 1-caproyl LPC and LPE, at equimolar concentrations of 10 μg/ml of 1-palmitoyl LPC, had no effect on BK-induced Ca^{2+} transients. Palmitoyl-l-carnitine, but not palmitic acid, had a similar inhibitory effect.

**LPC Inhibited BK-Induced Accumulation of Inositol Phosphates**

Incubation of cultured BAECs with 1 μM BK induced accumulation of IP_1, IP_2, and IP_3 as shown in Figure 7. IP_2 and IP_3 production was rapid and reached

<table>
<thead>
<tr>
<th>Table 1. Structural Specificity of Lysophospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agents</td>
</tr>
<tr>
<td>LPC (l-stearyl)</td>
</tr>
<tr>
<td>LPC (1-palmitoyl)</td>
</tr>
<tr>
<td>LPC (l-caproyl)</td>
</tr>
<tr>
<td>LPC (1-palmiroyl)</td>
</tr>
<tr>
<td>Palmitic acid</td>
</tr>
<tr>
<td>PC (1-palmitoyl, 2-oleoyl)</td>
</tr>
<tr>
<td>Palmitoyl-l-carnitine</td>
</tr>
</tbody>
</table>

LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine. For Ca^{2+} transients: +, increase in [Ca^{2+}]; –, no increase in [Ca^{2+}]. For inhibition: +, inhibition of bradykinin (10 nM)–induced Ca^{2+} transients; –, no inhibition of bradykinin (10 nM)–induced Ca^{2+} transients.
FIGURE 5. Tracings showing the effects of bradykinin (BK) and lysophosphatidylcholine (LPC) on $[Ca^{2+}]_{i}$, in the absence of extracellular $Ca^{2+}$. F indicates fluorescence. Panel A: In $Ca^{2+}$-free physiological salt solution (PSS) with 1 mM EGTA, 10 µg/ml 1-palmitoyl LPC induced a transient increase in $[Ca^{2+}]_{i}$, in bovine aortic endothelial cells (BAECs). Panel B: In $Ca^{2+}$-free PSS with 1 mM EGTA. 10 nM BK was added to the monolayer of BAECs. When $[Ca^{2+}]_{i}$ had returned to the basal level, 10 nM BK was repeatedly added to the cells. Panel C: In $Ca^{2+}$-free PSS with 1 mM EGTA, BAECs were first stimulated with 10 nM BK, and when $[Ca^{2+}]_{i}$ had returned to the basal level, 10 µg/ml 1-palmitoyl LPC was added. Panel D: In $Ca^{2+}$-free PSS with 1 mM EGTA, BAECs were first stimulated with 10 µg/ml 1-palmitoyl LPC and subsequently by 10 nM BK. Each tracing is a representative of five independent experiments.

A peak at 30 seconds with approximately threefold increases and thereafter declined. The decline of accumulated IP$_2$ and IP$_3$ might be due to the conversion of IP$_2$ to IP$_1$ and IP$_3$ to IP$_2$, as demonstrated in other cell types. On the other hand, without BK, no significant increase in any of the inositol phosphates was induced in BAECs preincubated with serum-depleted medium even in the presence of LiCl. Figure 8 shows the dose–response relation of BK for the accumulation of IP$_3$. The threshold concentration and the mean half-maximum effective concentration value of BK were 0.1 and 3 nM, respectively. Identical values were obtained for IP$_2$.

We examined the effects of phospholipids on the phospholipase C–mediated reaction in BAECs. Figure 9 shows the time course of the LPC-induced accumulation of inositol phosphates for 20 minutes. 1-Palmitoyl LPC (10 µg/ml) induced a small but significant amount of accumulation of IP$_2$ and IP$_3$ for 20 minutes. In contrast, the accumulation of IP$_3$ was not observed at any time. PC (1–20 µg/ml) itself had no effect on phospholipase C–mediated phosphoinositide hydrolysis. 1-Palmitoyl LPC (1–20 µg/ml) pretreatment for 10 minutes markedly inhibited accumulation of inositol phosphates promoted by 100 nM BK. As shown in Figure 10, the half-maximum inhibitory concentration value of LPC for BK-stimulated IP$_3$ accumulation was approximately 5 µg/ml. An identical value was obtained for IP$_2$. In contrast, PC (1–20 µg/ml) did not attenuate the BK-induced accumulation of inositol phosphates.

**Discussion**

The present study demonstrates that LPC inhibits the production and/or release of EDRF from BAECs in bioassay experiments (Figure 2). In our previous report, modified LDL with copper oxidation or phospholipase A$_2$ treatment inhibited endothelium-dependent relaxation of rabbit thoracic aortas, and LPC generated during the modification was the main substance in the impairment of endothelium-dependent relaxation. From these results, LPC did not affect the degradation of EDRF, but it inhibited the production and/or release of EDRF. It has been proposed that the production and release of EDRF are mediated by intracellular $Ca^{2+}$ signal transduction through a receptor-mediated phospholipase C–coupled system. To clarify the cellular mechanism of the inhibitory effect of LPC, we examined the effect of LPC on intracellular $Ca^{2+}$ signal transduction and phosphoinositide hydrolysis in endothelial cells.
TABLE 2. Summary of the Effects of Lysophosphatidyicholine and Phosphatidyicholine on Bradykinin-Induced Increases in [Ca++], of Bovine Aortic Endothelial Cells

<table>
<thead>
<tr>
<th>[Ca++]</th>
<th>(nM)</th>
<th>Initial component</th>
<th>Sustained component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,050±220</td>
<td>950±240</td>
<td></td>
</tr>
<tr>
<td>LPC (1 μg/ml)</td>
<td>1,100±230</td>
<td>1,050±280</td>
<td></td>
</tr>
<tr>
<td>LPC (5 μg/ml)</td>
<td>520±200*</td>
<td>480±200*</td>
<td></td>
</tr>
<tr>
<td>LPC (10 μg/ml)</td>
<td>250±120*</td>
<td>420±120*</td>
<td></td>
</tr>
<tr>
<td>PC (20 μg/ml)</td>
<td>1,020±170</td>
<td>900±90</td>
<td></td>
</tr>
</tbody>
</table>

LPC, lysophosphatidylcholine (1-palmitoyl); PC, phosphatidylcholine. Values are mean±SEM of 10 independent experiments.

The peak values during the initial and sustained components are shown for the cells incubated without phospholipids (control) and with 1, 5, or 10 μg/ml LPC and 20 μg/ml PC.

*P<0.05 compared with control.

We demonstrated that in cultured BAECs LPC induced a biphasic response of [Ca++]>, which consisted of a rapid increase followed by a sustained increase, and that the initial component was mobilized from intracellular Ca++ stores that overlapped the IP3-sensitive Ca++ stores. We also demonstrated that LPC itself did not induce the accumulation of IP3 in BAECs. The other major finding in this study is that LPC inhibits both Ca++ mobilization from intracellular Ca++ stores and Ca++ influx from extracellular space evoked by BK. We also demonstrated that LPC inhibited BK-stimulated phosphoinositide hydrolysis.

The Ca++ transient evoked by LPC as well as BK was composed of two components, the Ca++ mobilization from intracellular Ca++ stores and the Ca++ influx across the plasma membrane from the extracellular space. The mechanisms of LPC-induced Ca++ mobilization from intracellular Ca++ stores in BAECs are still unknown. In the absence of extracellular Ca++, the depletion of the IP3-sensitive Ca++ store abolished the Ca++ mobilization evoked by LPC (Figure 5), and LPC itself did not induce the detectable accumulation of IP3 (Figure 5). Thus, our findings indicate that LPC elicited Ca++ mobilization from IP3-sensitive intracellular Ca++ stores by mechanisms independent of phospholipase C-mediated systems. LPC was reported to move rapidly across the sarcolemmal reticulum, which has a specific transfer system, “flipase.” Although no documentation exists whether the plasma membrane of BAECs has this transfer system or not, it may be possible for LPC to move across the plasma membrane and induce Ca++ mobilization from the intracellular Ca++ stores. Previous studies showed that Ca++ mobilization from intracellular Ca++ stores without phosphoinositide hydrolysis was similarly induced by unsaturated free fatty acids in T cells. In another study of fibroblasts, LPC did not induce Ca++ mobilization from intracellular Ca++ stores. The discrepancy may be due to the difference of cell types. As shown in Figure 9, LPC did not induce the accumulation of IP3, but the levels of IP1 and IP2 were slightly but significantly increased. The reason for this observation might be related to accelerated degradation of IP3, conversion to other inositol compounds, or the heterogeneity of phospholipase C.

LPC induced Ca++ influx across the plasma membrane. In several cell types including cardiac myocytes,
LPC increased the membrane permeability and augmented Ca\(^{2+}\) influx across the plasma membrane. In the case of BAECs, LPC-induced Ca\(^{2+}\) influx might also be due to the alteration of the membrane permeability. As shown in Table 1, LPC with a long aliphatic chain, but not LPC with a short aliphatic chain, could induce Ca\(^{2+}\) transients. Neither LPE nor PC had an effect. These results suggest that the structure of a phosphate-containing polar head as well as the length of the fatty acid chain in the 1\(^\text{st}\) carbon region of glycerol backbone may play an important role in Ca\(^{2+}\) transients induced by lysophospholipids. It was reported that LPC itself induced endothelium-dependent relaxation of rabbit aorta through its weak detergent action.\(^{32}\) Our finding that LPC itself induced an increase in \([Ca^{2+}]_i\) in endothelial cells may be related to these findings.

LPC inhibited both the Ca\(^{2+}\) mobilization from the intracellular Ca\(^{2+}\) stores and the Ca\(^{2+}\) influx across the plasma membrane from the extracellular space evoked by BK. LPC also inhibited BK-stimulated phosphoinositide hydrolysis. The inhibitory effect of LPC on Ca\(^{2+}\) mobilization from intracellular Ca\(^{2+}\) stores results from the suppression of BK-induced IP\(_3\) accumulation. However, LPC itself induced Ca\(^{2+}\) transients, and the stores required refilling from extracellular space after Ca\(^{2+}\) was released into the cytosol. These findings indicate that the inhibitory effect of LPC on Ca\(^{2+}\) mobilization from Ca\(^{2+}\) stores may be partially due to the depletion of the IP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores.

The mechanism of the inhibitory effect of LPC on BK-stimulated Ca\(^{2+}\) influx is unknown, because the precise mechanism of the Ca\(^{2+}\) influx stimulated by agonists in endothelial cells is not fully established. Previous studies suggest that in several cells receptor-mediated Ca\(^{2+}\) influx may be mediated by 1) a channel that is activated secondarily by the rise in [Ca\(^{2+}\)], as observed in human neutrophils or internal second messengers other than Ca\(^{2+}\),\(^{33}\) 2) lipoxygenase intermediates,\(^{34}\) or 3) the empty state of the intracellular Ca\(^{2+}\) pool, i.e., the capacitative calcium entry.\(^{35}\) It is reported that BK-stimulated Ca\(^{2+}\) influx in endothelial cells is not related to voltage-operated Ca\(^{2+}\) channels.\(^{36}\) Amphiphilic compounds such as lysophospholipids are known to alter the kinetics of transmembrane ion transport, the activity of membrane-bound enzymes,\(^{37,38}\) and sarcosommal molecular dynamics.\(^{39}\) In Purkinje fibers, exogenous LPC was reported to be incorporated primarily into the plasma membrane within 10 minutes.\(^{40}\) According to the previous studies, an increase in lysophospholipids in the plasma membrane may increase the increased membrane fluidity and permeability. It is also possible that alteration of membrane fluidity may modulate the kinetics of membrane-bound proteins. The inhibitory effect of LPC is probably due to the direct interaction with the plasma membrane of endothelial cells. The changes of the membrane property and the perturbation of LPC into the membrane may

**FIGURE 7.** Time course of bradykinin-induced accumulation of inositol phosphates. Bovine aortic endothelial cells were stimulated with (○) and without (●) 1 μM bradykinin. Panel A: Accumulation of inositol 1-monophosphate. Panel B: Accumulation of inositol 1,4-bisphosphate. Panel C: Accumulation of inositol 1,4,5-trisphosphate. Results are expressed as mean±SEM of three independent experiments.
cause the inhibitory effects of LPC on phospholipase C-mediated phosphoinositide hydrolysis, the generation of second messengers, and Ca\(^{2+}\) influx across the plasma membrane evoked by BK. LPC also inhibited ATP-induced Ca\(^{2+}\) transients. This result indicates that the action of LPC is not as a receptor antagonist at endothelial cells.

Lypospholipids are known to influence several enzyme systems including adenylate cyclase, guanylate cyclase,\(^{38}\) and protein kinase C.\(^{41}\) Oishi et al.\(^{41}\) have demonstrated that LPC stimulated the protein kinase C purified from pig brain in vitro. More recently, we have demonstrated that LPC modulated the activity of protein kinase C purified from BAECs in a biphasic manner in vitro, i.e., stimulation at a lower concentration (3–10 \(\mu\)M) and inhibition at a higher concentration (>20 \(\mu\)M) (authors’ unpublished data). In general, the desensitization of the receptor is mediated at the level of the receptor, G protein, or phospholipase C.\(^{42}\) Ryan et al.\(^{43}\) showed that phorbol myristate acetate, but neither forskolin nor nitroprusside, inhibited the histamine-induced Ca\(^{2+}\) transient in cultured human aortic endothelial cells. These results suggest that a negative feedback mechanism, which blocks the intracellular Ca\(^{2+}\) signal transduction mediated by receptor agonists, is triggered through the activation of protein kinase C but not through cyclic nucleotides in endothelial cells. Moreover, prolonged exposure to phorbol 12,13-dibutyrate, another activator of protein kinase C, inhibited endothelium-dependent relaxation evoked by histamine in the pig pulmonary artery and by acetylcholine or substance P in the rabbit aorta.\(^{44,45}\) These results suggest that protein kinase C activation suppressed receptor-mediated processes linked to the synthesis of EDRF. Although it remains to be determined whether exogenously applied LPC can stimulate the protein kinase C system in intact endothelial cells, it is possible to speculate that the inhibitory effect of LPC may be mediated by the mechanisms activated by protein kinase C.

It is known that LPC also has detergent-like or cytotoxic properties. In general, below the critical micelle concentration (CMC), the amphiphilic molecule exists as a monomer. Above the CMC, the addition of more amphiphiles to the solution leads to the formation of micelles, but the concentration of free monomers does not change. It is reported that, at the concentration below the CMC, the membrane alteration in red blood cells is related to incorporation of exogenous LPC into the plasma membrane and that, at the concentration above the CMC, cell lysis occurs because of the detergent properties of micelles.\(^{46}\) The CMC for amphiphiles is dependent on the condition of the solution, and the CMC of LPC in a physiological solution is reported to be 40–50 \(\mu\)M (20–25 \(\mu\)g/ml).\(^{46}\) In this study, although

**FIGURE 9.** Time course of lysophosphatidylcholine (LPC)–induced accumulation of inositol phosphates. Bovine aortic endothelial cells were stimulated with (○) and without (●) 10 \(\mu\)g/ml 1-palmitoyl LPC for various periods of time. Panel A: Accumulation of inositol 1-monophosphate. Panel B: Accumulation of inositol 1,4-bisphosphate. Panel C: Accumulation of inositol 1,4,5-trisphosphate. Results are expressed as mean±SEM of five independent experiments. *\(p<0.05\) compared with the control value.

**FIGURE 10.** Concentration dependence of the inhibitory effects of phosphatidylcholine (PC, ●) and lysophosphatidylcholine (LPC, ○) on bradykinin (BK)–induced accumulation of inositol 1,4,5-trisphosphate. Bovine aortic endothelial cells were preincubated with various concentrations of 1-palmitoyl LPC for 10 minutes and then stimulated with 100 nM BK for 30 seconds. Results are expressed as mean±SEM of five independent experiments. *\(p<0.001\) compared with PC.
high doses (>20 μg/ml) of LPC induced cytotoxic changes such as cytoplasmic vacuolation and the abrupt leakage of the dye, these changes were not observed in the lower concentrations (<20 μg/ml). In the preliminary study, lactate dehydrogenase, a cytosolic enzyme, was not released from BAECs during the incubation of these concentrations of LPC for 1 hour (data not shown). Therefore, the observed effect of LPC on [Ca\textsuperscript{2+}]) and phosphoinositide hydrolysis in BAECs was not due to cytotoxic effects, and cell viability was preserved in these concentrations of LPC.

In atherosclerotic aorta, severalfold increases of LPC were demonstrated in nutritionally induced atherosclerosis.\textsuperscript{17} Furthermore, oxidized LDL has been shown to be present in atherosclerotic arterial lesions in the human and rabbit.\textsuperscript{12} In oxidized LDL, approximately 40% of PC was converted to LPC during oxidative modification.\textsuperscript{13} The estimated contents of LPC in 1 mg LDL were 20 μg/ml in native LDL and 220 μg/ml in oxidized LDL.\textsuperscript{13} The concentration of LPC used in the present study corresponded to the concentrations in which it has the inhibitory effects on endothelium-dependent relaxation,\textsuperscript{15,14} and they were within the biological range. The difference between the concentrations of LPC in the LDL particle and the concentrations of exogenous LPC to induce comparable effects in this study may reflect the limited availability of free LPC from LDL in the interaction of LPC with endothelial cells. We reported that oxidized LDL inhibited phosphoinositide hydrolysis and Ca\textsuperscript{2+} transients induced by BK.\textsuperscript{48,49} The effects of exogenous LPC in the present study are comparable to those of LPC in oxidized LDL.

From the present investigation, it is speculated that the LPC-induced impairment of the endothelium-dependent relaxation is related to the inhibitory actions of LPC on phosphoinositide hydrolysis and calcium increases in endothelial cells. These results suggest that the LPC that accumulates in oxidized LDL and atherosclerotic arteries may modify the function of vascular endothelial cells.

Acknowledgment

The authors would like to thank Miss Noriko Hamana for preparation of the cell cultures and typing the manuscript.

References


11. Carew TE, Schwenke DC, Steinberg D: Antiatherogenic effect of probucol unrelated to its hypolcholesterolemic effect: Evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. \textit{Proc Natl Acad Sci U S A} 1987;84:7722–7729


Lysophosphatidylcholine inhibits bradykinin-induced phosphoinositide hydrolysis and calcium transients in cultured bovine aortic endothelial cells.

N Inoue, K Hirata, M Yamada, Y Hamamori, Y Matsuda, H Akita and M Yokoyama

doi: 10.1161/01.RES.71.6.1410

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/71/6/1410

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/