Original Paper

Journal of Biomedical Science

J Biomed Sci 2002;9:327–333

Received: December 19, 2001
Accepted: April 2, 2002

Lysophosphatidylcholine Alters Vascular Tone in Rat Aorta by Suppressing Endothelial $[Ca^{2+}]_i$ Signaling

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Key Words
Calcium · Endothelium · Lysophosphatidylcholine · Vasodilatation

Abstract
The detailed mechanism of how lysophosphatidylcholine (LPC) suppresses endothelium-dependent vasodilatation is unclear at present. We investigated the effects of LPC on endothelial intracellular calcium (EC $[Ca^{2+}]_i$) signaling and vascular tone simultaneously using a new technique we developed. Fura-2-labeled rat aortic specimens were mounted in a tissue flow chamber and precontracted with phenylephrine (5×10^{-8} *M*). Under either basal or agonist-stimulated conditions, the EC $[Ca^{2+}]_i$ level was calculated from fura 2 fluorescence ratio images, and the vascular tone was estimated by measuring the relative displacement of the fluorescence images. Although both acetylcholine (ACh)-induced EC $[Ca^{2+}]_i$ elevation and the concomitant vasorelaxation were partially suppressed in specimens pretreated with LPC (20 μ*M*), the quantitative relationship between EC $[Ca^{2+}]_i$ elevation and the corresponding vasorelaxation was unaffected. A high concentration of LPC (40 μ*M*) completely eliminated ACh-evoked $[Ca^{2+}]_i$ elevation and vasodilatation. It has been reported that exposing vascular tissue to a calcium-free buffer causes a reduction in the EC $[Ca^{2+}]_i$ level and the accompanying vasoconstriction. Pretreatment with 20 μ*M* LPC reduced the basal EC $[Ca^{2+}]_i$ level and abolished the calcium-free solution-induced EC $[Ca^{2+}]_i$ reduction and vasoconstriction. We conclude that LPC impairs endothelium-dependent vasorelaxation mainly by reducing the basal EC $[Ca^{2+}]_i$ level and suppressing agonist-evoked EC $[Ca^{2+}]_i$ signaling.

Introduction

Lysophosphatidylcholine (LPC), a major bioactive component of oxidized low-density lipoprotein [2, 11], has been shown to exert multiple effects on cardiovascular functions, including perturbation of membrane permeability [10], induction of the expression of many adhesion molecules [9, 13], inhibition of endothelial cell migration and proliferation [14], modification of G-protein signaling [3, 21], repression of endothelium-dependent vasodilatation [1, 11], and stimulation of endothelial cell apoptosis [15]. Because of these multiple effects, it has been suggested that LPC may play an etiological role in atherosclerosis. Suppression of agonist-induced endothelium-dependent vasorelaxation (EDR) is one of the earliest pathological manifestations of atherosclerosis [16].

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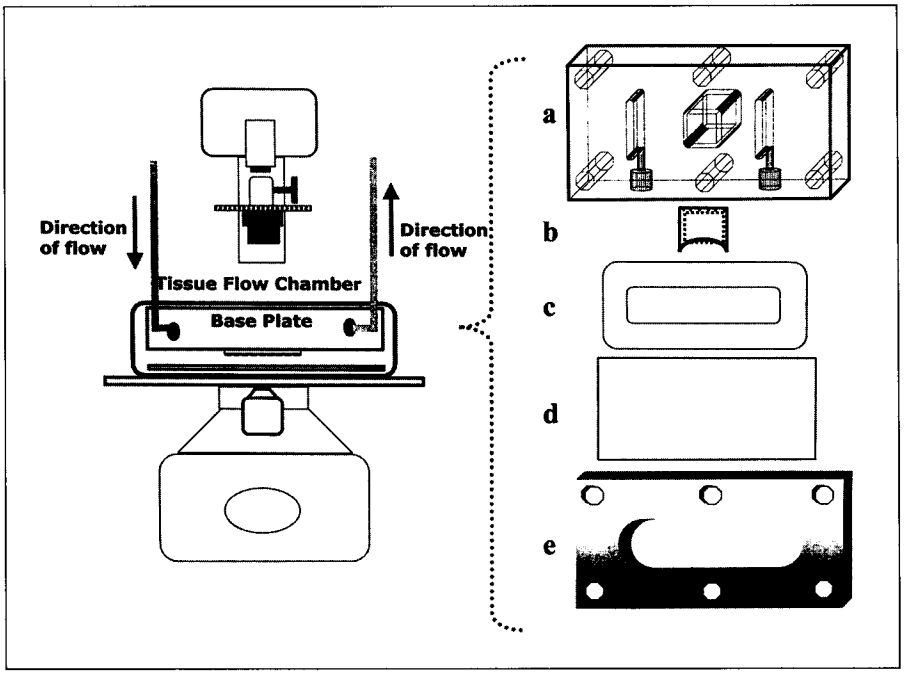


Fig. 1. Experimental setup.

It has been shown that the EDR evoked by endothelium-derived relaxation factors is impaired in vessel segments pretreated with LPC [1]. Although LPC-suppressed EDR has been examined in many studies, details of its mechanism are not clear at present. LPC may perturb either the efficacy or production of endothelial nitric oxide (NO), a well-known vasodilator. For example, enhanced superoxide production in the presence of LPC converts NO to peroxynitrite, which is a less-potent vasodilator than NO [2, 7, 20]. Moreover, LPC inhibits the elevation of endothelial intracellular free calcium concentrations (EC $[Ca^{2+}]_i$) and vasodilatation [12]. Recently we showed that EC $[Ca^{2+}]_i$ is an integrating signal for vascular tone modulation [5]. In this study, our aim was to clarify the role of EC $[Ca^{2+}]_i$ in LPC suppression of agonist-evoked EDR in rat aortae. Moreover, since the basal EC $[Ca^{2+}]_i$ level is crucial in maintaining the basal tone [5], the effect of LPC on the basal EC $[Ca^{2+}]_i$ level was also investigated.

To achieve our goal, we used a method that allows simultaneous measurements of EC $[Ca^{2+}]_i$ and vascular contraction/relaxation [5]. Our results confirm that EC $[Ca^{2+}]_i$, under both basal and acetylcholine (ACh)-stimulated conditions, plays an important role in modulating vascular tone [5] and that LPC suppresses both parameters under stimulated conditions [12]. Additionally, we also found that the effects of LPC were observed only when the ACh-evoked elevation in EC $[Ca^{2+}]_i$ was suppressed to less than 250 n*M*. Although both ACh-induced EC $[Ca^{2+}]_i$ elevation and vasorelaxation were suppressed by LPC pretreatment, the quantitative relationship between these two parameters remained the same. Finally, the basal EC $[Ca^{2+}]_i$ level was also suppressed by LPC, and this prevented further contraction when the specimen was exposed to a calcium-free buffer. Therefore, EC $[Ca^{2+}]_i$ appears to be the primary target of LPC-suppressed vasodilatation.

Methods

Vessel Preparation and Fura 2 Loading

This study was conducted in conformity with the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publ. No. 85-23, revised 1996). Male Wistar rats (6–8 weeks old) were anesthetized by ether inhalation, and their thoracic aortae were immediately dissected out. Aortic rings (5 mm long) were immersed in an organ chamber containing Krebs-Ringer solution (118.0 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 24 $NaHCO_3$, 0.03 Na_2-EDTA, and 11.0 glucose all in m*M*, pH 7.4) and gassed with 95% O_2-5% CO_2. The rings were labeled for 1 h at room temperature with 10 μ*M* fura 2-AM in the presence of 0.025% Pluronic F-127 [18]. In some experiments, the labeling procedure was carried out in the presence of 20 or 40 μ*M* LPC for 1 h. Excess chemicals were washed away with Krebs-Ringer solution.

Experimental Setup

The basic experimental setup for endothelial $[Ca^{2+}]_i$ measurement previously described [6] was modified during vessel mounting in order to allow simultaneous measurement of vascular endothelial $[Ca^{2+}]_i$ and vascular smooth muscle contraction [5]. One side of the longitudinally opened vessel segment was fixed in the direction of blood flow with insect pins. The corners on the opposite side were passively stretched to an optimal tension (4 g) and pinned onto the base plate (fig. 1b). This arrangement allows free vessel movement in response to the addition of vasoactive chemicals. The base plate was then covered with a gasket (fig. 1c), cover glass (fig. 1d), and metal plate (fig. 1e). After vessel mounting, the flow chamber was placed on a microscope stage and perfused with Krebs-Ringer buffer at 30 °C under a constant flow rate of 700 µl/min (estimated shear stress 3 dyn/cm^2) for an equilibration period of 1 h (fig. 1).

An initial series of experiments was performed on aortic en face preparations for simultaneous measurements of endothelial $[Ca^{2+}]_i$ and vasodilatation in the presence of phenylephrine (PE). Our previous study showed that the EC_{50} of PE is approximately 50 n*M*; this concentration was thereafter used in the following experiments to test the vascular responses to different vasodilators. Experiments were carried out in the presence of PE (50 n*M*), followed by subsequent exposure to cumulative ACh concentrations (10^{-9} to 10^{-5} *M*). The relative movement of endothelial cells was used as an index of vascular displacement, whereas fluorescence ratio images from fura-2-labeled endothelial cells provided quantitative information about EC $[Ca^{2+}]_i$.

Determination of in situ Endothelial $[Ca^{2+}]_i$ and Vascular Displacement

Calibration of endothelial $[Ca^{2+}]_i$ in each preparation was described previously [6]. Briefly, at the end of each experiment, EC $[Ca^{2+}]_i$ was calibrated by applying ionomycin (5 µ*M*) in the presence of 5 m*M* EGTA, followed by 10 m*M* $CaCl_2$. Finally, 5 m*M* of manganese was added in order to quench the cytosol fura 2 fluorescence. Endothelial $[Ca^{2+}]_i$ was estimated after subtracting the background and autofluorescence according to the established formula [4]. Axon image workbench software (Axon Instruments, Foster City, Calif., USA) was used for digital image acquisition and EC $[Ca^{2+}]_i$ analysis.

To calculate the vascular displacement, MetaMorph software (Universal Imaging, Downingtown, Pa., USA) was used to trace and analyze a series of images. The fluorescence image was sharply focused, and a particular endothelial cell in the initial image was selected as a location marker. In the presence of PE, this image was forced to shift inward during the experiments. After the marker had reached its final equilibrium location, the total displacement represented the extent of vascular contraction induced by PE. When the preparation was subsequently exposed to either ACh or ionomycin, the marker moved back towards its initial location, indicating vasorelaxation. The percent changes in vascular tone were normalized against the PE-precontraction value, regardless of whether LPC was present or absent.

Reagents

Reagents for preparing Krebs-Ringer solution were purchased from Merck (Darmstadt, Germany). Other reagents were obtained from Sigma (St. Louis, Mo., USA).

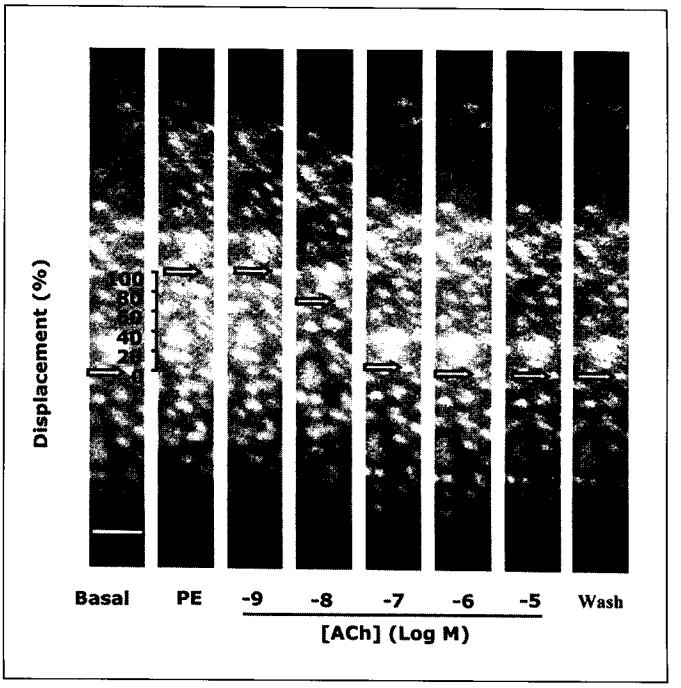


Fig. 2. An example of vascular displacement revealed under epifluorescence optics. A specific strip (65 × 480 pixels) from a series of calcium images (640 × 480 pixels) was selected to show an arbitrary EC marker. In this example, an EC marker moved upwards in response to PE stimulation and moved back downwards when subjected to the cumulative addition of ACh. Bar equals 100 µm.

Statistical Analysis

Results are expressed as the mean ± SEM. Sample sizes are indicated by n. Dose responses of agonist-induced $[Ca^{2+}]_i$ elevation were analyzed by ANOVA with a repeated measures design. Differences between two groups were compared using unpaired Student's t test with $p < 0.05$ considered statistically significant.

Results

In order to investigate the relationship between EC $[Ca^{2+}]_i$ and the concomitant vasodilatation, we simultaneously measured both parameters in our image processing system. While the fluorescence ratio image of endothelium can be used to calculate the EC $[Ca^{2+}]_i$, the displacement of fluorescence images serves as an index for vascular tone. A typical recording of the aortic displacement response to PE and ACh is shown in figure 2. The fluorescence image shifted inward during smooth muscle contraction when the specimen was exposed to PE. Subsequent exposure to cumulatively added concentrations of

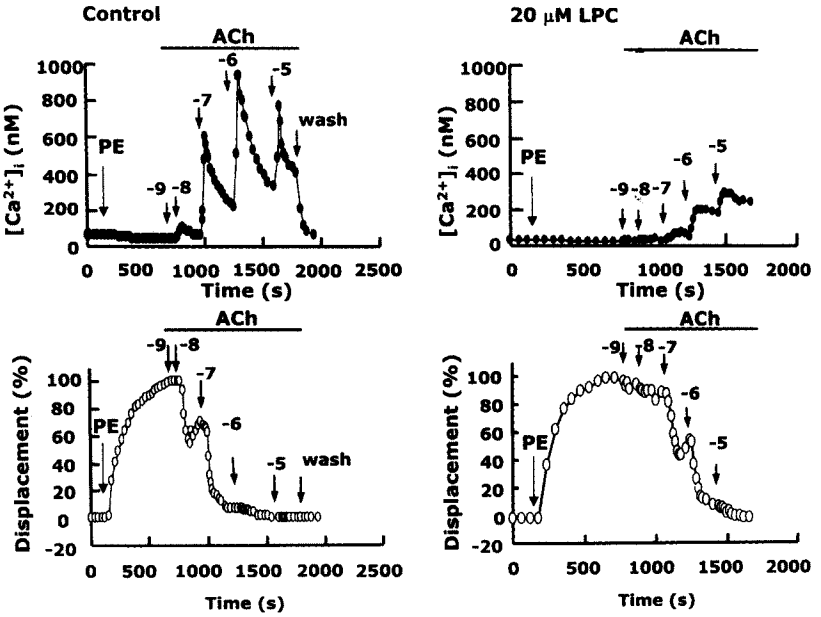
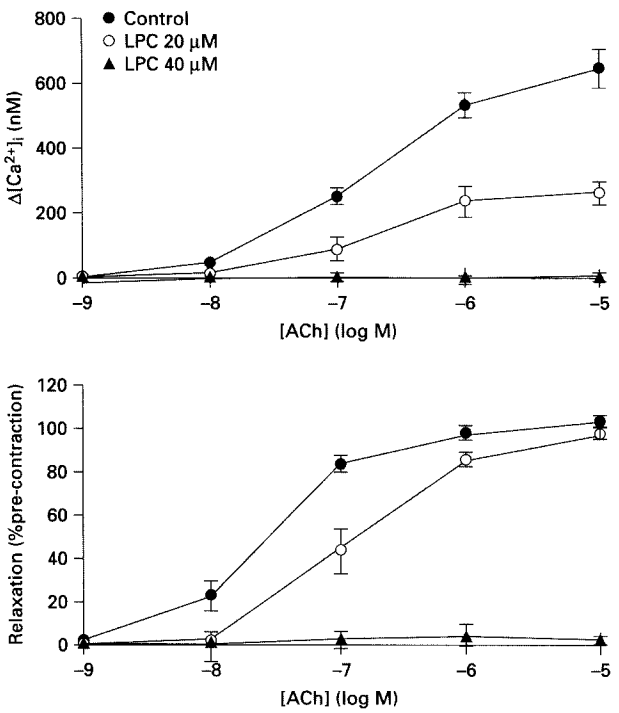


Fig. 3. Simultaneous recording of EC $[Ca^{2+}]_i$ and vascular displacement from aortic specimens with or without LPC pretreatment. Vasodilatation was initiated by low-level EC $[Ca^{2+}]_i$ elevation under either condition. When compared with the control group, the ACh-evoked EC $[Ca^{2+}]_i$ elevation was drastically suppressed in specimens pretreated with 20 μM LPC. However, at high concentrations of ACh, the relatively small elevations in EC $[Ca^{2+}]_i$ were sufficient to cause maximal vasodilatation in these LPC-pretreated specimens.



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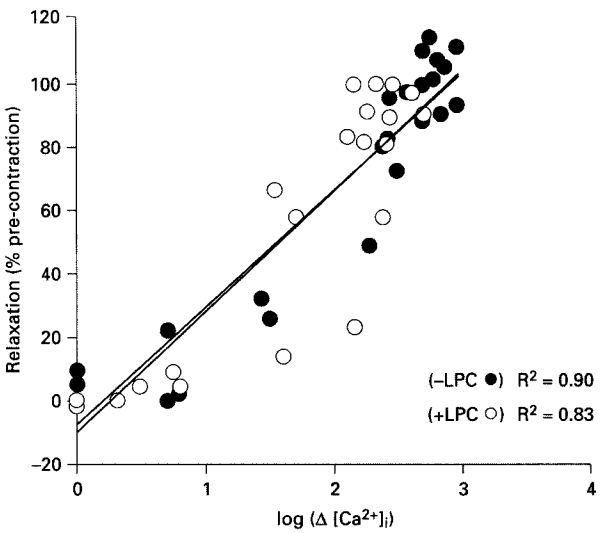

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Fig. 4. Statistical results of ACh-evoked EC $[Ca^{2+}]_i$ elevation and vasodilatation in specimens with or without LPC pretreatment. Both EC $[Ca^{2+}]_i$ level and vasodilatation increased in dose-dependent manners when subjected to the cumulative addition of ACh. While 20 μM of LPC significantly suppressed the calcium responses, it shifted the dose-response curve of vasodilatation slightly to the right. The maximal vasodilatation value remained the same. Both EC $[Ca^{2+}]_i$ elevation and the concomitant vasodilatation were abolished in specimens pretreated with 40 μM LPC (n = 5).

Fig. 5. Relationship between ACh-induced EC $[Ca^{2+}]_i$ elevation (abscissa) and smooth muscle relaxation (ordinate) with (○) or without (●) 20 μM LPC pretreatment in PE-precontracted vascular preparations. The lines are log-linear fitting curves of these two experimental groups.

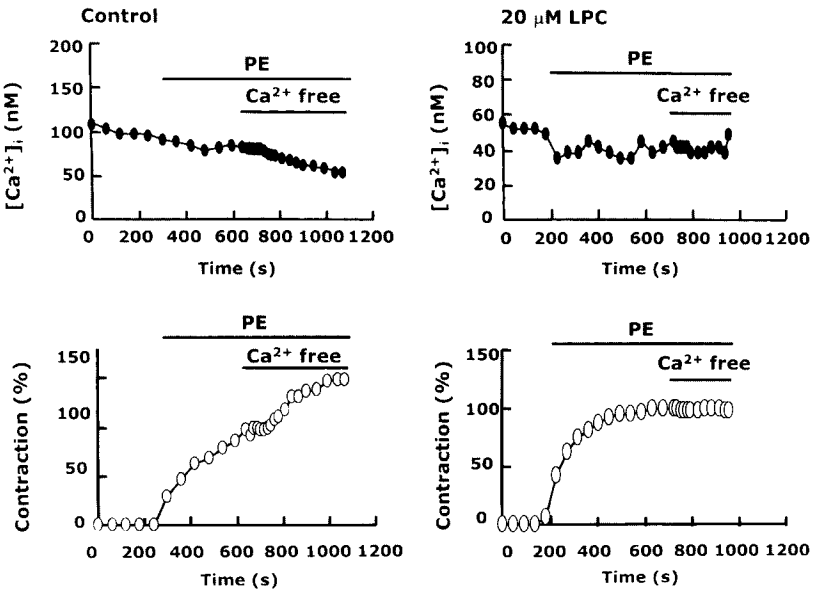

Fig. 6. Effects of calcium-free solution-induced changes in EC $[Ca^{2+}]_i$ and vascular displacement for specimens with or without LPC pretreatment. In the control group, the basal endothelial $[Ca^{2+}]_i$ level was suppressed when subjected to the calcium-free solution. This suppression of the EC $[Ca^{2+}]_i$ level was accompanied by vasoconstriction. When using specimens pretreated with 20 μM LPC, we found that the basal EC $[Ca^{2+}]_i$ level was reduced, and calcium-free solution-induced vasoconstriction was blocked.

ACh dilated the specimen and caused the fluorescence image to move in the opposite direction. Displacement of an arbitrary marker between the basal and PE-contracted conditions was defined as 100%, i.e. the maximal displacement. The relative vasorelaxation in the presence of various concentrations of ACh was expressed as a percentage of maximal displacement.

The left panel in figure 3 shows the control tracings of EC $[Ca^{2+}]_i$ and vascular displacement when specimens were initially exposed to PE and subsequently exposed to various concentrations of ACh. While PE induced rapid vascular displacement (contraction) without altering the EC $[Ca^{2+}]_i$, ACh induced both EC $[Ca^{2+}]_i$ elevation and vascular displacement (relaxation) in a dose-dependent manner. We then performed similar experiments using specimens incubated with LPC. Treatment with LPC did not alter the extent of PE-induced vasoconstriction. Although specimens pretreated with 20 μM LPC showed tracings similar to those of the controls, the actual responses at most ACh concentrations were obviously reduced (fig. 3). While specimens pretreated with 40 μM LPC showed PE-induced vasoconstriction, they no longer responded to ACh, as judged by the little ACh-evoked EC $[Ca^{2+}]_i$ elevation and vasorelaxation seen (tracings not shown).

Statistical results of ACh-evoked EC $[Ca^{2+}]_i$ elevation and concomitant vasorelaxation with or without 20 μM LPC pretreatment are shown in figure 4. In the control group, the EC $[Ca^{2+}]_i$ level progressively increased with increasing ACh concentrations, while vasorelaxation became saturated at ACh concentrations $>1 \times 10^{-7}$ M. As a comparison, LPC pretreatment drastically suppressed ACh-induced EC $[Ca^{2+}]_i$ elevation and shifted the dose-response curve of vasodilatation to the right without changing the maximal vasorelaxation value.

Original data from figure 4 were used to construct the quantitative relationship between EC $[Ca^{2+}]_i$ and vasorelaxation. When the ACh-evoked elevation of EC $[Ca^{2+}]_i$ was plotted against the extent of the corresponding vascular relaxation, the curve appeared to be log-linear (fig. 5). Since there was a significant increase in vasorelaxation which occurred with a minimal increase in EC $[Ca^{2+}]_i$, these curves showed no apparent threshold value of EC $[Ca^{2+}]_i$ elevation. The half-maximal relaxation induced by ACh application was accompanied by a less than 100-nM increase in EC $[Ca^{2+}]_i$. Moreover, even though the ACh-evoked EC $[Ca^{2+}]_i$ elevation in the LPC group rarely exceeded 300 nM, the correlation curves were almost identical regardless of whether specimens had been pretreated with 20 μM LPC.

We also noticed that LPC was able to suppress the basal EC $[Ca^{2+}]_i$ level (fig. 3). LPC (20 μM) reduced basal EC $[Ca^{2+}]_i$ values from 91.4 ± 14.0 to 46.3 ± 9.4 nM (n = 5). Under normal conditions, a reduction in the EC $[Ca^{2+}]_i$

Table 1. Changes in EC $[Ca^{2+}]_i$ and vasoconstriction induced by the calcium-free solution

	PE ($5 \times 10^{-8} M$)	
	normal	Ca^{2+}-free
EC $[Ca^{2+}]_i$, nM		
Control	91.4 ± 14.0	55.5 ± 3.7*
+ LPC	46.3 ± 9.4**	39.2 ± 8.5
Contraction, %		
Control	100	180.5 ± 19.8*
+ LPC	100	103.8 ± 2.5**

Data are expressed as the mean ± SEM (n = 5). * $p < 0.05$ (Ca^{2+}-free buffer vs. normal buffer), ** $p < 0.05$ (LPC-pretreated groups vs. the control).

level and concomitant vasoconstriction were observed when the calcium-free solution was applied to the vessel specimens, indicating a reduction in the basal release of endothelium-derived relaxing factors (fig. 6). In LPC-pretreated preparations, the basal EC $[Ca^{2+}]_i$ was lower, and the calcium-free solution caused little if any further decrease in EC $[Ca^{2+}]_i$. Moreover, the vasoconstriction induced by the calcium-free solution was also abolished. Results are summarized in table 1.

Discussion

In this study, we demonstrate the application of our newly developed method, one that allows simultaneous measurements of both EC $[Ca^{2+}]_i$ level and vascular displacement, in order to examine the mechanism of LPC-induced suppression of EDR at the tissue level. An advantage of our system is that it provides good temporal and spatial resolution for quantifying EC $[Ca^{2+}]_i$ levels in rat aortic specimens. Moreover, with this system, the fura 2 fluorescence signal almost entirely originates from the vascular endothelium [6]. This is particularly important when vascular specimens are either contracting or relaxing during experimental manipulations. Since $[Ca^{2+}]_i$ levels in vascular smooth muscle cells change with vascular tone, quantification of EC $[Ca^{2+}]_i$ levels would be rendered very difficult if part of the fluorescence signal actually came from the underlying smooth muscle cells. In addition, both EC $[Ca^{2+}]_i$ and vascular contractility were measured under the condition of both endothelial cells and smooth muscle cells remaining intact.

This study reveals the determining factor(s) for vascular responses that is(are) impaired in LPC-pretreated rat aortae by investigating the quantitative relationship between EC $[Ca^{2+}]_i$ levels and vascular displacement using LPC-pretreated vascular specimens. Our results support the notion that EC $[Ca^{2+}]_i$ is not only a major modulator in the regulation of vascular tone but also the major target of LPC-suppressed EDR. Regardless of whether or not aortic specimens were pretreated with LPC, agonist-induced EDR was similarly correlated with EC $[Ca^{2+}]_i$ levels, suggesting that factors downstream of EC $[Ca^{2+}]_i$ signaling are relatively unaltered by LPC. Although LPC may enhance superoxide formation in cultured endothelial cells as well [2, 7, 20], this mechanism may not be so important in vascular tissues. Reduced production of endothelium-derived relaxing factors in LPC-pretreated vascular tissues is likely due to inhibition of agonist-evoked EC $[Ca^{2+}]_i$ elevation and suppression of the basal EC $[Ca^{2+}]_i$ level.

LPC suppresses not only ACh-evoked EC $[Ca^{2+}]_i$ elevation but also the basal EC $[Ca^{2+}]_i$ level. Our previous study showed that both ACh-induced EC $[Ca^{2+}]_i$ elevation and EDR are completely abolished in rat aortae pretreated with 40 μM BAPTA which chelates EC $[Ca^{2+}]_i$ [5]. In the present study, pretreatment with 40 μM LPC also blocked ACh-induced EC $[Ca^{2+}]_i$ elevation and EDR. Moreover, the basal EC $[Ca^{2+}]_i$ level was also suppressed by LPC. Although LPC may impair some factor(s) upstream of EC $[Ca^{2+}]_i$ signaling, the exact mechanism of how LPC achieves this is still unknown. Lysophosphatidic acid (LPA), a molecule related to LPC, was also used in this study. LPA has been reported to exert some deleterious effects on endothelial cells [17]. However, pretreatment with LPA affected neither EC $[Ca^{2+}]_i$ signaling nor EDR in our system (data not shown). Recently, LPC receptors have been identified in lymphocytes [8]. It has been suggested that LPC inhibits G protein and causes the inhibition of agonist-induced EC $[Ca^{2+}]_i$ elevation [3]. Whether or not LPC binding to its receptors disturbs G-protein-coupled calcium signaling needs to be further investigated in vascular endothelium.

Our previous study also showed that when rat aortic tissue is exposed to a calcium-free solution, both a drop in the basal EC $[Ca^{2+}]_i$ level and concomitant vasoconstriction occur [5]. Moreover, the calcium-free solution-induced vasoconstriction can be blocked by a combination of inhibitors of various endothelium-derived relaxing factors. Among all relaxation factors, NO has gained wide acceptance as a cardiovascular protector, as it has many anti-atherogenic effects including inhibition of the follow-

ing: smooth muscle proliferation and migration, platelet aggregation, oxidation of low-density lipoproteins, monocyte and platelet adhesion, and synthesis of inflammatory cytokines [19]. The mechanism of basal NO release being suppressed by the inhibitory effects of LPC on the basal EC $[Ca^{2+}]_i$ level can partially explain the atherogenic role of LPC. To our knowledge, we are the first to address the physiological role of the EC basal $[Ca^{2+}]_i$ level and the suppression effects of LPC on the EC basal $[Ca^{2+}]_i$ level.

If the vascular responses to LPC are to interrupt the EC $[Ca^{2+}]_i$ signaling in both basal and stimulated conditions, then LPC should not affect EDR caused by EC $[Ca^{2+}]_i$-independent mechanisms. We tested another agonist, clonidine, which induces vasodilatation in PE-precontracted vessels without elevating EC $[Ca^{2+}]_i$ levels in this system (data not shown). LPC did not affect clonidine-induced vasodilatation either (data not shown). This evidence further strengthens our hypothesis that LPC affects EDR mainly by suppressing EC $[Ca^{2+}]_i$ signaling. Finally, our findings are also consistent with the fact that 10 μM LPC does not affect EDR induced by various concentrations of A23187 [1], a receptor-independent calcium ionophore.

Acknowledgments

This study was supported by grants from the National Science Council of the ROC (NSC 90-2320-B-006-047, 90-2320-B-006-077) and from the Ministry of Education (91-B-FA09-2-4), Taiwan, ROC.

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