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Marked dissociation between intracellular Ca^{2+} level and contraction on exposure of rat aorta to lysophosphatidylcholine

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Abstract

We investigated the relationship between tension development and the cytosolic free Ca^{2+} level ($[Ca^{2+}]_i$) on exposure of the endothelium-denuded isolated rat aorta to palmitoyl-L-α-lysophosphatidylcholine. Lysophosphatidylcholine concentration-dependently induced a gradual increase in $[Ca^{2+}]_i$. Application of 10^{-4} M lysophosphatidylcholine induced a large and sustained tonic increase in $[Ca^{2+}]_i$ (the peak $[Ca^{2+}]_i$ was $125.2 \pm 11.5\%$ of the 80 mM K^+-induced response) but only a small contraction ($4.0 \pm 1.4\%$ of the 80 mM K^+ induced contraction). The sustained increase in $[Ca^{2+}]_i$ was attenuated when extracellular Ca^{2+} was removed but it was unaffected by verapamil or 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7). Digitonin also produced a gradual increase in $[Ca^{2+}]_i$ but with a pronounced contraction. Triton X-100 (0.1%) produced a marked elevation in $[Ca^{2+}]_i$ with no detectable contraction. Triton X-100, however, caused a rapid leakage of fura PE-3. Treatment with 10^{-4} M lysophosphatidylcholine for 1 or 2 h did not affect the contractile response induced by 80 mM K^+ and this treatment did not release lactate dehydrogenase from the rat aorta. Treatment with lysophosphatidylcholine did not affect either the cyclic AMP level or the cyclic GMP level in endothelium-denuded aortic tissues. These results show that in the rat aorta lysophosphatidylcholine produces a large increase in $[Ca^{2+}]_i$ (possibly in a non-contractile compartment) which does not induce contraction. Thus, the increase in $[Ca^{2+}]_i$ induced by lysophosphatidylcholine (i) requires external Ca^{2+} but is not due to an increased Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels, (ii) is not primarily due to protein kinase C activation and (iii) is probably not due to a detergent action (like those of digitonin and triton X-100). The relative lack of a contractile response to lysophosphatidylcholine is not due to formation of cyclic AMP or cyclic GMP.

Keywords: Lysophosphatidylcholine; Ca^{2+} level, cytosolic; Contraction; Detergent; Aorta, rat

1. Introduction

Lysophosphatidylcholine is considered to play a causative role in the development of atherosclerosis and ischaemic heart disease (Corr et al., 1984; Das et al., 1986; Steinberg et al., 1989). It has been reported that this substance is transferred from oxidatively modified low-density lipoprotein (LDL) to the endothelial surface membrane and that both lysophosphatidylcholine and oxidized LDL impair endothelium-dependent relaxation in isolated arteries (Kugiyama et al., 1990, 1992; Rajavashisth et al., 1990; Witztum and Steinberg, 1991; Flavahan, 1992). Lysophosphatidylcholine accumulates in myocardial tissue during ischaemia (Corr et al., 1982; Kinnaird et al., 1988) and exogenous application of lysophosphatidylcholine produces Ca^{2+} overload in isolated or cultured ventricular myocytes (Liu et al., 1991; Ver Donck et al., 1992; Chen et al., 1996). Lysophosphatidylcholine also causes Ca^{2+} influx in cultured vascular smooth muscle cells (Locher et al., 1992; Stoll and Spector, 1993; Tokumura et al., 1994; Chen et al., 1995).

It is well known that protein kinase C mediates the signal transduction underlying a variety of cellular functions (Nishizuka, 1986; Asaoka et al., 1992) and that activation of protein kinase C produces contraction in several arteries (Danthuluri and Deth, 1984; Rasmussen et al., 1984, 1987; Itoh and Lederis, 1987; Bazan et al., 1993; Suenaga et al., 1993). Lysophosphatidylcholine is capable

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of activating protein kinase C, and protein kinase C activation is involved in the mechanism leading to endothelial dysfunction (Kugiyama et al., 1992). Moreover, it has also been reported that in the permeabilized small mesenteric artery, lysophosphatidylcholine increases Ca^{2+} sensitivity via protein kinase C activation (Jensen et al., 1996).

Since lysophosphatidylcholine produces a Ca^{2+} influx and activates protein kinase C, it would be expected to be a strong vasoconstrictor. Indeed, Murohara et al. (1994) reported that lysophosphatidylcholine elicited a further contraction during the plateau contraction evoked by prostaglandin $F_{2\alpha}$ in the pig coronary artery. However, this contraction was found to be caused by a lysophosphatidylcholine-mediated inhibition of endothelium-derived nitric oxide release.

Few studies have assessed the effect of lysophosphatidylcholine on an endothelium-denuded artery. Recently, we reported that lysophosphatidylcholine potentiated the vascular contractile responses induced by high K^+, UK14,304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) (a selective α_2-adrenoceptor agonist) and phorbol ester in the endothelium-denuded rat aorta (Suenaga and Kamata, 1998). However, in that study, we noted that lysophosphatidylcholine did not produce a contraction when given alone. The aim of the present study was to determine whether lysophosphatidylcholine itself produces an increase in $[Ca^{2+}]_i$ in the isolated rat aorta, as it does in cultured vascular smooth muscle cells, and to examine the relationship between intracellular free Ca^{2+} and contractile force during exposure to lysophosphatidylcholine.

2. Materials and methods

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Sciences, Sports and Culture, Japan).

2.1. Preparation of aortic strips

Male Wistar rats, 8–10 weeks old, were anaesthetized with sodium pentobarbitone (50 mg/kg, i.p.), then killed by decapitation. The thoracic aorta was rapidly dissected out and placed into modified Krebs–Henseleit solution (KHS, composition in mM: NaCl 118; KCl 4.7; $CaCl_2$ 1.8; $NaHCO_3$ 25.0; $MgSO_4$ 1.2; NaH_2PO_4 1.2; dextrose 11.0). It was then cleaned of loosely adhering fat and connective tissue and cut into helical strips 2 mm in width and 10 mm in length. The endothelium was removed by rubbing the intimal surface with a cotton swab, successful removal being functionally confirmed by the absence of a relaxation to 10 μM acetylcholine.

2.2. Measurement of intracellular free Ca^{2+} and tension

Tension and cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) were measured by the method of Sato et al. (1988) with the minor modifications described previously (Suenaga and Kamata, 1998). The aortic strips were exposed to 10^{-5} M fura PE3-acetoxymethyl ester (fura PE3-AM) in the presence of 0.04% cremophor EL in the dark for 5 h at 26°C. The tissue was then rinsed with normal KHS and placed in the organ bath of a fluorometer (Japan Spectroscopic, CAF 110, Tokyo, Japan) containing KHS at 37°C. The tissue was held in a horizontal configuration under a resting tension of 0.5 g (which is the optimal tension for inducing a maximal contraction) and equilibrated for 30 min. One end of the tissue was connected to a force transducer for the measurement of isometric tension (Nihon Kohden, TB-611, Tokyo, Japan) and the other end was fixed. The intimal surface of the fura-PE3-loaded tissue was alternately (frequency, 128 Hz) subjected to excitation wavelengths of 340 and 380 nm. The fluorescence emissions at a wavelength of 500 nm for each excitation light were separated by means of a dichroic mirror and measured with the aid of a photomultiplier. The ratio (F_{340}/F_{380}) of the emitted fluorescence signals was used to provide an index of $[Ca^{2+}]_i$. The contractile responses and the emitted fluorescence signals were displayed on a pen recorder (Yokogawa, Model 3021, Tokyo, Japan).

After equilibration, each aortic strip was first exposed to 80 mM K^+ (made by iso-osmotic substitution for NaCl). The fluorescence ratio (F_{340}/F_{380}) in the resting muscle and that in muscle depolarized by 80 mM K^+ were taken as 0 and 100%, respectively. After washing, the aortic strips were exposed to lysophosphatidylcholine, digitonin, triton X-100 or 80 mM K^+. To exclude the possible influence of extracellular Ca^{2+}, some preparations were exposed for 5 min to Ca^{2+}-free or low Ca^{2+} KHS each solution containing 0.5 mM ethylene glycol-bis-(beta-aminoethyl ether) *N,N,N′,N′*-tetra-acetic acid (EGTA), before lysophosphatidylcholine was added to the bath. When the response to lysophosphatidylcholine had stabilized, Ca^{2+} (2 mM) was added. Calculations of the concentration of extracellular free Ca^{2+} were made with allowance being made for the temperature and pH of the solution (Goldstein, 1979).

2.3. Influence of lysophosphatidylcholine on vascular contraction, and assessment of cytotoxicity

Endothelium-denuded aortic strips were first contracted by 80 mM K^+ and these responses were taken as 100%. After washing and equilibrating for 1 h, the aortic strips were incubated for 1, 2 or 24 h with or without 10^{-4} M lysophosphatidylcholine. After the incubation period, the aortic strips were washed with fresh KHS and then exposed to 80 mM K^+. In this study, aortic strips were

pretreated with 3×10^{-4} M N^{ω}-nitro-L-arginine to avoid the influence of inducible NO synthase.

The cytotoxic effect of lysophosphatidylcholine on the aortic strips was assessed by measuring lactate dehydrogenase (LDH) release. For this, we used a commercially available method (LDH-UV test kit; Wako, Osaka, Japan) based on the procedure described by Wrobleski and LaDue (1955). Aortic strips (2 mm in width and 40 mm in length) were put into a test tube containing 3 ml KHS at 37°C and incubated for 1, 2 or 24 h with or without 10^{-4} M lysophosphatidylcholine. After the incubation period, 0.05 ml of the incubation solution was removed and the LDH in the solution determined. The aortic strip itself, in the remaining incubation solution (2.95 ml), was treated with 1% triton X-100 for 30 min to enable total LDH activity to be measured.

2.4. Assay of cyclic nucleotides

Endothelium-denuded aortic tissues were equilibrated in KHS for 1 h, then incubated with lysophosphatidylcholine, sodium nitroprusside or forskolin for 1 or 10 min. After incubation, the preparations were quickly frozen in liquid nitrogen, then homogenized in ice-cold 6% trichloroacetic acid containing 10^{-4} M 3-isobutyl-1-methyl-xanthine (IBMX) in a Potter glass–glass homogenizer. The homogenates were centrifuged at 3000 rpm for 10 min at 4°C and the supernatants were extracted with three volumes of water-saturated ether. Cyclic AMP and cyclic GMP were measured by radioimmunoassay using Yamasa cyclic AMP and cyclic GMP kits. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. The amount of each cyclic nucleotide was expressed as pmol/mg protein.

2.5. Drugs

Bovine serum albumin (Fraction V), cremophor EL, digitonin, EGTA, forskolin, H-7, IBMX, L-α-lysophosphatidylcholine (palmitoyl), N^{ω}-nitro-L-arginine, noradrenaline, triton X-100 and verapamil were purchased from

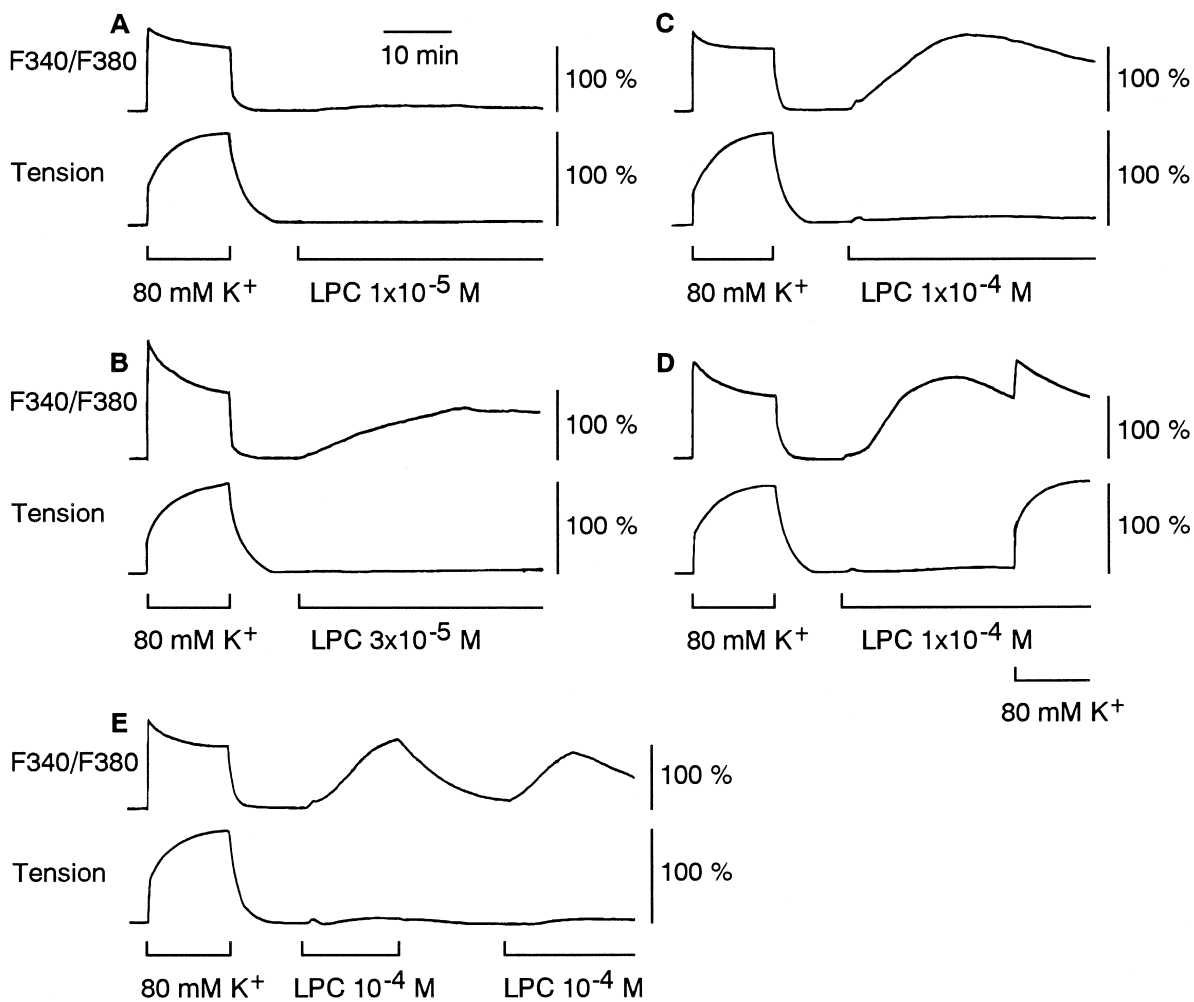

Fig. 1. Typical traces showing changes in fluorescence ratio (F340/F380) and force development induced by various concentrations of lysophosphatidylcholine (LPC) in the rat aorta (A–C). (D) The aortic strip was exposed to 80 mM K^+ (second application) in the continued presence of 10^{-4} M lysophosphatidylcholine. (E) The aortic strip was exposed to 10^{-4} M lysophosphatidylcholine for 15 min, then washed with flash solution for 15 min before 10^{-4} M lysophosphatidylcholine was applied again. The resting level is taken as 0% and the 80 mM K^+-stimulated level as 100%.

Sigma (St. Louis, MO, USA). Acetylcholine chloride was purchased from Daiichi Pharmaceuticals (Tokyo, Japan). Fura PE3-AM and sodium nitroprusside were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fura PE3-AM was dissolved in dimethylsulfoxide (DMSO) and mixed with cremophor EL. The final concentrations of DMSO and cremophor EL were 1% and 0.04%, respectively. At these concentrations, DMSO and cremophor EL had no detectable effect on $[Ca^{2+}]_i$ or tension in the aortic strips. Forskolin was dissolved in DMSO and diluted in distilled water. All the other drugs were dissolved in distilled water.

2.6. Statistics

The results shown in the text and figures are expressed as the mean ± S.E. Statistical differences were assessed by Dunnett's multiple comparison test following a One-way analysis of variance.

3. Results

3.1. Intracellular free Ca^{2+} and tension development in response to lysophosphatidylcholine

Typical changes evoked by palmitoyl-L-α-lysophosphatidylcholine in muscle tension development and intracellular free Ca^{2+} in fura-PE3-loaded aortic strips are illustrated in Fig. 1. Lysophosphatidylcholine concentration-dependently induced a gradual increase in $[Ca^{2+}]_i$ in normal medium. The application of 10^{-4} M lysophosphatidylcholine induced a large and sustained increase in $[Ca^{2+}]_i$ (the peak $[Ca^{2+}]_i$ was 125.2 ± 11.5% of the 80 mM K^+-induced response). However, this concentration of lysophosphatidylcholine produced only a slight contraction (4.0 ± 1.4% of the 80 mM K^+-induced contraction). During the lysophosphatidylcholine response, a second application of 80 mM K^+ induced a contraction that was 98.2 ± 2.5% ($n = 4$) of that induced by the first application of 80 mM K^+ (Fig. 1D). The second application of 80 mM K^+ also elicited a further increase in $[Ca^{2+}]_i$ on top of the increase in $[Ca^{2+}]_i$ evoked by lysophosphatidylcholine. As a comparison, in lysophosphatidylcholine-untreated aortic strips, a second application of 80 mM K^+ induced a contraction that was 99.9 ± 0.5% of the contraction evoked by the first application of 80 mM K^+ (Fig. 2). In contrast, lysophosphatidylcholine (10^{-4} M) reduced the contractile response to 10^{-5} M noradrenaline by 15% (from 103.4 ± 2.1% to 88.0 ± 5.7% of the 80 mM K^+-induced response; $p < 0.05$, $n = 4$). The lysophosphatidylcholine-evoked increase in $[Ca^{2+}]_i$ was repeatable following a washout (Fig. 1E), the second administration of lysophosphatidylcholine (10^{-4} M) evoking a substantial increase in $[Ca^{2+}]_i$ (the peak $[Ca^{2+}]_i$ was 79.9 ± 7.2% of the 80 mM K^+-induced response). When a lower concentration of lysophosphatidylcholine (3×10^{-5} M) was used (as the first administration), it evoked a substantial increase in $[Ca^{2+}]_i$ (the peak $[Ca^{2+}]_i$ was 44.9 ± 5.5% of the 80 mM K^+-induced response) with no contraction at all (Fig. 1B). These results are summarized in Fig. 2.

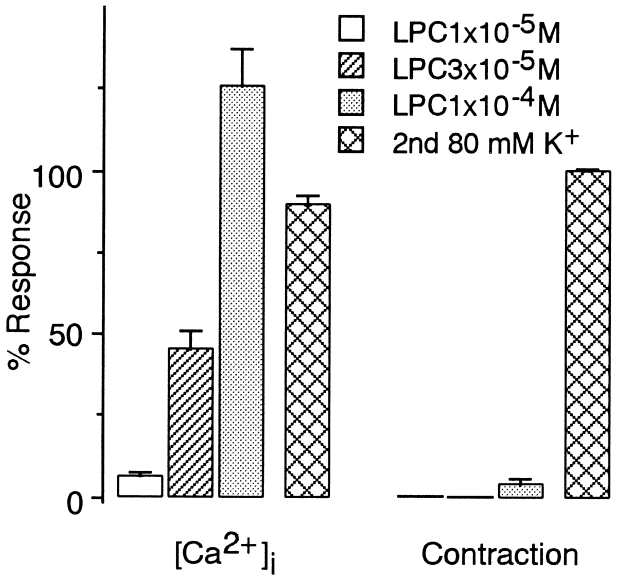

Fig. 2. Changes in $[Ca^{2+}]_i$ level and contraction induced by various concentrations of lysophosphatidylcholine (LPC) in the rat aorta. The peak increase in $[Ca^{2+}]_i$ evoked by lysophosphatidylcholine was taken to be the response. For comparison, the cross-hatched columns show the changes in $[Ca^{2+}]_i$ level and contraction induced by a second application of 80 mM K^+ in the absence of lysophosphatidylcholine. The resting level is taken as 0% and the 80 mM K^+-stimulated level (first application) as 100%. Values represent the mean ± S.E. for 4–5 smooth muscle preparations, each isolated from a different animal.

To test whether the increase in $[Ca^{2+}]_i$ induced by lysophosphatidylcholine depended on Ca^{2+} influx, we investigated the relationship between the extracellular free Ca^{2+} concentration and the lysophosphatidylcholine-induced increase in $[Ca^{2+}]_i$. The magnitude of the 10^{-4} M lysophosphatidylcholine-evoked sustained increase in $[Ca^{2+}]_i$ was proportional to the concentration of Ca^{2+} in the extracellular medium (Fig. 3). In Ca^{2+}-free solution, 10^{-4} M lysophosphatidylcholine induced only a small and very transient increase in $[Ca^{2+}]_i$ (9.1 ± 1.7% of the 80 mM K^+-induced response) followed by a return to the resting level, and no detectable contraction (Fig. 4A). Addition of Ca^{2+} (2 mM) to the Ca^{2+}-free solution in the continued presence of lysophosphatidylcholine induced a sustained increase in $[Ca^{2+}]_i$ (80.5 ± 8.2% of the 80 mM K^+-induced response) with a slight contraction (4.3 ± 0.6% of the 80 mM K^+-induced response) (Fig. 4A). Verapamil (10^{-5} M) did not affect the increase in $[Ca^{2+}]_i$ induced by 10^{-4} M lysophosphatidylcholine, but it strongly inhibited the 80 mM K^+-induced contraction (Fig. 4B). The protein kinase C inhibitor, 1-(5-isoquinolinesulphonyl)-2-methyl-

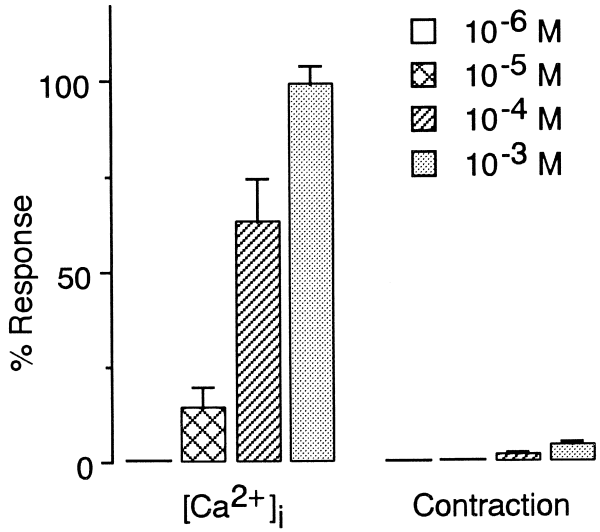

Fig. 3. Effect of varying the concentration of extracellular Ca^{2+} on the 10^{-4} M lysophosphatidylcholine-evoked sustained increase in $[Ca^{2+}]_i$. The resting level is taken as 0% and the 80 mM K^+-stimulated level as 100%. When aortic strips were exposed to lysophosphatidylcholine in 10^{-6} M Ca^{2+} Krebs solution, a sustained increase in $[Ca^{2+}]_i$ was not obtained. Values represent the mean ± S.E. for 4 smooth muscle preparations, each isolated from a different animal.

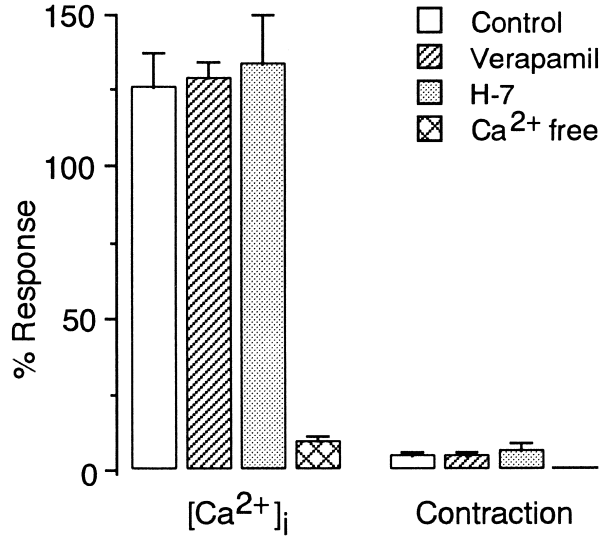

Fig. 5. Effects of removal of extracellular Ca^{2+}, application of 10^{-5} M verapamil and application of 10^{-5} M H-7 on changes in $[Ca^{2+}]_i$ level and contraction induced by 10^{-4} M lysophosphatidylcholine in the rat aorta. ''Control'' represents response to 10^{-4} M lysophosphatidylcholine in normal medium. The resting level is taken as 0% and the 80 mM K^+-stimulated level as 100%. Values represent the mean ± S.E. for 4–5 smooth muscle preparations, each isolated from a different animal.

piperazine dihydrochloride (H-7) at a concentration of 10^{-5} M also failed to affect the increase in $[Ca^{2+}]_i$ induced by 10^{-4} M lysophosphatidylcholine. These results are summarized in Fig. 5.

3.2. Influence of detergent action on intracellular free Ca^{2+} and tension

It seemed possible that the dissociation between the intracellular Ca^{2+} level and contraction on exposure of the rat aorta to lysophosphatidylcholine could be due to a detergent action. The application of detergent may produce morphological changes such as cytoplasmic vacuolation and an abrupt leakage of some of the cytosolic proteins that are related to the contractile response. Under these conditions, leakage of fluorescence dye may also occur.

Lysophosphatidylcholine (10^{-4} M) caused an increase in the 500 nm fluorescence emission at 340 nm excitation and a simultaneous decrease in the 500 nm fluorescence

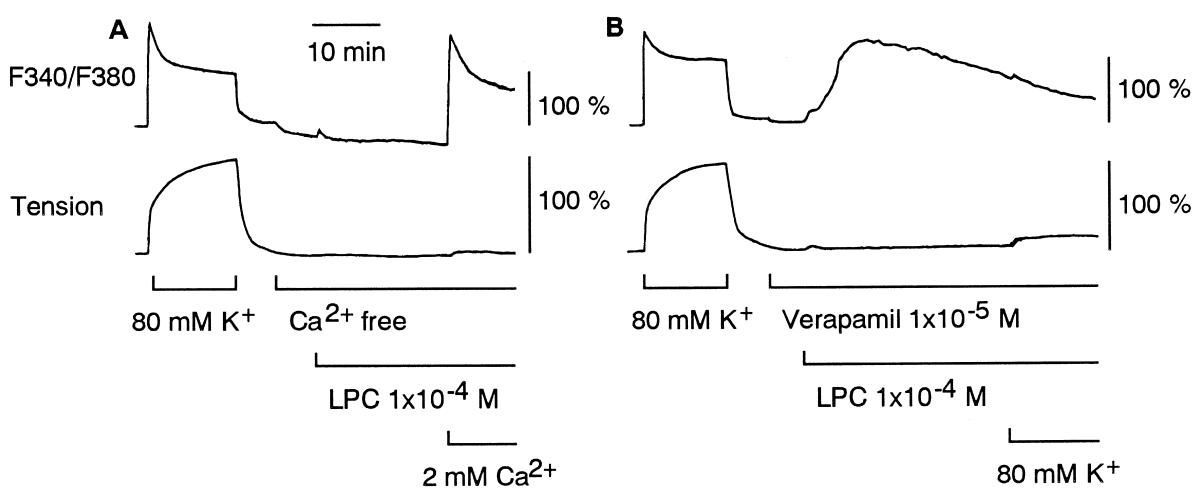

Fig. 4. Typical traces showing effects of removal of extracellular Ca^{2+} (A) or application of 10^{-5} M verapamil (B) on changes in fluorescence ratio (F340/F380) and force development induced by 10^{-4} M lysophosphatidylcholine (LPC) in the rat aorta. In panel A, lysophosphatidylcholine was still present when Ca^{2+} (2 mM) was added. The resting level is taken as 0% and the 80 mM K^+-stimulated level as 100%.

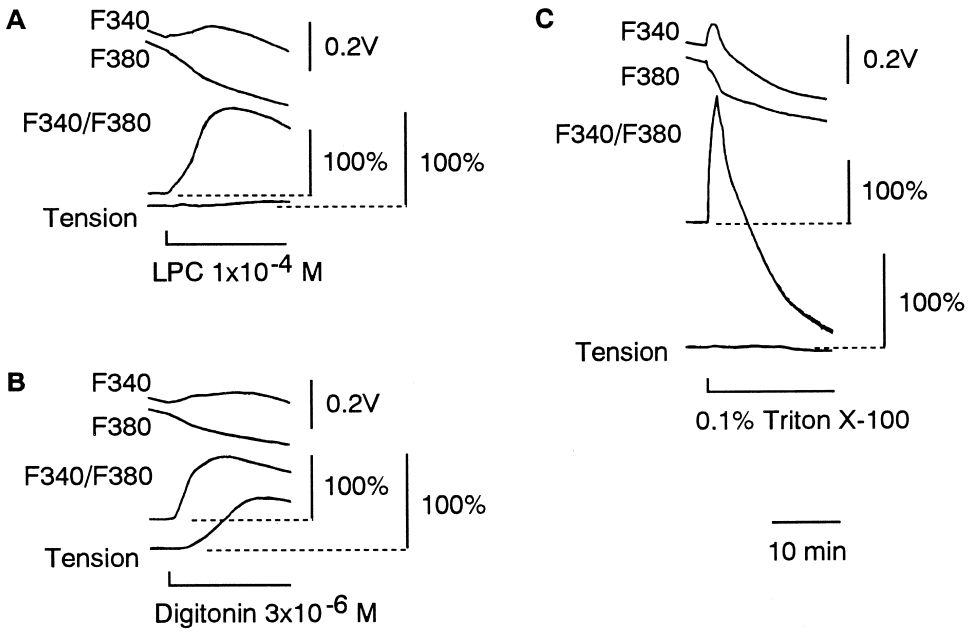

Fig. 6. Typical traces showing changes in fluorescence and force development induced by 10^{-4} M lysophosphatidylcholine (LPC), 3×10^{-6} M digitonin and 0.1% triton X-100 in the rat aorta. The traces show changes in fluorescence intensity obtained at 340 nm (F340) and 380 nm (F380) excitation and the ratio (F340/F380). The resting levels of both tension and fluorescence ratio are taken as 0% and the 80 mM K^+-stimulated levels as 100%.

emission at 380 nm excitation (Fig. 6A). The ratio of the fluorescence emissions at these two wavelengths was substantially increased (Fig. 6A). Digitonin (3×10^{-6} M) also caused an increase in the 500 nm fluorescence emission at 340 nm excitation and a simultaneous decrease in the 500 nm fluorescence emission at 380 nm excitation, so the ratio showed a substantial increase (Fig. 6B). However, digitonin produced a pronounced contraction that was concentration-dependent (Figs. 6B and 7). Triton X-100 (0.1%) induced a large increase in $[Ca^{2+}]_i$ (increase in the ratio of the fluorescence signals) with no detectable contraction (Fig. 6C). However, following the peak of the $[Ca^{2+}]_i$ response, the fluorescence level decreased rapidly and dropped to a level lower than that seen prior to the administration of triton X-100. During this period, the fluorescence intensities obtained at 340 nm and 380 nm excitation each showed a decrease. When an aortic strip was exposed to a high dose (10^{-5} M) of digitonin, the fluorescence ratio also dropped to a level lower than that seen prior to its administration (data not shown). In the presence of 0.1% triton X-100, a high K^+ (80 mM)-induced contraction was not observed ($n = 3$).

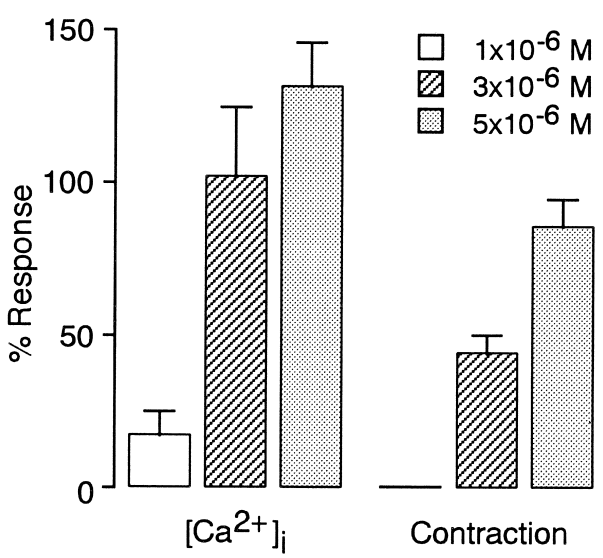

Fig. 7. Changes in $[Ca^{2+}]_i$ level and contraction induced by various concentrations of digitonin in the rat aorta. The resting level is taken as 0% and the 80 mM K^+-stimulated level as 100%. Values represent the mean ± S.E. for 5 smooth muscle preparations, each isolated from a different animal.

3.3. Influence of lysophosphatidylcholine on vascular contraction, and assessment of cytotoxicity

Treatment with 10^{-4} M lysophosphatidylcholine for 1 or 2 h did not significantly attenuate the contractile response induced by 80 mM K^+ (Fig. 8A). However, long-term exposure (24 h) to 10^{-4} M lysophosphatidylcholine strongly attenuated the 80 mM K^+-induced contraction.

Treatment with 10^{-4} M lysophosphatidylcholine for 1 or 2 h also caused no significant increase in the LDH activity in the incubation solution (Fig. 8B). Long-term exposure (24 h) to 10^{-4} M lysophosphatidylcholine caused a significant release of LDH activity, the level in the

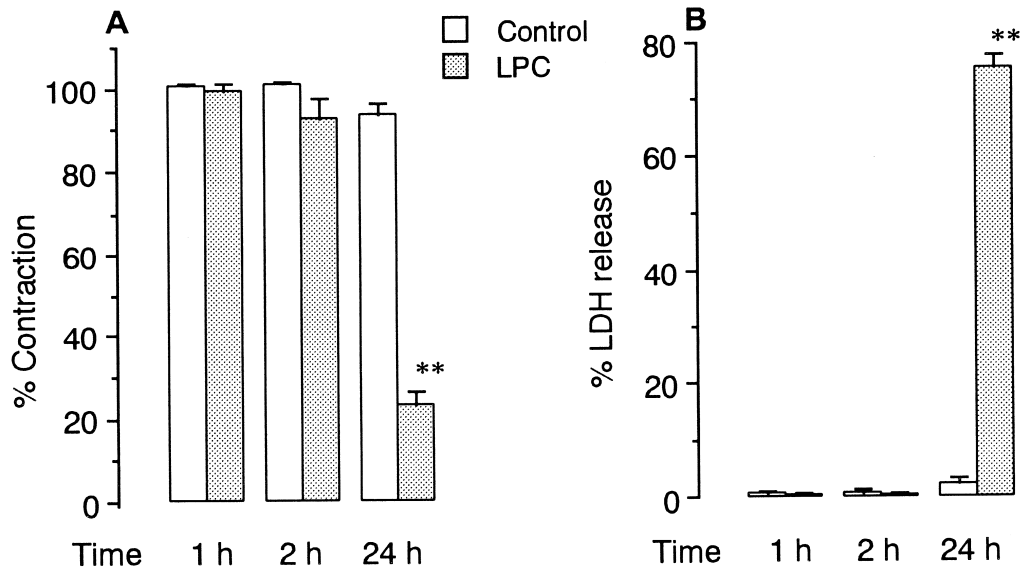

Fig. 8. Time-dependent effects of 10^{-4} M lysophosphatidylcholine (LPC) on 80 mM K^+-induced contractile responses (A) and release of lactate dehydrogenase (LDH) from rat aorta (B). ''Control'' represents responses recorded in the absence of lysophosphatidylcholine. Values represent the mean ± S.E. for 4–5 smooth muscle preparations, each isolated from a different animal. Values are expressed as a percentage of the tension developed as a result of pretreatment with 80 mM K^+ in each strip (A) or expressed as a percentage of the total LDH activity released from each tissue after treatment with 1% triton X-100 (B). $^{**}P < 0.01$ vs. control in the absence of lysophosphatidylcholine.

incubation solution reaching 75.1 ± 2.2% of the total LDH activity in the aortic strip.

3.4. Influence of lysophosphatidylcholine on cyclic nucleotide levels in aortic strips

The basal levels of cyclic AMP and cyclic GMP in endothelium-denuded aortic strips were 3.70 ± 0.18 pmol/mg protein and 0.33 ± 0.01 pmol/mg protein, respectively (Table 1). Treatment with 10^{-4} M lysophosphatidylcholine for 10 min or with 3×10^{-5} M lysophosphatidylcholine for 10 min had no effect on either the cyclic AMP level or the cyclic GMP level. A short exposure (1 min) to 10^{-4} M lysophosphatidylcholine also failed to affect the cyclic GMP level. Forskolin (10^{-5} M) significantly increased the cyclic AMP content, while sodium nitroprusside (10^{-6} M) significantly increased the cyclic GMP content.

4. Discussion

In the present study, lysophosphatidylcholine produced a sustained increase in $[Ca^{2+}]_i$ in the endothelium-denuded rat aorta. The magnitude of the sustained increase in $[Ca^{2+}]_i$ was proportional to the concentration of Ca^{2+} in the extracellular medium and no increase was evoked in Ca^{2+}-free solution. However, verapamil had no effect on the sustained increase in $[Ca^{2+}]_i$. These results suggest that while the increase in $[Ca^{2+}]_i$ induced by lysophosphatidylcholine requires external Ca^{2+}, it is not due to an increased Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels. These results are similar to those obtained previously

Table 1
Effects of lysophosphatidylcholine (LPC), sodium nitroprusside (SNP) and forskolin on cyclic nucleotide content of endothelium-denuded rat aorta
NT, not tested.

Treatment	Incubation time (min)	cAMP (pmol/mg protein)	cGMP (pmol/mg protein)
Control		3.70 ± 0.18	0.33 ± 0.01
LPC 3×10^{-5} M	10	3.98 ± 0.07	0.34 ± 0.03
LPC 1×10^{-4} M	1	NT	0.33 ± 0.01
LPC 1×10^{-4} M	10	3.71 ± 0.09	0.35 ± 0.02
SNP 1×10^{-6} M	1	NT	115.76 ± 12.47[a]
Forskolin 1×10^{-5} M	10	88.06 ± 14.77[a]	NT

[a] $p < 0.01$ significantly different from control value.

in cultured rat aortic smooth muscle cells (Chen et al., 1995). In the present study, lysophosphatidylcholine produced a gradual increase in $[Ca^{2+}]_i$ in normal solution. However, in Ca^{2+}-free solution addition of Ca^{2+} in the presence of lysophosphatidylcholine (the aortic strip was exposed to the compound for 20 min) induced a rapid increase in $[Ca^{2+}]_i$. These results suggest that lysophosphatidylcholine may gradually induce an increase in Ca^{2+} permeability.

Despite the large increase in $[Ca^{2+}]_i$ it evoked, lysophosphatidylcholine induced only a small contraction. To our knowledge, this is the first report showing a lack of correlation between the rise in intracellular free Ca^{2+} and the contractile response on exposure of the isolated rat aorta to lysophosphatidylcholine.

One explanation for the dissociation between $[Ca^{2+}]_i$ and contraction on exposure to lysophosphatidylcholine is that it is due to some injury to the contractile apparatus. Since lysophosphatidylcholine is an amphiphilic metabolite, it can exist in fluids as monomers or micelles, or both. Although the critical micelle concentration for amphiphiles is dependent on the type of solution, the critical micelle concentration for lysophosphatidylcholine in a physiological solution is reported to be 40–50 μM (Bergmann et al., 1981). In the present study, the lysophosphatidylcholine might have existed in the Krebs solution as both monomers and micelles when 100 μM lysophosphatidylcholine was applied. However, low concentrations of lysophosphatidylcholine (10 or 30 μM) also produced an increase in $[Ca^{2+}]_i$ in the present experiments, suggesting that the increase in $[Ca^{2+}]_i$ is probably not due to some non-specific detergent effects of lysophosphatidylcholine micelles.

Cell injury is often assessed by measuring the release of intracellular LDH. It has been reported that 100 μM lysophosphatidylcholine causes a time-dependent release of LDH from vascular smooth muscle cells (Tokumura et al., 1994). We found (Fig. 8) that treatment with 100 μM lysophosphatidylcholine for 1 or 2 h (much longer than the time for which it was normally applied in the present study) did not cause a release of LDH from the aorta, suggesting that the dissociation between $[Ca^{2+}]_i$ and the contractile response evoked by lysophosphatidylcholine may not be due to a cytotoxic effect. In the present study, lysophosphatidylcholine inhibited the noradrenaline-induced contraction, a result consistent with our previous data (Suenaga and Kamata, 1998). However, we consider that this effect is unlikely to be due to a cytotoxic action since the noradrenaline-induced contraction was almost restored following removal of the lysophosphatidylcholine (the previous study) and the 80 mM K^+-induced contraction was not affected by lysophosphatidylcholine (the present study).

Triton X-100 has a pronounced detergent action and is widely used to produce chemical skinning of muscle preparations. In the present study, triton X-100 also produced a marked elevation in $[Ca^{2+}]_i$ (increase in the ratio of the fluorescence signals) with no contraction. However, following the peak in the $[Ca^{2+}]_i$ response, a marked decrease in fluorescence occurred. This decrease may have been the result of a leak of fluorescent dye from the aorta since the fluorescence dropped to a level lower than that seen prior to the administration of triton X-100. In the triton X-100-treated aorta, high K^+ failed to produce a contraction, suggesting that triton X-100 may induce a leak of some of the cytosolic proteins involved in contractile responses. In contrast, lysophosphatidylcholine induced a slow but progressive increase in $[Ca^{2+}]_i$ with a subsequent return towards the level seen prior to its administration. These observations suggest that lysophosphatidylcholine may not cause a leakage of fluorescent dye and that the dissociation between $[Ca^{2+}]_i$ and contraction seen with this compound may not be due to a detergent action like that of triton X-100. Digitonin also has a detergent action. In the present study, a high dose (10 μM) of digitonin evoked a leakage of fluorescent dye from the aorta. However, a low concentration of digitonin (1 μM) produced an increase in $[Ca^{2+}]_i$ without such a leak and it may have mimicked the dissociative effect of lysophosphatidylcholine, although it is difficult to be sure because the evoked increase in $[Ca^{2+}]_i$ was quite small ($16.9 \pm 7.7\%$ of the 80 mM K^+-induced response). Ishida et al. (1993) also reported that a low dose (0.2 μM) of digitonin caused an increase in $[Ca^{2+}]_i$ without a leakage of dye (in their case, from cardiac cells). However, digitonin (at 3 or 5 μM), unlike lysophosphatidylcholine, simultaneously produced a contractile response in the present study. We consider that a low concentration of digitonin may selectively increase Ca^{2+} permeability and that this concentration of digitonin may not cause leakage of fluorescent dye or of cytosolic proteins involved in producing contractile responses. The above results also suggest that the observed dissociative effect of lysophosphatidylcholine may not be due to a digitonin-like action.

It needs to be considered whether the dissociation between $[Ca^{2+}]_i$ and contraction seen with lysophosphatidylcholine might be due to a decrease in Ca^{2+}-sensitivity. Increases in cyclic AMP and cyclic GMP levels in vascular smooth muscle are both known to cause a decrease in Ca^{2+}-sensitivity (Karaki et al., 1988, 1997; Balwierczak, 1991). However, in the present study, lysophosphatidylcholine did not increase either the cyclic AMP or cyclic GMP level in the rat aorta.

Another possibility we need to consider is that lysophosphatidylcholine might evoke an increase in $[Ca^{2+}]_i$ in a non-contractile Ca^{2+} compartment within the rat aortic smooth muscle cell. Indeed, recent studies have suggested that the distribution of intracellular free Ca^{2+} is not uniform and that a lack of association between the $[Ca^{2+}]_i$ measured with a Ca^{2+} indicator and the contractile response might result from a local increase in $[Ca^{2+}]_i$. In fact, a few years ago Abe et al. (1995) were able to

demonstrate the existence of just such a non-contractile Ca^{2+} compartment in vascular smooth muscle. This non-contractile Ca^{2+} compartment may not contain contractile elements and it may be separated from the contractile Ca^{2+} compartment by a diffusion barrier. Reports that cyclopiazonic acid and ATP can evoke an increase in $[Ca^{2+}]_i$ with little or no contraction also suggest that a non-contractile Ca^{2+} compartment is present in vascular smooth muscle cells (Abe et al., 1996; Kitajima et al., 1996). This potential mechanism seems at present to provide the most plausible explanation for the absence of contraction during the lysophosphatidylcholine-evoked increase in $[Ca^{2+}]_i$ seen in the present study. At present, it seems most likely that it is due to the evoked increase in $[Ca^{2+}]_i$ being confined to a non-contractile component within the smooth muscle cell. Whatever the underlying mechanism might actually be, the present study demonstrates that the large increase in $[Ca^{2+}]_i$ evoked by lysophosphatidylcholine differs in some way from those induced by high K^+ and other vasoconstrictors.

Lysophosphatidylcholine is considered to play a role in signal transduction via a regulation of protein kinase C (Bells and Burns, 1991). However, in the present study, H-7 did not inhibit the increase in $[Ca^{2+}]_i$ evoked by lysophosphatidylcholine in the rat aorta, suggesting that the observed effect of lysophosphatidylcholine on $[Ca^{2+}]_i$ may not be due to protein kinase C activation. Moreover, lysophosphatidylcholine produced only a small contraction. This result suggests that lysophosphatidylcholine probably does not strongly activate the protein kinase C that mediates vascular contraction in rat aortic smooth muscle.

Since the concentration of lysophosphatidylcholine is increased in atherosclerotic arterial walls (Portman et al., 1970; Vidaver et al., 1985), atherosclerotic arteries are presumably chronically exposed to a high concentration of lysophosphatidylcholine. We recently reported that 10 μM lysophosphatidylcholine potentiated a vascular contractile response (without affecting the maximal contraction) by enhancing the vasoconstrictor-induced increase in $[Ca^{2+}]_i$ and that this augmentation effect was strongly inhibited by Ca^{2+}-channel blockade (Suenaga and Kamata, 1998). In the present study, a high dose (100 μM) of lysophosphatidylcholine given alone produced a large increase in $[Ca^{2+}]_i$ in the rat aorta. Since the increase in $[Ca^{2+}]_i$ was not inhibited by verapamil and did not lead to contraction, the effect observed with lysophosphatidylcholine in the present study and the augmentation effect of lysophosphatidylcholine on vascular contractile responses may be mediated by different mechanisms.

In conclusion, in endothelium-denuded rat aortic strips lysophosphatidylcholine produces a large increase in $[Ca^{2+}]_i$ which does not induce the expected contraction. Although the increase in $[Ca^{2+}]_i$ requires external Ca^{2+}, it is not due to an increased Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels. The effect of lysophosphatidylcholine on $[Ca^{2+}]_i$ is not due to any extent to protein kinase C activation, nor does it seem to be due to a detergent action like that of digitonin and triton X-100. The relative lack of a contractile response to lysophosphatidylcholine is not due to a decrease in Ca^{2+} sensitivity secondary to the formation of cyclic AMP or cyclic GMP.

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References

Abe, F., Mitsui, M., Karaki, H., Endoh, M., 1995. Calcium compartments in vascular smooth muscle cells as detected by aequorin signal. Br. J. Pharmacol. 116, 3000–3004.

Abe, F., Karaki, H., Endoh, M., 1996. Effects of cyclopiazonic acid and ryanodine on cytosolic calcium and contraction in vascular smooth muscle. Br. J. Pharmacol. 118, 1711–1716.

Asaoka, Y., Nakamura, S., Yoshida, K., Nishizuka, Y., 1992. Protein kinase C, calcium and phospholipid degradation. Trends Biol. Sci. 17, 414–417.

Balwierczak, J.L., 1991. The relationship of KCl- and prostaglandin $F_{2\alpha}$-mediated increases in tension of the porcine coronary artery with changes in intracellular Ca^{2+} measured with fura-2. Br. J. Pharmacol. 104, 373–378.

Bazan, E., Campball, A.K., Rapoport, R.M., 1993. Effects of phorbol esters on contraction and protein kinase C activation in rabbit aorta. Life Sci. 54, 29–33.

Bells, R.M., Burns, D.J., 1991. Lipid activation of protein kinase C. J. Biol. Chem. 266, 4661–4664.

Bergmann, S.R., Ferguson, T.B. Jr., Sobel, B.E., 1981. Effects of amphiphiles on erythrocytes, coronary arteries, and perfused hearts. Am. J. Physiol. 240, H229–H237.

Chen, Y., Morimoto, S., Kitano, S., Koh, E., Fukuo, K., Jiang, B., Chen, S., Yasuda, O., Hirotani, A., Ogihara, T., 1995. Lysophosphatidylcholine causes Ca^{2+} influx, enhanced DNA synthesis and cytotoxicity in cultured vascular smooth muscle cells. Atherosclerosis 112, 69–76.

Chen, M., Hashizume, H., Abiko, Y., 1996. Effects of β-adrenoceptor antagonists on Ca^{2+}-overload induced by lysophosphatidylcholine in rat isolated cardiomyocytes. Br. J. Pharmacol. 118, 865–870.

Corr, P.B., Snyder, D.W., Lee, B.I., Gross, R.W., Keim, C.R., Sobel, B.E., 1982. Pathophysiological concentration of lysophosphatides and the slow response. Am. J. Physiol. 243, H187–H195.

Corr, P.B., Greer, R.W., Sobel, B.E., 1984. Amphipathic metabolites and membrane dysfunction in ischemic myocardium. Circ. Res. 55, 136–154.

Danthuluri, N.R., Deth, R.C., 1984. Phorbol ester-induced contraction of arterial smooth muscle and inhibition of α-adrenergic response. Biochem. Biophys. Res. Commun. 125, 1103–1109.

Das, D.K., Engelman, R.M., Rousou, J.A., Breyer, R.H., Otani, H., Lemeshow, S., 1986. Role of membrane phospholipids in myocardial injury induced by ischemia and reperfusion. Am. J. Physiol. 251, H71–H79.

Flavahan, N.A., 1992. Atherosclerosis of lipoprotein-induced endothelial dysfunction: potential mechanisms underlying reduction in EDRF/nitric oxide activity. Circulation 85, 1927–1937.

Goldstein, D.A., 1979. Calculation of the concentrations of free cations

and cation–ligand complexes in solutions containing multiple divalent cations and ligands. Biophys. J. 26, 235–242.

Ishida, H., Hirota, Y., Nakazawa, H., 1993. Effect of sub-skinning concentrations of saponin on intracellular Ca2+ and plasma membrane fluidity in cultured cardiac cells. Biochim. Biophys. Acta 1145, 58–62.

Itoh, H., Lederis, K., 1987. Contraction of rat thoracic aorta strips induced by phorbol 12-myristate 13-acetate. Am. J. Physiol. 252, C244–C247.

Jensen, P.E., Ohanian, J., Stausbøl-Grøn, B., Buus, N.H., Aalkjaer, C., 1996. Increase by lysophosphatidylcholines of smooth muscle Ca^{2+} sensitivity in α-toxin-permeabilized small mesenteric artery from the rat. Br. J. Pharmacol. 117, 1238–1244.

Karaki, H., Sato, K., Ozaki, H., Murakami, K., 1988. Effects of sodium nitroprusside on cytosolic calcium level in vascular smooth muscle. Eur. J. Pharmacol. 156, 259–266.

Karaki, H., Ozaki, H., Hori, M., Mitsui-Saito, M., Amano, K., Harada, K., Miyamoto, S., Nakazawa, H., Won, K.J., Sato, K., 1997. Calcium movements, distribution, and functions in smooth muscle. Pharmacol. Rev. 49, 157–230.

Kinnaird, A.A.A., Choy, P.C., Man, R.Y.K., 1988. lysophosphatidylcholine accumulation in the ischemic canine heart. Lipids 23, 32–35.

Kitajima, S., Harada, K., Hori, M., Ozaki, H., Karaki, H., 1996. Dissociation of P_2 purinoceptor-mediated increase in intracellular Ca^{2+} level from myosin light chain phosphorylation and contraction in rat aorta. Br. J. Pharmacol. 118, 543–548.

Kugiyama, K., Kerns, S.A., Morrisett, J.D., Roberts, R., Henry, P.D., 1990. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. Nature 344, 160–162.

Kugiyama, K., Ohgushi, M., Sugiyama, S., Murohara, T., Fukunaga, K., Miyamoto, E., Yasue, H., 1992. Lysophosphatidylcholine inhibits surface receptor-mediated intracellular signals in endothelial cells by a pathway involving protein kinase C activation. Circ. Res. 71, 1422–1428.

Liu, E., Goldhaber, J.I., Weiss, J.N., 1991. Effects of lysophosphatidylcholine on electrophysiological properties and excitation–contraction coupling in isolated guinea-pig ventricular myocytes. J. Clin. Invest. 88, 1819–1832.

Locher, R., Weisser, B., Mengden, T., Brunner, C., Vetter, W., 1992. Lysolecithin action on vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 183, 156–162.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Murohara, T., Kugiyama, K., Ohgushi, M., Sugiyama, S., Ohta, Y., Yasue, H., 1994. LPC in oxidized LDL elicits vasocontraction and inhibits endothelium-dependent relaxation. Am. J. Physiol. 267, H2441–H2449.

Nishizuka, Y., 1986. Studies and perspectives of protein kinase C. Science 233, 305–312.

Portman, O.W., Soltys, P., Alexander, M., Osuga, T., 1970. Metabolism of lysolecithin in vivo: effects of hyperlipemia and atherosclerosis in squirrel monkeys. J. Lipid Res. 11, 596–604.

Rajavashisth, T.A., Andalibi, A., Territo, M.C., Berliner, J.A., Navab, M., Fogelman, M.A., Lusis, A.J., 1990. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature 344, 254–257.

Rasmussen, H., Forder, J., Kojima, I., Scriabine, A., 1984. TPA-induced contraction of isolated rabbit vascular smooth muscle. Biochem. Biophys. Res. Commun. 122, 776–784.

Rasmussen, H., Takuwa, Y., Park, S., 1987. Protein kinase C in the regulation of smooth muscle contraction. FASEB J. 1, 177–185.

Sato, K., Ozaki, H., Karaki, H., 1988. Changes in cytosolic calcium level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent calcium indicator fura-2. J. Pharmacol. Exp. Ther. 246, 294–300.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., Witztum, J.L., 1989. Beyond cholesterol, modifications of low-density lipoprotein that increase its atherogenicity. New Engl. J. Med. 320, 915–924.

Stoll, L., Spector, A.A., 1993. Lysophosphatidylcholine causes cGMP-dependent verapamil-sensitive Ca^{2+} influx in vascular smooth muscle cells. Am. J. Physiol. 264, C885–C893.

Suenaga, H., Kamata, K., 1998. Lysophosphatidylcholine potentiates vascular contractile responses by enhancing vasoconstrictor-induced increase in cytosolic free Ca^{2+} in rat aorta. Eur J. Pharmacol. 361, 217–226.

Suenaga, H., Kamata, K., Kasuya, Y., 1993. Phorbol ester (PMA)-induced biphasic contraction of the rat aorta and effects of sodium nitroprusside and forskolin on the contraction. Res. Commun. Chem. Pathol. Pharm. 79, 313–324.

Tokumura, A., Iimori, M., Nishioka, Y., Kitahara, M., Sakashita, M., Tanaka, S., 1994. Lysophosphatidic acids induce proliferation of cultured vascular smooth muscle cells from rat aorta. Am. J. Physiol. 267, C204–C210.

Ver Donck, L., Verellen, G., Geerts, H., Borgers, M., 1992. Lysophosphatidylcholine-induced Ca^{2+}-overload in isolated cardiomyocytes and effect of cytoprotective drugs. J. Mol. Cell. Cardiol. 24, 977–988.

Vidaver, G.A., Ting, A., Lee, J.W., 1985. Evidence that lysolecithin is an important causal agent of atherosclerosis. J. Theor. Biol. 115, 27–41.

Witztum, J.L., Steinberg, D., 1991. Role of oxidized low density lipoprotein in atherogenesis. J. Clin. Invest. 88, 1785–1792.

Wrobleski, I., LaDue, J.S., 1955. Lactic dehydrogenase activity in blood. Proc. Soc. Exp. Biol. Med. 90, 210–213.