# Stimulatory and inhibitory actions of lysophosphatidylcholine, depending on its fatty acid residue, on the phospholipase C/$Ca^{2+}$ system in HL-60 leukaemia cells

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We examined the mechanism of action of lysophosphatidylcholine (LPC), which is suggested to be involved in the pathogenesis of atherosclerosis and inflammatory disorders, in HL-60 leukaemia cells. Extracellular 1-palmitoyl LPC increased the intracellular $Ca^{2+}$ concentration in association with production of inositol phosphate. These actions of LPC were markedly inhibited by treatment of the cells with pertussis toxin and U73122, a phospholipase C inhibitor. The lipid-induced stimulation of the phospholipase C/$Ca^{2+}$ system was also attenuated in the dibutyryl cAMP-induced differentiated (neutrophil-like) cells, in which phospholipase C activation induced by NaF or formyl-Met-Leu-Phe was enhanced. In contrast with the stimulatory action of 1-palmitoyl LPC, 1-stearoyl LPC was inhibitory for the phospholipase C/$Ca^{2+}$ system stimulated by NaF as well as by 1-palmitoyl LPC or other $Ca^{2+}$-mobilizing agonists. In a cell-free system, only an inhibitory effect on phospholipase C activity was observed even by 1-palmitoyl LPC; 1-stearoyl LPC was more inhibitive than 1-palmitoyl LPC. Taken together, these results suggest that atherogenic and inflammatory LPC exerts both stimulatory and inhibitory actions on the phospholipase C/$Ca^{2+}$ system depending on the species of fatty acid residue of the lipid; the stimulatory effect is possibly mediated through G-protein-coupled receptors; the inhibitory effect might be caused by dysfunction of the components involved in the enzyme system owing to the amphiphilic nature of the lipid. 1-Palmitoyl LPC prefers the former receptor stimulation at least in intact cells, but 1-stearoyl LPC preferentially exerts the latter inhibitory action.

## INTRODUCTION

It has been shown that secretory phospholipase $A_2$ ($PLA_2$) released into the circulation can contribute to the development of haemorrhagic and inflammatory disorders, including acute pancreatitis, arthritis and septic shock [1,2]. Recent study revealed that the enzymic generation of lysophosphatidylcholine (LPC) is essential for the secretory $PLA_2$-mediated inhibition of platelet activation, which can result in haemorrhagic diseases [3,4]. The secretory $PLA_2$ might also be present in low-density lipoprotein (LDL) in the circulation and activated during oxidation [2]. Oxidized LDL has been shown to be an atherogenic lipoprotein [5–7], and LPC generated by $PLA_2$ has recently been suggested to be a major component inducing atherosclerosis [2]. Thus LPC is accumulated in inflammatory and atherosclerotic lesions and involved in the pathogenesis of a variety of inflammatory disorders and vascular atherosclerosis. Actually, LPC acts on several types of cell involved in atherosclerosis and inflammation. For example, the lipid is a chemoattractant for monocytic cells [8] and T-lymphocytes [9] and is also a regulator of proliferation of T-lymphocytes [10] and macrophages [11,12]. In endothelial cells it induces the expression of several growth factors [13] and adhesion molecules such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1 and P-selectin [14–16]. The lipid also impairs the endothelial release of nitric oxide, resulting in the inhibition of arterial relaxation in response to hormones and neurotransmitters [17–21], although the opposite actions, namely the stimulation of nitric oxide synthesis [22–24] and nitric oxide synthase induction [25,26], have also been reported in response to the lipid in the same type of cells. In platelets, the lipid induces an increase in cAMP levels, resulting in the inhibition of aggregation [4]. LPC also increases cytoplasmic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in several types of cell including endothelial cells [18,24], smooth-muscle cells [27,28], leucocytes [29] and macrophages [30], although the lipid has been reported to inhibit agonist-induced $[Ca^{2+}]_i$ increase [18,19].

Thus a variety of actions have been reported in response to the lipid. However, as mentioned above, they seem to be contradictory in some cases, i.e. synthesis of nitric oxide and regulation of cellular $Ca^{2+}$. In addition, the action mechanism of LPC has not yet been characterized. The lipid is amphiphilic, so it can incorporate into the plasma membranes or penetrate into the cells. Thus the lipid might alter membrane fluidity or interact directly with intracellular signalling molecules, resulting in the modification of cellular functions. However, the ability of LPC to incorporate into plasma membranes or to penetrate into the cells does not always mean that the lipid acts primarily on the intracellular target molecules to exert its biological actions. Recent studies have suggested that amphiphilic lysosphingolipids such as sphingosine 1-phosphate (S1P) and sphingosylphos-

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free $Ca^{2+}$ concentration; FMLP, formyl-Met-Leu-Phe; GTP[S], guanosine 5′-[γ-thio]triphosphate; $IP_1$, inositol monophosphate; $IP_2$, inositol bisphosphate; $IP_3$, inositol trisphosphate; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; PAF, platelet-activating factor; $PLA_2$, phospholipase $A_2$; PTX, pertussis toxin; S1P, sphingosine 1-phosphate; SPC, sphingosylphosphocholine.

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phocholine (SPC) can stimulate cell surface receptors to modulate a variety of signalling pathways [29,31–37]. For LPC as well, results favouring the presence of cell surface receptors have also been reported. For example, LPC activated adenylate cyclase by a mechanism involving $G_s$-protein in platelets [4]. In mouse macrophages [38] or HL-60 leukaemia cells [29], oxidized LDL or LPC induced $Ca^{2+}$ mobilization and these $Ca^{2+}$ responses were markedly inhibited by treatment of the cells with pertussis toxin (PTX), suggesting an involvement of PTX-sensitive G-proteins in lipid signalling. In relation to this, a G-protein-coupled receptor with LDL-binding motifs has been cloned [39]. However, in these studies, the mechanism by which LPC increased $[Ca^{2+}]_i$ has not yet been characterized. Furthermore, whether LPC activated the G-proteins directly or indirectly has not been examined yet. In the present study we focused on the cellular $Ca^{2+}$ regulatory action of LPC and extended the previous HL-60 study [29] on its action mechanism.

We found that LPC-induced $Ca^{2+}$ mobilization in HL-60 cells was associated with activation of phospholipase C. Several lines of evidence suggested that the enzyme activation was mediated through G-protein-coupled receptors. Furthermore we showed that LPC also inhibited the phospholipase C/$Ca^{2+}$ system in some cases. Whether the lipid induces stimulation or inhibition depends on the species of fatty acid residue of LPC; 1-palmitoyl ($C_{16:0}$) LPC is stimulatory but 1-stearoyl ($C_{18:0}$) or 1-oleoyl ($C_{18:1}$) LPC is inhibitory for the phospholipase C/$Ca^{2+}$ system.

## EXPERIMENTAL

### Materials

1-Stearoyl ($C_{18:0}$) LPC, 1-oleoyl ($C_{18:1}$) LPC, 1-palmitoyl ($C_{16:0}$) LPC, 1-myristoyl ($C_{14:0}$) LPC, 1-lauroyl ($C_{12:0}$) LPC, 1-decanoyl ($C_{10:0}$) LPC, 1-hexanoyl ($C_{6:0}$) LPC, SPC, thapsigargin, formyl-Met-Leu-Phe (FMLP), 1-oleoyl-*sn*-glycerol 3-phosphate (lysophosphatidic acid) and platelet-activating factor (PAF) were purchased from Sigma; Fura 2 acetoxymethyl ester was from Dojindo (Tokyo, Japan); and *myo*-[2-$^3$H]inositol (23.0 Ci/mmol) was from Du Pont–New England Nuclear. U73122 and U73343 were generously provided by Upjohn Co. (Kalamazoo, MI, U.S.A.). The sources of all other reagents were the same as described previously [29,31,40–42].

### Cell cultures

HL-60 leukaemia cells were routinely cultured in an RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum (Life Technologies) in a humidified air/$CO_2$ (19:1) atmosphere. Two days before the experiments, the cells were sedimented (250 ***g*** for 5 min) and transferred to fresh medium for measurement of $[Ca^{2+}]_i$. For the inositol phosphate response and membrane phospholipase C assay, the cells were transferred to an inositol-free RPMI 1640 medium containing 10% (v/v) fetal calf serum and *myo*-[2-$^3$H]inositol (4 $\mu$Ci/ml). Unless specified otherwise, these undifferentiated cells were used. In some experiments (those shown in Figures 3, 4b, 7 and 9), however, HL-60 cells were cultured for 5 days (unless specified otherwise) in a medium containing 500 $\mu$M dibutyryl cAMP to differentiate into neutrophil-like cells. Other culture conditions were the same as those for undifferentiated cells. Treatment of the cells with PTX was performed by adding the toxin (100 ng/ml) to the medium 16 h before the experiments.

### Measurement of [$^3$H]inositol phosphate production

The [$^3$H]inositol-labelled cells were washed by sedimentation (250 ***g*** for 5 min) and resuspension with Hepes-buffered medium consisting of 20 mM Hepes, pH 7.5, 134 mM NaCl, 4.7 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 5 mM glucose and 0.1% BSA (fraction V). The washing procedure was repeated and the cells were finally resuspended in the same medium. The cells (approx. $2 \times 10^6$) were preincubated for 10 min with 10 mM LiCl in polypropylene vials (20 ml) in a final volume of 1.5 ml. The test agents (from 100-fold concentrated stock solutions) were then added to the medium and the cells were incubated for 10 min unless stated otherwise. The cell suspension (0.5 ml) in duplicate was transferred to tubes containing 1 ml of $CHCl_3$/MeOH/HCl (100:100:1, by vol.). $^3$H-labelled total inositol phosphates [inositol monophosphate ($IP_1$) + inositol bisphosphate ($IP_2$) + inositol trisphosphate ($IP_3$)] were separated from other labelled compounds including glycerophosphoinositol and inositol as described previously [40,41]. In the experiments shown in Figure 1(c) the cells were incubated for the indicated duration and $^3$H-labelled inositol polyphosphates ($IP_2 + IP_3$) were measured. Where indicated, the results were normalized to $10^5$ d.p.m. total radioactivity incorporated into the cellular inositol lipids. The radioactivity of the trichloroacetic acid (5%)-insoluble fraction was measured as the total radioactivity.

### Measurement of $[Ca^{2+}]_i$

The cells were sedimented, resuspended in Ham's 10 medium containing 0.1% BSA and then incubated for 20 min with 1 $\mu$M Fura 2 acetoxymethyl ester. $[Ca^{2+}]_i$ was estimated from the change in the fluorescence of the Fura 2-loaded cells as described previously [40,41].

### Membrane preparation and assay of phospholipase C

This was performed by a procedure similar to that for the enzyme assay of FRTL-5 thyroid cell membranes as described previously [42]. The cells cultured with [$^3$H]inositol were washed twice with $Ca^{2+}$- and $Mg^{2+}$-free PBS containing 1 mM EGTA. The cells were then suspended in 50 mM Hepes, pH 7.4, containing 50 mM sucrose, 1 mM EGTA and 100 units/ml aprotinin and homogenized in a Physcotron homogenizer (NS-310E; Nition, Tokyo, Japan) for 30 s. The homogenate was then centrifuged at 500 ***g*** for 5 min, the supernatant was recentrifuged at 10000 ***g*** for 15 min, and the resultant pellet was used as the crude plasma membranes. These membranes (approx. 100 $\mu$g of protein), containing $3 \times 10^5$ d.p.m. in 100 $\mu$l, were incubated at 37 °C for 10 min in a final volume of 200 $\mu$l in the incubation medium [final concentrations: 700 $\mu$M $CaCl_2$ (unless otherwise specified), 1 mM EGTA, 50 mM sucrose, 100 units/ml aprotinin, 2.5 mM $MgCl_2$, 100 mM KCl, 10 mM LiCl, 0.1 mg/ml BSA, 50 mM Hepes, pH 7.4, and various agents tested]. Free $Ca^{2+}$ concentration was measured by Quin 2 fluorescence and was approx. 200 nM under these conditions. In some experiments, the membranes were incubated with 2 mM $Ca^{2+}$ instead of 700 $\mu$M $Ca^{2+}$ in the absence of any guanine nucleotide to estimate the catalytic activity of the enzyme. Under these conditions, the free $Ca^{2+}$ concentration was estimated to be approx. 1 mM. The reaction was terminated by adding first 1 ml of $CHCl_3$/MeOH/HCl (100:100:1, by vol.) and then 0.3 ml of water. Because in a preliminary experiment we noticed that the radioactive $IP_2$ and $IP_3$ were changed in a similar fashion in response to various agents, the sum of the production of labelled $IP_2$ and $IP_3$ was measured as the phospholipase C activity. Data were normalized to $10^5$ d.p.m. radioactivity in the membranes.

### Data presentation

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the representative or means ± S.E.M. for at least three separate experiments unless otherwise stated.

## RESULTS

### Extracellular LPC increases $[Ca^{2+}]_i$ depending on phospholipase C activation

As shown in Figure 1(a), 1-palmitoyl LPC induced a biphasic increase in $[Ca^{2+}]_i$, i.e. a rapid increase followed by a sustained increase, in undifferentiated HL-60 cells. An addition of excess EGTA over 2 mM extracellular $Ca^{2+}$ to the incubation medium inhibited the response of the sustained phase but hardly affected the peak response induced by LPC, suggesting that the $[Ca^{2+}]_i$ increase in the early phase was derived predominantly from intracellular pools.

One of the mechanisms of intracellular $Ca^{2+}$ mobilization is the activation of phospholipase C. Actually, LPC increased inositol phosphate production (Figure 1c). In this experiment we measured inositol polyphosphates ($IP_2 + IP_3$), which were transiently increased by LPC with a peak at approx. 1–2 min. In contrast, $IP_1$ increased gradually in response to LPC; $IP_1$ accumulation was 220 ± 9% and 261 ± 12% of the initial value at 5 and 10 min respectively (four observations). To confirm an involvement of phospholipase C activation in the LPC-induced $[Ca^{2+}]_i$ increase, we examined the effect of U73122, a potent phospholipase C inhibitor, on these LPC actions. The inhibitor markedly inhibited the increase in $[Ca^{2+}]_i$ (Figures 1a and 1b) and phospholipase C activation (Figure 1c) as induced by LPC. The inhibition seems to be related to the nature of the drug to inhibit phospholipase C. First, U73343, an inactive analogue of U73122, failed to inhibit the LPC actions on the increase in $[Ca^{2+}]_i$ (Figures 1a and 1b) and phospholipase C activation (Figure 1c). Secondly, U73122, with a potency similar to that for the LPC action, inhibited the $[Ca^{2+}]_i$ increase as induced by UTP, a $P_2$-purinergic agonist, whose effect has already been shown to be due to phospholipase C activation (Figure 1b). These results clearly indicate that phospholipase C is involved in the LPC-induced increase in $[Ca^{2+}]_i$, at least at the early phase.

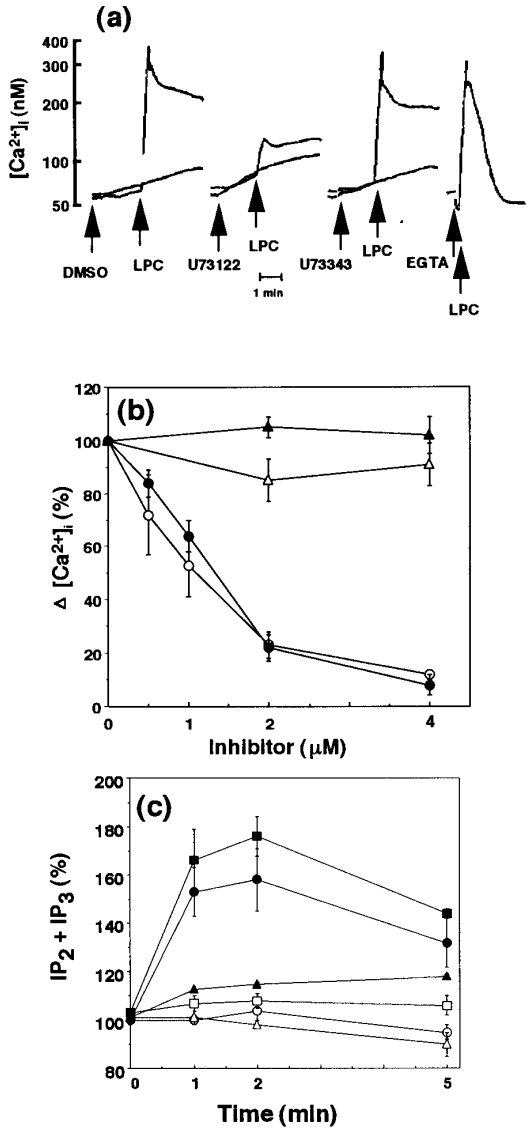


**Figure 1 Effect of a phospholipase C inhibitor on LPC-induced $Ca^{2+}$ mobilization and inositol phosphate production**

Undifferentiated HL-60 cells were used. (**a**) Representative traces of time-dependent $[Ca^{2+}]_i$ changes. The cells were first incubated with the indicated agents [DMSO, U73122 (4 $\mu$M), U73343 (4 $\mu$M) or EGTA (5 mM)], and 2 min or 10 s (for the EGTA experiment) later 1-palmitoyl LPC (30 $\mu$M) or vehicle (water) was then added to the incubation medium. (**b**) $Ca^{2+}$ experiments performed similarly to those in (**a**) in the cells treated with the indicated doses of U73122 (○,●) or U73343 (△,▲). UTP (300 nM; ●,▲) was also employed for the comparison with 1-palmitoyl LPC (30 $\mu$M; ○,△). The results are expressed as percentages of the maximal increment (peak value minus basal value) obtained at approx. 10 s after the addition of LPC or UTP in the absence of the enzyme inhibitor or its derivative. The 100% values were 224 ± 14 nM and 206 ± 21 nM for LPC and UTP respectively. (**c**) Time course of change in inositol phosphate ($IP_2 + IP_3$) production in response to 1-palmitoyl LPC (30 $\mu$M) (●,▲,■) or vehicle (○,△,□) in the cells treated with vehicle (○,●), 4 $\mu$M U73122 (△,▲) or 4 $\mu$M U73343 (□,■). The enzyme inhibitor or its derivative was added 2 min before LPC addition. Results are expressed as percentages of the initial value taken as 100%. The normalized initial value was 475 ± 14 d.p.m. Results in (**b**) and (**c**) are means ± S.E.M. for three separate experiments.

### Suppression of 1-palmitoyl LPC-induced actions by PTX treatment

The effect of PTX on the 1-palmitoyl LPC-induced actions is examined in Figure 2. The toxin markedly suppressed the LPC-induced increase in $[Ca^{2+}]_i$ (Figure 2a) and the lipid-induced inositol phosphate production (Figure 2b). The toxin effect was not due to a non-specific action, because NaF-induced inositol phosphate production was hardly affected by treatment with toxin (see Figure 6d). These results suggest an involvement of PTX-sensitive G-proteins in the 1-palmitoyl LPC-induced activation of the enzyme system.

### Suppression of 1-palmitoyl LPC-induced actions by dibutyryl cAMP-induced differentiation

Differentiation into neutrophil-like cells has been reported to be associated with an increase in content of PTX-sensitive G-proteins [29,43]. We next examined whether LPC actions were influenced under such conditions in which the content of G-proteins was increased. The cells were treated with dibutyryl cAMP. Differentiation under these conditions was evidenced by the induction of responses of $Ca^{2+}$ (Figure 3a) and inositol phosphate (Figure 3b) to FMLP. The effects of thapsigargin, an inhibitor of $Ca^{2+}$-ATPase, on $[Ca^{2+}]_i$ (Figure 3a) and NaF, a non-selective G-protein activator, on inositol phosphate production (Figure 3b) were also slightly enhanced by dibutyryl cAMP-

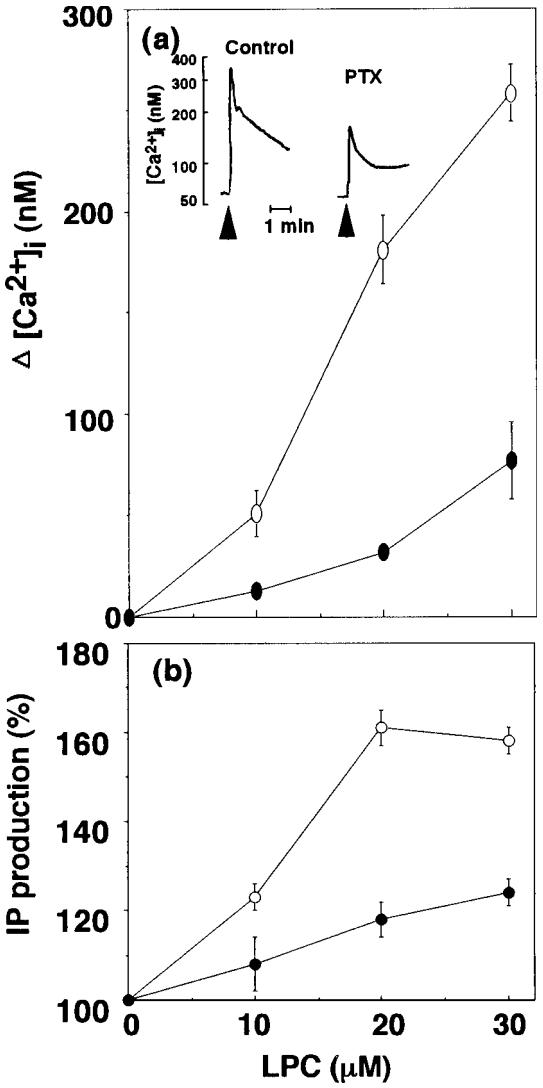


**Figure 2 Effect of PTX on 1-palmitoyl LPC-induced increase in $[Ca^{2+}]_i$ and production of inositol phosphate**

Undifferentiated HL-60 cells were used. (**a**) Control cells (○) or PTX-treated cells (●) were incubated with the indicated concentrations of 1-palmitoyl LPC to monitor $[Ca^{2+}]_i$. The maximal increment (peak value minus basal value) was plotted. The inset shows representative traces of the $[Ca^{2+}]_i$ change in control cells and PTX-treated cells. At the arrow, 30 $\mu$M LPC was added to the incubation medium. (**b**) Control cells (○) or PTX-treated cells (●) were incubated for 10 min with the indicated concentrations of 1-palmitoyl LPC to measure inositol phosphate ($IP_1 + IP_2 + IP_3$) production. The results are expressed as percentages of the respective basal value. The normalized basal value was $1157 \pm 26$ and $939 \pm 89$ d.p.m. for control cells and toxin-treated cells respectively.

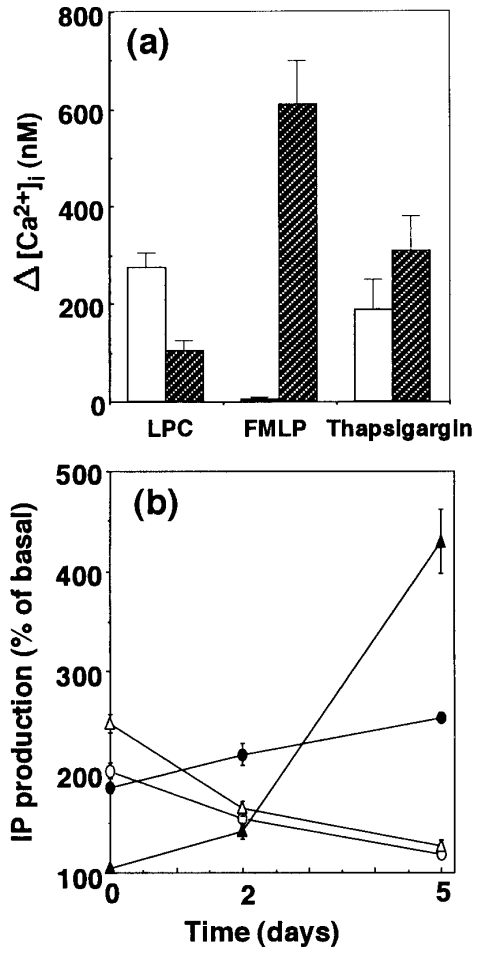


**Figure 3 Differentiation into neutrophil-like cells attenuates 1-palmitoyl LPC-induced activation of the phospholipase C/$Ca^{2+}$ system**

(**a**) Undifferentiated cells (open column) or differentiated cells (hatched column) cultured for 5 days with dibutyryl cAMP were incubated with the indicated agents (30 $\mu$M 1-palmitoyl LPC, 3 nM FMLP or 300 nM thapsigargin) to monitor $[Ca^{2+}]_i$. The maximal increment of $[Ca^{2+}]_i$, i.e. the peak value (at approx. 10 s for LPC, at approx. 30 s for FMLP or at approx. 3 min for thapsigargin) minus the basal value, is shown. (**b**) Cells were cultured with dibutyryl cAMP for the indicated durations and assayed for inositol phosphate response. The cells were incubated for 10 min with 30 $\mu$M 1-palmitoyl LPC (○), 30 $\mu$M SPC (△), 20 mM NaF (●) or 3 nM FMLP (▲) to measure the production of inositol phosphate ($IP_1 + IP_2 + IP_3$). The results are expressed as percentages of the respective basal value obtained without any addition. The normalized basal value was $1088 \pm 97$ d.p.m. at zero time, $1046 \pm 97$ d.p.m. at 2 days and $686 \pm 69$ d.p.m. at 5 days after treatment with dibutyryl cAMP.

induced differentiation, possibly reflecting an increase in the content of G-proteins. Thus the activities of the steps beyond G-proteins in the signalling pathways leading to phospholipase C activation and $Ca^{2+}$ mobilization seem to be normal under the differentiated conditions. Nevertheless the LPC-induced $[Ca^{2+}]_i$ increase (Figure 3a) and inositol phosphate production (Figure 3b) were not enhanced but were markedly suppressed by dibutyryl cAMP treatment. These results ruled out the possibility that 1-palmitoyl LPC activates G-proteins directly and therefore suggested that the LPC target is located before the G-protein step in the signalling pathway.

### LPC-induced increase in $[Ca^{2+}]_i$ seems to be independent of other putative lipid receptors

Several novel lipid receptors have recently been identified [35–37, 44,45]. We therefore examined the possibility that LPC activates the phospholipase C/$Ca^{2+}$ system through the putative receptors for other lipids that have chemical structures similar to that of LPC. In undifferentiated HL-60 cells (Figure 4a), S1P and lysophosphatidic acid were less effective than 1-palmitoyl LPC for induction of the increase in $[Ca^{2+}]_i$, excluding the putative S1P and lysophosphatidic acid receptors as possible action sites of LPC. PAF clearly increased $[Ca^{2+}]_i$ at 10–30 $\mu$M, although the response at 30 $\mu$M was significantly weaker than the response at the same concentration of LPC (Figure 4a). Furthermore, in contrast with the $Ca^{2+}$ response to LPC, the response to PAF was

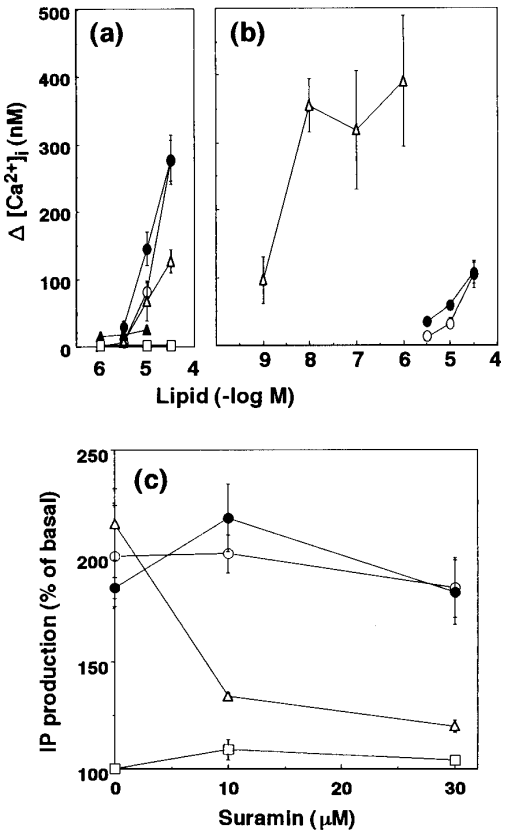


**Figure 4 1-Palmitoyl LPC seems to activate the phospholipase C/$Ca^{2+}$ system independently of the putative receptors for other lipids with similar chemical structures**

Undifferentiated cells (**a**) or the neutrophil-like cells differentiated by dibutyryl cAMP (**b**) were incubated with the indicated doses of 1-palmitoyl LPC (○), SPC (●), PAF (△), S1P (▲) or lysophosphatidic acid (□) to monitor $[Ca^{2+}]_i$ change. The maximal increment at 10–30 s after the addition of these agents is plotted. (**c**) The [$^3$H]inositol-labelled undifferentiated cells were incubated for 10 min without addition (□) or with 30 $\mu$M 1-palmitoyl LPC (○), 30 $\mu$M SPC (△) or 20 mM NaF (●) in the presence of the indicated concentrations of suramin. The production of total inositol phosphate ($IP_1 + IP_2 + IP_3$) was measured and is expressed as percentages of the basal value obtained without any addition. The normalized basal value was 976 ± 123 d.p.m.

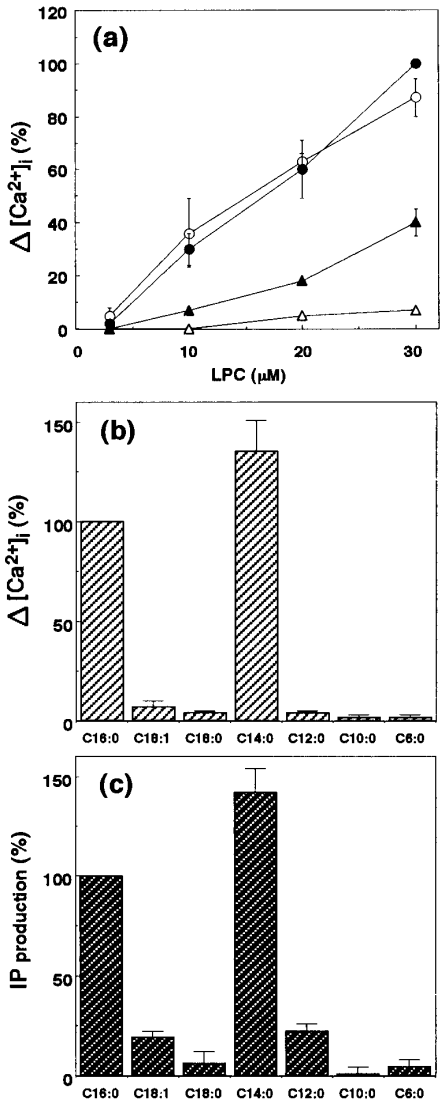


**Figure 5 Role of the fatty acid residue in the LPC molecule in lipid-induced activation of the phospholipase C/$Ca^{2+}$ system**

Undifferentiated HL-60 cells were used. (**a**) Dose-dependent change in $[Ca^{2+}]_i$ caused by SPC (○), 1-palmitoyl ($C_{16:0}$) LPC (●), 1-stearoyl ($C_{18:0}$) LPC (△) or egg yolk LPC (▲, composed predominantly of 1-palmitoyl LPC and 1-stearoyl LPC). Results are expressed as percentages of the maximal increment as induced by 30 $\mu$M 1-palmitoyl LPC. The 100% value was 276 ± 37 nM. (**b**) Cells were incubated with the indicated species of LPC [1-palmitoyl ($C_{16:0}$) LPC, 1-oleoyl ($C_{18:1}$) LPC, 1-stearoyl ($C_{18:0}$) LPC, 1-myristoyl ($C_{14:0}$) LPC, 1-lauroyl ($C_{12:0}$) LPC, 1-decanoyl ($C_{10:0}$) LPC or 1-hexanoyl ($C_{6:0}$) LPC] at 30 $\mu$M to monitor $[Ca^{2+}]_i$ change. Results are expressed as percentages of the maximal increment induced by 1-palmitoyl ($C_{16:0}$) LPC. The 100% value was 236 ± 31 nM. (**c**) [$^3$H]Inositol-labelled cells were incubated for 10 min with the indicated species of LPC [symbol and concentration as shown in (**b**)] and total inositol phosphate ($IP_1 + IP_2 + IP_3$) production was measured. The normalized inositol phosphate production under basal conditions without any addition was 1827 ± 43 d.p.m.; this was increased to 3142 ± 73 d.p.m. by 1-palmitoyl ($C_{16:0}$) LPC. Results are expressed as percentages of the 1-palmitoyl LPC-induced response.

markedly increased by treatment with dibutyryl cAMP (Figure 4b). These results also excluded the PAF receptor as a possible target for LPC. Finally, we examined whether LPC shares the same receptor with SPC, as both LPC and SPC have a phosphorylcholine residue in their molecule. The responses of $Ca^{2+}$ (Figures 4a and 4b) and inositol phosphate (Figure 3b) to SPC were also suppressed by dibutyryl cAMP-induced differentiation or by treatment with PTX [29]. Therefore the LPC and SPC signalling pathways are very similar. However, as shown in Figure 4(c), the action of SPC was inhibited by suramin, a non-selective receptor antagonist [45,46], whereas LPC- and NaF-induced actions were insensitive to the drug. This also suggests the SPC receptor-independent action of LPC.

## LPC also inhibits the phospholipase C/$Ca^{2+}$ system depending on its fatty acid residue

In the studies described above, we used 1-palmitoyl ($C_{16:0}$) LPC to characterize the LPC action. This species of LPC was as effective as SPC for activation of the phospholipase C/$Ca^{2+}$ system (Figures 4 and 5a). We have previously shown that egg yolk LPC increased $[Ca^{2+}]_i$ but was less effective than SPC in HL-60 cells [29]; this is confirmed in Figure 5(a). Egg yolk LPC is composed of predominantly 1-palmitoyl LPC and 1-stearoyl ($C_{18:0}$) LPC, suggesting that the ability to increase $[Ca^{2+}]_i$ is dependent on the fatty acid constituent in the lipid molecule. Actually, 1-stearoyl LPC was a very weak $Ca^{2+}$ mobilizer in HL-

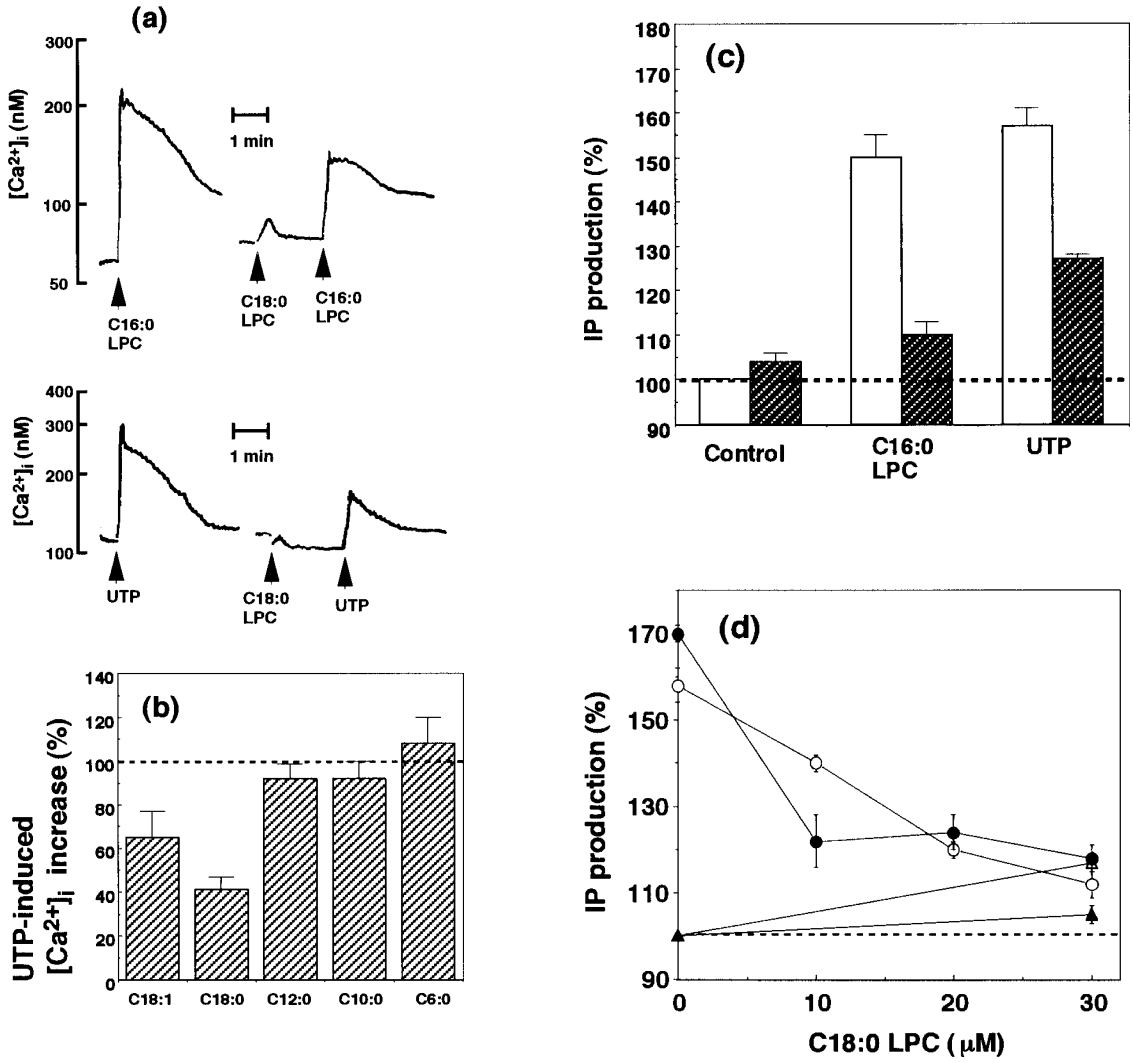


**Figure 6 1-Stearoyl LPC and 1-oleoyl LPC are inhibitory for 1-palmitoyl LPC, UTP and NaF-induced activation of the phospholipase C/$Ca^{2+}$ system**

Undifferentiated HL-60 cells were used. (**a**, **b**) $Ca^{2+}$ responses. (**a**) Representative traces of the $[Ca^{2+}]_i$ change: at the arrow, 1-palmitoyl ($C_{16:0}$) LPC (30 $\mu$M), 1-stearoyl ($C_{18:0}$) LPC (30 $\mu$M) or UTP (300 nM) was added to the incubation medium. (**b**) Cells were incubated for 2 min with the indicated LPC with a different species of fatty acid in its molecule at 30 $\mu$M, and the effect of UTP (300 nM)-induced $[Ca^{2+}]_i$ was then examined. The symbol for LPC is the same as in Figure 5. The results are expressed as percentages of the maximal increment obtained by UTP without LPC pretreatment. The maximal value was $245 \pm 24$ nM. (**c**, **d**) The [$^3$H]inositol-labelled cells were used for measurement of total inositol phosphate ($IP_1 + IP_2 + IP_3$). (**c**) Cells were incubated for 10 min without (control) or with the indicated agent (30 $\mu$M 1-palmitoyl LPC or 300 nM UTP) in the presence (hatched column) or absence (open column) of 30 $\mu$M 1-stearoyl LPC. (**d**) Control cells (○,△) or toxin-treated cells (●,▲) were incubated for 10 min without (△,▲) or with (○,●) 20 mM NaF in the presence of the indicated concentrations of 1-stearoyl LPC. The results are expressed as percentages of the respective basal value obtained without any addition. The normalized basal value was $941 \pm 52$ and $676 \pm 48$ d.p.m. for control cells and PTX-treated cells respectively.

60 cells (Figure 5a). The effect on the increase in $[Ca^{2+}]_i$ of other species of LPC with different fatty acid moieties is examined in Figure 5(b). 1-Myristoyl ($C_{14:0}$) LPC was as effective as 1-palmitoyl LPC but other LPCs, including 1-oleoyl ($C_{18:1}$) LPC, 1-lauroyl ($C_{12:0}$) LPC, 1-decanoyl ($C_{10:0}$) LPC and 1-hexanoyl ($C_{6:0}$) LPC, were very weak $Ca^{2+}$ mobilizers, like 1-stearoyl LPC. Their ability to mobilize $Ca^{2+}$ was parallel to their ability to activate phospholipase C (Figure 5c).

LPC has previously been reported to inhibit the thrombin- or bradykinin-induced activation of phospholipase C and mobilization of $Ca^{2+}$ in endothelial cells [18,19]. This led us to examine the effect of these inactive species of LPC on the $[Ca^{2+}]_i$ increase induced by active 1-palmitoyl LPC. A prior treatment of the cells with 1-stearoyl LPC clearly inhibited the 1-palmitoyl LPC-induced increase in $[Ca^{2+}]_i$ (Figure 6a). This inhibition was not specific to 1-palmitoyl LPC; treatment with 1-stearoyl LPC also inhibited the $Ca^{2+}$ response to UTP, a $P_2$-purinergic agonist (Figures 6a and 6b). 1-Oleoyl LPC ($C_{18:1}$) was also effective but other species of LPC possessing a lauroyl, decanoyl or hexanoyl substituent were ineffective in inhibiting UTP-induced action (Figure 6b). The inhibition by 1-stearoyl LPC was also observed for the phospholipase C activation induced by 1-palmitoyl LPC (Figure 6c), UTP (Figure 6c) and NaF (Figure 6d). In contrast with the stimulatory action of 1-palmitoyl LPC (Figure 2), the inhibitory action by 1-stearoyl LPC was hardly affected by PTX treatment (Figure 6d).

In undifferentiated HL-60 cells, evaluation of the ability of 1-palmitoyl LPC to inhibit phospholipase C is difficult because of

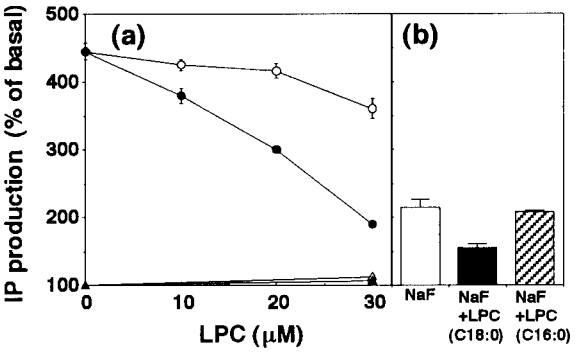


**Figure 7 Comparison of the effects of 1-palmitoyl LPC and 1-stearoyl LPC on the FMLP- and NaF-induced inositol phosphate production in differentiated HL-60 cells**

The cells were treated with dibutyryl cAMP to differentiate them into neutrophil-like cells. (**a**) [$^3$H]Inositol-labelled cells were incubated for 10 min without (△,▲) or with (○,●) 3 nM FMLP in the presence of the indicated concentrations of 1-palmitoyl LPC (○,△) or 1-stearoyl LPC (●,▲). (**b**) Cells were similarly incubated with 20 mM NaF in the absence or presence of the indicated species of LPC at 30 $\mu$M. The production of inositol phosphate ($IP_1 + IP_2 + IP_3$) was measured. The results are expressed as percentages of the basal value obtained without any addition. The normalized basal value was 594 ± 19 d.p.m.

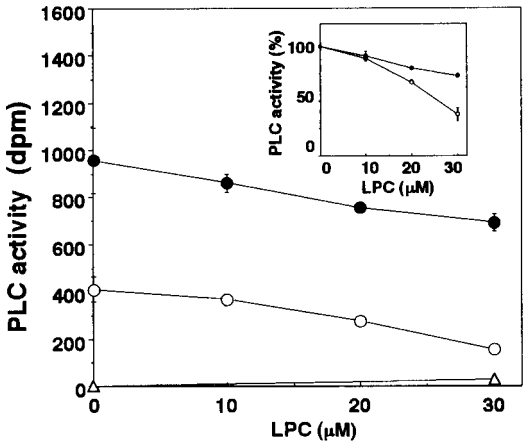


**Figure 8 Effect of 1-palmitoyl LPC on phospholipase C (PLC) activity in membrane preparation**

The crude membranes were prepared from [$^3$H]inositol-labelled undifferentiated cells and assayed for phospholipase C. The membrane preparations were incubated with (○) or without (△) 1 $\mu$M GTP[S] in the presence of 700 $\mu$M $Ca^{2+}$ to estimate guanine nucleotide-dependent activity or without any guanine nucleotide in the presence of 2 mM $Ca^{2+}$ (●) to estimate the catalytic activity of the enzyme. The incubation medium was supplemented with the indicated doses of 1-palmitoyl LPC. The inset shows the enzyme activity expressed as percentages of the respective control activity in the absence of LPC.

its stimulatory action. To estimate this activity, experiments were also performed in differentiated HL-60 cells, in which a stimulatory mechanism of LPC was markedly attenuated (see Figure 3). As shown in Figure 7(a), 1-stearoyl LPC at 10 $\mu$M significantly inhibited FMLP-induced inositol phosphate production but 1-palmitoyl LPC was not effective below 30 $\mu$M. The greater inhibitory action of 1-stearoyl LPC than that of 1-palmitoyl LPC was also observed for NaF-induced action (Figure 7b). Therefore, in contrast with the stimulatory action, 1-stearoyl LPC has a stronger inhibitory action than 1-palmitoyl LPC.

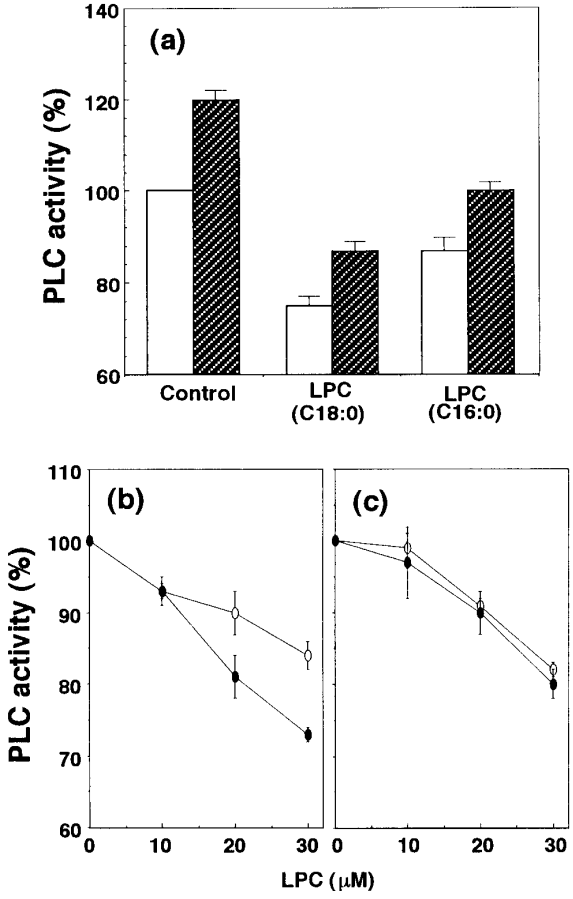


**Figure 9 Comparison of the effects of 1-palmitoyl LPC and 1-stearoyl LPC on phospholipase C (PLC) activity in membranes prepared from differentiated HL-60 cells**

The crude membranes were prepared from [$^3$H]inositol-labelled differentiated cells and assayed for phospholipase C. (**a**) Membrane preparations were incubated with (hatched column) or without (open column) 100 nM FMLP in the presence of 0.3 $\mu$M GTP[S]. In the absence of GTP[S], the enzyme activity was marginal and FMLP alone exerted no significant effect. The incubation medium also included, variously, vehicle (control), 30 $\mu$M 1-stearoyl ($C_{18:0}$) LPC or 30 $\mu$M 1-palmitoyl ($C_{16:0}$) LPC. The results are expressed as percentages of the activity obtained by GTP[S] alone in the control experiments. The normalized 100% value was 615 ± 78 d.p.m. (**b**) Membranes were incubated with the indicated concentrations of 1-palmitoyl LPC (○) or 1-stearoyl LPC (●) in the presence of 1 $\mu$M GTP[S]. (**c**) Membranes were incubated with LPC similarly to the experiments shown in (**b**), but without any guanine nucleotide in the presence of 2 mM $Ca^{2+}$. The results in (**b**) and (**c**) are expressed as percentages of the activity in the absence of LPC. The normalized 100% value was 974 ± 109 d.p.m. in (**b**) and 1497 ± 99 d.p.m. in (**c**).

## Analysis of LPC-induced inhibition of phospholipase C in a cell-free system

Finally, we analysed LPC actions in membranes prepared from undifferentiated cells (Figure 8) and differentiated cells (Figure 9). As shown in Figure 8, the enzyme activity increased in response to guanosine 5′-[γ-thio]triphosphate (GTP[S]) in the membrane preparations, suggesting that this system is at least good enough for investigating the G-protein-mediated enzyme regulation. We expected 1-palmitoyl LPC to activate enzyme activity in a guanine nucleotide-dependent manner; however, it was inhibitory for the GTP[S]-induced activation. 1-Stearoyl LPC was also inhibitory for the enzyme activity and a more effective inhibitor than 1-palmitoyl LPC (results not shown). The

enzyme activity at high $Ca^{2+}$ concentration, which might reflect the catalytic activity of the enzyme, was also inhibited by 1-palmitoyl LPC; however, the inhibition rate was smaller than for the GTP[S]-induced activation (Figure 8, inset). Thus 1-palmitoyl LPC was inhibitory for the activity of G-proteins as well as phospholipase C itself in membranes prepared from undifferentiated cells.

In the study shown in Figure 9 we employed membranes prepared from differentiated cells to compare the ability of 1-palmitoyl LPC and 1-stearoyl LPC to inhibit the enzyme activity. In this membrane preparation, even though a higher dose of FMLP was used, the action of FMLP was small compared with that in the intact cells; 3 nM FMLP activated phospholipase C approx. 300–400 % over basal in the intact cells (Figure 7a) but 100 nM FMLP only approx. 20 % over basal in cell membranes (Figure 9a). Thus the receptor-mediated enzyme activation is not easily detected in the membrane preparation. This might explain why we could not detect an effect of 1-palmitoyl LPC in the membrane preparation in Figure 8. In any event, we could observe a significant guanine nucleotide-dependent FMLP action, albeit small, on phospholipase C. This FMLP action was significantly attenuated by either 1-palmitoyl LPC or 1-stearoyl LPC (Figure 9a). In this case, however, the net increase in the activity induced by FMLP was not significant between 1-palmitoyl LPC-treated and 1-stearoyl LPC-treated membranes. A significant difference was observed for the GTP[S]-induced action: 1-stearoyl LPC was more inhibitory than 1-palmitoyl LPC (Figures 9a and 9b), whereas both species of LPC inhibited the $Ca^{2+}$-induced activation to a similar extent (Figure 9c). Thus the greater inhibitory effect of 1-stearoyl LPC than of 1-palmitoyl LPC on the phospholipase C activity in intact cells as well as in the cell-free system might be accounted for partly by its stronger inhibitory action on G-proteins.

## DISCUSSION

In this paper we present evidence that LPC activates the phospholipase C/$Ca^{2+}$ system, possibly through receptors coupling to one or more PTX-sensitive G-proteins. In addition we show that the LPC-induced modulation of phospholipase C/$Ca^{2+}$ system is dependent on its fatty acid constituent; 1-palmitoyl ($C_{16:0}$) LPC activates it, but 1-stearoyl ($C_{18:0}$) or 1-oleoyl ($C_{18:1}$) LPC suppresses its activation to some extent as induced by $Ca^{2+}$-mobilizing agonists. The stimulatory and inhibitory actions of LPC on the phospholipase C/$Ca^{2+}$ system might be important from a pathophysiological view of inflammatory disorders and vascular atherosclerosis.

An involvement of G-protein-coupled receptors in the 1-palmitoyl LPC-induced activation of the phospholipase C/$Ca^{2+}$ system was suggested from experiments with PTX; the LPC-induced activation of the system was markedly suppressed by a prior treatment of the cells with the toxin, which ADP-ribosylates $G_i/G_o$-proteins and thereby blocks the communication between the receptors and the effector enzymes (Figure 2). Therefore it is reasonable to assume that the LPC actions are mediated through $G_i/G_o$-protein-coupled receptors. It is still possible, however, that amphiphilic LPC is incorporated into the plasma membrane or penetrates into the cells and then directly activates $G_i/G_o$-proteins. If this were the case as well, PTX would block the lipid-induced actions. This possibility, however, is unlikely on the basis of the following observations. First, in HL-60 crude cell membranes, 1-palmitoyl LPC never stimulated but inhibited either $Ca^{2+}$-induced (possibly reflecting catalyst activity) or GTP[S]-induced (possibly reflecting the sum of the activities of G-proteins and catalyst) phospholipase C activity. In this case, the latter GTP[S]-induced activity was more sensitive to LPC than the $Ca^{2+}$-induced one (Figure 8), suggesting that the lipid suppresses the functions of G-proteins as well as the phospholipase C enzyme itself. Therefore it is unlikely that 1-palmitoyl LPC is an activator for G-proteins or phospholipase C in intact cells even if it were to enter the cells. Consistently with this result, LPC has been suggested to inhibit the function of $G_i$-proteins [20,21]. Secondly, 1-palmitoyl LPC-induced actions were inhibited by dibutyryl cAMP-induced differentiation. Under these differentiated conditions, the downstream region of the G-protein-mediated signalling cascade leading to phospholipase C activation and $Ca^{2+}$ mobilization is fortified (Figure 3). If 1-palmitoyl LPC were to activate G-proteins directly, the lipid responses would also be enhanced in the differentiated cells. Therefore the results in differentiated cells might rule out the possibility that the lipid directly activates G-proteins and suggest that 1-palmitoyl LPC acts on the target molecule (possibly receptors) present in the step before G-proteins in the signalling pathway. Finally, the stimulatory LPC action was cell-type specific; in preliminary experiments we observed a similar PTX- and U73122-sensitive LPC-induced $Ca^{2+}$ rise in THP-1 monocytic leukaemia cells or human endothelial cells but not in FRTL-5 thyroid cells, human aortic smooth-muscle cells nor 3T3 fibroblasts.

Thus 1-palmitoyl LPC seems to stimulate phospholipase C through a G-protein-coupled receptor, which might in turn produce $IP_3$ and mobilize $Ca^{2+}$ from the intracellular pool. For phospholipase C-coupled receptor agonists, the dose–response curve for $Ca^{2+}$ response usually lies to the left of the dose–response curve for inositol phosphate production [47]. However, the situation was opposite with 1-palmitoyl LPC: even though inositol phosphate production was saturated at 20 mM LPC, $[Ca^{2+}]_i$ increased further at the higher dose (30 mM) of LPC (Figure 2). Therefore, especially at higher doses of 1-palmitoyl LPC, we might not completely rule out the participation of phospholipase C-independent $Ca^{2+}$ mobilization.

PAF receptor has recently been proposed as an LPC receptor in mouse macrophages [30]. In undifferentiated HL-60 cells as well, we observed a $Ca^{2+}$ response to PAF; however, the response was less than that to LPC (Figure 4a). In addition, in contrast with the action of LPC, which was markedly attenuated by dibutyryl cAMP-induced differentiation, the response to PAF was markedly enhanced by the same differentiation of the cells (Figure 4b). These results ruled out the PAF receptor as a possible LPC receptor, at least in HL-60 cells. The affinity of PAF for its receptor has usually been reported to be of nanomolar order as shown in the differentiated cells (Figure 4b). Thus, the micromolar response to PAF in undifferentiated cells (Figure 4a) might be mediated through other lipid receptors. Similarly, on the basis of the pharmacological characterization (Figure 4) (such as a long-term differentiation effect or a short-term suramin effect on the LPC actions), we conclude that the putative receptors for LPC might be different from lysophosphatidic acid receptors [45] and lysosphingolipid receptors [35–37] such as S1P and SPC in HL-60 cells. In addition, the following observations also support this conclusion. In the previous HL-60 cell study [31] we detected significant $Ca^{2+}$ responses to S1P and lysophosphatidic acid. In the present study we used a different batch of HL-60 cells in which these lipid responses were marginal (Figure 4a), whereas responses to SPC and LPC were unchanged between different batches of the cells. SPC was an effective $Ca^{2+}$-mobilizing agent in other cell types including Swiss 3T3 cells and FRTL-5 cells, whereas LPC was ineffective in these cells (results not shown).

In addition to the activation of the phospholipase C/$Ca^{2+}$ system by LPC, we also observed that the lipid in some cases

inhibited $Ca^{2+}$ mobilizing agonist-induced activation of the enzyme system depending on its fatty acid constituents; 1-palmitoyl ($C_{16:0}$) LPC is stimulatory for phospholipase C/$Ca^{2+}$ system, but 1-stearoyl ($C_{18:0}$) or 1-oleoyl ($C_{18:1}$) LPC was inhibitory for 1-palmitoyl ($C_{16:0}$) LPC or other $Ca^{2+}$-mobilizing agonist-induced stimulation of the system. Recent studies have shown that LPC activates protein kinase C [48,49]; this enzyme stimulation has been suggested to be involved in some of the LPC actions including the inhibition of $Ca^{2+}$-mobilizing hormone-induced phospholipase C/$Ca^{2+}$ system activation [19], the stimulation of P-selectin expression [16], the activation of phospholipase D [50] and the stimulation of expression of intercellular adhesion molecule 1 [15]. However, protein kinase C assay with a mixed micellar assay system showed that the enzyme was activated by 1-palmitoyl LPC and 1-oleoyl LPC but not by 1-stearoyl LPC [48]. Moreover, in our preliminary experiment we failed to reverse the 1-stearoyl LPC-induced inhibition of FMLP-induced $Ca^{2+}$ increase by GF109203, a potent inhibitor of protein kinase C, whereas this enzyme inhibitor effectively reversed the phorbol ester-induced inhibition of the $Ca^{2+}$ response. These results make it unlikely that the inhibition of the phospholipase C/$Ca^{2+}$ system by 1-stearoyl LPC is mediated by protein kinase C activation in HL-60 cells.

In the cell-free system in which ATP, a phosphate donor for protein kinase C, was not included, the inhibitory action of LPC was also observed. This further excludes the possibility of an involvement of protein kinase C in the inhibitory action of LPC. As discussed above, in this cell-free system, LPC seems to affect at least two components in the enzyme system: phospholipase C itself and G-proteins. In this cell-free system, 1-palmitoyl LPC was also inhibitory for the enzyme activity; this species of LPC inhibited the former intrinsic enzyme activity to the same extent as 1-stearoyl LPC, whereas it was a weaker inhibitor than 1-stearoyl LPC for G-protein-induced enzyme activation. Therefore when LPC enters into the cells, the lipid exerts an inhibitory action only on the enzyme system. Although the mechanism of action of this inhibitory action of LPC remains unclear, the detergent action of the lipid might participate. This inhibitory action of 1-palmitoyl LPC might be overcome by the receptor-mediated stimulatory action in intact cells. With 1-stearoyl LPC, however, only an inhibitory action is exhibited because of its weak activity as a receptor agonist.

Here we have focused on the early signalling pathway of LPC leading to the change in $Ca^{2+}$ metabolism in leukaemia cells; therefore the physiological and pathophysiological roles of the LPC-induced activation and inhibition of the phospholipase C/$Ca^{2+}$ pathway have not been characterized. However, the actions of LPC on the $Ca^{2+}$ metabolism might be important from a pathophysiological view of inflammatory disorders and vascular atherosclerosis; the normal LPC concentration in serum or plasma is reported to be approx. 100 $\mu$M [27,51,52]; this value would increase in these disorders. It should be noticed, however, that this concentration does not mean free LPC concentration. LPC is usually present in lipoproteins such as LDL in serum or plasma and, even when released from the proteins, it binds easily to albumin. In endothelial cells, LPC-induced increase in $[Ca^{2+}]_i$ and inhibition of the agonist-induced phospholipase C/$Ca^{2+}$ system have already been reported. In preliminary experiments in the same type of cells, we observed a similar PTX-sensitive increase in $[Ca^{2+}]_i$. Inhibition of the phospholipase C/$Ca^{2+}$ system might explain the LPC-induced dysfunction of endothelium-dependent relaxation of arteries possibly through nitric oxide production [17–21]. In contrast, an increase in $Ca^{2+}$ might induce nitric oxide synthesis. Although this is contradictory to the view of LPC as an atherogenic compound, a few reports have shown that LPC actually stimulates nitric oxide synthesis in endothelial cells [22–24]. In relation to this, LPC has also been reported to induce the transcriptional expression of nitric oxide synthase in these cells [25,26]. In leucocytes, especially monocytes and T-lymphocytes, LPC has been reported to be a chemoattractant factor [8,9]. The change in $Ca^{2+}$ metabolism might be involved in the chemotaxis. In macrophages, $Ca^{2+}$ metabolism might be involved in the regulation of a cytokine synthesis [38]. The discovery of the different pharmacological specificity of LPC depending on its fatty acid constituent might help to explain the multifunctional nature of the lipid and the apparent contradiction such as the opposite directions of the responses to the lipid that have previously been reported. In any event, the newly identified LPC signalling mechanisms might provide novel insights into the molecular mechanisms of the multiple actions of LPC in the cells involved in the inflammatory disorders and vascular atherosclerosis.

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