Lysophosphatidic acid activation of phosphatidylcholine-hydrolysing phospholipase D and actin polymerization by a pertussis toxin-sensitive mechanism

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Incubation of IIC9 fibroblasts with lysophosphatidic acid (LPA) induced an increase in the amount of filamentous actin (F-actin), which was concentration-dependent with a maximal effect at 100 ng/ml. Phosphatidic acid (PA) also produced a concentration-dependent increase of F-actin, but it was less potent than LPA. The LPA-induced increase in F-actin was rapid and sustained for at least 60 min. LPA rapidly increased the levels of PA and choline, with maximal increases at 5 min and 30 s respectively. LPA also caused a monophasic increase in diacylglycerol (DAG) which lagged behind the increases in PA and choline. LPA stimulated phosphatidylbutanol formation in the presence of butanol and produced a small increase in inositol phosphates that was much less than that induced by α-thrombin. Pretreatment of cells with pertussis toxin (PTX) caused greater than 50% inhibition of the LPA-stimulated increases in PA, DAG and choline. PTX increased the LPA concentration required to induce half-maximal actin polymerization by about 10-fold. PTX caused a similar shift in the dose–response curve for LPA-induced PA formation. These results suggest that LPA induces an increase in PA by activating a phosphatidylcholine-hydrolysing phospholipase D via a PTX-sensitive G-protein and that the increase in PA is involved in the activation of actin polymerization.

INTRODUCTION

Lysophosphatidic acid (LPA) is now considered to act as an extracellular messenger [1,2]. It produces several cellular responses, such as phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, resulting in a cytosolic Ca^{2+} increase [1,3–5], activation of mitogen-activated protein kinase (MAP kinase) [1,6–8], inhibition of adenylate cyclase [1,3], and induction of mitogenesis [3,9,10]. Recently, a photoreactive LPA analogue has been used to identify a putative LPA receptor as a membrane protein of 38–40 kDa in various cells including A431 cells, mouse neuroblastoma cells and normal fibroblasts [11]. LPA is also known to activate phosphatidylcholine (PC) hydrolysis by phospholipase D (PLD) in Rat-1 fibroblasts [12].

Recently, LPA has been reported to activate actin polymerization in Swiss 3T3 fibroblasts [13]. Furthermore, microinjection of the cells with C3 ADP ribosyltransferase from *Clostridium botulinum*, a Rho inhibitor, blocks stress fibre formation induced by LPA [13,14]. There is also a recent report that LPA activates phosphorylation of focal adhesion kinase [8], which is a tyrosine kinase concentrated in the focal adhesions on plasma membrane from which stress fibres originate [13,15]. However, the mechanisms by which LPA activates Rho and focal adhesion kinase in the formation of stress fibres are unknown.

In this manuscript, we report that LPA activates actin polymerization and also PC-hydrolysing PLD in IIC9 fibroblasts. This report provides a possible mechanism of actin polymerization induced by LPA and also provides additional evidence for the conclusion in our previous report [16] that phosphatidic acid (PA) derived from PC activates actin polymerization in IIC9 cells.

MATERIALS AND METHODS

Materials

Culture medium components were purchased from Gibco/BRL. PA, LPA (oleoyl), *Streptomyces chromofuscus* PLD and α-thrombin (approx. 3000 units/mg of protein) were obtained from Sigma. [9,10-^{3}H]Myristic acid (33.5 Ci/mmol), [*methyl*-^{14}C]choline chloride (53 mCi/nmol) and *myo*-[2-^{3}H(n)]inositol (12.3 Ci/nmol) were from DuPont–New England Nuclear. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin (NBD-phallacidin) was from Molecular Probes (Eugene, OR, U.S.A.) and pertussis toxin (PTX) was from List Biological Laboratories (Campbell, CA, U.S.A.). AG 1-X8 resin (200–400 mesh, formate form) was from Bio-Rad and Silica gel t.l.c. plates (pre-absorbed and channelled) were from Whatman. Suramin was from Biomol (Plymouth Meeting, PA, U.S.A.).

Cell Culture

IIC9 cells, a subclone of Chinese hamster embryo fibroblasts (kindly given by Dr. Daniel M. Raben, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.), were grown and maintained according to Ha and Exton [17]. Briefly, cells were grown on 6-well plates or 100-mm-diam. culture dishes for 2 days in medium containing serum, and subconfluent cultures were incubated for 2 days in serum-free medium.

Abbreviations used: DAG, diacylglycerol; F-actin, filamentous actin; IP_3, inositol 1,4,5-trisphosphate; LPA, lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; NBD-phallacidin, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PBut, phosphatidylbutanol; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PTX, pertussis toxin.

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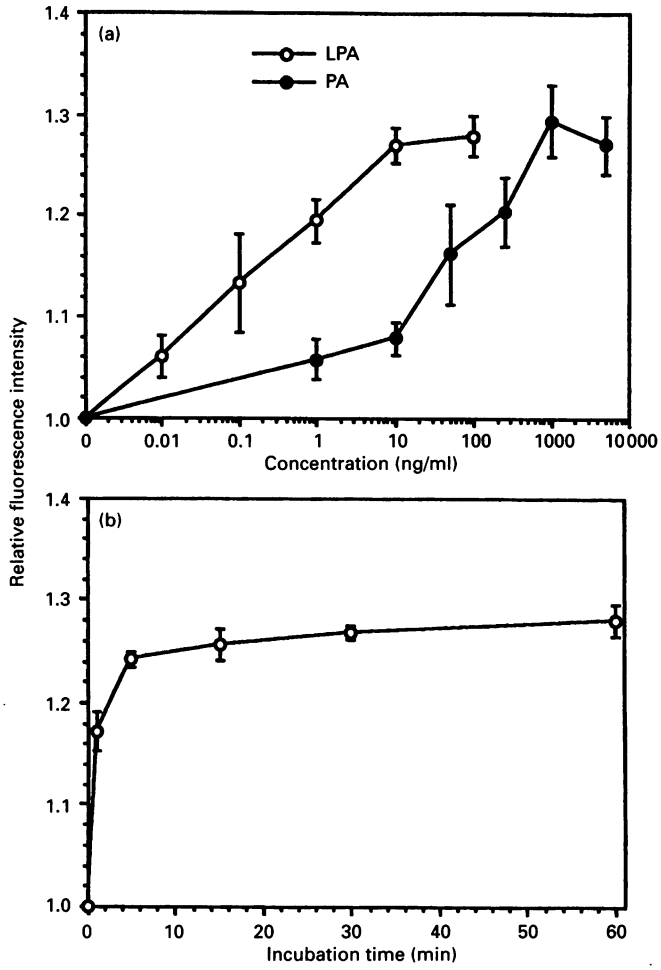

Figure 1 Effect of LPA and PA on F-actin content

Cells, grown and serum-starved, were incubated with various concentrations of LPA and PA for 15 min (**a**) or incubated with 100 ng/ml LPA for the indicated times (**b**). F-actin content was measured as described in the Materials and methods section. Data are means ± S.E.M. from three independent experiments.

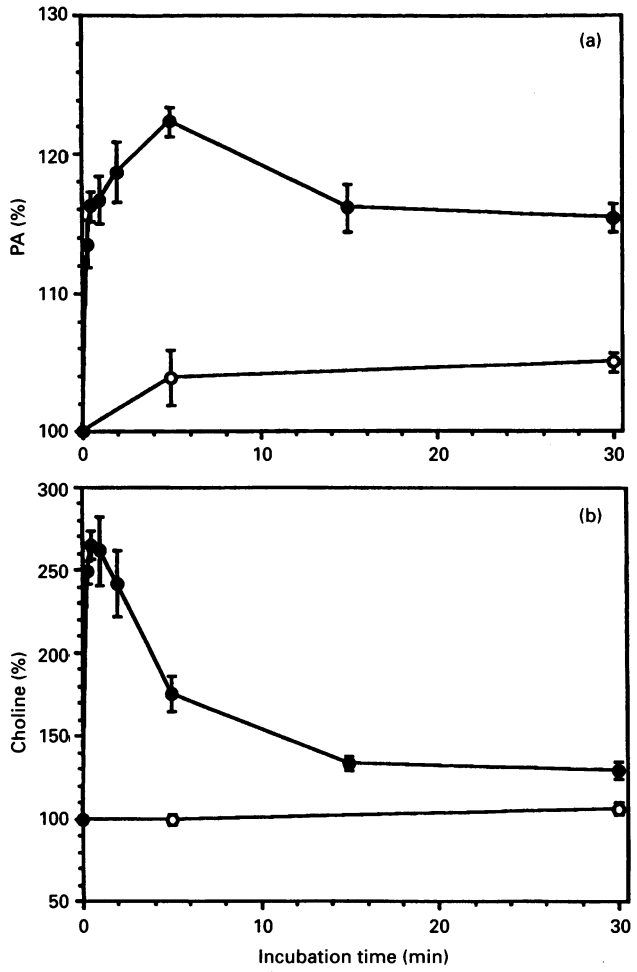

Figure 2 Changes of PA (a) and choline (b) levels induced by LPA

Cells, grown for 2 days, were labelled with 10 μCi/dish of [^{3}H]myristic acid (**a**) or 2 μCi/dish of [^{14}C]choline chloride (**b**) for 2 days in serum-free medium and then incubated with 100 ng/ml LPA for the times indicated. PA and choline were isolated and quantified as described in the Materials and methods section. Data are means ± S.E.M. from three independent experiments. ○, Control; ●, LPA induced.

Filamentous actin (F-actin) measurement

F-actin content was measured by the procedures of Ha and Exton [16]. Briefly, cells were grown on 6-well plates, serum-starved, and then incubated with various concentrations of LPA or PA. In some experiments, cells were preincubated with 100 ng/ml PTX for 2 h or various concentrations (0.02–0.4 mg/ml) of suramin for 5 min. The treated cells were fixed, stained with NBD-phallacidin, and the fluorescence intensity was measured using an SPEX Fluorog (1681, 0.22 m spectrometer) with an excitation wavelength of 465 nm and an emission wavelength of 535 nm.

Measurement of PA, phosphatidylbutanol (PBut) and diacylglycerol (DAG)

Cells were labelled with 10 μCi/100-mm-diam. dish of [^{3}H]myristic acid for 2 days in serum-free medium and were incubated with various concentrations of LPA for the times indicated in the figure legends. In the case of PBut formation, 0.3% (v/v) butan-1-ol was added 10 min before LPA and incubation was for 15 min. In some experiments, cells were preincubated with 100 ng/ml PTX for 2 h followed by incubation with LPA. Following extraction of lipids from the cells [18], PA and DAG were separated and measured according to the methods of Ha and Exton [17] and PBut was separated by t.l.c. using Silica Gel 6A (Whatman) and a solvent system of ethylacetate/iso-octane/acetic acid/H_2O (10:5:2:10, by vol.). PBut was identified by its co-migration with a standard prepared using PLD from *S. chromofuscus*.

Measurement of intracellular inositol phosphates, choline and phosphocholine

Cells were labelled for 2 days with 3 μCi/dish of [^{3}H]*myo*-inositol in inositol- and serum-free medium, or 2 μCi/dish of [^{14}C]choline chloride in serum-free medium, and then activated with 100 ng/ml LPA or 500 ng/ml α-thrombin. Cells were scraped in ice-cold methanol and the aqueous layer was separated according to Bligh and Dyer [18]. Inositol phosphates including inositol 1-monophosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate (IP_3) were separated using 1 ml of AG 1-X8 resin

and counted in a scintillation counter [17]. Choline and phosphocholine were separated and measured according to the procedures of Ha and Exton [17].

RESULTS

Activation of actin polymerization by LPA

Incubation of IIC9 fibroblasts with LPA increased the amount of F-actin. When measured at 15 min, there was a significant increase at 0.1 ng/ml and a maximal effect at 100 ng/ml (Figure 1a). PA also induced a concentration-dependent increase of F-actin in these cells, in agreement with previous findings [16], but PA was much less potent than LPA (Figure 1a). The increase in F-actin induced by LPA was accompanied by cell elongation (results not shown), as seen when the cells are treated with PA or *S. chromofuscus* PLD [16]. Figure 1(b) shows that the increase in F-actin levels caused by LPA occurred rapidly, with a significant increase observed at 1 min and a maximal effect at 5 min.

Activation of PC hydrolysis by LPA

To determine if activation of PC hydrolysis by PLD was involved in the effect of LPA, levels of PA and choline were measured. Figure 2(a) shows that LPA rapidly increased radiolabelled PA in [^{3}H]myristic acid-labelled cells, with a significant increase within 15 s and a maximal increase at 5 min. The PA level remained elevated for at least 30 min (Figure 2a). LPA also elicited a rapid rise in cellular choline (Figure 2b), confirming activation of PC-hydrolysing PLD. A maximal increase in choline was observed at 30 s, after which time it decreased but still remained above control levels. There were no significant changes of phosphocholine (results not shown), suggesting that PC-hydrolysing phospholipase C (PLC) was not activated. To confirm that PLD was activated, [^{3}H]myristic acid-labelled cells were incubated with butanol and increasing concentrations of LPA. Figure 3 shows that LPA caused a dose-dependent stimulation of PBut formation, with a half-maximal effect at 4 ng/ml. Similar dose–response curves were seen for PA and DAG formation (results not shown).

Changes in inositol phosphates with LPA

The effect of LPA on PIP_2 hydrolysis in IIC9 fibroblasts was also examined. Previous studies [17,19,20] have demonstrated that α-thrombin elicits a rapid, but transient, activation of phosphatidylinositol (PI)-specific PLC and a sustained increase in PC breakdown in these cells. As a result, a biphasic increase in DAG levels is observed [17,19,20]. Figure 4(a) shows that LPA caused a monophasic increase in DAG which lagged behind the increases in PA and choline, suggesting that DAG was mainly derived from PA generated by PC hydrolysis. To test more directly if activation of PI-specific PLC was absent or minimal, changes in inositol phosphates were measured in [^{3}H]inositol-prelabelled cells. As shown in Figure 4(b), LPA produced a small increase in inositol phosphates, which was much less than that elicited by α-thrombin.

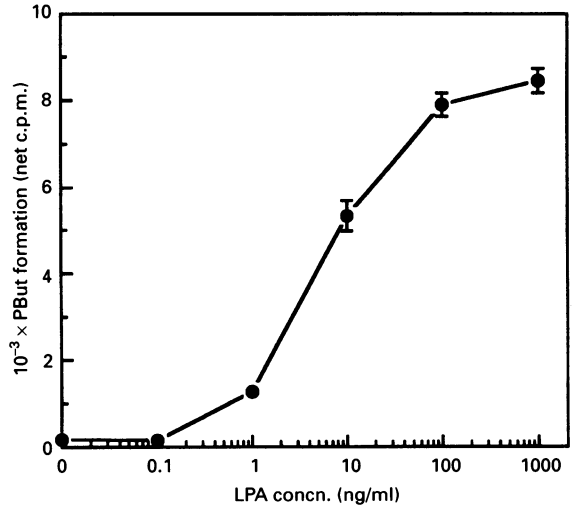

Figure 3 Effect of LPA on PLD activity as assessed by PBut formation

Cells were labelled for 2 days with 10 μCi/dish of [^{3}H]myristic acid in serum-free medium. After incubation with 0.3% (v/v) of butan-1-ol for 10 min, the cells were exposed to increasing concentrations of LPA for 15 min and [^{3}H]PBut was isolated as described in the Materials and methods section. Data are from an experiment that is representative of three and assayed in duplicate.

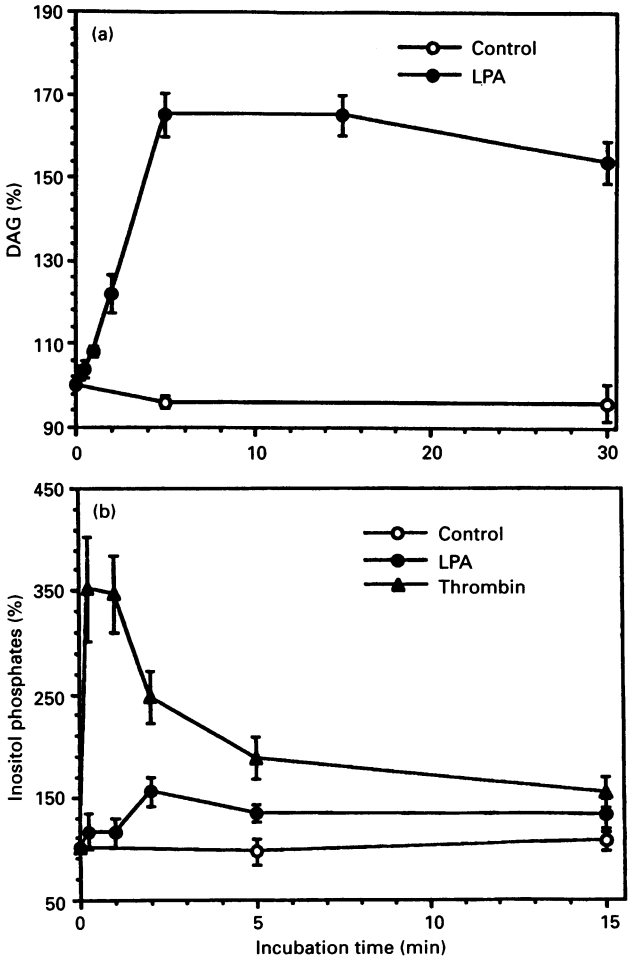

Figure 4 Time course of changes in DAG (a) and inositol phosphates (b)

Cells were labelled for 2 days with 10 μCi/dish of [^{3}H]myristic acid in serum-free medium (**a**) or 3 μCi/dish of [^{3}H]*myo*-inositol in inositol- and serum-free medium. After incubation with 100 ng/ml LPA or 500 ng/ml α-thrombin for the indicated times, DAG and inositol phosphates were separated and quantified as described in the Materials and methods section. Data are means ± S.E.M. from three independent experiments.

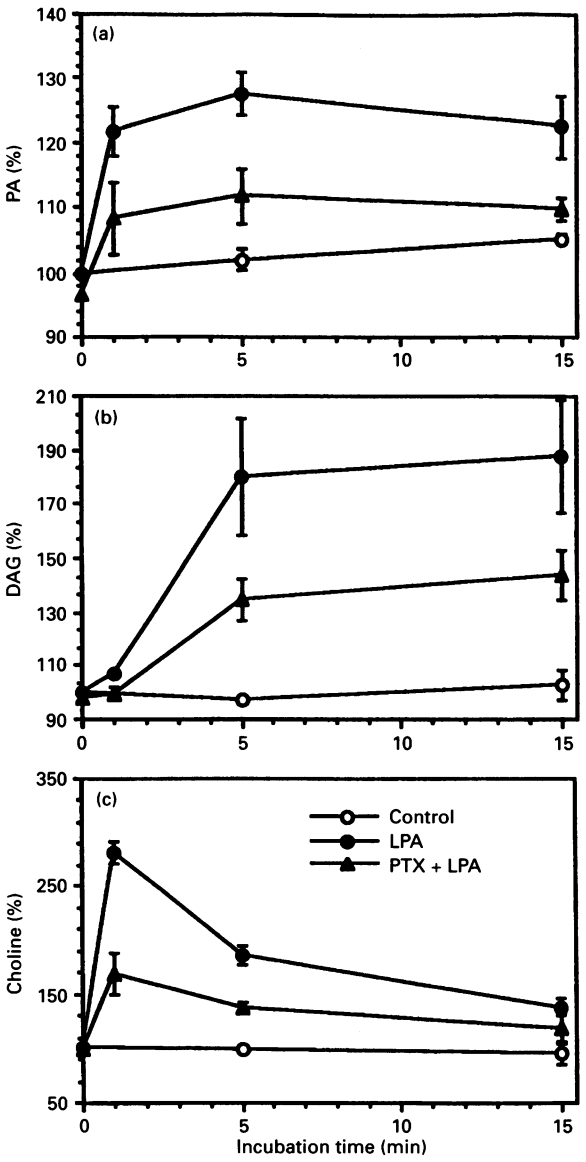

Figure 5 Effect of PTX on the amounts of PA (a), DAG (b), and choline (c) increased by LPA

Cells were labelled with 10 μCi/dish of [^{3}H]myristic acid (**a** and **b**) or 2 μCi/dish of [^{14}C]choline chloride (**c**) for 2 days in serum-free medium. Following preincubation with 100 ng/ml PTX for 2 h, cells were stimulated with 100 ng/ml LPA for the indicated times. The amounts of PA, DAG and choline were measured as described in the Materials and methods section. Data are means ± S.E.M. from three independent experiments.

Effects of PTX

Previous investigations [3,12] have revealed that PTX pretreatment of cells can inhibit LPA effects. Pretreatment of IIC9 cells with PTX for 2 h caused greater than 50% inhibition of LPA-stimulated PA, DAG and choline levels, whereas basal levels were unaffected (Figure 5). In view of our previous conclusion that α-thrombin-induced actin polymerization resulted from increased production of PA via PLD activation [16], we expected that the partial reduction in PA concentration induced by PTX would shift the dose–response curve for the effect of LPA on actin polymerization to the right. Figure 6

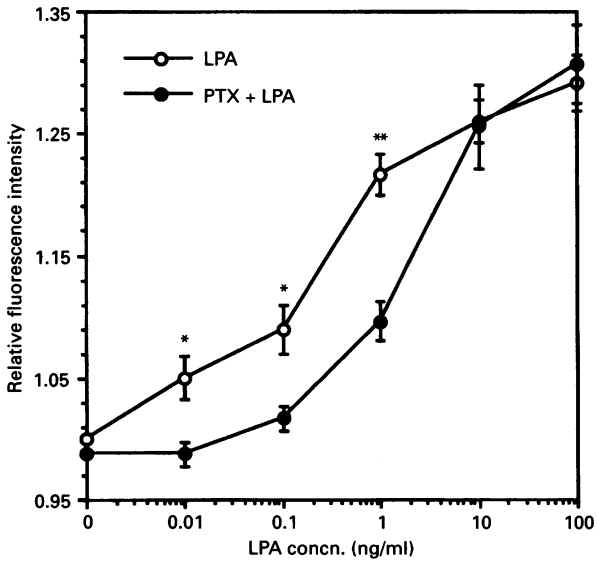

Figure 6 Effect of PTX on actin polymerization induced by LPA

Cells, grown and serum-starved, were preincubated with 100 ng/ml PTX and then stimulated with various concentrations of LPA for 15 min. F-actin content was measured as described in the Materials and methods section. Data are means ± S.E.M. from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$.

shows that the LPA concentration which induced half-maximal actin polymerization was increased about 10-fold by PTX. However, PTX had no significant effect on actin polymerization induced by PA or *S. chromofuscus* PLD (results not shown). As expected, PTX also caused a shift to the right in the dose–response curve for PA formation (results not shown).

DISCUSSION

Addition of LPA to cells produces numerous effects including calcium mobilization [1,3–5], activation of MAP kinase [1,6–8], stimulation of mitogenesis [3,9,10], inhibition of adenylate cyclase [1,3], and activation of actin polymerization [13,14]. Recently, a putative cell-surface receptor for LPA was identified [11]. We report here that LPA rapidly stimulates actin polymerization in IIC9 fibroblasts and provide evidence that this effect is mediated by an increase in PA levels produced by PLD. Previously, we showed that IIC9 cells treated with exogenous PA, *S. chromofuscus* PLD, or α-thrombin responded with an increase in F-actin [16]. It is thus possible that PA, which increases in many cell types after stimulation by agonists [21], is involved in agonist-induced actin polymerization.

Several reports indicate the existence of a PA-hydrolysing phospholipase A_2 in cells [2,10,22], raising the possibility that PA produced in response to agonists may be hydrolysed to LPA. In IIC9 cells, however, neither α-thrombin nor *S. chromofuscus* PLD significantly altered LPA levels, while both increased PA [16]. This argues against the idea that LPA is downstream of PA in the pathway leading to actin polymerization.

The activation of PLD by LPA was demonstrated by increases in PA, PBut and choline (Figures 2 and 3). The rise in DAG lagged behind the increases in PA and choline, suggesting that DAG was derived from PA by the action of PA phosphohydrolase. Consistent with this mechanism of DAG formation is the lack of an effect of LPA on phosphocholine levels (results not

shown), which would be increased if DAG were produced by stimulation of PC-hydrolysing PLC.

The mechanism by which LPA activates PLD in IIC9 cells is unclear. Recently, van der Bend et al. [12] showed that LPA activates both PC-hydrolysing PLD and PI-specific PLC in Rat-1 fibroblasts. The stimulation of PLD occurred secondarily to stimulation of PI-specific PLC and appeared to involve protein kinase C (PKC) activation, since PKC downregulation abolished the LPA effect. Our results with IIC9 cells differ from the results observed for Rat-1 fibroblasts in two respects. First, LPA produced only a weak activation of PIP_2 hydrolysis in IIC9 cells, but gave a large (10-fold) response in Rat-1 cells. Secondly, PTX preincubation partly blocked PLD activation in IIC9 cells but had no effect in Rat-1 cells. These results suggest differences in the mechanisms involved in PLD activation by LPA in the two cell types.

Stimulation of PIP_2 hydrolysis by LPA is apparently cell type-specific. LPA increases cytosolic Ca^{2+}, presumably by increasing IP_3, in Rat-1 and HF fibroblasts [2–4], but not in neutrophils and Jurkat T cells [4]. Increases in inositol phosphates in Rat-1 and HF cells occur at 1–100 μM LPA [3,4,12,23], whereas LPA binding to the putative cell-surface receptor occurs at 2–200 nM LPA [11]. The stimulation of PLD described in this manuscript takes place at concentrations of LPA (1–200 μM) that are similar to those at which PI-specific PLC stimulation occurs, suggesting that both effects may be mediated by a receptor that is different from that identified by photolabelling.

The mechanisms by which LPA activates PLD and actin polymerization are unknown. As shown in our previous study [17], actin polymerization in IIC9 cells is not mediated by changes in inositol phosphates, Ca^{2+}, DAG or PKC, but appears to be due to PLD activation and PA formation. Numerous studies have implicated a G-protein in PLD activation, but its identity remains unknown [21]. Partial inhibition of LPA-induced PLD activity by PTX suggests the involvement of a G_i-like G-protein. In addition, the low-molecular-mass G-protein Rho is known to be involved in activation of actin polymerization [13,14] and recently has been implicated in PLD activation in neutrophils [24]. Thus it is interesting to speculate that Rho may be involved in the pathway of actin polymerization induced by activation of PLD in IIC9 cells. Consistent with this possibility is the finding that PA inhibits the GTPase activity of Rho [25], although it should be recognized that this has only been shown *in vitro*. Activation of actin polymerization by LPA may involve both heterotrimeric and low-molecular-mass G-proteins.

We have found that the concentration of LPA which induces a half-maximal increase in actin polymerization (0.2 ng/ml, Figures 1 and 6) is 20-fold less than that which gives a half-maximal activation of PLD. This suggests that actin polymerization is sensitive to small changes in PA. The mechanism by which PA activates actin polymerization may involve mechanisms in addition to those described above. Stress fibres originate from electron-dense regions of plasma membrane known as focal adhesions, which contain cytoskeletal proteins such as vinculin, talin, integrin, tensin, paxillin and α-actinin [13,26–28]. Recently, it has been reported that focal adhesion kinase, a tyrosine kinase that is localized in focal adhesions [15], is activated by LPA [8]. It is possible that this effect of LPA may also be mediated by PA, which may induce formation of stress fibres by interacting with focal adhesion kinase or other cytoskeletal proteins. This possibility needs further exploration.

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