Role of lysophosphatidic acid in the regulation of uterine leiomyoma cell proliferation by phospholipase D and autotaxin

Emmanuelle Billon-Denis, Zahra Tanfin,[1] and Philippe Robin

University Paris Sud, Institut de Biochimie et Biophysique Moléculaire et Cellulaire Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8619, Équipe Signalisation et Régulations Cellulaires, Orsay, France

Abstract **Phospholipase D (PLD) hydrolyzes phosphatidylcholine into phosphatidic acid (PA), a lipidic mediator that may act directly on cellular proteins or may be metabolized into lysophosphatidic acid (LPA). We previously showed that PLD contributed to the mitogenic effect of endothelin-1 (ET-1) in a leiomyoma cell line (ELT3 cells). In this work, we tested the ability of exogenous PA and PLD from *Streptomyces chromofuscus* (scPLD) to reproduce the effect of endogenous PLD in ELT3 cells and the possibility that these agents acted through LPA formation. We found that PA, scPLD, and LPA stimulated thymidine incorporation. LPA and scPLD induced extracellular signal-regulated kinase ($ERK_{1/2}$) mitogen-activated protein kinase activation. Using Ki16425, an LPA_1/LPA_3 receptor antagonist and small interfering RNA targeting LPA_1 receptor, we demonstrated that scPLD acted through LPA production and LPA_1 receptor activation. We found that scPLD induced LPA production by hydrolyzing lysophosphatidylcholine through its lysophospholipase D (lysoPLD) activity. Autotaxin (ATX), a naturally occurring lysoPLD, reproduced the effects of scPLD. By contrast, endogenous PLD stimulated by ET-1 failed to produce LPA. These results demonstrate that scPLD stimulated ELT3 cell proliferation by an LPA-dependent mechanism, different from that triggered by endogenous PLD. These data suggest that in vivo, an extracellular lysoPLD such as ATX may participate in leiomyoma growth through local LPA formation.**—Billon-Denis, E., Z. Tanfin, and P. Robin. **Role of lysophosphatidic acid in the regulation of uterine leiomyoma cell proliferation by phospholipase D and autotaxin.** ***J. Lipid Res.*** **2008.** 49: **295–307**

Supplementary key words extracellular signal-regulated kinase • lysophosphatidylcholine • phosphatidic acid • endothelin-1 • uterine tumor

Uterine leiomyomas (or fibroids) are benign tumors of the uterine smooth muscle (myometrium). It is well established that leiomyoma growth is under hormonal control by ovarian steroids and growth factors but also by G protein-coupled receptor (GPCR) agonists (1). An Eker rat leiomyoma tumor-derived cell line (ELT3) was characterized and has been used successfully to investigate the hormonal modulation in association with the disease pathogenesis (2, 3). We recently demonstrated that endothelin-1 (ET-1), a 21-amino acid peptidic hormone, induced the survival and proliferation of ELT3 cells (4, 5). The proliferative effect of ET-1 was stronger in ELT3 cells than in normal myometrial cells, and this increased effect was correlated with an increase in phospholipase D (PLD) activity in ELT3 cells (5). Mammalian PLD is implicated in diverse physiological functions, including proliferation, migration, inflammation, and secretion (6). Moreover, the enhancement of PLD activity has been described in association with some cancer tumors (7). PLD hydrolyzes phosphatidylcholine (PC) to produce phosphatidic acid (PA) and free choline. PA is a bioactive phospholipid involved in many intracellular biological functions attributed to PLD (8). It can function in signal transduction events by interacting with several signaling proteins, such as Raf-1, PKCζ and PKCϵ, PLCγ, and mTOR (9, 10). PA has also been proposed to act through the binding to the orphan receptor GPR63/PSP24β, which belongs to the GPCR family (10, 11).

Another possibility is that PA does not act by itself but serves as a precursor for the synthesis of other bioactive compounds. Indeed, PA can be converted into diacylglycerol by lipid phosphate phosphatases or can be deacylated by a phospholipase A (PLA) to form lysophosphatidic acid (LPA) (10, 12, 13).

LPA is a bioactive lysophospholipid present in diverse biological fluids. Serum is the best-characterized source of LPA. During blood coagulation, platelet activation participates in LPA production. LPA is also produced by a lysophospholipase D (lysoPLD) activity, present in serum, that hydrolyzes lysophosphatidylcholine (LPC) to form

Manuscript received 6 April 2007 and in revised form 10 September 2007.

Published, JLR Papers in Press, November 16, 2007.
DOI 10.1194/jlr.M700171-JLR200

[1] To whom correspondence should be addressed.
e-mail: zahra.tanfin@u-psud.fr

LPA and free choline (12). Recently, autotaxin (ATX) was identified as a circulating lysoPLD, and it is thought to be the main source of LPA in serum and other body fluids (14, 15). ATX and LPA stimulate cell proliferation, migration, and survival (13, 16, 17). LPA is also involved in the physiology of the uterus. In human myometrium, LPA exerts a mitogenic effect and stimulates stress fiber formation (18, 19). Moreover, it has been demonstrated that LPA plays an important role in embryo implantation in mice (20). LPA and ATX are also involved in the development and spreading of malignant tumors, particularly ovarian and prostate cancers (16, 17, 21, 22). LPA is also a biological marker for prostate and ovarian cancers as well as for endometrial and cervical carcinomas (16).

LPA mediates its effects by interacting with membrane receptors of the GPCR family. To date, five LPA receptors, termed LPA_1–LPA_5, have been identified. LPA_1, LPA_2, and LPA_3 belong to the endothelial differentiation gene (edg) family, like the sphingosine-1-phosphate receptors (23). In contrast, the recently described LPA_4 and LPA_5 show limited homology to LPA_1–LPA_3 (23, 24). LPA receptors are coupled to different G proteins and signaling pathways, such as Gi/phosphoinositide 3-kinase, Gi/Ras, Gq/phospholipase C, $G_{12/13}$/RhoA, and Gs/adenylyl cyclase (23).

Bacterial PLD from *Streptomyces chromofuscus* (scPLD), as mammalian PLD, catalyzes the hydrolysis of PC into PA (25). It has often been used to induce PA synthesis in membranes of living cells to mimic the effects of endogenous mammalian PLD. Recently, we demonstrated that scPLD increased PA production, activated the extracellular signal-regulated kinase ($ERK_{1/2}$) pathway, and stimulated the proliferation of ELT3 cells (5). This may indicate that scPLD and endogenous PLD act through a common pathway. However, in addition to its PLD activity, scPLD possesses lysoPLD activity and is able to catalyze the synthesis of LPA from LPC, as does ATX (25). In the present study, we investigated the involvement of PA and LPA in the cellular effects of scPLD, ET-1, and ATX. Moreover, we tested the possibility that LPA can be synthesized from PA or by an alternative mechanism. This study is also an attempt to identify new lipid mediators and related pathways that may be involved in the control of leiomyoma growth.

MATERIALS AND METHODS

Chemicals

[^{14}C]oleoyl-coenzyme A (50 mCi/mmol), [^{3}H]methyl-choline (30–40 Ci/mmol), and [^{3}H]thymidine (6.7 Ci/mmol) were purchased from Perkin-Elmer. scPLD was from Sigma-Aldrich, and 1 IU liberates 1.0 μM choline from L-α-phosphatidylcholine per hour at pH 8.0 and 30°C. 4β-Phorbol-12,13-dibutyrate (PDBu), fatty acid-free BSA (ffBSA), methyl-α-D-mannopyranoside, 1-oleoyl-2-*sn*-lysophosphatidylcholine, bromoenol lactone (BEL), and quinacrine were obtained from Sigma-Aldrich. 1-Oleoyl-2-*sn*-lysophosphatidic acid and 1,2-dioleoyl phosphatidic acid were from Avanti Polar Lipids. Phosphonofluoridic acid methyl-5,8,11,14-eicosatetraenyl ester (MAFP) and arachidonyl-trifluoromethyl ketone (AACOCF3) were from Biomol. Lipofectamine 2000 and all of the media and reagents for the cell culture were from Invitrogen. ET-1 was from NeoMPS. Concanavalin-A Sepharose was from GE-Healthcare. Platelet-derived growth factor (PDGF) was from Peprotech. Ki16425 was a generous gift from Kirin Brewery Co., Ltd. (Gunma, Japan). Anti-active $ERK_{1/2}$ antibody, U0126, deoxyribonucleotides, Moloney murine leukemia virus reverse transcriptase, and *Taq* DNA polymerase were from Promega Corp. Anti-rabbit HRP-conjugated IgG was from Cell Signaling. Anti-ERK_2 antibody was from Santa Cruz. PCR primers and control small interfering RNA (siRNA) were from MWG-Biotech. Rat LPA_1/edg2 receptor-directed siRNA (siGENOME ON-TARGETplus SMARTpool L-098917-01-0010) came from Dharmacon. Cell line Nucleofector kit V was from Amaxa. The pCDNA-ATX expression vector encoding wild-type murine ATX (mATX) and the pTrcHis-AGPAT vector encoding 1-acyl-*sn*-glycerol-3-phosphate acyltransferase were kind gifts from Dr. J. S. Saulnier-Blache (Institut National de la Santé et de la Recherche Médicale U317, University Paul Sabatier, Toulouse, France).

Cell culture and transfection

The Eker rat tumor-derived ELT3 uterine leiomyoma cell line was kindly provided by Dr. C. L. Walker (M. D. Anderson Cancer Center, University of Texas, Smithville, TX). ELT3 cells were maintained in DF8 medium supplemented with 10% (v/v) fetal calf serum, as described previously (5), and 5% CO_2-95% humidified air at 37°C. Cells were placed in serum-free medium overnight before experiments. Cos-7 cells were cultured in DMEM containing 10% fetal calf serum. ELT3 and Cos-7 cells were transfected with pCDNA-ATX expression vector or pCDNA empty vector using Lipofectamine 2000 according to the manufacturer's instructions.

RNA interference

ELT3 cells cultured in 75 cm^2 flasks were harvested by trypsinization and electroporated with an Amaxa Nucleofector device (program T-030) at a density of 10^6 cells in 100 μl of buffer V containing 2.5 μM of control or LPA_1 receptor-directed siRNA according to the manufacturer's instructions. Ten hours after electroporation, cells were serum-starved for 16 h before each experiment.

Semipurification of ATX

The semipurification of mATX from Cos-7 cells was performed as described by Pradere et al. (26) with some modifications. Twenty-four hours after transfection with ATX expression vector (or empty vector), Cos-7 cells were placed in DMEM without serum (5 ml/100 mm diameter plate) and incubated for an additional period of 24 h. Culture media were then centrifuged (300 *g* for 10 min) to remove floating cells and incubated overnight in the presence of concanavalin A-Sepharose beads. After three washings, ATX was eluted from the beads by incubation for 2 h at 37°C in PBS containing 0.3 M methyl-α-D-mannopyranoside.

LPC assay

LPC neosynthesis was determined by [^{3}H]choline incorporation in ELT3 cells. Confluent, serum-starved ELT3 cells, on 12-well plates, were incubated for various times in MEM containing 5 μCi of [^{3}H]choline (or [^{3}H]myristic acid in some experiments). At the end of the incubation, the media were removed and placed in glass tubes containing 1 ml of methanol. Cells were scraped in 1 ml of cold (−20°C) methanol and transferred to glass tubes containing 1 ml of water. In each tube, 800 μl of cold chloroform/methanol/HCl (149:48:3, v/v/v) was added. After a

vigorous mixing, the monophase was split by the addition of 0.6 ml of 2 M KCl and 0.6 ml of chloroform. The chloroform extract was dried under vacuum, suspended in 25 μl of methanol-chloroform (1:1, v/v), and spotted on thin-layer chromatography silica gel plates. Plates were developed in a mixture of chloroform-methanol-28% ammoniac (65:35:4, v/v/v) and analyzed with a computerized Berthold radiochromascanner. When [^{3}H]myristic acid was used instead of [^{3}H]choline, the chromatography solvent was composed of chloroform-methanol-acetic acid-water (50:30:8:4, v/v/v/v) to allow a better separation of LPC from LPA. The position of [^{3}H]LPC on the plates was determined by comparison with the position of nonradioactive 1-oleoyl-LPC standard, as revealed by iodine vapor staining.

LPA radioenzymatic quantification

Radioenzymatic quantification of LPA was performed essentially as described by Saulnier-Blache et al. (27). This method is based on the use of recombinant 1-acyl-2-*sn*-glycerol-3-phosphate acyltransferase (LPAAT), which catalyzes the transfer of the acyl moiety of an acyl-CoA molecule to the *sn*-2 position of 1-acyl LPA.

Preparation of recombinant LPAAT

The cDNA encoding an LPAAT enzyme was previously cloned in the bacterial expression vector pTrcHis (28). DH5α-competent cells were transformed with pTrcHis-AGPAT plasmid, encoding LPAAT, and grown in Luria broth medium containing 50 μg/ml ampicillin. When the absorbance at 600 nm reached 0.6, bacteria were cultured in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. Bacteria were collected by centrifugation at 3,000 *g* for 10 min, and the pellet was disrupted by sonication in 0.2 M Tris-HCl (pH 7.4). After 20 min of centrifugation at 10,000 *g*, the supernatant was recovered and further centrifuged at 100,000 *g* for 90 min. The pellet was resuspended in 0.2 M Tris-HCl (pH 7.4) to a final protein concentration of 1 μg/μl and used for radioenzymatic quantification of LPA.

Lipid extract preparation

Serum-starved confluent ELT3 cells seeded on six-well plates were rinsed twice with Hanks' balanced salt solution containing 20 mM HEPES (pH 7.5) and 1 mg/ml ffBSA and incubated in 1 ml of the same medium. Cells were then exposed to the agents tested. At the end of the incubation, the media were removed and placed in glass tubes containing 1 ml of water-saturated butanol. Cells were scraped in 1 ml of water-saturated butanol and transferred to the same glass tubes containing the media. After the addition of 1 ml of water, the tubes were shaken vigorously and centrifuged (10 min at 300 *g*). The upper butanol phase was collected and evaporated under vacuum.

LPA radioenzymatic quantification assay

Dry lipid extracts were resuspended in 200 μl of reaction medium containing 161 μl of Tween 20 (1 mg/ml in water), 8 μl of orthovanadate (500 μM), 20 μl of Tris (200 mM; pH 7.5), 1 μl of [^{14}C]oleoyl-CoA, and 10 μl of recombinant LPAAT. The mixture was incubated for 2 h at 20°C and vortexed every 15 min. The reaction was stopped by adding 400 μl of $CHCl_3$/methanol/HCl (40:40:0.26, v/v/v). After vigorous shaking and 5 min of centrifugation at 3,000 *g*, the lower chloroformic phase was collected and evaporated under vacuum. Dry lipid extracts were resuspended in 25 μl of $CHCl_3$/methanol (1:1, v/v), spotted on thin-layer chromatography silica gel plates, and separated with $CHCl_3$/methanol/NH_4OH/water (65:25:0.9:3, v/v/v/v) as solvent. Quantification of [^{14}C]PA was performed using a Storm phosphorimager (Molecular Dynamics).

Western blot analysis of phosphorylated $ERK_{1/2}$

Serum-starved confluent ELT3 cells seeded on 12-well plates were exposed to the agents tested. Reactions were stopped by aspiration of the incubation medium followed by the addition of 50 μl of cold solubilization buffer [50 mM HEPES, pH 7.4, 150 mM NaCl, 100 mM NaF, 10% (v/v) glycerol, 10 mM $Na_4P_2O_7$, 200 μM Na_3VO_4, 10 mM EDTA, 1% (v/v) Triton-X100, 10 μg/ml aprotinin and leupeptin, and 0.5 mM PMSF]. Cells were detached by scraping on ice and centrifuged at 10,000 *g* for 5 min at 4°C. Detergent-extracted proteins were analyzed by 10% (w/v) SDS-PAGE. The separated proteins were transferred to nitrocellulose sheets and probed with polyclonal anti-active $ERK_{1/2}$ antibodies (1:5,000, v/v) and HRP-conjugated anti-rabbit IgG secondary antibody. Blots were revealed by ECL technique and exposed on X-ray films. The blots were then stripped and reprobed with an anti-ERK_2 antibody and HRP-conjugated anti-rabbit IgG secondary antibody. Quantification of the developed films was performed with a Personal Densitometer (Molecular Dynamics).

[^{3}H]thymidine incorporation

Serum-starved ELT3 cells (50% confluent) on 24-well plates were incubated for 48 h with the various agents to be tested and 1.5 μCi/ml [^{3}H]thymidine. Reactions were stopped by aspiration of the incubation medium and addition of 0.5 ml of cold TCA (10%, w/v). Radioactivity incorporated into TCA-precipitated material was recovered with 0.5 ml of 0.1 N NaOH and quantified by liquid scintillation counting.

LPA receptors and ATX mRNA expression

Detection of transcripts encoding the five LPA receptors and ATX was performed using RT-PCR technique. Total cellular RNA from ELT3 cells and rat lung, kidney, and ovary were isolated with Insta-Pure reagent (Eurogentec) according to the manufacturer's protocol, and 5 μg of total RNA was reverse-transcribed into cDNA using 200 units of Moloney murine leukemia virus-reverse transcriptase, 0.2 mM deoxynucleoside triphosphates, and 10 μM random hexamer primers. Target cDNA was amplified using one-tenth of the reverse-transcribed cDNA prepared, 0.2 mM deoxynucleoside triphosphates, 2 mM $MgCl_2$, 2.5 units of Taq DNA polymerase, and 100 pmol of each primer in PCR buffer (final volume = 25 μl). The primer sets used are listed in **Table 1**. The mixture was amplified in a thermal cycler (iCycler; Bio-Rad) under the following conditions: 94°C (15 s), 60°C (30 s), and 72°C (30 s) for 30 (LPA receptors) or 35 (ATX) cycles. The resulting PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Quantitative PCR

Quantitative real-time PCR was performed on the Lightcycler 2.0 (Roche). PCR was carried out using the FastStart DNA Master SYBR Green I reagents according to the manufacturer's instructions under the following conditions: 95°C (10 s), 57°C (10 s), and 72°C (10 s) for 40 cycles. Melting curve analyses were performed at the end of the amplification reactions. The relative amounts of LPA_1 and LPA_3 cDNA in each sample were determined using a standardization curve performed in the same PCR run. The standardization curves were obtained by amplification of successive dilutions of a PCR product resulting from conventional PCR. GAPDH transcript was used as standard for the relative quantification of the LPA_1 transcript in siRNA-treated cells.

TABLE 1. Description of primer sets used for PCR amplification

Sample	Forward Primer	Reverse Primer	Size *(bp)*	Accession Number
LPA_1	TCTTCTGGGCCATTTTCAAC	TGCCTGAAGGTGGCGCTCAT	350	AF090347
LPA_2	CCTACCTCTTCCTCATGTTC	TAAAGGGTGGAGTCCATCAG	806	XM_573887
LPA_3	GGAATTGCCTCTGCAACATCT	GAGTAGATGATGGGGTTCA	382	NM_023969
LPA_4	ACCACCACTTGCTTTGAAGG	AGAGTTGCAAGGCACAAGGT	353	XM_228501
LPA_5	TCCTACTGGCCAACCTCATC	GAAGTAGCCTCTGGCTGGTG	348	XM_575667
Autotaxin	ATTACGATGGCCTACGTGACACTG	CAGTTGCTAAGACTACACTGCCCA	403	BC081747.1
GAPDH	CTGCACCACCAACTGCTTAG	ACCACCCTGTTGCTGTAGCC	530	XR_009097.1

LPA, lysophosphatidic acid.

Statistical analysis

All quantitative data were analyzed by ANOVA. $P < 0.05$ was considered significant.

RESULTS

scPLD, PA, and LPA stimulate DNA synthesis

Incubation of ELT3 cells with scPLD stimulated [^{3}H]thymidine incorporation (**Fig. 1A**) in a dose-dependent manner. This result is consistent with previous data showing that scPLD increased DNA synthesis in ELT3 cells (5). Because PLD produces PA by hydrolyzing PC, we tested the ability of PA to reproduce the scPLD effect. As shown in Fig. 1B, PA was able to stimulate DNA synthesis. The effect of 10 μM PA was comparable to that obtained with 10 IU/ml scPLD. The concentration of 30 μM was more effective and corresponded to a 2-fold increase in thymidine incorporation over the basal value. It has been shown that PA could act by itself on intracellular targets and/or specific membrane receptors (10, 11). PA could be also deacylated into LPA, which in turn would bind to LPA receptors (12, 13). To determine whether scPLD and PA acted through LPA receptors, we used the specific $LPA_{1/3}$ receptor antagonist Ki16425 (29). The results in Fig. 1B show that Ki16425 fully inhibited the thymidine incorporation induced by both scPLD and PA, suggesting that these two agents acted through LPA receptors. This interpretation was supported by the results presented in Fig. 1C, which show that exogenous LPA was able to stimulate DNA synthesis. The effect of PA cannot be attributed to contaminating traces of LPA in commercial PA preparations, because we found that commercial PA contained at most 1% LPA (data not shown).

The stimulatory effect of LPA was dose-dependent, and a 2-fold stimulation was obtained with a concentration of 30 μM. The stimulatory effects of PA and scPLD were inhibited by the MAP kinase ERK kinase inhibitor U0126 (Fig. 1B), indicating the involvement of the $ERK_{1/2}$ pathway in their proliferative effects. This inhibition was not attributable to a toxic effect of U0126, because we previously verified that in ELT3 cells this inhibitor induced neither apoptosis (4) nor cell lysis (data not shown). As for scPLD and PA, the stimulation induced by LPA was fully inhibited by Ki16425 (Fig. 1C). The inhibitory effect of Ki16425 was not attributable to a toxic effect on cells, because it had no effect on the PDGF-induced DNA synthesis (Fig. 1B). Furthermore, Ki16425 had no inhibitory effect on scPLD activity (data not shown). These results suggest that the scPLD and PA effects involved the synthesis of bioactive LPA, which acted through the same membrane receptors as those activated by exogenous LPA.

Activation of $ERK_{1/2}$ by scPLD, LPA, and PA

Because thymidine incorporation as a result of scPLD and PA was sensitive to U0126, we tested their effects on $ERK_{1/2}$ activation, an essential process for DNA synthesis induction by mitogenic agents. As shown in **Fig. 2A**, scPLD stimulated $ERK_{1/2}$ phosphorylation in a transient manner. $ERK_{1/2}$ phosphorylation was maximal at ~3–5 min and then declined to an almost basal value after 20 min of stimulation. Unexpectedly, compared with scPLD, PA induced only a very low activation of $ERK_{1/2}$ (Fig. 2B). In contrast, LPA markedly stimulated $ERK_{1/2}$ activation at 3 min (Fig. 2C, D). This effect was dose-dependently inhibited by Ki16425 (Fig. 2C), with a strong effect observed with 10 nM. The activation of $ERK_{1/2}$ induced by scPLD was also inhibited by Ki16425, indicating that both LPA and scPLD stimulated $ERK_{1/2}$ through $LPA_{1/3}$ receptors. Ki16425 did not alter the stimulation of $ERK_{1/2}$ by PDGF, confirming the specificity of this antagonist. These results indicate that scPLD stimulated $ERK_{1/2}$ activation and proliferation of ELT3 cells by generating LPA, which in turn acted through $LPA_{1/3}$ receptors.

The LPA_1 receptor is involved in scPLD, LPA, and PA cellular effects

The expression of LPA receptor mRNA in ELT3 cells was determined by RT-PCR using specific primers. The results obtained indicated that the transcripts encoding the five known LPA receptors were detected (**Fig. 3A**). The results obtained with Ki16425 indicated that LPA_1 and/or LPA_3 was responsible for the LPA effects; therefore, we quantified the corresponding transcripts by real-time quantitative PCR. We found that LPA_1 receptor transcript was 11.2 ± 2.4 times more abundant than LPA_3 transcript. These results suggest that the LPA_1 receptor was mainly responsible for the cellular effects of LPA. To verify this hypothesis, we tested the effect of LPA_1 receptor down-regulation by RNA interference technique using specific siRNA targeted to the rat LPA_1 receptor. In the conditions tested, treatment of ELT3 cells with LPA_1-directed siRNA

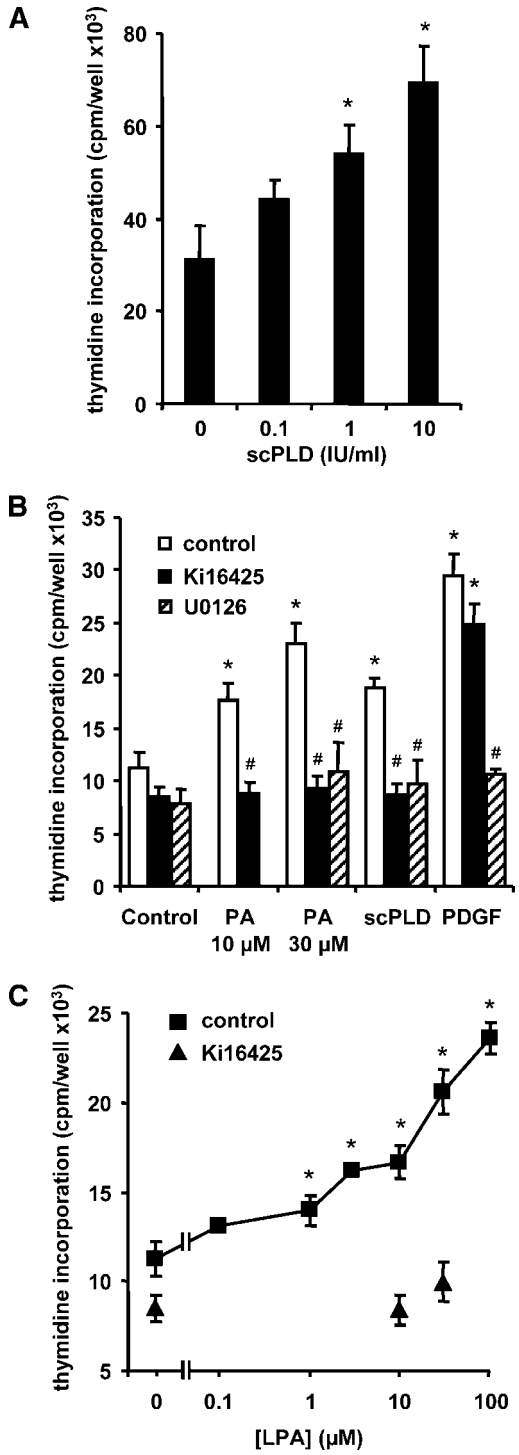

Fig. 1. Effect of phospholipase D from *Streptomyces chromofuscus* (scPLD), phosphatidic acid (PA), and lysophosphatidic acid (LPA) on DNA synthesis. Cells were treated for 48 h with the indicated concentrations of scPLD (A), with scPLD (10 IU/ml), PA (10 and 30 μM), or platelet-derived growth factor (PDGF) (25 ng/ml) in the presence or absence of 10 μM Ki16425 or 2 μM U0126 (preincubated for 10 min) (B), or with indicated concentrations of LPA in the presence or absence of 10 μM Ki16425 (C). All incubations were performed in the presence of 1.5 μCi/ml [^{3}H]thymidine. Incorporation of [^{3}H]thymidine was measured as described in Materials and Methods. Results are expressed as cpm/well and are means ± SEM of three independent experiments. * Significantly different from the untreated control; # significantly different from the respective control.

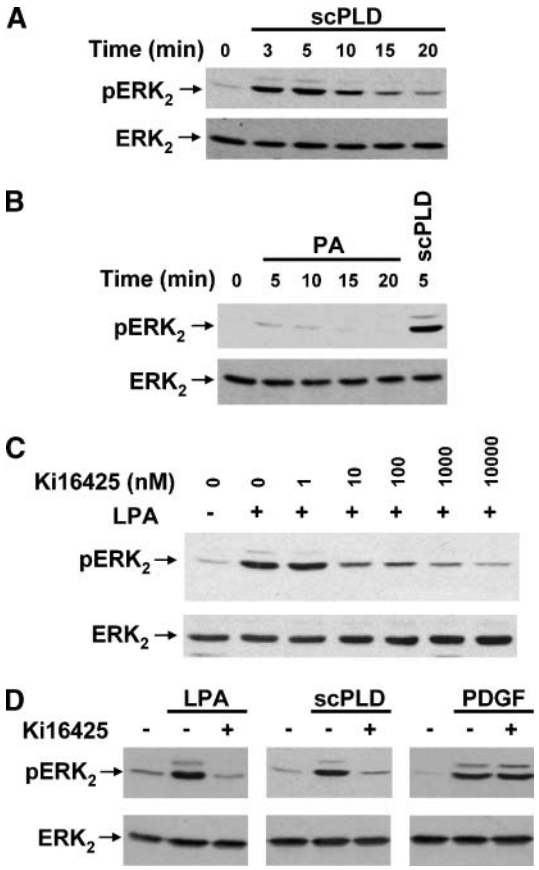

Fig. 2. Effects of scPLD, PA, and LPA on extracellular signal-regulated kinase ($ERK_{1/2}$) activation. A: Cells were incubated in the presence of 10 IU/ml scPLD for the indicated times. B: Cells were incubated in the presence of 10 μM PA or 10 IU/ml scPLD for the indicated times. C: Cells were incubated for 3 min with or without 10 μM LPA in the presence or absence of the indicated concentrations of Ki16425. D: Cells were incubated for 3 min with 10 μM LPA, 10 IU/ml scPLD, or 25 ng/ml PDGF in the presence or absence of 10 μM Ki16425. Cells were lysed, and detergent-extracted proteins were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-active $ERK_{1/2}$ ($pERK_2$). The blots were stripped and reprobed with anti-total ERK_2 antibody (ERK_2). Results are representative of three independent experiments.

decreased the amount of LPA_1 receptor transcript by 9-fold (9.34 ± 0.59; $P < 0.05$). The data presented in Fig. 3B show that in these conditions, LPA_1-specific siRNA treatment strongly inhibited LPA as well as scPLD-induced $ERK_{1/2}$ activation. This inhibition was specific, because the PDGF response was not affected by LPA_1 siRNA treatment (Fig. 3C). Moreover, treatment of ELT3 cells with LPA_1-directed siRNA also inhibited the scPLD, LPA, and PA stimulatory effects on DNA synthesis (Fig. 3D). These results show that the LPA_1 receptor is mainly responsible for the mitogenic effects of scPLD, PA, and LPA in ELT3 cells.

scPLD synthesizes LPA

The synthesis of LPA in response to scPLD was investigated using a radioenzymatic assay. In this assay, LPA is converted into [^{14}C]PA in the presence of LPAAT and [^{14}C]oleoyl-CoA. **Figure 4A, B** show that LPA was not

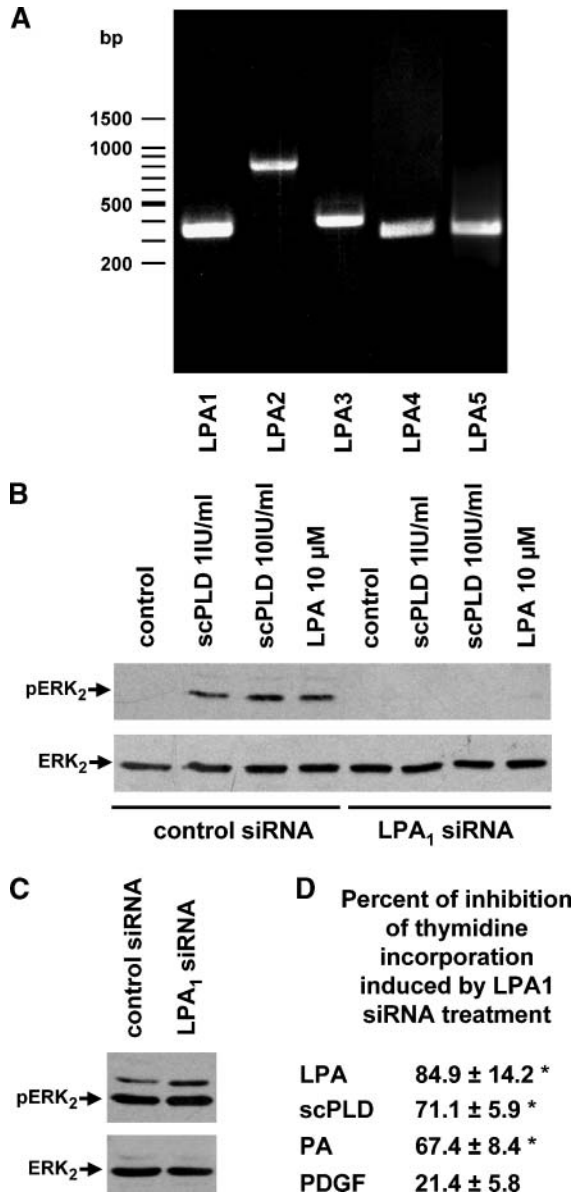

Fig. 3. Involvement of the LPA_1 receptor in scPLD, LPA, and PA effects. A: The expression of LPA receptor subtypes was examined by RT-PCR analysis as described in Materials and Methods. The image is representative of three independent experiments. B, C: One day after nucleofection with control or LPA_1-targeted small interfering RNA (siRNA), ELT3 cells were incubated for 3 min with 10 μM LPA and 1 or 10 IU/ml scPLD (B) or 25 ng/ml PDGF (C). Cells were lysed, and detergent-extracted proteins were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-active $ERK_{1/2}$ ($pERK_2$) antibody. The blots were stripped and reprobed with anti-total ERK_2 antibody (ERK_2). D: One day after nucleofection with control siRNA or LPA_1-targeted siRNA, cells were treated for 48 h with 10 IU/ml scPLD, 10 μM LPA, 10 μM PA, or 25 ng/ml PDGF. All incubations were performed in the presence of 1.5 μCi/ml [^{3}H]thymidine. The values represent the percentages of inhibition of thymidine incorporation attributable to LPA_1 siRNA nucleofection compared with control siRNA nucleofection. The results are means ± SEM of four independent experiments. * Significantly different from the respective control siRNA-treated cells.

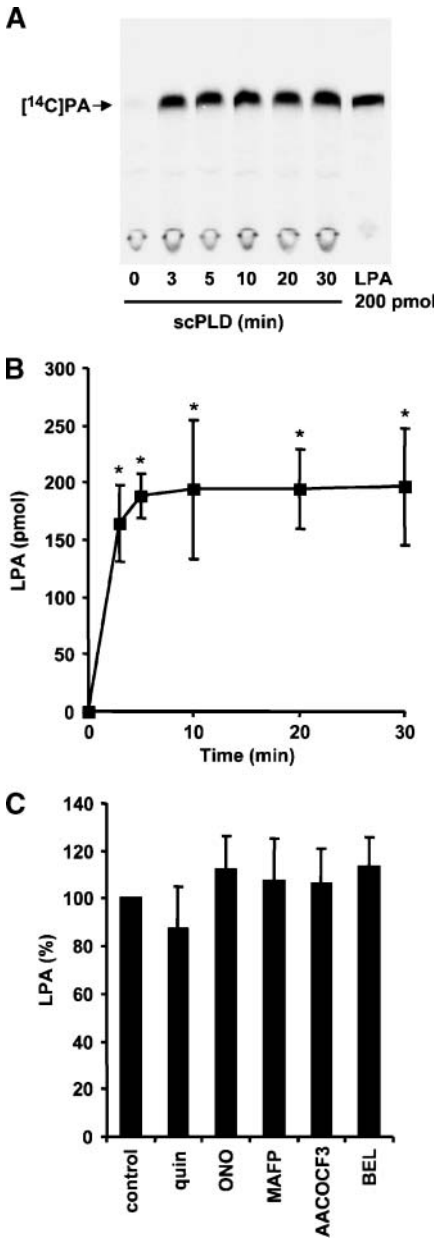

Fig. 4. scPLD induces LPA formation. Serum-starved cells were incubated in the presence of 10 IU/ml scPLD for the indicated times. LPA contained in each well (cells + medium) was quantified with a radioenzymatic assay as described in Materials and Methods. In this assay, LPA was transformed into [^{14}C]PA in the presence of recombinant 1-acyl-*sn*-glycerol-3-phosphate acyltransferase and [^{14}C]oleoyl-CoA. A: Autoradiography of a TLC plate showing [^{14}C]PA bands. A standard assay was performed with 200 pmol of exogenous LPA. B: The radioactivity of [^{14}C]PA was quantified. The results are expressed in pmol/well and are means ± SEM of three independent experiments. C: Cells were incubated for 20 min with 10 IU/ml scPLD in the presence or absence of the following phospholipase A_2 (PLA_2) inhibitors: 500 μM quinacrine (quin), 10 μM ONO-RS-082 (ONO), 10 μM phosphonofluoridic acid methyl-5,8,11,14-eicosatetraenyl ester (MAFP), 10 μM arachidonyltrifluoromethyl ketone (AACOCF3), or 25 μM bromoenol lactone (BEL) (added 20 min before scPLD). LPA contained in each well (cells + medium) was quantified by radioenzymatic assay. Results are expressed as percentage of the LPA amount in the control experiment performed in the absence of inhibitors. Values are means ± SEM of three independent experiments. * Significantly different from the time 0 value.

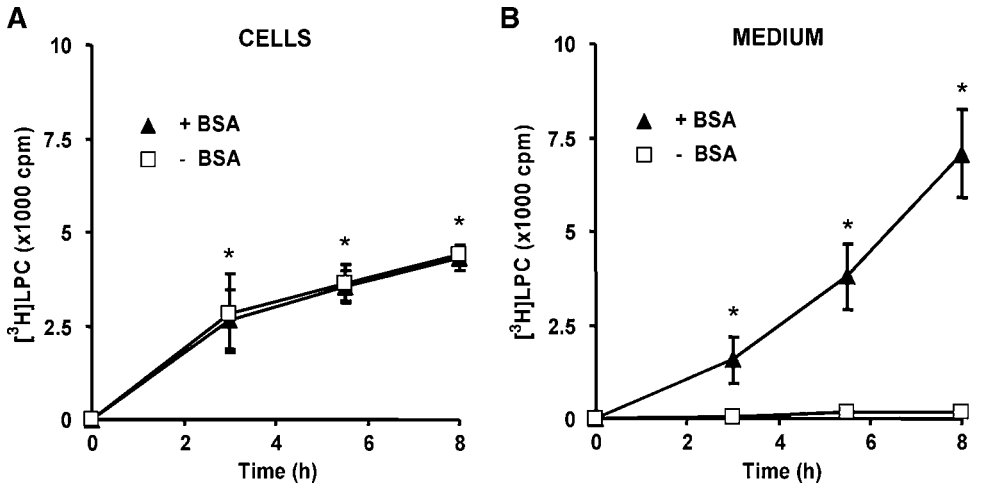

Fig. 5. ELT3 cells synthesize lysophosphatidylcholine (LPC). Serum-starved cells were incubated for the indicated times with 5 μCi/ml [^{3}H]choline in the presence or absence of 0.1% fatty acid-free BSA (ffBSA). Lipids were extracted from cells (A) and media (B) and analyzed by TLC, and LPC radioactivity was quantified. Values, expressed as cpm/well, are means ± SEM of three independent experiments. * Significantly different from the time 0 value.

detected in resting ELT3 cells. Incubation of ELT3 cells in the presence of scPLD resulted in a rapid generation of 1-acyl-2-*sn*-LPA (Fig. 4A, B). The amount of LPA reached a plateau after 10 min, which corresponded to a value of ~200 pmol per well. Because scPLD is known to produce PA by hydrolyzing PC, the possibility that LPA could be synthesized by deacylation of PA through a PLA_2 activity was tested. The nonselective PLA_2 inhibitors quinacrine and ONO-RS-082 (Fig. 4C) had no effect on the formation of LPA catalyzed by scPLD. Similarly, the more specific PLA_2 inhibitors MAFP, AACOCF3, and BEL (cytosolic phospholipase A2/calcium insensitive phospholipase A2 inhibitors) failed to inhibit the generation of LPA, indicating that the hydrolysis of PA by a PLA_2 activity was not the source of LPA produced by scPLD.

ELT3 cells synthesize LPC

Because PLA_2 did not appear to be involved in the synthesis of LPA, we tested the possibility that LPA could be produced directly by the hydrolysis of LPC by scPLD. Indeed, this enzyme has been shown to possess lysoPLD activity (25). Thus, we first tested whether ELT3 cells were able to synthesize LPC. **Figure 5A** shows that ELT3 cells synthesized LPC and that its amount increased with time. LPC was found only in cells (Fig. 5A) and was absent from the culture medium (Fig. 5A, B). ffBSA is often used to extract lysophospholipids from membranes. Treatment of ELT3 cells with ffBSA increased LPC production (Fig. 5). In these conditions, LPC appeared in the medium and its level increased with time (Fig. 5B). In contrast, the level of LPC in cells was not affected by ffBSA (Fig. 5A). After 8 h of incubation with ffBSA, the amount of [^{3}H]LPC was ~2-fold higher in the medium compared with the cells. In these conditions, only [^{3}H]LPC and [^{3}H]PC were detected in the medium; [^{3}H]PC amount in the medium was <25% of [^{3}H]LPC and <1% of cellular [^{3}H]PC; [^{3}H]LPC corresponded to <2% of total cellular [^{3}H]PC. Moreover, [^{3}H]sphingomyelin level was ~5% of total cellular [^{3}H]PC. These data indicated that ELT3 cells synthesized LPC and that this synthesis was enhanced by ffBSA.

scPLD hydrolyzes LPC produced by ELT3 cells to generate LPA

We next tested whether the LPC synthesized by the cells could be hydrolyzed by scPLD to produce LPA. To this end, cells were [^{3}H]choline-labeled in the presence of ffBSA and then incubated in the presence of scPLD. We observed that the amount of [^{3}H]LPC present in the medium declined with time after the addition of scPLD (**Fig. 6A**, closed bars). Moreover, when the conditioned medium containing [^{3}H]LPC was incubated with scPLD in the absence of cells, the hydrolysis of LPC also occurred and was even more efficient (Fig. 6A, open bars). In these conditions, [^{3}H]LPC almost disappeared from the medium within 5 min. To demonstrate that scPLD synthesized LPA from LPC, ELT3 cells were labeled overnight with [^{3}H]myristic acid in the presence of ffBSA. In these conditions, the medium contained mainly [^{3}H]LPC. When such a conditioned medium was incubated with scPLD, [^{3}H]LPC level decreased with time and concomitantly [^{3}H]LPA level increased (Fig. 6B). The sum of [^{3}H]LPC and [^{3}H]LPA was constant throughout the experiment, indicating that [^{3}H]LPC was the unique source of [^{3}H]LPA.

Insofar as LPC is hydrolyzed by scPLD to generate LPA, one would expect that ffBSA, which increases LPC amount in the medium, would also increase the formation of LPA. Therefore, the effect of ffBSA on LPA production was tested. Figure 6C shows that LPA production triggered by scPLD was increased by ~4-fold when cells were preincubated overnight with ffBSA. We observed in Fig. 4C that

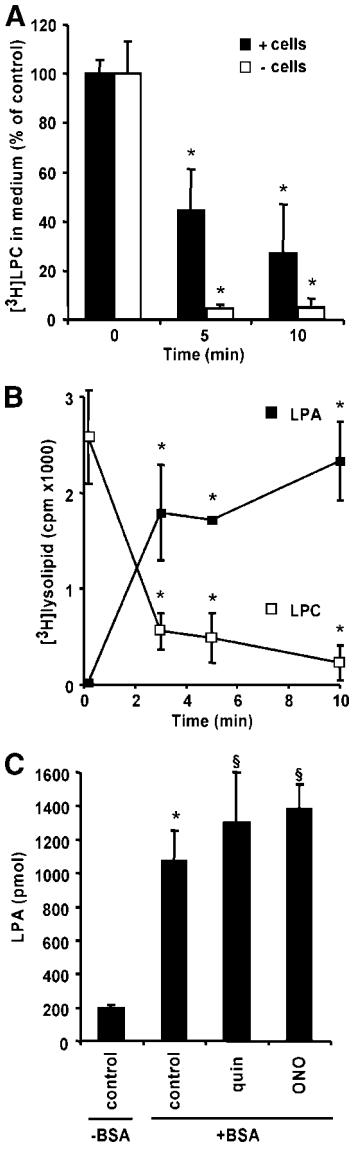

Fig. 6. scPLD synthesizes LPA from LPC. A: Cells were serum-starved and labeled for 16 h in the presence of 0.1% ffBSA and 5 μCi/ml [^{3}H]choline. scPLD (10 IU/ml) was added either to cultured cells (closed bars) or to conditioned medium without cells (open bars) for the indicated times. Lipids were extracted from media and analyzed by TLC. LPC radioactivity was quantified and expressed as percentage of the control value (time 0). Values are means ± SEM of three independent experiments. * Significantly different from the time 0 value. B: Cells were serum-starved and labeled for 16 h in the presence of 0.1% ffBSA and 5 μCi/ml [^{3}H]myristic acid. The medium was incubated with 10 IU/ml scPLD for the indicated times, and lipids were extracted. [^{3}H]LPA and [^{3}H]LPC were separated by TLC and quantified. Results are expressed as cpm and are means ± SEM of three independent experiments. * Significantly different from the time 0 value. C: Cells were serum-starved overnight in the presence or absence of 0.1% ffBSA. Cells were then incubated in the presence of 10 IU/ml scPLD for 20 min in the presence or absence of the following PLA_2 inhibitors: 500 μM quinacrine (quin) or 10 μM ONO-RS-082 (ONO). PLA_2 inhibitors were added at 20 min before the addition of scPLD. LPA contained in each well (cells + medium) was quantified with a radioenzymatic assay. The results are expressed as pmol/well and are means ± SEM of three independent experiments. * Significantly different from control − BSA; § significantly different from control − BSA but not significantly different from control + BSA.

LPA production, induced by scPLD in the absence of ffBSA, was insensitive to PLA_2 inhibitors. The results presented in Fig. 6C show that these inhibitors remained without effect even in the presence of ffBSA. These results indicate that the presence of ffBSA increased LPA production only by enhancing LPC level, but not by stimulating an additional process.

Effect of ffBSA on $ERK_{1/2}$ activation

Because ffBSA increased LPA formation, it should potentiate the effect of scPLD on $ERK_{1/2}$ activation. **Figure 7A** shows the dose-dependent activation of $ERK_{1/2}$ by scPLD in the absence or presence of ffBSA in the medium. The presence of ffBSA in the medium clearly increased $ERK_{1/2}$ phosphorylation, especially at scPLD doses of >1 IU/ml. Kinetic experiments show that, in the presence of ffBSA, $ERK_{1/2}$ activation induced by scPLD was stronger and more sustained (Fig. 7B). The potentiating effect of ffBSA was reproduced by incubating the cells in the presence of exogenous LPC (Fig. 7C). Note that ffBSA and LPC, used alone, had no

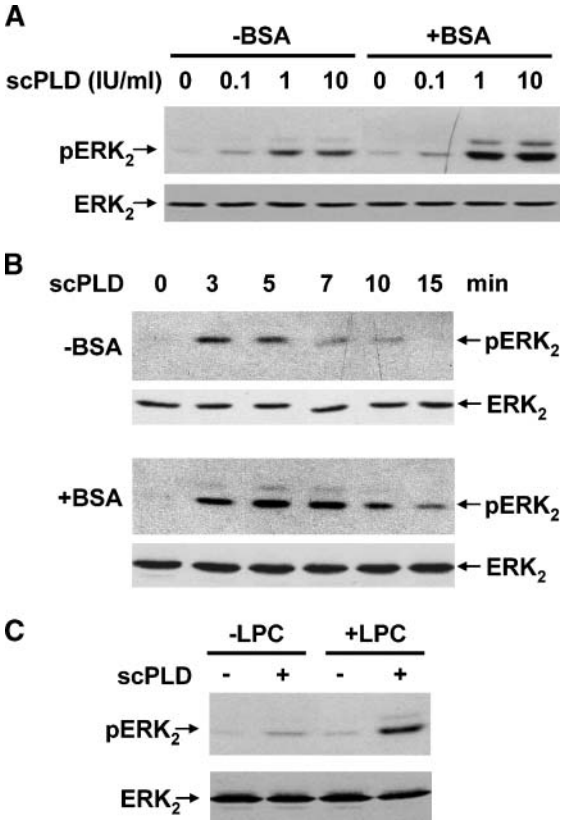

Fig. 7. Effects of ffBSA and LPC on scPLD-induced $ERK_{1/2}$ activation. A: Cells were incubated in the presence of the indicated concentrations of scPLD for 5 min in the presence or absence of 0.1% ffBSA. B: Cells were incubated in the presence of 10 IU/ml scPLD for the indicated times in the presence or absence of 0.1% ffBSA. C: Cells were treated with or without 10 IU/ml scPLD for 5 min in the presence or absence of 10 μM exogenous LPC. Cells were lysed, and detergent-extracted proteins were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-active $ERK_{1/2}$. Results are representative of three independent experiments.

effect on $ERK_{1/2}$ activation. Moreover, ffBSA did not influence $ERK_{1/2}$ activation induced by PDGF (data not shown). These data support our hypothesis that ffBSA enhanced $ERK_{1/2}$ activation through the increase in LPC production. Additionally, these data provide evidence that scPLD's effect involved its lysoPLD activity.

Effects of ATX on ELT3 cells

To confirm the involvement of lysoPLD activity in the scPLD-mediated effects in ELT3 cells, we tested whether ATX, a physiological mammalian lysoPLD, was able to reproduce the effects of scPLD on ELT3 cells. To this end, mATX was heterologously expressed in Cos-7 cells and purified from conditioned culture media. As shown in **Fig. 8A**, incubation of ELT3 cells in the presence of mATX induced the synthesis of LPA. ATX also induced a transient activation of $ERK_{1/2}$, with a maximal activation at 5 min (Fig. 8B). This activation of $ERK_{1/2}$ was abolished by Ki16425, indicating that LPA was responsible for this effect of ATX (Fig. 8B). Moreover, mATX purified from Cos-7 cell medium was able to stimulate DNA synthesis in ELT3 cells (Fig. 8C). This effect was also inhibited by Ki16425 (Fig. 8C). All of these effects, including LPA production, $ERK_{1/2}$ activation, and thymidine incorporation, were not observed in control experiments, in which ELT3 cells were incubated in the presence of conditioned media of Cos-7 cells transfected with pCDNA empty vector (Fig. 8A–C, control). Finally, we tested the effect of mATX expression directly in ELT3 cells, which do not express endogenous ATX (Fig. 8D). As shown in Fig. 8E, transfection of ELT3 cells with mATX expression vector increased the rate of DNA synthesis compared with cells transfected with the empty vector.

Activation of endogenous PLD does not activate LPA-dependent processes

In a previous study, we showed that ET-1 stimulated endogenous PLD activity, which contributes to $ERK_{1/2}$ activation and cell proliferation in ELT3 cells but not in normal myometrial cells (5). One hypothesis was that the PA produced by endogenous PLD may be metabolized into LPA. Therefore, we tested the effect of Ki16425 on $ERK_{1/2}$ activation induced by ET-1 and PDBu. We found that the activation of $ERK_{1/2}$ by ET-1 and PDBu was not affected by the LPA receptor antagonist (**Fig. 9A**). Furthermore, Ki16425 also had no effect on thymidine incorporation induced by ET-1 (Fig. 9B). Moreover, stimulation of cells in the presence of ET-1 failed to induce the formation of LPA (Fig. 9C). The presence of LPA remained undetectable after ET-1 stimulation for up to 16 h (data not shown).

DISCUSSION

In this study, we investigated the mechanism by which scPLD and endogenous PLD contributed to the increase of $ERK_{1/2}$ activity and thymidine incorporation in ELT3 cells. Like other PLDs, scPLD catalyzes the hydrolysis of PC into PA, a phospholipid that has been reported to play a role in $ERK_{1/2}$ activation and proliferation in several models (9). However, in ELT3 cells, exogenous PA did not efficiently activate $ERK_{1/2}$, and the effects of scPLD were fully inhibited by the $LPA_{1/3}$ receptor antagonist Ki16425. VPC 32183, which is also a $LPA_{1/3}$ receptor antagonist (30), gave similar results (data not shown). These data suggested that PA was certainly not responsible for the effects of scPLD.

We next tested the possibility that the PA produced by scPLD was deacylated to generate LPA. This conversion of PA into LPA could be catalyzed by PLA_1 and PLA_2 family members (12). In ELT3 cells, we observed the synthesis of LPA acylated on the *sn*-1 position of glycerol, suggesting the participation of a PLA_2 family enzyme. Calcium-independent as well as secretory PLA_2 have been shown to hydrolyze PA (31–33). However, in ELT3 cells, the PLA_2 inhibitors tested (quinacrine, ONO-RS-082) had no effect on the generation of LPA induced by scPLD. Consistent with these results, the $iPLA_2$ or $cPLA_2$ inhibitors BEL, MAFP, and AACOCF3 were also without effect on LPA formation. Thus, the LPA production pathway involving PA hydrolysis by PLA_1 or PLA_2 likely does not operate in ELT3 cells. Another possibility for the synthesis of LPA is related to the lysoPLD activity of scPLD. Indeed, scPLD possesses an additional lysoPLD activity and is able to hydrolyze LPC directly into LPA (25).

Lysophospholipids, unlike other phospholipids, are hydrophilic lipids that can be extracted from cellular membranes by binding to ffBSA. We found that ELT3 cells synthesized LPC and that this synthesis was enhanced when cells were treated with ffBSA. Comparable results were found by Robinson, Baisted, and Vance (34) in rat hepatocytes. Incubation of conditioned medium of ffBSA-treated ELT3 cells with scPLD decreased LPC amount and concomitantly increased LPA level, confirming that LPC was the substrate for the LPA production. These results are in agreement with those obtained by van Dijk et al. (35) in fibroblasts. These cumulative data demonstrated that in ELT3 cells, scPLD synthesizes bioactive LPA by direct hydrolysis of LPC contained in the plasma membrane or present in the medium as an ffBSA-bound form. The synthesis of LPA from LPC is a physiological process catalyzed by ATX in plasma and other body fluids (14, 15). In contrast to other cell types, such as ovarian cells, ELT3 cells do not express ATX; therefore, they are unable to synthesize LPA from LPC in vitro. However, our results showed that mATX, purified from culture media of ATX expressing Cos-7 cells, reproduced all of the effects of scPLD on ELT3 cells. Indeed, ATX induced the generation of LPA, $ERK_{1/2}$ activation, and [^{3}H]thymidine incorporation almost as efficiently as scPLD in ELT3 cells. Moreover, the expression of mATX in ELT3 cells also stimulated thymidine incorporation, but with a somewhat lesser effect. This strongly suggests that, in vivo, circulating ATX may locally synthesize LPA from leiomyoma-derived LPC.

LPA produced by scPLD and ATX, as well as exogenous LPA, activated $ERK_{1/2}$. Bacterial scPLD stimulated $ERK_{1/2}$ phosphorylation in a transient manner, with peak activity

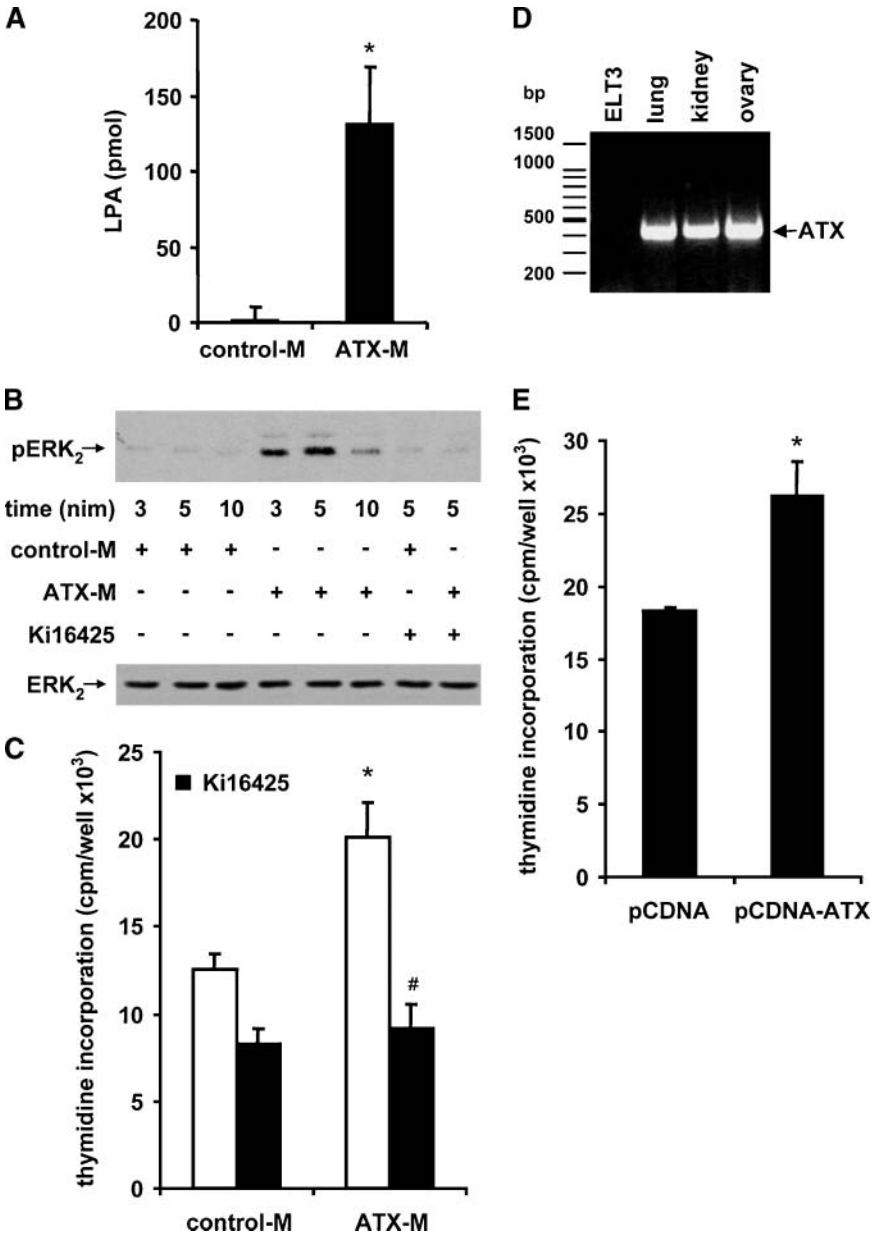

Fig. 8. Effects of murine autotaxin (mATX) on ELT3 cells. A: Cells were incubated in the presence of conditioned media from Cos-7 cells transfected with pCDNA-ATX (ATX-M) or empty pCDNA vector (control-M). After 1 h of incubation, LPA contained in each well (cells + medium) was quantified as described in Materials and Methods. Results are expressed as pmol/well of a six-well plate and are means ± SEM of three independent experiments. B: Cells were incubated in the presence of conditioned media from Cos-7 cells transfected with pCDNA-ATX (ATX-M) or empty pCDNA vector (control-M) for the indicated times in the presence or absence of 10 μM Ki16425. Cells were lysed, and detergent-extracted proteins were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-active $ERK_{1/2}$. Results shown are representative of four experiments. C: Cells were incubated in the presence of conditioned media from Cos-7 cells transfected with pCDNA-ATX (ATX-M) or empty pCDNA vector (control-M) for 48 h in the presence or absence of 10 μM Ki16425. All incubations were performed in the presence of 1.5 μCi/ml [^{3}H]thymidine. Incorporation of [^{3}H]thymidine was measured as described above. Results are expressed as cpm/well and are means ± SEM of three independent experiments. D: The expression of ATX was tested by RT-PCR as described in Materials and Methods. The image is representative of three independent experiments. E: ELT3 cells (50% confluent) were transfected with pCDNA-ATX or pCDNA empty vector and then incubated for 48 h in the presence of 1.5 μCi/ml [^{3}H]thymidine. Incorporation of [^{3}H]thymidine was measured as described above. Results are expressed as cpm/well and are means ± SEM of three independent experiments. * Significantly different from the control value; $^{\#}$ significantly different from the respective control.

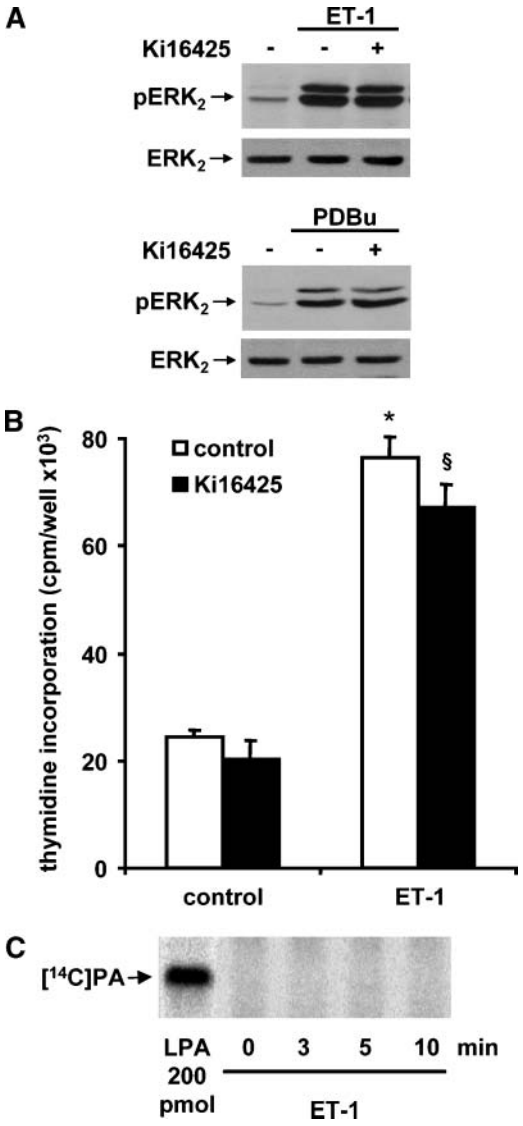

Fig. 9. Role of LPA in endogenous PLD-dependent effects. A: Cells were incubated in the presence of 50 nM endothelin-1 (ET-1) or 1 μM 4β-phorbol-12,13-dibutyrate (PDBu) in the presence or absence of 10 μM Ki16425. Cells were lysed, and detergent-extracted proteins were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-active $ERK_{1/2}$. B: Cells were treated with or without 50 nM ET-1 for 48 h in the presence or absence of 10 μM Ki16425. All incubations were performed in the presence of 1.5 μCi/ml [^{3}H]thymidine. Incorporation of [^{3}H]thymidine was measured as described above. Results are expressed as cpm/well and are means ± SEM of three independent experiments. * Significantly different from the untreated control; § significantly different from the untreated control but not significantly different from ET-1 alone. C: Cells were incubated in the presence of 50 nM ET-1 for the indicated times. LPA contained in each well (cells + medium) was quantified. A typical autoradiographic result is shown.

at ~5 min. After 5 min, $ERK_{1/2}$ phosphorylation began to decline, even though the level of LPA was stable for at least 30 min. This observation is certainly a consequence of the desensitization of the LPA receptors. Indeed, it has been shown that desensitization- or internalization-defective mutants of GPCRs were able to induce a more sustained activation of $ERK_{1/2}$ (36). Moreover, the LPA_1 receptor has been shown to undergo rapid internalization in the presence of LPA (37).

The expression of LPA receptor mRNA has been studied by RT-PCR experiments in ELT3 cells. Although the five known transcripts were detected by RT-PCR, the use of Ki16425 indicated that the effects of LPA on $ERK_{1/2}$ activation and DNA synthesis were only mediated through LPA_1 and/or LPA_3 receptor subtypes. Ki16425 has been shown to antagonize LPA_1 and LPA_3 receptors and to have no significant effect on LPA_2 and LPA_5 receptors (24, 29). There is also indirect evidence that it does not inhibit LPA_4 receptor signaling (29). The expression of LPA_1–LPA_3 had been described previously in normal human myometrial cells (18). Real-time PCR experiments indicated that, in ELT3 cells, LPA_1 mRNA was expressed at >10-fold higher levels than LPA_3 mRNA, suggesting that LPA signals mainly through the LPA_1 receptor. This interpretation was further supported by the finding that Ki16425, which is more specific for LPA_1 receptors (29), significantly inhibited the scPLD effect at doses as low as 10 nM. Finally, using RNA interference, we showed that LPA_1 receptor downregulation inhibited the LPA and scPLD activation of $ERK_{1/2}$ and the PA, scPLD, and LPA stimulation of thymidine incorporation. These latter results demonstrate that the LPA_1 receptor was the main LPA receptor responsible for the effects of LPA in ELT3 cells and strongly suggest that scPLD and exogenous PA acted through LPA generation.

In ELT3 cells, scPLD appeared to act through its lysoPLD activity; however, this PLD is also able to hydrolyze PC to produce PA, which may participate in $ERK_{1/2}$ activation and cell proliferation. To determine the potential role of PA, we used exogenous PA. We first observed that exogenous PA stimulated thymidine incorporation as efficiently as LPA. Similar results have been obtained in Rat-1 fibroblasts, leading the authors to raise the question of the existence of PA receptors (38). More recently, it was suggested that PA may be an agonist for the orphan receptor GPR63/PSP24β, which is able to stimulate calcium mobilization when expressed in CHO cells (11). Surprisingly, the effect of PA was inhibited by the $LPA_{1/3}$ antagonist Ki16425 and siRNA targeted toward the LPA_1 receptor, indicating the involvement of this receptor. Unexpectedly, exogenous PA had only a very small effect on $ERK_{1/2}$ activation, even after incubations as long as 6 h (data not shown). However, the PA effect on DNA synthesis was inhibited by U0126, indicating that $ERK_{1/2}$ mitogen-activated protein kinases were nevertheless involved in DNA synthesis. To explain this unexpected phenomenon, it can be hypothesized that a weak hydrolysis of PA could occur via an eventual PLA activity, which could produce a low but sustained level of LPA. This production of LPA would be too weak to generate a high level of $ERK_{1/2}$ activity but would be sufficient to stimulate DNA synthesis. Unfortunately, this point has not been tested successfully, because PLA inhibitors had toxic effects on ELT3 cells after treatment for several hours. Therefore, the mechanism by which exogenous PA

stimulated the LPA_1 receptor to induce DNA synthesis remains to be established.

In a previous study, we showed that ET-1 stimulated $ERK_{1/2}$ and the proliferation of ELT3 cells in a PLD-dependent manner (5). Because these effects were reproduced by scPLD, we concluded that PA, synthesized by endogenous or exogenous PLD, regulated $ERK_{1/2}$ activation and proliferation. In the present work, we demonstrated that, in fact, the two kinds of PLD acted through different mechanisms to regulate $ERK_{1/2}$ and cell proliferation. Indeed, scPLD was found to act via the synthesis of LPA; by contrast, endogenous mammalian PLD activated by ET-1 did not lead to LPA formation. Therefore, endogenous PLD likely acted through PA generation. This implies that the PA generated in the outer leaflet of the plasma membrane by scPLD and the intracellular PA synthesized by endogenous PLD act via differential mechanisms converging on the activation of $ERK_{1/2}$ and cell proliferation. Diverse mechanisms by which endogenous PA contributes to $ERK_{1/2}$ activation have been reported (9). PA can interact with different proteins of the $ERK_{1/2}$ signaling pathway, such as Raf-1, PKCζ and PKCϵ, and PLCγ, to promote translocation and/or their activation (9, 10). PA is also able to bind and activate mTOR (39), a protein kinase involved in cell proliferation; however, this kinase is constitutively active in ELT3 cells (40) and thus may not be regulated by PA. More interestingly, a recent report demonstrated that sphingosine kinase 1 (SphK1) is an intracellular effector of PA (41). This lipid kinase phosphorylates sphingosine to produce sphingosine-1-phosphate (S1P) (42). This lipid mediator acts via membrane receptors and regulates the $ERK_{1/2}$ signaling pathway and many physiological and pathological processes, including proliferation, survival, and contraction (42, 43). Recent data from our group demonstrated that ET-1 activates SphK1, which contributes to ET-1-mediated myometrial contraction (44). Furthermore, we recently reported that in ELT3 cells, SphK1 and S1P exerted an antiapoptotic effect (4) and that exogenous S1P stimulated DNA synthesis (unpublished results). In agreement with these observations, Jeng et al. (45) found that overexpression of SphK1 in ELT3 cells increased cell proliferation. In light of these data, it became possible to postulate that the SphK1/S1P pathway may be one mechanism by which endogenous PA contributes to ET-1-mediated $ERK_{1/2}$ activation and cell proliferation in ELT3 cells. This hypothesis now needs to be tested.

In conclusion, our results show for the first time that LPA is a mitogenic factor for leiomyoma cells; thus, it has to be considered a potential progression factor for these tumors, as it is for diverse cancer tumors. Furthermore, our data indicate that scPLD acts in a comparable manner with ATX, a lysoPLD naturally present in the serum and other biological fluids of mammals (17). Indeed, ATX is an extracellular lysoPLD that hydrolyzes LPC, present in plasma in an albumin- or lipoprotein-bound form, to generate LPA (12, 17). Interestingly, it has been proposed that in human, ATX activity regulates uterine functions (14, 46). Because ELT3 leiomyoma cells synthesize LPC, it can be proposed that ATX may also participate in leiomyoma development via LPA production. The relevance of this proposal is further supported by the involvement of ATX and LPA in angiogenic processes and tumor development. Finally, our data indicate that LPA needs to be considered in the context of leiomyoma growth and provide new perspectives into the potential role of lipidic mediators in smooth muscle cell pathophysiology.

The authors thank Dr. H. Ohta and Dr. T. Sakai (Kirin Brewery Co., Ltd., Pharmaceutical Division, Gunma, Japan) for the kind gift of Ki16425, Dr. J. S. Saulnier-Blache (Institut National de la Santé et de la Recherche Médicale U317, University Paul Sabatier, Toulouse, France) for providing them with the LPAAT and the mATX expression plasmids, and Dr. C. Walker (University of Texas M. D. Anderson Cancer Center, Science Park Research Division, Smithville, TX) for providing them with the ELT3 cell line. The authors are grateful to Dr. O. Guittet for his expert advice on real-time PCR quantification and to G. Vilain for helpful technical assistance. This work was supported by grants from the Centre National de la Recherche Scientifique and the Université Paris Sud. E.B-D. was the recipient of a fellowship from the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche.

REFERENCES

1. Walker, C. L., and E. A. Stewart. 2005. Uterine fibroids: the elephant in the room. *Science.* **308:** 1589–1592.
2. Everitt, J. I., D. C. Wolf, S. R. Howe, T. L. Goldsworthy, and C. Walker. 1995. Rodent model of reproductive tract leiomyomata. Clinical and pathological features. *Am. J. Pathol.* **146:** 1556–1567.
3. Howe, S. R., M. M. Gottardis, J. I. Everitt, T. L. Goldsworthy, D. C. Wolf, and C. Walker. 1995. Rodent model of reproductive tract leiomyomata. Establishment and characterization of tumor-derived cell lines. *Am. J. Pathol.* **146:** 1568–1579.
4. Raymond, M. N., C. Bole-Feysot, Y. Banno, Z. Tanfin, and P. Robin. 2006. Endothelin-1 inhibits apoptosis through a sphingosine kinase 1-dependent mechanism in uterine leiomyoma ELT3 cells. *Endocrinology.* **147:** 5873–5882.
5. Robin, P., S. Chouayekh, C. Bole-Feysot, D. Leiber, and Z. Tanfin. 2005. Contribution of phospholipase D in endothelin-1-mediated extracellular signal-regulated kinase activation and proliferation in rat uterine leiomyoma cells. *Biol. Reprod.* **72:** 69–77.
6. Exton, J. H. 2002. Phospholipase D—structure, regulation and function. *Rev. Physiol. Biochem. Pharmacol.* **144:** 1–94.
7. Foster, D. A., and L. Xu. 2003. Phospholipase D in cell proliferation and cancer. *Mol. Cancer Res.* **1:** 789–800.
8. Jenkins, G. M., and M. A. Frohman. 2005. Phospholipase D: a lipid centric review. *Cell. Mol. Life Sci.* **62:** 2305–2316.
9. Rizzo, M., and G. Romero. 2002. Pharmacological importance of phospholipase D and phosphatidic acid in the regulation of the mitogen-activated protein kinase cascade. *Pharmacol. Ther.* **94:** 35–50.
10. Stace, C. L., and N. T. Ktistakis. 2006. Phosphatidic acid- and phosphatidylserine-binding proteins. *Biochim. Biophys. Acta.* **1761:** 913–926.
11. Niedernberg, A., S. Tunaru, A. Blaukat, A. Ardati, and E. Kostenis. 2003. Sphingosine 1-phosphate and dioleoylphosphatidic acid are low affinity agonists for the orphan receptor GPR63. *Cell. Signal.* **15:** 435–446.
12. Aoki, J. 2004. Mechanisms of lysophosphatidic acid production. *Semin. Cell Dev. Biol.* **15:** 477–489.
13. Luquain, C., V. A. Sciorra, and A. J. Morris. 2003. Lysophosphatidic acid signaling: how a small lipid does big things. *Trends Biochem. Sci.* **28:** 377–383.
14. Tokumura, A., E. Majima, Y. Kariya, K. Tominaga, K. Kogure, K. Yasuda, and K. Fukuzawa. 2002. Identification of human plasma

lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. *J. Biol. Chem.* **277:** 39436–39442.
15. Umezu-Goto, M., Y. Kishi, A. Taira, K. Hama, N. Dohmae, K. Takio, T. Yamori, G. B. Mills, K. Inoue, J. Aoki, et al. 2002. Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J. Cell Biol.* **158:** 227–233.
16. Mills, G. B., and W. H. Moolenaar. 2003. The emerging role of lysophosphatidic acid in cancer. *Nat. Rev. Cancer.* **3:** 582–591.
17. Xie, Y., and K. E. Meier. 2004. Lysophospholipase D and its role in LPA production. *Cell. Signal.* **16:** 975–981.
18. Nilsson, U. K., and S. P. Svensson. 2003. Inhibition of Ca^{2+}/calmodulin-dependent protein kinase or epidermal growth factor receptor tyrosine kinase abolishes lysophosphatidic acid-mediated DNA-synthesis in human myometrial smooth muscle cells. *Cell Biol. Int.* **27:** 341–347.
19. Gogarten, W., C. W. Emala, K. S. Lindeman, and C. A. Hirshman. 2001. Oxytocin and lysophosphatidic acid induce stress fiber formation in human myometrial cells via a pathway involving Rho-kinase. *Biol. Reprod.* **65:** 401–406.
20. Ye, X., K. Hama, J. J. Contos, B. Anliker, A. Inoue, M. K. Skinner, H. Suzuki, T. Amano, G. Kennedy, H. Arai, et al. 2005. LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature.* **435:** 104–108.
21. Birgbauer, E., and J. Chun. 2006. New developments in the biological functions of lysophospholipids. *Cell. Mol. Life Sci.* **63:** 2695–2701.
22. Gardell, S. E., A. E. Dubin, and J. Chun. 2006. Emerging medicinal roles for lysophospholipid signaling. *Trends Mol. Med.* **12:** 65–75.
23. Anliker, B., and J. Chun. 2004. Lysophospholipid G protein-coupled receptors. *J. Biol. Chem.* **279:** 20555–20558.
24. Lee, C. W., R. Rivera, S. Gardell, A. E. Dubin, and J. Chun. 2006. GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J. Biol. Chem.* **281:** 23589–23597.
25. Imamura, S., and Y. Horiuti. 1979. Purification of *Streptomyces chromofuscus* phospholipase D by hydrophobic affinity chromatography on palmitoyl cellulose. *J. Biochem. (Tokyo).* **85:** 79–95.
26. Pradere, J. P., E. Tarnus, S. Gres, P. Valet, and J. S. Saulnier-Blache. 2007. Secretion and lysophospholipase D activity of autotaxin by adipocytes are controlled by N-glycosylation and signal peptidase. *Biochim. Biophys. Acta.* **1771:** 93–102.
27. Saulnier-Blache, J. S., A. Girard, M. F. Simon, M. Lafontan, and P. Valet. 2000. A simple and highly sensitive radioenzymatic assay for lysophosphatidic acid quantification. *J. Lipid Res.* **41:** 1947–1951.
28. Kume, K., and T. Shimizu. 1997. cDNA cloning and expression of murine 1-acyl-sn-glycerol-3-phosphate acyltransferase. *Biochem. Biophys. Res. Commun.* **237:** 663–666.
29. Ohta, H., K. Sato, N. Murata, A. Damirin, E. Malchinkhuu, J. Kon, T. Kimura, M. Tobo, Y. Yamazaki, T. Watanabe, et al. 2003. Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. *Mol. Pharmacol.* **64:** 994–1005.
30. Heasley, B. H., R. Jarosz, K. R. Lynch, and T. L. Macdonald. 2004. Initial structure-activity relationships of lysophosphatidic acid receptor antagonists: discovery of a high-affinity LPA1/LPA3 receptor antagonist. *Bioorg. Med. Chem. Lett.* **14:** 2735–2740.
31. Carnevale, K. A., and M. K. Cathcart. 2001. Calcium-independent phospholipase A(2) is required for human monocyte chemotaxis to monocyte chemoattractant protein 1. *J. Immunol.* **167:** 3414–3421.
32. Fourcade, O., M. F. Simon, C. Viode, N. Rugani, F. Leballe, A. Ragab, B. Fournie, L. Sarda, and H. Chap. 1995. Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell.* **80:** 919–927.
33. Tang, J., R. W. Kriz, N. Wolfman, M. Shaffer, J. Seehra, and S. S. Jones. 1997. A novel cytosolic calcium-independent phospholipase A2 contains eight ankyrin motifs. *J. Biol. Chem.* **272:** 8567–8575.
34. Robinson, B. S., D. J. Baisted, and D. E. Vance. 1989. Comparison of albumin-mediated release of lysophosphatidylcholine and lysophosphatidylethanolamine from cultured rat hepatocytes. *Biochem. J.* **264:** 125–131.
35. van Dijk, M. C., F. Postma, H. Hilkmann, K. Jalink, W. J. van Blitterswijk, and W. H. Moolenaar. 1998. Exogenous phospholipase D generates lysophosphatidic acid and activates Ras, Rho and Ca^{2+} signaling pathways. *Curr. Biol.* **8:** 386–392.
36. Kranenburg, O., and W. H. Moolenaar. 2001. Ras-MAP kinase signalling by lysophosphatidic acid and other G protein-coupled receptor agonists. *Oncogene.* **20:** 1540–1546.
37. Murph, M. M., L. A. Scaccia, L. A. Volpicelli, and H. Radhakrishna. 2003. Agonist-induced endocytosis of lysophosphatidic acid-coupled LPA1/EDG-2 receptors via a dynamin2- and Rab5-dependent pathway. *J. Cell Sci.* **116:** 1969–1980.
38. van Corven, E. J., A. van Rijswijk, K. Jalink, R. L. van der Bend, W. J. van Blitterswijk, and W. H. Moolenaar. 1992. Mitogenic action of lysophosphatidic acid and phosphatidic acid on fibroblasts. Dependence on acyl-chain length and inhibition by suramin. *Biochem. J.* **281:** 163–169.
39. Fang, Y., M. Vilella-Bach, R. Bachmann, A. Flanigan, and J. Chen. 2001. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science.* **294:** 1942–1945.
40. Goncharova, E. A., D. A. Goncharov, A. Eszterhas, D. S. Hunter, M. K. Glassberg, R. S. Yeung, C. L. Walker, D. Noonan, D. J. Kwiatkowski, M. M. Chou, et al. 2002. Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis (LAM). *J. Biol. Chem.* **277:** 30958–30967.
41. Delon, C., M. Manifava, E. Wood, D. Thompson, S. Krugmann, S. Pyne, and N. T. Ktistakis. 2004. Sphingosine kinase 1 is an intracellular effector of phosphatidic acid. *J. Biol. Chem.* **279:** 44763–44774.
42. Hait, N. C., C. A. Oskeritzian, S. W. Paugh, S. Milstien, and S. Spiegel. 2006. Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases. *Biochim. Biophys. Acta.* **1758:** 2016–2026.
43. Watterson, K. R., P. H. Ratz, and S. Spiegel. 2005. The role of sphingosine-1-phosphate in smooth muscle contraction. *Cell. Signal.* **17:** 289–298.
44. Leiber, D., Y. Banno, and Z. Tanfin. 2007. Exogenous sphingosine 1-phosphate and sphingosine kinase activated by endothelin-1 induced myometrial contraction through differential mechanisms. *Am. J. Physiol. Cell Physiol.* **292:** C240–C250.
45. Jeng, Y. J., V. R. Suarez, M. G. Izban, H. Q. Wang, and M. S. Soloff. 2006. Progesterone-induced sphingosine kinase-1 expression in the rat uterus during pregnancy and signaling consequences. *Am. J. Physiol. Endocrinol. Metab.* **292:** 1110–1121.
46. Tokumura, A., Y. Kanaya, M. Miyake, S. Yamano, M. Irahara, and K. Fukuzawa. 2002. Increased production of bioactive lysophosphatidic acid by serum lysophospholipase D in human pregnancy. *Biol. Reprod.* **67:** 1386–1392.