Exogenous phospholipase D generates lysophosphatidic acid and activates Ras, Rho and Ca^{2+} signaling pathways

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Background: Phospholipase D (PLD) hydrolyzes phospholipids to generate phosphatidic acid (PA) and a free headgroup. PLDs occur as both intracellular and secreted forms; the latter can act as potent virulence factors. Exogenous PLD has growth-factor-like properties, in that it induces proto-oncogene transcription, mitogenesis and cytoskeletal changes in target cells. The underlying mechanism is unknown, although it is generally assumed that PLD action is mediated by PA serving as a putative second messenger.

Results: In quiescent fibroblasts, exogenous PLD (from *Streptomyces chromofuscus*) stimulated accumulation of the GTP-bound form of Ras, activation of mitogen-activated protein (MAP) kinase and DNA synthesis, through the pertussis-toxin-sensitive inhibitory G protein G_i. Furthermore, PLD mimicked bioactive lysophospholipids (but not PA) in inducing Ca^{2+} mobilization, membrane depolarization and Rho-mediated neurite retraction. PLD action was mediated by lysophosphatidic acid (LPA) derived from lysophosphatidylcholine acting on cognate G-protein-coupled LPA receptor(s). There was no evidence for the involvement of PA in mediating the effects of exogenous PLD.

Conclusions: Our results provide a molecular explanation for the multiple cellular responses to exogenous PLDs. These PLDs generate bioactive LPA from pre-existing lysophosphatidylcholine in the outer membrane leaflet, resulting in activation of G-protein-coupled LPA receptors and consequent activation of Ras, Rho and Ca^{2+} signaling pathways. Unscheduled activation of LPA receptors may underlie, at least in part, the known pathogenic effects of exogenous PLDs.

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Background

Phospholipase D (PLD) hydrolyzes phospholipids to generate phosphatidic acid (PA) and the relevant headgroup. PLDs occur in both intracellular and secreted forms. Whereas intracellular PLD has been implicated in eukaryotic signal transduction and membrane traffic [1–3], PLDs secreted by bacteria can act as potent virulence factors [4–7]. A pathogenic PLD enzyme, which hydrolyzes lysophosphatidylcholine (LPC) and sphingomyelin, is also found in spider venom [4,7–9]. Although they arise in diverse organisms, exogenous PLDs evoke similar clinical effects, notably severe inflammatory responses, platelet aggregation and an increase in vascular permeability, without evidence for a purely cytotoxic effect [4,6–10]. In no case is the molecular basis for pathogenesis known [4].

When added to cultured mammalian cells, bacterial PLD (from *Streptomyces chromofuscus*) mimics hormones and growth factors in inducing such diverse responses as Ca^{2+} mobilization, muscle contraction, c-*fos* and c-*myc* proto-oncogene transcription, protein tyrosine phosphorylation, secretion, actin polymerization and DNA synthesis [11–17]. The underlying signaling mechanism is not understood, although it is generally believed that PLD action is mediated by PA serving as a putative second messenger [11–17]. Although PA may modulate certain enzyme activities *in vitro* [1,2], no molecular target of PA has been rigorously defined to date.

In the present study, we have re-examined the signaling mechanisms activated in response to exogenous PLD. We found that PLD acts by producing bioactive lysophosphatidic acid (LPA) [18] from pre-existing LPC in the outer leaflet of the plasma membrane. Newly produced LPA then activates its cognate G-protein-coupled receptor(s) to trigger the signaling cascades mediated by Ras, Rho and phospholipase C (PLC); there is no need to invoke a signaling role for PA produced at the outer membrane leaflet. Our results solve the long-standing question of how exogenous PLD activates mammalian cells, and they provide a biochemical basis for its known pathogenic effects.

Results and discussion

To determine how exogenous PLD activates quiescent cells, we analyzed changes in mitogenic signaling pathways, the cytoskeleton, Ca^{2+} mobilization and electro-physiological responses in PLD-treated cells. We also analyzed PLD-induced phospholipid metabolism in these cells.

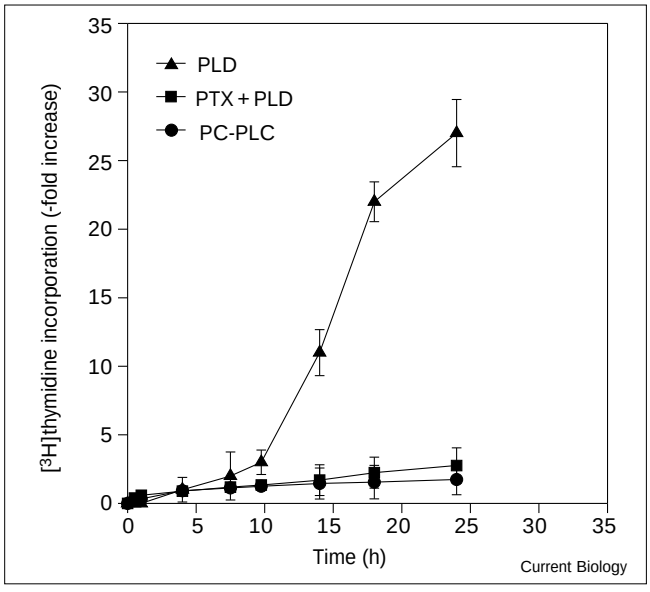
G_i-mediated mitogenic signaling

When added to serum-deprived Rat-1 cells, PLD (from *S. chromofuscus* [19]) evoked a significant mitogenic response (Figure 1). In contrast, exogenous phospholipase C (PC-PLC from *Bacillus cereus*), which generates diacylglycerol from phosphatidylcholine (PC) [20], failed to induce DNA synthesis in these cells (Figure 1; see also [20]). The kinetics of PLD-induced DNA synthesis are similar to those observed with growth factors, with S-phase entry starting after about 8 hours from the start of induction; there was no sign of long-term cytotoxicity. As shown in Figure 1, PLD-induced DNA synthesis is almost completely inhibited by pertussis toxin (PTX), indicating a key role for a heterotrimeric G protein of the G_i subfamily.

Mitogenic agonists that signal through G_i rapidly activate the Ras GTPase [21] and the downstream Raf and mitogen-activated protein (MAP) kinase cascade. We measured the activation state of Ras in Rat-1 cells using two distinct assays: Ras–GTP accumulation and stimulation of the Ras–Raf interaction. Figure 2 shows that PLD treatment of quiescent Rat-1 cells leads to Ras activation within minutes. Similar Ras activation was observed in response to LPA, but not PA (Figure 2) [21]. Pretreatment of the cells with PTX inhibited Ras activation in response to PLD and LPA, but not epidermal growth factor (EGF) (Figure 2) [21].

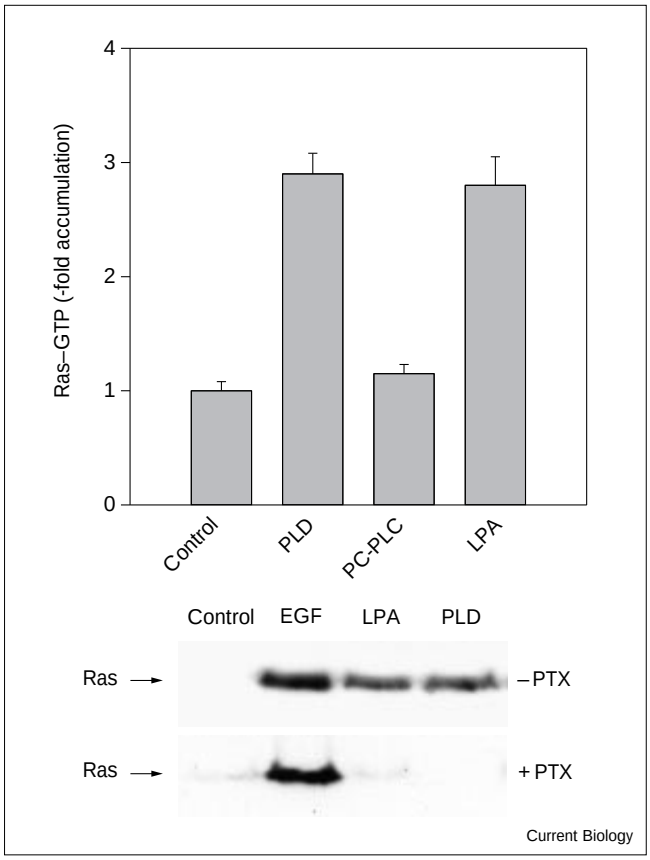
As shown in Figure 3a, PLD-induced Ras activation was followed by a long-lasting (over 4 hours) activation of MAP kinase (p42/p44 ERK) in a PTX-sensitive manner. In contrast, non-mitogenic PC-PLC failed to activate Ras (Figure 2) and induced only transient (less than 1 hour)

Figure 1



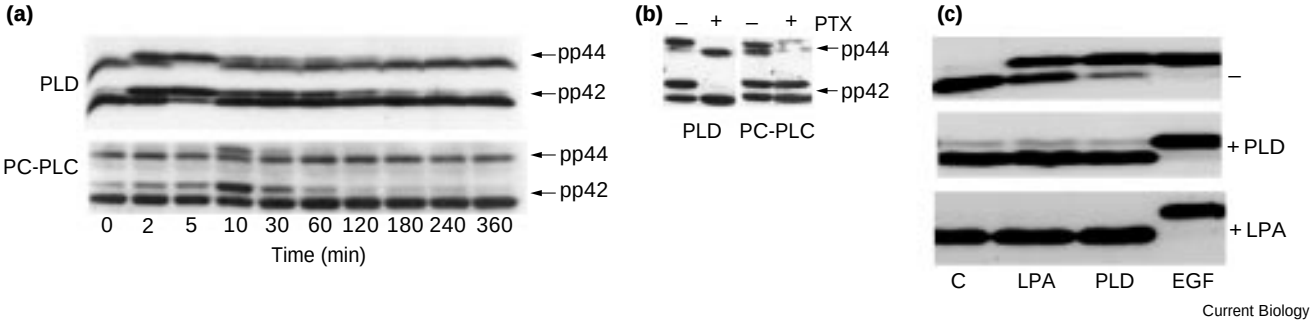
PLD-induced DNA synthesis in Rat-1 cells. Thymidine incorporation (an indicator of DNA synthesis) was measured as a function of time. Serum-deprived cells were exposed to PLD (derived from *S. chromofuscus*; 5 U/ml), in the absence or presence of PTX (200 ng/ml), or to PC-PLC (derived from *B. cereus*; 1 U/ml), as indicated. Error bars represent SD (n = 4).

Figure 2



Ras activation. (a) Accumulation of the GTP-bound form of Ras (Ras–GTP) as a function of time. Cells labeled with ^{32}P were stimulated for 5 min with PLD (5 U/ml), PC-PLC (1 U/ml) or LPA (1 μM), and the guanine nucleotides bound to immunoprecipitated Ras were assayed (see Materials and methods). Error bars indicate s.e.m. (n = 3). (b) Detection of the GTP-bound form of Ras using a glutathione-S-transferase (GST) fusion protein containing the minimal Ras-binding domain (RBD) of Raf1. Rat-1 cells were stimulated for 2 min with EGF (10 ng/ml), LPA (1 μM) or PLD (5 U/ml) in the presence or absence of PTX (200 ng/ml; overnight pretreatment) as indicated. Ras was identified by precipitation using the GST–RBD fusion protein followed by immunoblotting using anti-Ras antibody Y13–259. Control indicates no addition.

Figure 3



MAP kinase activation. (a) Cells were stimulated for different periods of time with PLD (5 U/ml) or PC-PLC (1 U/ml), and total cell lysates were immunoblotted with anti-p42 MAP kinase antibodies that recognize the p42 and p44 isoforms of MAP kinase. Activated (phosphorylated) forms of MAP kinase (pp42 and pp44) show reduced electrophoretic mobility. (b) Sensitivity of PLD-induced MAP kinase activation to PTX (200 ng/ml; cells were stimulated with PLD for 10 min). Note that the response to PC-PLC is resistant to PTX. (c) Cross-desensitization. Activation of p42 MAP kinase induced by LPA (1 μM), PLD (5 U/ml) or EGF (10 ng/ml) in quiescent Rat-1 cells, compared with the response in cells preincubated for 30 min with either PLD or LPA. MAP kinase mobility shift was measured 10 min after addition of the agonist. C, non-stimulated control.

MAP kinase activation, which was insensitive to PTX (Figure 3a) and is thought to be mediated by protein kinase C-ζ [20]. These kinetics are consistent with mitogenesis requiring sustained rather than transient MAP kinase activation [22]. Collectively, these results strongly suggest that exogenous PLD acts through a G_i-coupled receptor to stimulate the Ras–Raf–MAP kinase cascade and ensuing DNA synthesis. Obviously, the most likely candidate is the LPA receptor, which is present in numerous cell types [18].

Cross-desensitization experiments

To examine the possibility that PLD acts through activation of LPA receptors, we carried out cross-desensitization experiments by monitoring MAP kinase activation and acute ionic responses to both LPA and PLD, notably Ca^{2+} mobilization and membrane depolarization. As shown in Figure 3b, LPA pretreatment of Rat-1 cells blocked PLD-induced MAP kinase activation, and *vice versa*, whereas EGF-induced MAP kinase activation was not affected by either pretreatment.

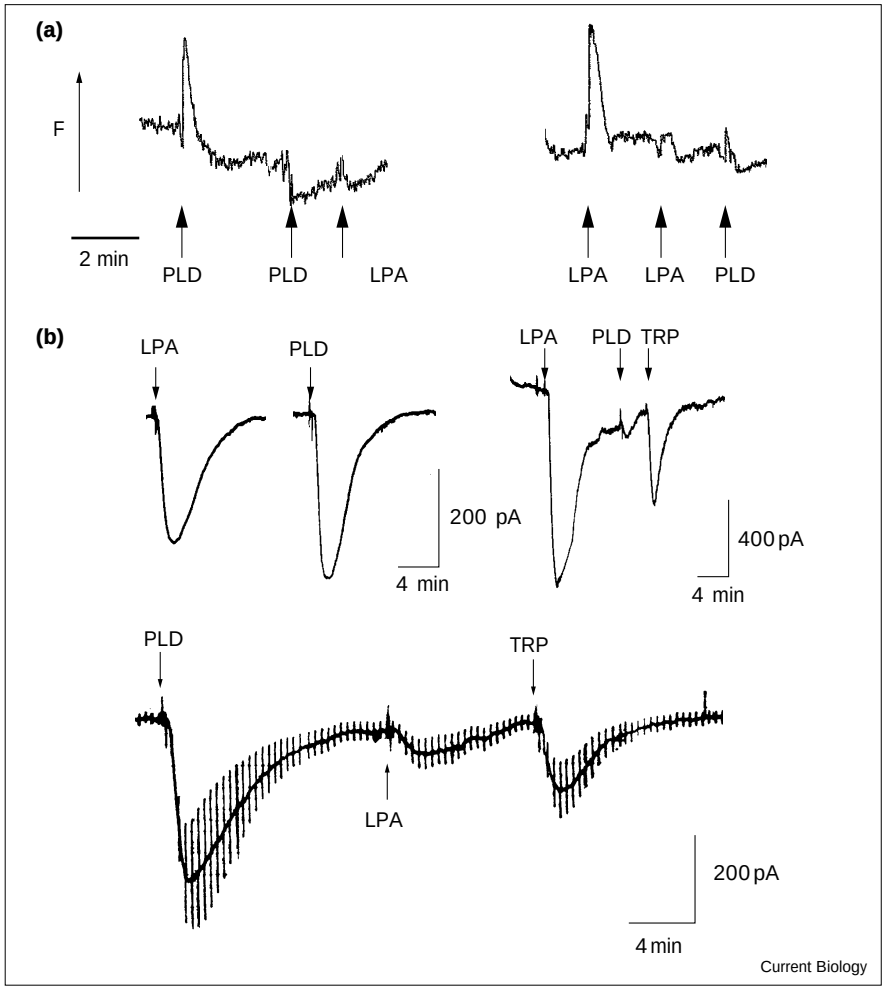
Figure 4a shows that PLD mimics LPA in evoking a rapid, transient Ca^{2+} signal in Rat-1 cells. Similar Ca^{2+} transients were observed in PLD-treated human diploid fibroblasts, A431 carcinoma cells and N1E-115 neuroblastoma cells ([12] and data not shown). PLD-induced Ca^{2+} mobilization is accompanied by enhanced inositol phosphate production [12], indicative of PLC activation. As shown in Figure 4a, prior addition of LPA prevented the Ca^{2+}-mobilizing action of PLD and *vice versa*, which supports the view that PLD acts through LPA receptors. In contrast, no such cross-desensitization was observed between the Ca^{2+}-mobilizing action of PLD and that of the lysosphingolipid sphingosine-1-phosphate (S1P), which acts on a distinct G-protein-coupled receptor [23,24] (data not shown). This finding rules out the possibility that PLD acts through S1P receptors.

LPA also evokes a transient membrane depolarization in target cells, which is caused by opening of a Cl^- channel [25] and accompanies Rho activation (F. Postma, W.H.M., unpublished observations). Figure 4b shows that PLD fully mimics LPA in evoking a transient inward current, reflecting membrane depolarization, in serum-deprived N1E-115 neuronal cells. LPA pretreatment desensitized the PLD-induced inward current, but not that induced by a thrombin receptor peptide agonist (TRP; Figure 4b) or S1P (data not shown). Conversely, PLD treatment inhibited the subsequent response to LPA (Figure 4b). None of the above ionic responses (Ca^{2+} mobilization and membrane depolarization) can be evoked by exogenously added PA, provided it is free of LPA contamination [25,26]. Collectively, the cross-desensitization experiments show that exogenous PLD signals through LPA receptors.

PLD mimics LPA in inducing Rho-mediated cytoskeletal contraction

We also examined the extent to which PLD mimics LPA in inducing rapid cytoskeletal contraction, using N1E-115 neuronal cells as a model. When treated with LPA or S1P, these cells undergo acute growth-cone collapse, neurite withdrawal and rounding of the cell body, caused by contraction of the actomyosin-based cytokeleton [24,27–29]. These dramatic shape changes are driven by the Ras-related Rho GTPase [24,28,29], a key regulator of the actin cytoskeleton [30]. As summarized in Table 1, exogenous PLD evoked neuronal shape changes that were

Figure 4



Ionic responses to PLD (1 U/ml), LPA (1 μM) and TRP (100 μg/ml). (a) Ca^{2+} mobilization in Rat-1 cells loaded with the fluorescent Ca^{2+} indicator Indo-1. F, fluorescence. (b) Electrophysiological recordings from single, serum-deprived N1E-115 neuronal cells using the perforated patch-clamp method (see Materials and methods). Agonist-induced inward currents represent Cl^--mediated membrane depolarization [25]. Current pulses in the lower recording reflect monitoring of membrane conductance.

indistinguishable from those induced by LPA; these effects were blocked by the Rho-inactivating C3 exotoxin. In contrast, no detectable shape changes were induced by addition of either PC-PLC (Table 1) or purified, LPA-free PA [27]. From these results, we conclude that exogenous PLD acts on LPA receptors to activate Rho signaling. Consistent with this, an earlier study has shown that exogenous PLD mimics LPA, rather than PA, in inducing Rho-mediated invasion of tumor cells *in vitro* [31,32].

PLD produces LPA from LPC

Having shown that exogenous PLD signals through LPA receptors, we next examined how LPA might be generated. First, we ruled out the formal possibility that PLD action is due to contaminating traces of LPA (or other bioactive lipids) by establishing that all cellular responses to PLD (but not those to LPA) were abolished after boiling the enzyme (data not shown). Next, we measured production of both PA and LPA in PLD-treated Rat-1 cells using two-dimensional thin layer chromatography (TLC). As shown in Figure 5a, PLD treatment of ^{32}P-labeled cells resulted in the rapid formation of PA and, to a lesser extent, LPA (relative increases were 11.1-fold and 3.3-fold, respectively). The newly produced LPA could arise either

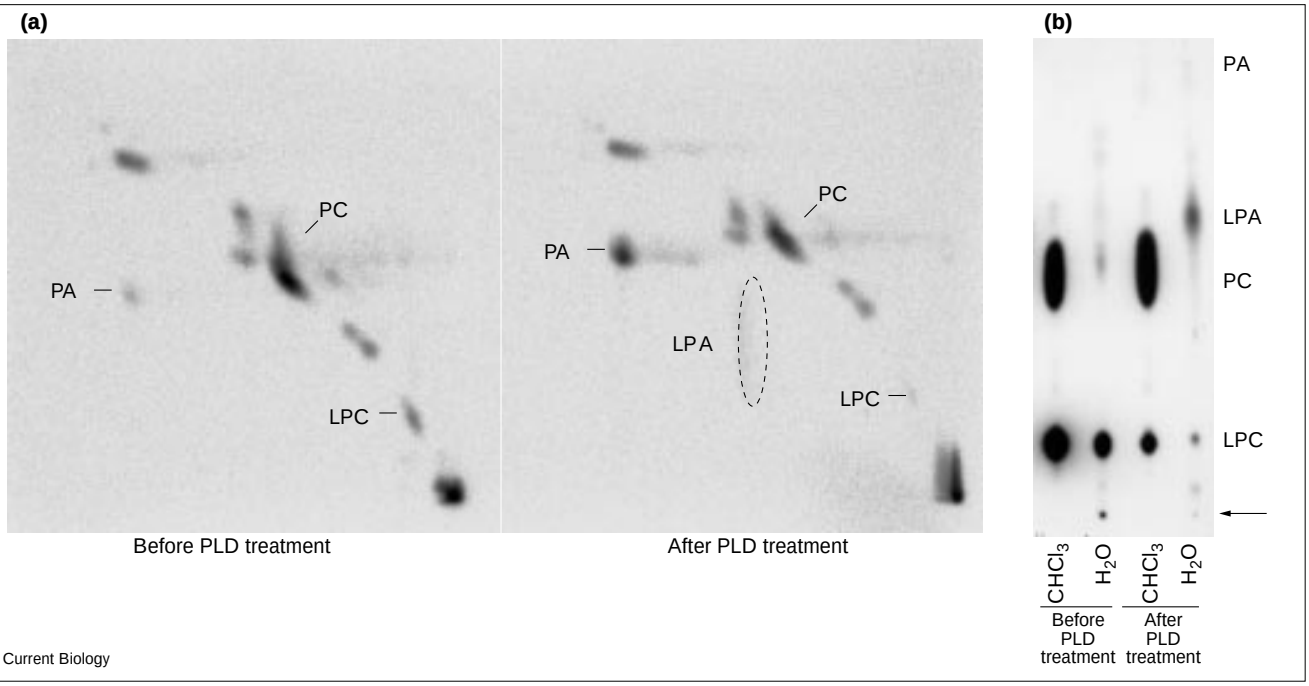
Table 1

PLD-induced and LPA-induced morphological changes in N1E-115 cells.

Treatment	Neurite retraction/cell rounding
LPA (0.5 μM)	++
PLD (1 U/ml)	++
PC-PLC (1 U/ml)	–
C3 exotoxin + PLD	–
C3 exotoxin + LPA	–

N1E-115 cells were incubated for 24 h in serum-free medium to induce cell flattening and neurite outgrowth [27,28]. Agonists were added and the cells were observed for shape changes [27]: ++, complete neurite retraction and cell rounding in > 90% of the flattened cells within 3 min; –, no detectable shape changes within 10 min. The Rho-inactivating C3 exotoxin (10 μg/ml) [28] was added 12 h prior to addition of PLD or LPA. The indicated responses were consistently observed in at least 12 separate experiments.

Figure 5

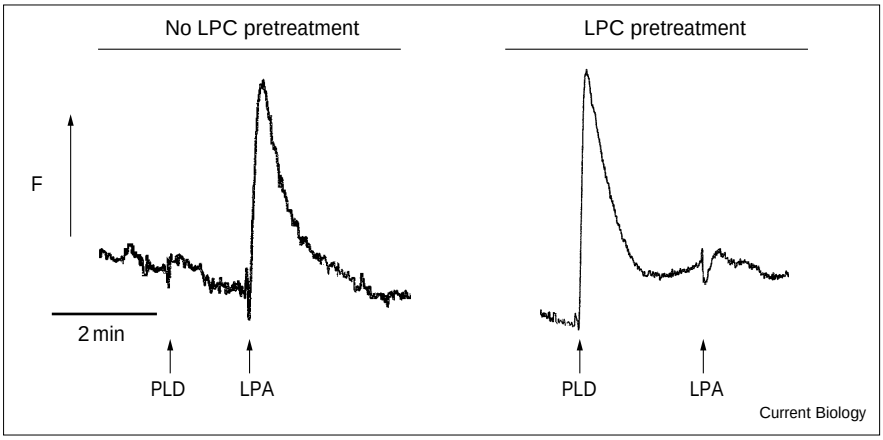


Phospholipid analysis of PLD-treated Rat-1 cells. (a) Two-dimensional TLC analysis of ^{32}P-labelled cells before and after treatment with PLD (5 U/ml) for 5 min. Note the appearance of LPA at the expense of LPC. Quantitative analysis revealed that PA levels increased 11.1-fold and LPA levels increased 3.3-fold after PLD treatment for 5 min. (b) TLC analysis of [^{32}P]LPC-labeled cells before and after treatment with PLD for 5 min, as indicated. $CHCl_3$, organic phase; H_2O, water phase. Note the appearance of LPA at the expense of LPC (water phase), whereas the radiolabeled PC pool remains unaltered (the latter pool is not accessible to PLD, as explained in the text).

from deacylation of PA (through phospholipase A_2 activity) or, more directly, through hydrolysis of pre-existing LPC. Regarding the latter possibility, we note that LPC might represent up to a few percent of total phospholipids in the plasma membrane, where it localizes preferentially to the outer leaflet [33–35]. Figure 5a shows that PLD-induced LPA formation was accompanied by a marked decrease in LPC, consistent with LPC serving as a precursor of LPA.

To further substantiate this precursor–product relationship, Rat-1 cells were incubated with [^{32}P]LPC, which readily inserts into the outer leaflet of the plasma membrane. PLD treatment of such [^{32}P]LPC-labeled cells

Figure 6



LPC-induced responsiveness to PLD in PLD-resistant COS-7 cells. The concentration of free Ca^{2+} in the cytosol (F) was monitored in Indo-1-loaded cells. Note that the lack of response to PLD was overcome after LPC pretreatment (15 min incubation with 10 μM LPC at 37°C, followed by removal of LPC prior to stimulation with PLD and LPA).

caused significant production of LPA at the expense of LPC (Figure 5b; note that PLD does not significantly affect the ^{32}P-labeled PC pool because this pool is localized to the cytosolic side of the plasma membrane and/or intracellular membranes, where it is generated through coenzyme-A-dependent acylation of [^{32}P]LPC [32]).

From these results, we conclude that, in addition to producing PA, PLD generates bioactive LPA through hydrolysis of LPC present in the outer leaflet of the plasma membrane. Newly produced LPA then activates its cognate G-protein-coupled receptor(s) [18]. Receptor activation might occur through lateral diffusion of LPA within the plane of the plasma membrane, because PLD-generated LPA was not detectable in the medium (data not shown). Further work is required to test this notion.

Unresponsiveness to PLD is overcome by LPC pretreatment

When testing various cell types for PLD-induced Ca^{2+} mobilization, we unexpectedly observed that COS-7 epithelial cells failed to respond to PLD, whereas they did respond to LPA. We reasoned that such resistance to PLD might be due to limited substrate availability and, if so, should be overcome by artificially enriching the plasma membrane with LPC. Indeed, pretreatment of COS-7 cells with exogenous LPC renders them fully responsive to PLD, as measured by Ca^{2+} mobilization (Figure 6). This result reinforces the notion that exogenous PLD acts by producing LPA from LPC, and strongly suggests that resistance to PLD is due to limited substrate availability in the outer membrane leaflet.

Conclusions

Our results show that the multiple cellular effects of exogenous PLD are elicited through activation of the G-protein-coupled LPA receptor(s), and that the widely held belief that newly produced PA is the primary mediator of the actions of exogenous PLD [11–17] is incorrect. Our data do not, of course, rule out the possibility that PA produced intracellularly by endogenous PLDs does have an as-yet unknown signaling function. There is an unequivocal role for bacterial and spider venom PLDs in the pathogenesis of disease, but the molecular basis for pathogenesis has been elusive to date [4]. Unscheduled activation of LPA receptors and consequent triggering of signaling cascades mediated by Ras, Rho and PLC, as shown here, may well account, at least in part, for the pathophysiological effects of PLD, which include inflammation, increased vascular permeability and platelet aggregation, and which overlap with the known biological responses to LPA [18]; it should be kept in mind, however, that exogenous PLDs may also generate sphingomyelin-derived messengers in addition to LPA. Future studies should examine the importance of the PLD/LPA receptor signaling system in the pathogenesis of bacterial disease.

Materials and methods

Materials

Phospholipids were obtained from either Sigma or Serdary Research Laboratories (London, Ontario). PLD (type VI; *S. chromofuscus*) was from Sigma, and PC-PLC (*B. cereus)* from Boehringer Mannheim. PTX and C3 exotoxin were from List laboratories. The GST–RafRBD fusion construct was obtained from J.L. Bos (Utrecht University).

DNA synthesis

Confluent, serum-deprived Rat-1 cells in 24-well culture plates were treated with agonists and exposed to [^{3}H]thymidine (0.5 μCi/ml) for various periods of time. Acid-precipitable material was dissolved in 0.1 M NaOH and quantified by liquid-scintillation counting.

Ras activation assays

Nearly confluent Rat-1 cells in six-well tissue culture plates were serum-starved overnight, labeled for 3 h in phosphate-free minimum essential medium (MEM) supplemented with 100 μCi/ml $^{32}P_i$, stimulated with agonists and washed with ice-cold PBS. Cells were lysed in 1% Triton X-114 buffer, and $p21^{ras}$ was immunoprecipitated with monoclonal Y13–259 precoupled via rabbit anti-rat IgG to protein A–Sepharose and then assayed for bound guanine nucleotides by TLC separation using polyethyleneimine-cellulose plates as described [21]. The amount of ^{32}P in the GDP and GTP spots was quantified using a Phosphoimager. In an alternative non-radioactive assay, we used the minimal Ras-binding domain (RBD) of Raf1 (amino acids 51–131) to identify activated Ras–GTP [33]. Ras was detected by precipitation with GST–RBD fusion protein followed by immunoblotting using monoclonal Y13–259 as described in [36].

MAP kinase activation assays

Activation of $p42^{MAPK}$ and $p44^{MAPK}$ was determined by mobility-shift assay. Stimulated Rat-1 cells were washed with cold PBS and lysed in SDS sample buffer. Proteins were separated by SDS–PAGE (10% acrylamide, 0.4% bisacrylamide gel) and transferred to nitrocellulose membranes. These were blocked for 30 min with 5% milk powder in TBST (10 mM Tris–HCl, pH 8.0; 150 mM NaCl containing 0.05% (v/v) Tween-20), incubated with anti-$p42^{MAPK}$ (ERK2) antibodies, washed in TBST and subsequently incubated with peroxidase-conjugated swine anti-rabbit antibody. Immuno-stained antibodies were visualized by enhanced chemiluminescence (Amersham).

Ca^{2+} mobilization

Confluent cells, grown on glass coverslips, were loaded with the fluorescent Ca^{2+} indicator Indo-1, and Ca^{2+}-dependent fluorescence was monitored as described [26].

Electrophysiology

Electrophysiological recordings were obtained from N1E-115 cells grown in 3 cm dishes using the whole-cell, perforated patch-clamp technique with amphotericin in the pipette solution, as described [25]. Micropipettes were fire-polished and filled with a high K^+, low Ca^{2+} buffer [23]. Data were collected using an EPC-7 amplifier (List-Medical), interfaced to a personal computer via an A/D converter (TL-1 DMA interface, Axon Instruments Inc.). Voltage-clamp protocols were generated, data were stored and analyzed using pClamp 6.0 (Axon Instruments Inc.).

Phospholipid analysis

[^{32}P]LPC was prepared by metabolic labeling of Rat-1 cells with $^{32}P_i$ for 20 h in MEM. Lipids were extracted [37] and separated by two-dimensional TLC as follows: two runs in the first dimension (chloroform/methanol/7 N ammonia: 60:60:5, v/v) with intermittent drying, followed by one run in the second dimension (chloroform/methanol/acetic acid/water: 50:30:8:4 (v/v)). The PC spot was scraped off and incubated in 0.5 ml 50 mM Tris/HCl pH 7.6, 1 mM Na deoxycholate, 10 mM $MgCl_2$ containing 50 units PLA_2 for 2 h at 37°C. Lipids were extracted and [^{32}P]LPC was isolated by one-dimensional TLC using chloroform/methanol/acetic acid/water: 50:30:8:4 (v/v) (two runs). For

analysis of PA/LPA generation *in vivo*, serum-starved Rat-1 cells were labeled with [^{32}P]lysoPC (10 μCi; 3 h) or with $^{32}P_i$ (50 μCi; 20 h), and then stimulated with PLD for 10 min. Cellular lipids were extracted, separated by one-dimensional or two-dimensional TLC (see above) and identified using comigrating marker lipids.

Acknowledgements

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