The biologically active phospholipid, lysophosphatidic acid, induces phosphatidylcholine breakdown in fibroblasts via activation of phospholipase D

Comparison with the response to endothelin

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Lysophosphatidic acid (LPA) is a simple phospholipid that possesses hormone- and growth-factor-like properties. LPA initiates its action by inducing GTP-dependent phosphoinositide hydrolysis and inhibiting adenylate cyclase [van Corven, Groenink, Jalink, Eichholtz & Moolenaar (1989) *Cell* **59**, 45–54]. Here we show that LPA stimulates rapid breakdown of phosphatidylcholine (PC) in Rat-1 fibroblasts. LPA-induced PC breakdown occurs through activation of phospholipase D (PLD), as measured by the formation of free choline and phosphatidic acid and by transphosphatidylation in the presence of butan-1-ol. LPA also stimulates generation of diacylglycerol, but there is no detectable formation of phosphocholine, suggesting that a PC-specific phospholipase C (PLC) is not involved. The response to LPA was compared with that to endothelin, a potent inducer of phospholipid hydrolysis but a poor mitogen for Rat-1 cells. Our results indicate that: (1) LPA is less efficient than endothelin in inducing phosphoinositide and PC breakdown; (2) LPA-induced PLD activation is short-lived, levelling off after 2 min, whereas the endothelin-stimulated increase in PLD activity persists for at least 1 h; (3) the effect of LPA on PLD, like that of endothelin, is blocked by long-term pretreatment of the cells with phorbol ester, suggesting that PLD activation occurs through a protein kinase C-dependent mechanism. Furthermore, our results support the notion that there is no simple causal relationship between the degree of agonist-induced phospholipid hydrolysis and the magnitude of the mitogenic response.

INTRODUCTION

The naturally occurring phospholipid lysophosphatidic acid (LPA) can elicit such diverse cellular responses as platelet aggregation [1], smooth-muscle contraction [2], and, in fibroblasts, cell proliferation [3,4]. LPA appears to initiate its action by stimulating phosphatidylinositol (PI)-specific phospholipase C (PLC) and inhibiting adenylate cyclase through G-proteins, as if LPA binds to its own G-protein-coupled receptor [3,5]. However, the signalling mechanisms underlying the biological activity of LPA still need further clarification. In addition to phosphoinositide turnover, rapid breakdown of phosphatidylcholine (PC) via a PLC and/or phospholipase D (PLD) has been observed in various agonist-stimulated cell types (for review see [6,7]). The net result of PLC/PLD-mediated breakdown of PC is formation of diacylglycerol (DG), the second messenger that activates protein kinase C (PKC). A sustained rise in DG derived from PC has been suggested to play an essential role in the mitogenic response to growth factors [8].

In the present study, we have examined PC breakdown induced by LPA in Rat-1 fibroblasts in comparison with that induced by the vasoconstrictive peptide endothelin, a known inducer of phosphoinositide [4,9–11] and PC [11] breakdown in fibroblasts. Unlike LPA, however, endothelin is hardly mitogenic by itself, but instead requires the presence of synergizing peptide growth factors for a full mitogenic response, at least in 3T3 cells [12]. We show that LPA and endothelin differentially evoke PC hydrolysis via activation of PLD in a PKC-dependent manner, with no

detectable involvement of a PC-specific PLC. Our results also show that there is no simple correlation between the degree of phospholipid hydrolysis and mitogenic potency.

EXPERIMENTAL

Materials

Oleoyl-3-*sn*-LPA was obtained from Sigma, endothelin (ET-1) from Cambridge Research Biochemicals (Cambridge, U.K.), and phorbol 12,13-dibutyrate (PDBU) and phorbol 12-myristate 13-acetate (PMA) were from Sigma. [³H]Thymidine, [³H]arachidonic acid, [¹⁴C]palmitic acid, [³H]myristic acid and ¹⁴C- and ³H-labelled choline were from Du Pont–New England Nuclear. Phosphatidylbutanol (PBut), as a reference compound for t.l.c., was synthesized from egg PC by transphosphatidylation with butan-1-ol by using cabbage PLD (Sigma) [13]. Pertussis toxin was obtained from List Biological Laboratories (Campbell, CA, U.S.A.), and epidermal growth factor from Collaborative Research (Lexington, MA, U.S.A.). Other lipids, used as reference compounds, were from Sigma.

Cell culture

Rat-1 fibroblasts were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal-calf serum (FCS). Rat-1 cells have acquired an infinite life-span in culture, but are otherwise apparently normal. Confluent cells were made quiescent by maintaining them in serum-free DMEM for 24 h.

Abbreviations used: DG, 1,2-diacyl-*sn*-glycerol; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; LPA, 3-*sn*-lysophosphatidic acid; PA, 3-*sn*-phosphatidic acid; PC, 3-*sn*-phosphatidylcholine; PDBU, phorbol dibutyrate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PI, 3-*sn*-phosphatidylinositol; IP_n, inositol phosphates; PBut, phosphatidylbutanol.

[³H]Thymidine incorporation

Quiescent cells in 24-well tissue culture dishes were incubated with the agonist to be tested. After 15 h, cells were exposed to [³H]thymidine (0.5 μ Ci/ml) for 6 h. Trichloroacetic acid-precipitable material was quantified by liquid-scintillation counting.

Determination of phospholipid hydrolysis

Cells on 55 mm-diam. glass dishes were labelled to steady state with [¹⁴C]palmitate (0.5 μ Ci/dish), [³H]choline or [¹⁴C]choline (1 μ Ci/dish) and [³H]arachidonate (1 μ Ci/dish) for 48 h in DMEM containing 0.1 % FCS. [³H]Myristate labelling (1 μ Ci/dish) was for 3 h (yielding 85 % labelling of PC). Stimulation of radiolabelled quiescent cells with agonist was performed in serum-free DMEM/Hepes (20 mM, pH 7.3). After stimulation, the medium was removed, followed by immediate addition of chloroform/methanol (2:1, v/v) together with lipid markers (20 μ g each). Lipids were extracted and subjected to Folch partitioning. Lipid metabolites, choline and phosphocholine were determined by t.l.c. and h.p.l.c. (for DG) as described in [14]. Transphosphatidylase activity of PLD was measured in [³H]-myristate-labelled cells in the presence of 0.05 % butan-1-ol as described in [15]. Control values in the absence of agonist did not vary significantly in time (up to 60 min) and were not affected by BSA (vehicle for LPA) or butan-1-ol. When cells were stimulated in the absence of butan-1-ol, no radiolabelled compounds were detectable on t.l.c. at the PBut position. Values, expressed in d.p.m., were normalized to the amount of radiolabel incorporated in the total phospholipid fraction (which varied by less than 10 % among the different samples). Values are expressed as the means of triplicates ($\pm$ S.D.).

Determination of inositol phosphates (IP_n)

Confluent cells in six-well tissue-culture dishes were labelled for 48 h with 2 μ Ci of *myo*-[³H]inositol/ml in DMEM containing 0.1 % FCS. Cells were stimulated with agonist in DMEM/Hepes for 30 min in the presence of 10 mM-LiCl. Reactions were stopped with 10 % (w/v) ice-cold trichloroacetic acid. After diethyl ether extraction, total [³H]inositol phosphates were determined as described in [16].

RESULTS

Effects of LPA and endothelin on DNA synthesis and inositol phosphate formation: lack of positive correlation

In the course of our studies to identify signal-transduction pathways in the action of LPA, we examined phospholipid metabolism in Rat-1 fibroblasts. The effects of LPA were compared with those of the vasoconstrictive peptide endothelin. The two agonists differ greatly in their potency to induce DNA synthesis, as measured by [³H]thymidine incorporation (Table 1). At saturating concentrations (100 μ M), LPA is a strong inducer of DNA synthesis (about 27-fold stimulation over control incorporation). In contrast, endothelin at optimal concentrations (100 nM) stimulates DNA synthesis only weakly (about 4-fold). A similar weak mitogenic response to endothelin has previously been found by others in 3T3 and Rat-1 cells [9,11,12].

Both LPA and endothelin have been reported to induce phosphoinositide hydrolysis in fibroblasts [3,5,9–11]. Strikingly, endothelin was found to be much more potent in evoking IP_n accumulation than was LPA (~80-fold versus ~10-fold over basal respectively) when tested at optimal mitogenic concentrations over a 30 min period, as illustrated in Fig. 1.

Stimulation of phospholipid breakdown

LPA-induced PC breakdown in Rat-1 cells was measured by labelling the cells to steady state with [³H]choline and [¹⁴C]palmitate for 48 h. Phosphoinositide breakdown was monitored simultaneously by including [³H]arachidonate, which is preferentially incorporated into PI [14], as a third label. As shown in Fig. 2 (two upper panels), LPA evokes a rapid increase in intracellular [³H]choline levels, with no significant increase in cellular [³H]-phosphocholine or in [³H]phosphocholine appearing in the medium (results not shown). This suggests that LPA induces PC breakdown through activation of PLD rather than PC-specific PLC, a conclusion supported by transphosphatidylase experiments described below. LPA also stimulates with similar kinetics the formation of [¹⁴C]palmitate-labelled phosphatidic acid (PA) and DG (Fig. 2, lower panels), again consistent with activation of PLD and subsequent dephosphorylation of the newly generated PA by a phosphohydrolase [7,15,17–19] to yield DG. Somewhat unexpectedly, there is little or no detectable increase in the levels of phosphoinositide-derived [*arachidonate*-³H]-DG and -PA in response to LPA (Fig. 2, lower panels) although a rapid 2-fold rise in [*arachidonate*-³H]monoacylglycerol was observed within 15 s (results not shown). Given the significant effect of LPA on phosphoinositide hydrolysis, InsP₃ formation and Ca²⁺ signalling ([3,5], and Fig. 1), it seems likely that LPA-induced formation of PI-derived DG escapes detection, owing to its rapid hydrolysis to monoacylglycerol. The level of [*palmitate*-¹⁴C]monoacylglycerol remained virtually unchanged (results not shown), suggesting

Table 1. Stimulation of DNA synthesis in quiescent Rat-1 cells

DNA synthesis in response to LPA, endothelin and epidermal growth factor (EGF) was measured by [³H]thymidine incorporation, as described in the Experimental section. Values are expressed as means of triplicates ($\pm$ S.D.).

Addition	[³ H]Thymidine incorporated (d.p.m./well)	Stimulation (fold)
None	5124 ($\pm$ 399)	1
Oleoyl-LPA (100 μ M)	140880 ($\pm$ 15814)	27
Endothelin (100 nM)	23029 ($\pm$ 1888)	4
EGF (10 ng/ml)	85836 ($\pm$ 1933)	17

Fig. 1. Phosphoinositide hydrolysis in Rat-1 fibroblasts, induced by endothelin and LPA

Phosphoinositide hydrolysis was monitored by the formation of [³H]inositol phosphates as described in the Experimental section, by using the indicated concentrations of endothelin. The concentration of oleoyl-LPA was 100 μ M.

Fig. 2. LPA-induced phospholipid breakdown

Phospholipid breakdown induced by oleoyl-LPA (100 μM) was monitored by the formation of choline, phosphocholine (P-choline), DG and PA in cells simultaneously labelled with [^3H]choline, [^{14}C]palmitate and [^3H]arachidonate, as described in the Experimental section. Upper panels: with (●) or without (○) LPA; lower panels: [^{14}C]palmitate metabolites (continuous lines) or [^3H]arachidonate metabolites (broken lines), with (black symbols) or without (white symbols) LPA.

that [*palmitate- ^{14}C*]DG, in contrast with [*arachidonate- ^3H*]DG, is not further converted by a DG lipase. This could be due to substrate specificity of this lipase or to compartmentalization in the cell.

Phospholipid hydrolysis induced by endothelin is illustrated in Fig. 3. In common with LPA, endothelin stimulates a rapid increase in free intracellular [^3H]choline levels, but not in [^3H]phosphocholine. The latter result is in agreement with previous findings [11]. As judged by changes in [^3H]choline levels, endothelin is at least twice as potent as LPA in stimulating PC breakdown (cf. Figs. 2 and 3, upper panels).

Endothelin-induced increases in PA and DG are also larger than those observed with LPA, and show kinetics distinct from those seen with LPA. The time course of endothelin-induced [*palmitate- ^{14}C*]DG is clearly biphasic (Fig. 3). The first peak, detected at 15 s, coincides with an even more significant generation of [^3H]arachidonate-labelled DG derived from PI and is followed by a second, longer lasting, rise in DG, which declines after 30 min. The first [^3H]arachidonate-enriched DG peak is closely followed by transient formation of [*arachidonate- ^3H*]PA, presumably owing to the action of DG kinase, whereas the slower increase in [*palmitate- ^{14}C*]DG (second peak) follows the formation of [*palmitate- ^{14}C*]PA, in agreement with a PA-DG precursor-product relationship mediated by a PA phosphohydrolase. This endothelin-induced and phased formation of phospholipid breakdown products, shown in Fig. 3, is remarkably similar to what we observed previously in bradykinin-stimulated human fibroblasts [14,15].

PLD activation as measured by transphosphatidylolation

PLD activity assays in living cells are hampered by the fact that the products, i.e. PA and choline, are subject to further

metabolism. This problem can be circumvented by measuring PLD activity via transphosphatidylolation (e.g. [15,19,20]) in the presence of a primary alcohol, such as butan-1-ol, generating a metabolically stable product, PBut. Fig. 4 shows the formation of [^3H]PBut induced by LPA and endothelin in [^3H]myristate-labelled Rat-1 cells as a function of time. Both stimuli induce PBut formation within 1 min, with endothelin being again more potent than LPA. With LPA, PBut formation levels off after 5 min, indicating that PLD activation is transient. By contrast, endothelin causes PBut to increase continuously during the assay period (20 min), albeit at a lower rate after 5 min.

Fig. 5 shows the dose-dependency of the LPA-induced PLD activity, as measured by transphosphatidylolation. Significant PLD activation is observed at concentrations as low as 50 nM-LPA, which is comparable with LPA-induced InsP_3 formation and Ca^{2+} mobilization in human fibroblasts [5].

Involvement of PKC

PKC-activating phorbol esters are known to stimulate PLD activity in many cells (for review see [6,7]). In a previous study, we have shown that the PLD response to PMA is relatively slow [15]. A 30 min PMA stimulation of Rat-1 cells results in a similar PBut formation to that with endothelin for 5 min (Fig. 6). When PKC was down-regulated by long-term pretreatment (24 h) of the cells with PDBU, formation of PBut and release of choline induced by LPA, endothelin or PMA was almost completely inhibited (Figs. 6a and 6b). In the case of PBut formation, long-term PDBU treatment also results in decreased basal values (Fig. 6b), probably reflecting basal PKC-dependent PLD activity.

Effect of pertussis toxin

A pertussis-toxin-sensitive G-protein is critically involved in

Fig. 3. Endothelin (100 nM)-induced phospholipid breakdown

For conditions and symbols, see Fig. 2 legend.

Fig. 4. Time course of LPA- and endothelin-induced PLD activation

Cells were labelled with $[^3\text{H}]\text{myristic acid}$ for 3 h. PLD activation was monitored by $[^3\text{H}]\text{PBut}$ formation (transphosphatidylolation) as described in the Experimental section. Concentrations used: oleoyl-LPA (■), 100 μM ; endothelin (ET; □), 100 nM.

Fig. 5. Dose-dependency of LPA-induced PLD activation

Cells were labelled with $[^3\text{H}]\text{myristic acid}$ for 3 h. PLD activity was measured by $[^3\text{H}]\text{PBut}$ formation (transphosphatidylolation) upon stimulation for 5 min with the indicated concentrations of oleoyl-LPA or 100 nM-endothelin (ET).

the mitogenic action of LPA [3]. To establish the possible involvement of such a toxin-sensitive G protein in PLD activation, we determined the effect of pertussis-toxin pretreatment (0.5 $\mu\text{g}/\text{ml}$, for 2 h) on PBut formation. No inhibition was found by either stimulus, nor did pertussis toxin affect basal PLD activity, but it was confirmed that the toxin (0.1 $\mu\text{g}/\text{ml}$) did inhibit LPA-induced mitogenesis (results not shown).

From studies on neutrophil activation it appears that agonist-induced PLD activation may be controlled by intracellular cyclic AMP levels [21]. Pretreatment of Rat-1 cells with dibutyl cyclic AMP (300 μM) or forskolin (50 μM), however, did not affect PLD activation by LPA or endothelin (results not shown).

DISCUSSION

LPA is an intriguing phospholipid agonist that can elicit such diverse cellular responses as platelet aggregation, smooth-muscle contraction and, in fibroblasts, mitogenesis. We have previously shown that LPA stimulates GTP-dependent phosphoinositide hydrolysis and inhibits adenylate cyclase in quiescent fibroblasts [3], but additional signalling pathways are likely to be involved in LPA action (reviewed in [22]). In the present study, we have shown that in Rat-1 cells LPA induces rapid PC breakdown, as evidenced by the formation of free choline and PA with similar

Fig. 6. Effect of down-regulation of PKC on agonist-induced PLD activation

PKC was down-regulated by addition of 100 nM-PDBU during labelling of the cells (24 h) with [^{14}C]choline and [^3H]myristic acid. After washing (three times), cells were stimulated with either 100 μM -oleoyl-LPA or 100 nM-endothelin (ET) for 5 min, or with 100 nM-PMA for 30 min. PLD activation was measured by the formation of free [^{14}C]choline (a) and [^3H]PBut (transphosphatidyl) (b). Control: 5 min incubation with butan-1-ol-containing medium alone. Key: ■, no PDBU; □, PDBU-down-regulated.

kinetics and by using transphosphatidyl assays. It is highly unlikely that LPA also activates a PC-specific PLC, since there is no detectable generation of phosphocholine during the first 30 min of stimulation. Given the fact that basal levels of phosphocholine are similar to those of choline (as judged by [^3H]choline incorporation under steady-state conditions), alterations in PLC-mediated phosphocholine formation would have been readily detected, in particular since [^3H]phosphocholine is not rapidly dephosphorylated upon stimulation of cells or cell lysates, as we have shown previously [14].

Rapid hydrolysis of PC via activation of PLD is also induced by the peptide endothelin, with no apparent involvement of a PC-specific PLC. This is in agreement with a previous report on agonist-induced formation of choline catabolites in fibroblasts [11]. So far, a biphasic DG response to endothelin (measured as DG 'mass') has only been described for smooth-muscle cells [23,24]. By using the double-fatty-acid radiolabelling technique we have now established an endothelin-induced biphasic DG response in fibroblasts, and have been able to assign the two DG peaks to originate from phosphoinositides and PC.

It is clear from the present results that endothelin is much more potent than LPA in stimulating both PC-specific PLD and PI-specific PLC. The differences between LPA and endothelin as phospholipid-hydrolysing agonists appear to be quantitative rather than qualitative. Thus LPA-induced formation of PA and DG via the PLD/PA phosphohydrolase pathway is less prominent than with endothelin as a stimulus. Moreover, LPA-induced PLD activation levels off after 2 min, whereas endothelin-stimulated PLD activity persists for at least 1 h. Essentially similar kinetic differences were observed with LPA- and endo-

thelin-induced IP_n accumulation (results not shown). It thus appears that there is a positive correlation between activation of PLC and PLD. Surprisingly, there is little or no detectable increase in phosphoinositide-derived DG (and PA) in LPA-stimulated cells, despite the appearance of PLC-mediated IP_n . By contrast, phosphoinositide-derived DG and PA are readily detected with endothelin stimulation. This suggests that DG metabolism is very efficiently controlled during LPA stimulation (through rapid activation of a DG lipase), whereas the greater intensity of the phosphoinositide-hydrolysing response to endothelin apparently exceeds the capacity of DG homeostatic events. Despite these quantitative differences between LPA and endothelin, we feel safe to conclude that both agonists utilize a common mechanism to stimulate PC breakdown, i.e. via activation of PLD in a PKC-dependent manner. In fact, PLD activation appears to be secondary to PKC stimulation, for example as a result of receptor-mediated phosphoinositide hydrolysis. Current evidence suggests that PC breakdown may be regulated by PKC, Ca^{2+} , as well as G-proteins, depending on cell types and agonists (for reviews see [6,7]).

An interesting result that emerges from the present studies is the lack of correlation between agonist-induced phospholipid hydrolysis (PLC- and PLD-catalysed) and the ultimate mitogenic response: the most potent inducer of phospholipid breakdown, endothelin, evokes only a poor mitogenic response, whereas the reverse is true for LPA. Furthermore, LPA-induced PLD activation as well as phosphoinositide hydrolysis occurs in a concentration range ($< 1 \mu\text{M}$) in which LPA has no mitogenic activity (mitogenic concentrations for LPA are above $5 \mu\text{M}$ [3]). Although LPA is taken up by cells and partially converted into monoacylglycerol [25], this breakdown product of LPA cannot account for its biological activity. These results suggest that, if phospholipid hydrolysis plays a role in the mitogenic response, there are at least other biochemical mechanisms essentially involved.

The poor mitogenic capacity of endothelin agrees with current insights in the role of the PLC/ Ca^{2+} /PKC pathway in growth-factor action (reviewed in [26]). Potent activators of phospholipid hydrolysis, such as bradykinin [14,15] or carbachol acting at M1 muscarinic receptors [27], fail to stimulate DNA synthesis significantly in 'normal' quiescent fibroblasts, and endothelin appears to be no exception to this rule. Only in the presence of synergizing peptide growth factors, such as epidermal growth factor or insulin, does the mitogenic potential of endothelin become evident [12].

Activation of PLD appears to be an ubiquitous early event in cell signal transduction. Although its predominant lipid product, PA, is likely to serve an important but as yet unknown function in short-term cellular regulation [28], the present results suggest that there is no direct causal relationship between agonist-induced PLD activity and the ultimate mitogenic response.

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