Mitogenic action of lysophosphatidic acid and phosphatidic acid on fibroblasts

Dependence on acyl-chain length and inhibition by suramin

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Lysophosphatidic acid (LPA) is a naturally occurring phospholipid with growth-factor-like activities [van Corven, Groenink, Jalink, Eichholtz & Moolenaar (1989) *Cell* **45**, 45–54]. We have examined various structural analogues of LPA for their ability to stimulate DNA synthesis in quiescent fibroblasts. When the acyl-chain length is varied, the rank order of mitogenic potency is: 1-oleoyl LPA $\approx$ 1-palmitoyl LPA > 1-myristoyl LPA > 1-lauroyl LPA > 1-decanoyl LPA; the last compound shows almost no activity over the concentration range tested (1–100 μ M). An ether-linked LPA (1-*O*-hexadecylglycerol 3-phosphate) has much decreased mitogenic activity as compared with the ester-linked analogue at concentrations < 25 μ M, and becomes cytotoxic at higher concentrations. Hexadecylphosphate, which lacks a glycerol backbone, has negligible activity. On a molar basis, diacyl phosphatidic acid (PA) is about equally potent as the corresponding LPA analogue, showing similar acyl-chain-length dependence; the data argue against the possibility that the mitogenic action of PA is due to contaminating traces of LPA. Although the short-chain analogues of LPA and PA fail to antagonize the action of long-chain (L)PAs, the polyanionic drug suramin inhibits LPA- and PA-induced DNA synthesis in a reversible and dose-dependent manner, at concentrations [IC_{50} (concn. giving 50% inhibition) $\approx$ 70 μ M] that do not affect epidermal-growth-factor-induced DNA synthesis. Suramin appears to act in the early G_0/G_1 phase of the cell cycle, blocking immediate responses to LPA such as phosphoinositide hydrolysis. We conclude that both LPA and PA can function as growth-promoting phospholipids, with the fatty acid chain length being a major determinant of mitogenic potency.

INTRODUCTION

Among the great variety of cellular lipids, PA and LPA are the focus of increasing attention. Apart from their precursor role in lipid biosynthesis *de novo* [1], these simple phospholipids are produced rapidly in activated cells (see, e.g., [2–5]), suggesting a potential role as intracellular second messenger. Furthermore, they can stimulate DNA synthesis and cell division when added extracellularly to fibroblasts or epithelial cells in culture [6–10]. Our recent studies suggest that LPA acts, at least in part, by activating a subset of the family of membrane-bound G-proteins, including the inhibitory G-protein (G_i) of adenylate cyclase and the stimulatory G-protein of phosphoinositide-specific phospholipase C [8,11,12]. We also presented evidence suggesting that previously reported Ca^{2+} -mobilizing effects of PA are actually due to LPA [11], a common contaminant of commercially available PA. However, the latter result does not necessarily imply that the long-term mitogenic effect of PA is similarly due to contaminating lyso-derivatives.

In the present study we have prepared LPA analogues with varying acyl-chain composition to establish the relationship between specific structural features of the LPA molecule and its mitogenic activity in quiescent fibroblasts. Insight into the structure–activity relationship should help to clarify further the mechanism of action of extracellularly added LPA, to solve the PA–LPA contamination problem, and also to discover potential LPA antagonists. Our results indicate that: (i) LPA and PA possess approximately equal mitogenic potencies; (ii) the ability to stimulate DNA synthesis correlates with the length of the acyl

chain(s); and (iii) suramin, a membrane-impermeant drug known to inhibit ligand–receptor interactions, is a selective antagonist of both short-term and long-term (L)PA action.

MATERIALS AND METHODS

Materials

PA, LPA, L - α -lysophosphatidylcholines and lyso-platelet-activating factor were obtained from Sigma, Avanti Polar Lipids (Pelham, AL, U.S.A.) or Serdary Research Laboratories (London, Ontario, Canada). Hexadecyl phosphate was kindly provided by Dr. R. C. Young (SmithKline Beecham Research Ltd., Welwyn, Herts., U.K.). Fatty-acid-free BSA, phospholipase D (cabbage, type 1), phospholipase A_2 (pig pancreas), RNAase A (1-AS, bovine pancreas), ethidium bromide, endothelin and insulin were purchased from Sigma; EGF was from Collaborative Research (Lexington, MA, U.S.A.). Bisbenzimidazole (Hoechst) H33258 was from Calbiochem (La Jolla, CA, U.S.A.), suramin from Bayer (Leverkusen, Germany) and pepsin from Merck (Darmstadt, Germany). [3H]Thymidine (6.7 Ci/mmol) and *myo*-[3H]inositol (23 Ci/mmol) were purchased from Amersham Corp. Silica-gel plates were from Merck, and Dowex resin was from Bio-Rad.

Cells

Rat-1 cells, Swiss 3T3 cells and human foreskin fibroblasts (HF cells) were routinely grown in DMEM containing 10% (v/v) FCS. Cells were made quiescent by growing them to

Abbreviations used: PA, L - α -phosphatidic acid; LPA, L - α -lysophosphatidic acid; EGF, epidermal growth factor; FCS, fetal-calf serum; G-protein, GTP-binding protein; IC_{50} , concn. giving 50% inhibition; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 , pH 7.4).

confluence and then maintaining them in DMEM + 0.1 % FCS (Rat-1) or serum-free DMEM (HF and Swiss 3T3) for 24 h.

DNA synthesis

Quiescent cells in 24-well tissue-culture dishes were incubated with the growth stimulus to be tested. At the required time, cells were exposed to [3 H]thymidine (0.5 μ Ci/ml) for periods of 1 to 6 h as indicated in the Results section. Trichloroacetic acid-precipitable material was dissolved in 0.1 M-NaOH and quantified by liquid-scintillation counting.

For measurements of DNA content, quiescent cells in six-well tissue-culture dishes were trypsin-treated, suspended in PBS and fixed in 70 % (v/v) ethanol. After fixation the cells were kept on ice for at least 3 h. Cells were then centrifuged (200 *g* for 5 min), ethanol was removed and 1 ml of RNAase A (0.5 mg/ml) dissolved in 100 mM-NaCl/100 mM-Tris (pH 7.5) was added. After incubation for 30 min at 37 °C, 1 ml of pepsin (2500 FIP U/g; 1 mg/ml) in 0.4 % HCl was added and incubation was continued for another 45 min. Next, 2 ml of ethidium bromide (20 μ g/ml) and Hoechst dye H33258 (8 μ g/ml) in 200 mM-Tris (pH 8–8.5) and 0.5 % BSA were added, and after a 15 min incubation period at room temperature, cells were centrifuged at 500 *g* for 10 min. The pellet was resuspended in 300 μ l of ethidium bromide/Hoechst dye solution and cell-cycle parameters were analysed with a fluorescence-activated cell sorter.

Preparation of LPAs

LPAs with various chain lengths were prepared enzymically either from the corresponding lysophosphatidylcholines by using phospholipase D, or from commercially available PA by using phospholipase A₂, essentially as described by Kates [13]. When the phospholipase D method was used, 5 mg of lysophosphatidylcholine (1-oleoyl, 1-palmitoyl, 1-myristoyl, 1-lauroyl, or 1-decanoyl) was added to 1 ml of 100 mM-potassium acetate (pH 5.6) and 10 mM-CaCl₂, followed by bath sonication. Phospholipase D (3–6 mg; 640 units/mg of protein) was then added and the reaction mixture was kept under N₂ at room temperature for 12–16 h. Phospholipids were extracted with 5 ml of chloroform/methanol (1:1, v/v) and 1.25 ml of 100 mM-HCl. The chloroform phase was evaporated and analysed by t.l.c. in the solvent system chloroform/methanol/water/acetic acid (50:30:4:2, by vol.) (in this system the *R_f* values for the long-chain lysophosphatidylcholine, LPA and PA are 0.09, 0.37 and 0.59 respectively; the shortest-chain-length LPA and PA tested, decanoyl, has a 15 % lower *R_f* value). LPAs were detected by water spraying, scraped into vials and extracted with three subsequent washes with the chloroform/methanol/HCl solution. LPAs were quantified by the phosphate-content assay of Morrison [14].

In the phospholipase A₂ method, 5 mg of PA (dioleoyl, dipalmitoyl, dimyristoyl, dilauroyl or didecanoyl) was dissolved in 1 ml of 1 mM-deoxycholate/50 mM-Tris (pH 7.5) by bath sonication (deoxycholate was used instead of diethyl ether, since this gave much better recoveries of LPA). Phospholipase A₂ (30 μ l of a 510 units/mg of protein stock) was then added, and the reaction conditions, lipid extraction and chromatographic procedures were identical with those described above. In general, both methods of LPA synthesis gave recoveries of 10–20 %, except for the synthesis of 1-decanoyl LPA, where the phospholipase D method was not efficient (approx. 5 % yield). (For unknown reasons, we were not successful in synthesizing significant quantities of 1-stearoyl LPA by either protocol.) Ether-linked LPA was synthesized from lyso-platelet-activating factor by the phospholipase D method described above.

Phospholipid purification

T.l.c.-purified lipids were kept in chloroform under N₂ at –20 °C. Stock solutions of the lipids were prepared by evaporating the chloroform and dissolving the lipids in water (concn. of 7.5 mg/ml) plus fatty-acid-free BSA (6.7 %, pH 7.4). Specific LPAs were further purified by isocratic reverse-phase h.p.l.c. LPAs purified from t.l.c. were dissolved in ethanol/water (1:1, v/v) to a concentration of 2–5 mg/ml. Samples (50–100 μ l) were chromatographed at room temperature on a μ Bondapak phenyl column (300 mm $\times$ 3.9 mm internal diam.; Waters Associates, Milford, MA, U.S.A.), with vacuum-degassed methanol/water/acetonitrile/acetic acid (260:140:8:1, by vol.) as mobile phase (flow rate 1 ml/min). Purified LPA, detected with a u.v. detector (206 nm), was dried under N₂ and kept in chloroform at –20 °C. H.p.l.c.-purified LPA was identified by t.l.c. as described above. The fatty acyl composition of LPA was determined by g.l.c. after trans-esterification with dry methanolic 0.2 M-NaOH for 90 min at 50 °C. After neutralization with acetic acid, the fatty acid methyl esters were extracted with chloroform, evaporated under nitrogen and taken up in n-hexane; 1–5 μ g of lipid was used for each analysis.

Depending on the supplier, 1-oleoyl LPA was found to contain more than 99 % oleate, and minor traces of palmitate, myristate and laurate (Sigma). 1-Myristoyl LPA contained approx. 97 % myristate and 0.2 % lipid material co-migrating with an oleate marker, and 0.3 % co-migrating with a palmitate marker. After h.p.l.c. purification, both 1-oleoyl and 1-myristoyl LPA induced the same mitogenic response as non-h.p.l.c.-purified samples. Although it cannot be rigorously excluded that some of the 1-acyl LPA undergoes acyl migration to yield 2-acyl LPA during prolonged incubation at 37 °C, the potential for acyl migration of 1-acyl glycerol is known to be very low under normal assay conditions [15].

Inositol phosphates

Confluent cells in six-well tissue-culture dishes were labelled for 48 h to near isotopic equilibrium with 2 μ Ci of *myo*-[3 H]inositol/ml in DMEM containing 0.1 % FCS. At 2 h before stimulation the cells were shifted to serum-free DMEM containing 20 mM-Hepes (pH 7.5). Cells were stimulated with agonist for 30 min in the presence of 10 mM-LiCl, and the reactions were stopped with ice-cold 10 % (w/v) trichloroacetic acid. After extraction of the supernatant with diethyl ether, samples were processed for analysis of total [3 H]inositol phosphates as described by Tilly *et al.* [16].

RESULTS

Growth-factor-like action of LPA and effect of acyl-chain length

Table 1 shows the relative mitogenic properties of 1-oleoyl LPA in the presence and absence of insulin (5 μ g/ml), as compared with various other mitogens (FCS, EGF, peptide hormones) in both Rat-1 and Swiss 3T3 cells. It appears that there is little or no synergism between LPA and insulin, contrary to the prominent synergistic effects observed between EGF and insulin or bombesin and insulin in Swiss 3T3 cells. In this cell type, a saturating concentration of LPA alone is about equally potent as the synergistic combination of EGF with insulin or bombesin with insulin (Table 1).

To examine how the acyl-chain length at the *sn*-1 position of the glycerol backbone affects mitogenic potency, LPA analogues containing various fatty acid moieties were prepared and tested for their ability to stimulate DNA synthesis in quiescent fibroblasts. Fig. 1(a) shows the mitogenic dose-response relationships of various 1-acyl LPAs for serum-deprived Rat-1 cells.

Table 1. Effects of LPA and other mitogens on DNA synthesis in Rat-1 and Swiss 3T3 fibroblasts

Quiescent cells were stimulated with the indicated agonists for 22 h. [^3H]Thymidine was present during the last 6 h of stimulation, and incorporation was assayed as described in the Materials and methods section. Values represent means $\pm$ S.E.M. of three to six independent experiments (each performed in triplicate). Basal incorporation of [^3H]thymidine was 2910 ± 373 d.p.m./well for Rat-1 cells and 975 ± 232 d.p.m./well for Swiss 3T3 cells. Abbreviation: n.d., not determined.

Addition	[^3H]Thymidine incorporation (fold stimulation)	
	Control	+ Insulin (5 $\mu\text{g}/\text{ml}$)
Rat-1 cells		
None	1.0	2.6
LPA (18:1) (20 μM)*	28 ± 2	36 ± 1
EGF (0.5 ng/ml)	28 ± 5	31 ± 1
Endothelin (100 nM)†	3.1 ± 0.3	n.d.
FCS (10%, v/v)	31 ± 2	n.d.
Swiss 3T3 cells		
None	1.0	16 ± 1
LPA (18:1) (100 μM)	79 ± 28	103 ± 23
EGF (10 ng/ml)	4 ± 1	71 ± 14
Bombesin (100 nM)	28 ± 7	86 ± 16
FCS (10%, v/v)	103 ± 19	n.d.

* A sub-optimal concentration of LPA was chosen to give a stimulation similar to that of EGF.

† Endothelin is a potent inducer of phosphoinositide hydrolysis in Rat-1 cells (cf. Table 4, and ref. [26]).

Qualitatively similar results were obtained in mouse Swiss 3T3 cells and human fibroblasts (not shown). It is clear that the acyl-chain length markedly influences the relative potency to stimulate DNA synthesis. Although 1-oleoyl LPA (LPA, 18:1) is as potent as 1-palmitoyl LPA (LPA, 16:0), with threshold concentrations of about 1 μM and maximal effects at 50–100 μM , a further decrease in the acyl chain length to myristoyl (14:0), lauroyl (12:0) and decanoyl (10:0) causes a progressive decrease in mitogenic potency: 1-myristoyl LPA has approximately half the potency of the 1-palmitoyl and 1-oleoyl analogues, whereas 1-decanoyl LPA lacks significant activity.

Effects of ether-linked LPA and hexadecyl phosphate

A small proportion of naturally occurring phospholipids are ether (alkyl) derivatives, i.e. the fatty acid in the *sn*-1 position is linked to the glycerol moiety by an ether rather than an ester bond [17]. We compared the mitogenic potency of 1-*O*-hexadecylglycerol 3-phosphate with that of 1-palmitoyl LPA in Rat-1 cells. This particular ether analogue of LPA is of interest, since it has been reported to be considerably more potent in promoting platelet aggregation than the corresponding 1-acylated compound [18]. As summarized in Table 2, 1-*O*-hexadecylglycerol 3-phosphate is hardly mitogenic when compared with 1-palmitoyl LPA at concentrations up to 25 μM ; however, high concentrations of ether-linked LPA were found to be cytotoxic, as judged by cell detachment and loss of viability. Hexadecyl phosphate, which corresponds to 1-palmitoyl LPA lacking the glycerol backbone, does not possess growth-factor-like activity (Table 2). From these data it appears that an ester-linked long-chain fatty acid and a glycerol backbone are essential for mitogenic activity of LPA.

Fig. 1. Dose-response curves of LPA- and PA-induced DNA synthesis in Rat-1 cells

(a) Quiescent Rat-1 cells were incubated in serum-free DMEM with the indicated concentrations of t.l.c.-purified LPA analogues for 22 h. [^3H]Thymidine was present during the last 6 h of stimulation, and incorporation was measured as described in the Materials and methods section. Basal [^3H]thymidine incorporation corresponds to 4500 ± 800 d.p.m./well. Values represent means $\pm$ S.E.M. of three to six independent experiments (each performed in triplicate). (b) Quiescent Rat-1 cells were incubated with 1,2-dioleoyl PA and assayed for [^3H]thymidine incorporation as in Fig. 1(a). The results are expressed as percentage of the response to 100 μM -1-oleoyl LPA. Basal [^3H]thymidine incorporation was 2270 ± 580 d.p.m./well (means $\pm$ S.E.M., $n = 7$).

Mitogenic effects of phosphatidic acids

To evaluate the mitogenic effects of the 1,2-diacyl form (PA), we tested various PA derivatives. Their relative potencies in evoking DNA synthesis are summarized in Table 3, and the dose-response curve for 1,2-dioleoyl PA is shown in Fig. 1(b). Again, decreasing the acyl chain length at both the *sn*-1 and the *sn*-2 position leads to a progressive decrease in mitogenic activity. The 1-stearoyl 2-arachidonoyl analogue of PA is also a potent inducer of DNA synthesis (Table 3). Because PA is known to contain contaminating traces of lyso-derivatives [11], care was taken to use freshly prepared PA solutions in which contamination was always less than approx. 1% as judged by t.l.c. Since the dose-response curves of 1-oleoyl LPA and 1,2-dioleoyl PA for inducing DNA synthesis were found to be very similar (Figs. 1a and 1b), with almost identical threshold concentrations, the observed mitogenic effect of PA cannot simply be the consequence of LPA contamination. Furthermore, when low doses of LPA (1–10 μM) were added in the presence of 100 μM -PA, no potentiating effect on DNA synthesis was observed. Also, addition of

Table 2. Mitogenic effects of ether-linked LPA and hexadecyl phosphate in Rat-1 cells

Quiescent Rat-1 cells were stimulated with the indicated agonists for 22 h. [^3H]Thymidine was present during the last 6 h of stimulation, and incorporation was assayed as described in the Materials and methods section. Values represent means $\pm$ S.E.M. of three different experiments (each performed in triplicate). Basal [^3H]thymidine incorporation was 4300 ± 426 d.p.m./well.

Addition	Concn. (μM)	[^3H]Thymidine incorporation (fold stimulation)
LPA (16:0)	100	22 ± 3
1- <i>O</i> -Hexadecylglycerol 3-phosphate	1	1.3
	10	2.1 ± 0.5
	25	3.3 ± 0.8
	50	Cytotoxic
Hexadecyl phosphate	1	1.1
	10	2.2
	100	1.8 ± 0.2

equimolar amounts of LPA and PA (10–50 μM) resulted in additive rather than synergistic effects on DNA synthesis (results not shown).

Kinetics of LPA- and PA-induced DNA synthesis

To compare further the mitogenic action of LPA with that of PA, the kinetics of entry into S-phase was monitored. Fig. 2 shows the time-dependent incorporation of [^3H]thymidine into Rat-1 cells incubated with 100 μM -1-oleoyl LPA, 100 μM -1,2-dioleoyl PA or 10 ng of EGF/ml. Both LPA and PA stimulate DNA synthesis with an almost identical time course. The peak of thymidine incorporation evoked by LPA and PA occurs at approx. 20 h after stimulation, whereas EGF-induced DNA synthesis reaches its maximum between 15 and 16 h.

The ability of LPA and PA to stimulate DNA synthesis, like that of EGF, requires their continuous presence in the culture medium. As shown in Fig. 3, removal of LPA at 3 h after stimulation inhibits the ability of the cells to enter S-phase. Similar results were obtained with PA (not shown). Rat-1 cell-cycle progression induced by LPA and PA was also monitored by flow-cytometric analysis. As expected, more than 90 % of untreated control cells had a G_0/G_1 -phase DNA content. After

Fig. 2. Kinetics of entry into S-phase (Rat-1 cells)

Quiescent Rat-1 cells were stimulated with 100 μM -1-oleoyl LPA (●), 100 μM -1,2-dioleoyl PA (□) or 10 ng of EGF/ml (○) for the indicated periods of time. [^3H]Thymidine was present during the last 1 h of stimulation, and incorporation was assayed as described in the Materials and methods section. Results of a typical experiment (performed five times, each in triplicate) are shown: S.E.M. < 5 %.

24 h incubation with LPA, approx. 40 % of the cells had doubled their DNA content.

Effect of short-chain LPA on long-chain (L)PA-induced DNA synthesis

Given the very weak mitogenic activity of 1-decanoyl (L)PA, we examined these short-chain analogues for possible antagonistic effects on 1-oleoyl LPA- and 1,2-dioleoyl PA-induced DNA synthesis. When added at relatively high concentrations (100 μM), the decanoyl analogue showed no detectable inhibition of 1-oleoyl LPA-induced DNA synthesis (tested at 1, 10 and 100 μM in three independent experiments). Similarly, didecanoyl-PA failed to attenuate the mitogenic activity of dioleoyl PA.

Inhibitory effect of suramin on DNA synthesis

The polyanionic naphthalenesulphonic acid derivative suramin is a known inhibitor of growth-factor action. Although its mode of action is poorly understood, suramin is membrane-impermeant [19], and is known to interfere with the interaction between peptide growth factors and their receptors, with differential dose-sensitivities [20–25].

Table 3. Mitogenic potency of 1,2-diacyl PA in Rat-1 cells

Quiescent Rat-1 cells were stimulated with agonist for 22 h. [^3H]Thymidine was present during the last 6 h of stimulation, and incorporation was assayed as described in the Materials and methods section. Concentration of all analogues was 70 μM . Values represent means $\pm$ S.E.M. for the numbers of experimental data points given in parentheses.

Addition	[^3H]Thymidine incorporation (% of response to 1-oleoyl LPA)
LPA (1-oleoyl) (100 μM)	100 ± 14 (45)
PA (1-stearoyl 2-arachidonoyl)	97 ± 6 (12)
PA (dioleoyl)	83 ± 16 (41)
PA (distearoyl)	90 ± 19 (11)
PA (dipalmitoyl)	91 ± 9 (14)
PA (dimyristoyl)	55 ± 9 (14)
PA (dilauroyl)	35 ± 6 (16)
PA (didecanoyl)	8.8 ± 0.3 (3)
None	3.4 ± 0.2 (60)

Fig. 3. Effect of mitogen removal on DNA synthesis

Quiescent Rat-1 cells were stimulated with 1-oleoyl LPA (100 μM), EGF (10 ng/ml) or FCS (10 %, v/v). Mitogens (added at zero time) were washed out either after 3 h (■) or after 22 h (control; □). [^3H]Thymidine was added after 16 h, and at 22 h cells were assayed for DNA synthesis as described in the Materials and methods section. Values represent means $\pm$ S.E.M. ($n = 3$).

Fig. 4. Effect of suramin on LPA-, PA-, and EGF-induced DNA synthesis

(a) Rat-1 cells were stimulated with 100 μ M-1-oleoyl LPA (●), 100 μ M-1,2-dioleoyl PA (□) or 10 ng of EGF/ml (○) for 22 h, in the presence of the indicated amounts of suramin. [3 H]Thymidine was present during the last 6 h of stimulation. Values represent means $\pm$ S.E.M. of three to six experiments (each performed in triplicate). (b) Rat-1 cells were stimulated at zero time with either 100 μ M-1-oleoyl LPA (●) or 10 ng of EGF/ml (○) for 22 h. Suramin (0.5 mg/ml) was added at 0, 3 or 6 h after the agonist. [3 H]Thymidine was present during the last 6 h of stimulation. Values represent means $\pm$ S.E.M. ($n = 3$).

Strikingly, suramin inhibits 1-oleoyl LPA- and 1,2-dioleoyl PA-induced DNA synthesis in Rat-1 cells in a dose-dependent manner, as shown in Fig. 4(a). Half-maximal inhibitory concentrations (IC_{50}) are approx. 0.1 mg of suramin/ml (70 μ M), and 80–85% inhibition is obtained at 1.0 mg/ml (0.7 mM). Suramin does not inhibit EGF-induced DNA synthesis, at least over the

concentration range affecting LPA action (Fig. 4a). This finding is consistent with a previous report that the dose of suramin required to inhibit EGF-induced mitogenicity is much higher than that for other mitogens [23]. The effect of suramin is fully reversible, as inferred from the finding that cells pretreated with 1 mg of suramin/ml for 24 h, followed by wash-out, show a normal mitogenic response upon subsequent treatment with 1-oleoyl LPA (results not shown). Exposure of Rat-1 cells to suramin at various times after LPA addition reveals that the drug exerts its inhibitory action in the early G_0/G_1 -phase of the cell cycle: Fig. 4(b) shows that suramin progressively loses its effect when it is added to the cells at increasingly later time points after LPA. At 6 h after LPA addition, suramin is almost completely ineffective in attenuating LPA action (Fig. 4b).

Suramin inhibits LPA-induced phosphoinositide hydrolysis

We also examined the ability of suramin to inhibit the earliest detectable cellular response to LPA, i.e. the breakdown of phosphoinositides [8,11]. Exposure of [3 H]inositol-labelled cells to 100 μ M-1-oleoyl LPA in the presence of suramin (0.5 mg/ml) results in complete inhibition of inositol phosphate accumulation as measured over a 30 min period (Table 4). As a control, suramin fails to affect inositol phosphate production evoked by the peptide endothelin, a highly potent inducer of phosphoinositide hydrolysis in Rat-1 cells (Table 4; see also [26]). Together, these results suggest that suramin antagonizes long-term (L)PA mitogenicity by intervening with an early step in the (L)PA-activity pathway, most likely at the level of the plasma membrane.

DISCUSSION

The present studies were undertaken to investigate the structure-activity relationship of LPA-induced DNA synthesis in quiescent fibroblasts and to search for pharmacological antagonists. The structural modifications made involved changes in the hydrophobic fatty acid moiety of the LPA molecule. The results indicate that the naturally occurring long-chain LPAs ($C_{18:1}$ and $C_{18:0}$) are by far the most potent in inducing DNA synthesis, and that mitogenic activity is progressively decreased with decreasing acyl-chain length; the C_{10} molecule (decanoyl LPA) is almost completely devoid of biological activity. Our results also indicate that the ester linkage between the fatty acid and the glycerol backbone at the *sn*-1 position is essential for mitogenic activity. Modification of the ester linkage to an ether linkage largely abolishes mitogenic activity and, in fact, turns it into a cytotoxic compound. Ether-linked phospholipids follow metabolic routes that are different from those utilized by ester-linked phospholipids [27]. One possibility is that ether-linked LPA yields metabolic products that interfere with long-term cellular viability in general and stimulation of DNA synthesis in particular.

We found that, on a molar basis, long-chain PAs are about equally potent as the corresponding LPAs, with the mitogenic potency again being correlated with the length of the acyl chains. The concentrations required for stimulation of DNA synthesis are within the 1–100 μ M range. This is in marked contrast with LPA-induced Ca^{2+} mobilization, which is observed at far lower concentrations (10–500 nM; ref. [11]). Furthermore, PA fails to mimic LPA in the early Ca^{2+} -signalling response, even when added at micromolar concentrations [11]. In fact, previously reported Ca^{2+} -mobilizing effects of PA [6] are most likely due to contaminating lyso-derivatives [11]. Yet the possibility that the mitogenic responses observed with various diacyl PAs (Table 3) are similarly due to contaminating traces of LPA seems highly

Table 4. Effect of suramin on agonist-induced inositol phosphate formation in Rat-1 cells

[3 H]Inositol-labelled Rat-1 cells were stimulated with agonist for 30 min in the presence or absence of suramin. Formation of [3 H]inositol phosphates was measured in the presence of 10 mM-LiCl as described in the Materials and methods section. Control value was 1016 ± 41 d.p.m./well. Values represent means $\pm$ S.E.M. ($n = 3$): n.d., not determined.

Addition	Total [3 H]inositol phosphates (fold stimulation)	
	Control	+ Suramin (0.5 mg/ml)
None	1.0	1.7 ± 0.3
LPA (18:1) (100 μ M)	3.9 ± 0.3	1.1 ± 0.1
FCS (10%, v/v)	4.4 ± 0.1	n.d.
Endothelin (100 nM)	49.7 ± 0.7	52.6 ± 5.9

unlikely. First, when PA was purified by t.l.c. in a system which clearly separates it from LPA, the mitogenic response still persists. Second, a LPA contamination of at most 1% (as can be observed in commercial PA operations) cannot explain the remarkable similarity of the dose-response curves of LPA and PA. Third, addition of LPA in the presence of saturating amounts of PA does not further increase the magnitude of the mitogenic response. For these reasons, we feel safe to conclude that both LPA and PA have growth-factor-like properties with about equal potencies on a molar basis. The structure-activity data suggest that the degree of partitioning into the plasma-membrane bilayer is a major determinant of the mitogenic potencies of these phospholipids.

In a search for potential antagonists, we first focused on (L)PA analogues possessing the weakest mitogenic activity, i.e. the decanoyl derivatives. Although dilauroyl-PA has been reported to antagonize certain biological activities of long-chain diacyl-PAs [10,28], our results clearly indicate that the short-chain (L)PA analogues lack significant antagonist activity when tested in mitogenic assays. The polysulphonated compound suramin was found to inhibit both LPA- and PA-induced DNA synthesis in a reversible and dose-dependent manner ($IC_{50} \approx 70 \mu M$). The drug appears to act at an early point in the pre-replicative phase of the cell cycle and inhibits early biochemical responses to LPA, such as phosphoinositide hydrolysis; importantly, suramin does not interfere with cell proliferation and signal transduction in general, as evidenced by the results obtained with EGF and endothelin respectively. Suramin has previously been reported to inhibit growth-factor-receptor interactions, particularly those of platelet-derived growth factor, transforming growth factor- β and insulin-like growth factor-1 [20-23,25]. Suramin also dissociates low-density lipoprotein from its receptor [29] and inhibits P_2 -purinergic-receptor-mediated signal transduction, presumably at the receptor level [30,31]. Suramin does not cross cell membranes [19] and, although the drug may be taken up by endocytosis, our results support the view that suramin acts extracellularly rather than intracellularly. Suramin can form complexes with proteins, thereby exerting profound effects on protein tertiary structure, which might explain its ligand-displacing properties [32]. In view of these considerations, our findings obtained with suramin would agree with the hypothesis that (L)PA action is mediated by a specific cell-surface receptor. Caution is needed, however, since polyanionic compounds such as suramin may exert their effects by non-specifically binding to the cell surface. Furthermore, we cannot rule out that suramin may interact with the (L)PA molecule itself, rendering it biologically inactive. In a conventional t.l.c. experiment, however, presence of excess suramin did not interfere with the migration behaviour of 1-oleoyl LPA to any extent (E. J. van Corven, unpublished work).

The 'receptor hypothesis' is, of course, not necessarily incompatible with a 'bilayer partitioning' model to explain (L)PA action. Inserted long-chain (L)PA might diffuse laterally within the plane of the lipid bilayer and then have access to a putative receptor which could recognize the glycerol phosphate moiety or the phosphate head group. Although the identification of a putative LPA 'receptor' awaits further studies, the biochemical mechanisms behind LPA action have been elucidated in some detail [8]. It was reported that LPA-induced DNA synthesis is blocked by pertussis toxin and is thought to be initiated by inhibition of adenylate cyclase and, to a lesser extent, by stimulation of phosphoinositide-specific phospholipases C through G-proteins. Given the similarity between the mitogenic activities of LPA and PA (e.g. acyl-chain-length dependence, dose-response curves, kinetics of entry into S-phase), and in view of their similar sensitivity to pertussis toxin (E. J. van Corven,

unpublished work) and suramin, both phospholipids are likely to act through the same signalling pathways, apparently involving (a) pertussis-toxin-sensitive G-protein(s). However, the results obtained to date raise a number of questions to which there are only partial answers as yet. For example: (i) why do cells require much higher concentrations of LPA for DNA synthesis than for evoking early responses (cf. ref. [11])?; (ii) apart from possible interaction with a putative cell-surface receptor, is uptake and/or metabolic conversion of LPA and PA essential for long-term mitogenicity? {however, the major metabolic breakdown products of LPA and PA (monoacylglycerol and diacylglycerol respectively; [33], and R. van der Bend, unpublished work) lack any mitogenic activity in our cell system [8]; (iii) does native endogenously produced (L)PA at the plasma membrane act in a similar manner to exogenously supplied (L)PA to stimulate DNA synthesis? By using (L)PA analogues with non-hydrolysable phosphonate headgroups, together with micro-injection studies, these questions can be examined in further detail.

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