Lysophosphatidic acid-induced Ca^{2+} mobilization in human A431 cells: structure–activity analysis

Kees JALINK,* Trudi HENGVELD,* Sipko MULDER,* Friso R. POSTMA,* Marie-Francoise SIMON,† Hugues CHAP,† Gijs A. van der MAREL,‡ Jacques H. van BOOM,‡ Wim J. van BLITTERSWIJK* and Wouter H. MOOLENAAR*§

*Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands, †INSERM Unite 326, Hôpital Purpan, Toulouse, France, and §Gorlaeus Laboratories, P.O. Box 9502, 2300 AA Leiden, The Netherlands

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycero-3-phosphate) is a platelet-derived lipid mediator that activates its own G-protein-coupled receptor to trigger phospholipase C-mediated Ca^{2+} mobilization and other effector pathways in numerous cell types. In this study we have examined the structural features of LPA that are important for activation of the Ca^{2+} -mobilizing receptor in human A431 carcinoma cells, which show an EC_{50} for oleoyl-LPA as low as 0.2 nM. When the acyl chain at the *sn*-1 position is altered, the rank order of potency is oleoyl-LPA > arachidonoyl-LPA > linolenoyl-LPA > linoleoyl-LPA > stearoyl-LPA = palmitoyl-LPA > myristoyl-LPA. The shorter-chain species, lauroyl- and decanoyl-LPA, show little or no activity. Ether-linked LPA (1-*O*-hexadecyl-*sn*-glycero-3-phosphate) is somewhat less potent than the corresponding ester-linked LPA; its stereoisomer is about equally active. Deletion of the glycerol backbone causes a 1000-fold decrease in potency. Replacement

of the phosphate group in palmitoyl-LPA by a hydrogen- or methyl-phosphonate moiety results in complete loss of activity. A phosphonate analogue with a methylene group replacing the oxygen at *sn*-3 has strongly decreased activity. All three phosphonate analogues induce cell lysis at doses > 15 μM . Similarly, the methyl and ethyl esters of palmitoyl-LPA are virtually inactive and become cytotoxic at micromolar doses. None of the LPA analogues tested has antagonist activity. Sphingosine 1-phosphate, a putative messenger with some structural similarities to LPA, elicits a transient rise in intracellular $[\text{Ca}^{2+}]$ only at micromolar doses; however, cross-desensitization experiments indicate that sphingosine 1-phosphate does not act through the LPA receptor. The results indicate that, although many features of the LPA structure are important for optimal activity, the phosphate group is most critical, suggesting that this moiety is directly involved in receptor activation.

INTRODUCTION

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycero-3-phosphate) is a novel lipid mediator with a wide range of biological activities, including stimulation of cell proliferation, smooth-muscle contraction, platelet aggregation, neurite retraction and cell motility (for review see [1]). On intravenous injection into animals, LPA elicits strong vasoactive effects and disturbances in respiration [2,3]. LPA is produced and released by activated platelets (and possibly other cells) and is a normal constituent of serum, where it is present at 1–5 μM in an albumin-bound form [1,4,5]. LPA activates its own G-protein-coupled receptor, resulting in stimulation of phospholipase C, with consequent Ca^{2+} mobilization from internal stores and activation of protein kinase C [6,7]. In addition, LPA inhibits adenylate cyclase and stimulates the formation of p21^{ras} -GTP in a pertussis-toxin-sensitive manner [6,8]. A candidate LPA cell-surface receptor of apparent molecular mass 38–40 kDa has been identified by photoaffinity labelling in various LPA-responsive cell types and in membranes from rat brain [9]. Labelling of the putative LPA receptor is competitively inhibited by unlabelled LPA with an IC_{50} of about 10 nM. Scatchard analysis of LPA binding to membranes from PC12 cells, 3T3 fibroblasts and rat brain has confirmed the presence of saturable binding sites for LPA, with calculated K_d values in the lower nanomolar range [10,11].

Although previous studies have revealed the specificity of LPA

as a physiological agonist in that related (lyso)lipids fail to mimic the rapid cellular effects of LPA [5,7], to date very little is known about the exact structural features of LPA that are necessary for receptor activation. Structure–activity analysis is an important first step toward elucidating how LPA interacts with its cognate receptor and, furthermore, should help to identify novel lipid mediators that are structurally similar to LPA but may have different biological potencies. Moreover, structure–activity studies are of obvious importance for the design or discovery of specific LPA antagonists.

The present study was undertaken to investigate the structure–activity relationship of LPA as a Ca^{2+} -mobilizing receptor agonist. To select a suitable model system, we first screened a variety of cell types for their ability to generate a transient Ca^{2+} signal in response to low doses of 1-oleoyl-LPA. Human A431 epidermoid carcinoma cells were found to be extremely sensitive (EC_{50} as low as 0.2 nM), and therefore were used for structure–activity analysis. We were particularly interested in testing how modifications at the 3-phosphate group affect LPA's activity. In addition, we have examined the effects of varying the length and nature of the fatty acid chain and changing the acyl ester bond into an ether linkage. We find that LPA tolerates various modifications in its acyl chain without losing activity, but that relatively small alterations in the phosphate group lead to inactive molecules. The results are discussed with respect to a model of LPA–receptor interaction.

Abbreviations used: LPA, lysophosphatidic acid; $[\text{Ca}^{2+}]_i$, intracellular $[\text{Ca}^{2+}]$; PA, phosphatidic acid; PAF, platelet-activating factor; L-HDGP, 1-*O*-hexadecyl-*sn*-glycero-3-phosphate; D-HDGP, 3-*O*-hexadecyl-*sn*-glycero-1-phosphate; HDMGP, 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphate; HDP, hexadecyl phosphate; diazirin-LPA, 11-[4-[3-(trifluoromethyl)-diaziriny]-phenyl]-undecanoyl-LPA; S-1-P, sphingosine 1-phosphate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum.

§ To whom correspondence should be addressed

EXPERIMENTAL

Materials

The various analogues of LPA and other lipids were either obtained from commercial sources (Sigma, Avanti Polar Lipids, or Serdary Research Laboratories, London, Ontario, Canada) or prepared enzymically by procedures described below. 1-*O*-Hexadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (HDMGP-choline) was from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Hexadecyl phosphate (HDP) was kindly provided by Dr. R. C. Young (SmithKline Beecham Research, Welwyn, U.K.). Fatty-acid-free BSA, sphingosylphosphocholine, phospholipase A₂ (from pig pancreas or *Naja naja*), phospholipase D (from cabbage, type 1, or from *Streptomyces chromofuscus*) and bradykinin were from Sigma, and Indo-1 acetoxymethyl ester (Indo-1/AM) was from Molecular Probes (Eugene, OR, U.S.A.). *myo*-[2-³H]inositol was from Amersham.

Cells

Human A431 epidermoid carcinoma cells and the various other cell types used were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% (v/v) fetal-calf serum (FCS).

Lipid synthesis and handling

LPAs with various acyl chain types were prepared enzymically either from the corresponding lysophosphatidylcholines by using phospholipase D, or from commercially available phosphatidic acid by using phospholipase A₂ as previously described [12]. The structures of the phosphonate analogues used in the present study are shown in Figure 1. Synthesis of 1,2-dipalmitoyl-*sn*-glycerol-3-H-phosphonate was carried out by the protocol described in Elie et al. [13], and that of 1,2-dipalmitoyl-*sn*-glycerol-

3-methylphosphonate and 1,2-dipalmitoyl-*sn*-glycerol-methylenephosphonate as described by Dreef et al. [14,15]. The corresponding lyso-phosphonates were prepared by phospholipase A₂ digestion, essentially similar to the protocol described by van Corven et al. [12]. The general procedure for preparing 1-*O*-hexadecyl-*sn*-glycero-3-phosphate (L-HDGP) and its enantiomer (D-HDGP) have been described previously [16]. HDMGP was prepared from HDMGP-choline by phospholipase D digestion. The methyl and ethyl esters of LPA were prepared by phospholipase D treatment of the corresponding lysophosphatidylcholine species in the presence of methanol or ethanol, respectively. Sphingosine 1-phosphate (S-1-P) was prepared by phospholipase D digestion of sphingosylphosphocholine as described in [17] and quantified by phosphorus determination. Diazirine-LPA was synthesized as previously described [9].

All analogues were surveyed for purity by TLC or GLC as detailed below. LPA preparations showed up to 2% acyl chain heterogeneity, with the relative amount of C₁₈-LPAs present in the other LPA species being less than 0.3%. To remove long-acyl-chain LPA from the bulk material, 1-myristoyl-LPA and 1-lauroyl-LPA were subjected to two consecutive runs of preparative HPLC (see below). This decreased the long-chain content of the preparations by 50–100-fold, as revealed by GLC. Other purifications were performed by TLC. Lipids were stored at –20 °C in chloroform or in chloroform/methanol (2:1, v/v) in glass vials under argon. For experiments, compounds were either dissolved to clear solutions or dispersed by bath-sonication at final concentrations of 1–10 mg/ml before delivery to the cells. We note that several precautions must be taken when using LPA-related lipids in bioassays. Adsorption of LPA to plastic and glassware is a serious source of variation when using diluted stock solutions. For example, 75% of a 5 μM stock solution of 1-oleoyl-[³²P]LPA was found stuck tightly to the wall of a plastic Eppendorf vial within 30 min. This radioactivity could not be dissolved with excess medium, but was completely recoverable with chloroform/methanol (K. Jalink, T. Hengeveld and R. van der Bend, unpublished work). Although LPA is quite stable in solution, stock solutions of other (lyso)lipids, including PA, are more prone to degradation and may contain significant amounts of LPA. Therefore, diluted stock solutions of LPA and its analogues were prepared immediately before experimentation.

TLC

Standard TLC was performed on activated precoated silica-gel 60 plates (Merck) with the solvent system chloroform/methanol/water/acetic acid (50:40:4:3, by vol.). Phospholipids were revealed by using either I₂ or primuline staining. LPA from commercial sources was found to be at least 99% pure, whereas the diacyl-phospholipid preparations often contained significant amounts of lyso-derivatives. Phospholipids were purified by TLC, scraped into glass vials and extracted with three subsequent washes with chloroform/methanol (2:1, v/v; acidified in the case of acidic phospholipids), followed by evaporation under nitrogen. Recovery of material was better than 95%, as determined by inclusion of a radio-labelled tracer.

GLC

For analysis of the acyl-chain composition, LPA was subjected to trans-esterification with 0.1 M dry methanolic NaOH at 80 °C for 4 h. The resulting fatty acid methyl esters were extracted with *n*-hexane and analysed with a Carlo Erba Strumentazione gas chromatograph (type HRGC 5300), equipped with a flame ionization detector. The stationary phase was 10% CP-Sil 58 in

Figure 1 Structures of the phosphonate analogues of LPA

R denotes the hydrocarbon chain at the *sn*-1 position. In all phosphonate analogues tested, R is palmitoyl (16:0).

a glass column (2500 cm $\times$ 0.32 cm). The peaks were identified by comparison with fatty acid methyl ester standards from Applied Science.

Reverse-phase HPLC

For HPLC purification of LPA preparations, 0.2–1 mg of material was chromatographed at room temperature on a μ Bondapak phenyl column (300 mm $\times$ 3.9 mm; Waters Associates, Milford, MA, U.S.A.). Isocratic elution was performed with methanol/water/acetonitrile/acetic acid (260:140:8:1, by vol.) at a flow rate of 1 ml/min. Lipids were detected by UV absorption at 206 nm, with a Spectroflow 757 UV detector.

Measurements of intracellular $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$)

Cells were plated on rectangular glass coverslips (30 mm $\times$ 8 mm) and cultured in DMEM containing 7.5% FCS for 2–4 days. When $> 70\%$ confluent, cells were serum-starved for 12–24 h. The cells were loaded with Indo-1 by incubating them in serum-free DMEM containing 5 μ M Indo-1/AM ester for 20–30 min. Cells were then transferred to quartz cuvettes and incubated in Hepes-buffered saline containing (mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Hepes (pH 7.40), at 37 $^{\circ}C$. Fluorescence measurements were carried out at 37 $^{\circ}C$ with a Perkin–Elmer LS3 spectrofluorimeter, with an excitation wavelength of 355 nm (5 nm slit) and emission wavelength of 405 nm (10 nm slit). $[Ca^{2+}]_i$ changes were expressed as the relative increase in Ca^{2+} -dependent Indo-1 fluorescence, $\Delta F/F$, as described previously [7]. These ratio values correlate well with peak $[Ca^{2+}]_i$ values and can be readily determined without time-consuming ionophore-based calibrations. For active LPA analogues, $\Delta F/F$ data ($n > 15$) were computer-fitted to a logistic sigmoid; 95% confidence intervals for calculated EC_{50} values ranged from $\pm 15\%$ to maximally $\pm 35\%$.

Determination of inositol phosphates

Nearly confluent A431 cells in six-well tissue-culture dishes were labelled to isotopic equilibrium with 2 μ Ci/ml of *myo*- $[^3H]$ inositol for 2 days in DMEM containing 1% FCS. At 2 h before stimulation the cells were shifted to serum-free DMEM and the cells were stimulated with agonist for 30 min in the presence of 10 mM LiCl. The reactions were stopped with ice-cold 10% (w/v) trichloroacetic acid and the supernatant was extracted with diethyl ether. Samples were processed for analysis of total $[^3H]$ inositol phosphates (IP_n) as previously described [18].

RESULTS

We paid particular attention to the choice of cell type for structure–activity analysis of LPA-induced Ca^{2+} mobilization. We wanted a cell type (i) that is highly sensitive to natural LPA and (ii) in which the fluorescent Ca^{2+} dye Indo-1 is readily taken up, hydrolysed and retained well within the cytosol. We set out to test a variety of cell types for their ability to generate a rapid, transient, Ca^{2+} signal in response to 1-oleoyl-LPA (0.1–1 μ M). Cells were deprived of serum for at least 4 h and then loaded with Indo-1. As summarized in Table 1, cells as diverse as normal and transformed fibroblasts, epithelial cells, carcinoma cells, osteoblasts and neuronal cells show a rapid Ca^{2+} signal after LPA addition. Cell types that failed to respond to LPA, even at doses as high as 10 μ M, include human neutrophils, HL60 leukaemic cells, rat mast cells and human HepG2 hepatoma cells [7]; K. Jalink and W. H. Moolenaar, unpublished work). Among the various cell types tested, we found human A431 epidermoid carcinoma cells to be at least 10-fold more sensitive to 1-oleoyl-

Table 1 LPA-responsive cell types, as determined by Ca^{2+} mobilization from internal stores

Cells were loaded with Indo-1 and then exposed to 0.1–1 μ M oleoyl-LPA. For details see the Experimental section. Unresponsive cell types are mentioned in the Results section.

Fibroblasts	Neuronal cells
Human foreskin	PC12 rat pheochromocytoma
Human lung (WI-38)	N1E-115 mouse neuroblastoma
Rat-1	NG108-15 neuroblastoma $\times$ glioma
v-Src-transformed Rat-1	IMR-32 human neuroblastoma
H-Ras-transformed Rat-1	
Mouse 3T3 (Swiss and NIH)	
Mouse C ₃ H/10T $\frac{1}{2}$	Human K562 erythroleukaemic cells
Chinese-hamster ovary (CHO) cells	Human Jurkat leukaemic cells
Monkey COS cells	Human mesangial cells
Chicken osteoblasts	
Human osteosarcoma cells	
Human 293 kidney cells	
Human A431 epidermoid carcinoma cells	
Human HeLa carcinoma cells	

LPA than were other cell types, such as human fibroblasts (see below). Furthermore, A431 cells were found to hydrolyse and retain Indo-1 very well. An additional advantage is that A431 cells express Ca^{2+} -mobilizing receptors for the bioactive peptide bradykinin [19], which serves as a convenient control agonist. We therefore used A431 cells for examining the structure–activity relationship of LPA.

The LPA-induced Ca^{2+} signal in A431 cells

The average resting $[Ca^{2+}]_i$ in confluent Indo-1-loaded A431 cells is 105 ± 27 nM ($n = 51$). Figure 2(a) shows a typical $[Ca^{2+}]_i$ transient evoked by addition of a saturating dose of 1-oleoyl-LPA (1 μ M). The Ca^{2+} signal is initiated within seconds and reaches its peak within 6–15 s of LPA addition. Over the next few minutes, $[Ca^{2+}]_i$ gradually returns to its pre-stimulation level. Peak $[Ca^{2+}]_i$ values induced by 1 μ M 1-oleoyl-LPA ranged from 530 to 2300 nM in 51 independent experiments (871 ± 270 nM, mean $\pm$ S.D.). The response to LPA is subject to homologous desensitization since a second challenge results in a greatly attenuated signal, whereas the response to the unrelated agonist bradykinin is not affected (Figure 2a). Conversely, prior stimulation of A431 cells with bradykinin did not affect the LPA-induced Ca^{2+} signal (not shown). As was previously found with human fibroblasts [7], freshly purified PA (dioleoyl or dipalmitoyl forms) fails to induce Ca^{2+} mobilization in A431 cells, either when suspended in water or when dissolved in a complex with fatty-acid-free BSA.

The LPA-induced increase in $[Ca^{2+}]_i$ is only marginally decreased when extracellular Ca^{2+} is chelated with excess EGTA (3 mM; results not shown). As expected, it is accompanied by enhanced phosphoinositide hydrolysis, as evidenced by a several-fold increase in the accumulation of total inositol phosphates in Li^+ -pretreated cells (control, 2350 ± 145 c.p.m.; after addition of 1 μ M LPA, 6490 ± 409 c.p.m.; $n = 3$). It thus appears that the LPA-induced Ca^{2+} signal in A431 cells, as in fibroblasts [6,7], is

Figure 2 LPA-induced Ca^{2+} mobilization in human A431 cells

(a) Typical Ca^{2+} signals in response to sequential additions of 1-oleoyl-LPA and bradykinin (BK) as indicated. (b) Dose-response relationship of 1-oleoyl-LPA-induced Ca^{2+} mobilization, as determined from peak $\Delta F/F$ values (see the Experimental section). Data are presented as means $\pm$ S.E.M.

Figure 3 Dependence of LPA-induced Ca^{2+} mobilization on acyl chain type

Peak values of $\Delta F/F$ as a function of concentration are shown for oleoyl-LPA (●), palmitoyl-LPA (○), myristoyl-LPA (◆), lauroyl-LPA (△) and decanoyl-LPA (□). Dose-responses for other analogues are omitted for sake of clarity. See Table 2 for EC_{50} values.

phospholipase C-mediated and originates largely from internal stores.

The magnitude of the 1-oleoyl-LPA-induced Ca^{2+} signal shows a dose-response relationship with a threshold as low as 20–30 pM, an EC_{50} of 0.2 nM and maximal responses observed at approx. 10 nM (Figure 2b). These values are 100–200-fold lower than those for human fibroblasts, which show an EC_{50} for 1-oleoyl-LPA of about 40 nM [7].

Variations in the acyl chain

To examine how the nature and length of the acyl chain affects the potency of LPA, we tested LPA analogues with different

Table 2 Activity of LPA as a function of the composition and conformation of the hydrophobic chain

For active LPA analogues, $\Delta F/F$ data ($n > 15$) were computer-fitted to a logistic sigmoid; 95% confidence intervals for calculated EC_{50} values ranged from $\pm 15\%$ to maximally $\pm 35\%$. For further details see the Experimental section.

Acyl chain	EC_{50}	Remarks
1-Arachidonoyl (20:4)	0.9 nM	
1-Stearoyl (18:0)	40	Mixed with fatty-acid-free BSA (1:1 molar ratio)
1-Oleoyl (18:1)	0.2	Independent of presence of BSA
1-Linoleoyl (18:2)	12	
1-Linolenoyl (18:3)	2	
1-Palmitoyl (16:0)	20	
1- <i>O</i> -Hexadecyl (L-HDGP)	90	
3- <i>O</i> -Hexadecyl (o-HDGP)	105	
1- <i>O</i> -Hexadecyl-2- <i>O</i> -methyl (HDMGP)	75	
1-Myristoyl (14:0)	140	
1-Lauroyl (12:0)	> 25 μM	Estimated by extrapolation; max. dose 500 μM
1-Decanoyl (10:0)		Inactive; max. dose 500 μM
1-Diazirine-undecanoyl (11:0)	12 nM	Photoreactive LPA

acyl-chain lengths and varying degrees of unsaturation for Ca^{2+} mobilization in A431 cells. From the dose-response curves in Figure 3 and the EC_{50} values in Table 2, it is clear that a decrease in chain length results in decreased potency. It is also evident that there is no strict correlation between the number of double bonds in the acyl chain and the potency of LPA. Of the various LPA analogues tested, 1-oleoyl-LPA is by far the most potent one. Because of the high potency of 1-oleoyl-LPA, care was taken to use freshly prepared solutions of the shorter-chain LPAs and, if necessary, to purify them by preparative HPLC (see the Experimental section). HPLC-purified preparations of myristoyl-LPA and lauroyl-LPA were tested for the induction of a Ca^{2+} response, and the EC_{50} value did not differ from that of the unpurified starting material, within experimental error. Therefore, the right-shifted dose-response relationship for short-chain LPAs indeed reflects a lower potency rather than contamination of an inactive LPA species with 1-oleoyl-LPA.

The observed rank order of potency is: 1-oleoyl-LPA (18:1) > 1-arachidonoyl-LPA (20:4) = 1-linolenoyl-LPA (18:3) > 1-linoleoyl-LPA (18:2) > 1-stearoyl-LPA (18:0) = 1-palmitoyl-LPA (16:0) > 1-myristoyl-LPA (14:0). Shorter-chain LPAs are almost devoid of activity (Figure 3 and Table 2). From these results it appears that there is no strict correlation between the number of double bonds in the acyl chain and LPA's potency. Maximally effective concentrations of shorter-chain LPAs desensitize the cells to subsequent addition of 1-oleoyl-LPA, and vice versa, indicative of identical sites of action (results not shown).

It should be noted that 1-stearoyl-LPA, unlike the other LPAs, is water-insoluble and yields turbid stock solutions even after sonication. In this physicochemical state, 1-stearoyl-LPA has no effect on $[\text{Ca}^{2+}]_i$ in A431 cells. A similar lack of effect has been reported in $\text{C}_3\text{H}/10\text{T}_1\frac{1}{2}$ fibroblasts [20], although in other cells 1-stearoyl-LPA readily elicits biological effects at nanomolar concentrations [5]. When 1-stearoyl-LPA was mixed with fatty-

Figure 4 Ca^{2+} mobilization by ether-linked LPAs

(a) Typical Ca^{2+} signals induced by sequential addition of L-HDGP, 1-oleoyl-LPA and bradykinin (BK), at the doses indicated. (b) Desensitization of the response to both the L- and D-stereoisomers of HDGP by prior addition of 1-oleoyl-LPA.

acid-free BSA (1:1 molar ratio) or a mild detergent (mono-acylglycerol, which lacks LPA-like activity), the lipid remained in solution, and evoked a Ca^{2+} response, with an EC_{50} of about 40 nM. Thus the mode of administration of 1-stearoyl-LPA may greatly influence its biological activity. In marked contrast, the presence of BSA did not significantly affect the EC_{50} value for 1-oleoyl-LPA (Table 2).

Ether-linked LPA analogues

A small proportion of natural glycerol-phospholipids contains an ether linkage at the *sn*-1 position rather than an ester bond. Of particular interest is 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor, PAF), a potent lipid mediator, and its precursor lyso-PAF. Both lyso-PAF and PAF are rapidly produced in certain stimulated cells and might serve as precursors for ether-linked LPA. We examined the effects of both lyso-PAF and its phospholipase D-generated breakdown product, 1-*O*-hexadecyl-*sn*-glycero-3-phosphate (HDGP).

Lyso-PAF (like PAF) is completely inactive, but HDGP induces a Ca^{2+} transient originating from intracellular stores in A431 cells with similar kinetics to that induced by 1-palmitoyl-LPA (Figure 4 and Table 2). The EC_{50} for HDGP is about 90 nM, which implies that ether-linked LPA is about 4-fold less potent than the corresponding ester-linked LPA (Table 2). The response to HDGP exhibits homologous desensitization (Figure 4), and the ether lipid specifically desensitizes the cells to subsequently added ester-linked LPA.

We also tested the enantiomer of 'natural' ether-linked LPA (3-*O*-hexadecyl-*sn*-glycero-1-phosphate, D-HDGP) for biological

activity. It was found that both isomers have similar potencies. In other words, reversion of the chirality at the *sn*-2 position of the glycerol backbone does not lead to altered activity. This result is in line with earlier studies on LPA-induced platelet aggregation, where it was found that ether-linked LPA (HDGP) does not display significant stereospecificity [16].

In addition, we studied the influence of replacing the free hydroxyl at the *sn*-2 position by an ether-linked methyl group, which yields 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphate (HDMGP). HDMGP was about equally potent as its lyso-derivative, HDGP, in inducing Ca^{2+} transients ($\text{EC}_{50} \sim 75$ nM; Table 2). Addition of HDMGP renders the cells unresponsive to subsequent addition of either ester-linked LPA or ether-linked LPA at their maximally effective concentrations. These results indicate that a free hydroxyl group at the *sn*-2 position is not an absolute requirement for activity.

Acyl chain tagged with a diazirine group

We reported previously that an LPA analogue containing a photoreactive group attached to a $\text{C}_{11:0}$ acyl chain, 11-[4-[3-(trifluoromethyl)diaziriny]-phenyl]-undecanoyl-LPA (diazirine-LPA), can evoke a Ca^{2+} signal in A431 cells [9]. Furthermore, pretreatment of the cells with diazirine-LPA fully inhibits the response to 1-oleoyl-LPA, but not to bradykinin [9]. In the present study, the EC_{50} for diazirine-LPA-induced Ca^{2+} mobilization in A431 cells was found to be 12 nM, which is very close to the value for 1-palmitoyl-LPA. It thus appears that the terminal four to six hydrocarbons in the fatty acid chain of natural LPA can be replaced by an aromatic ring structure without significant loss of biological activity. Taken together, our results show that the length of the acyl chain determines the potency of LPA as a receptor ligand, but that at a given chain length the exact nature of the acyl chain is not very critical.

Deletion of the glycerol backbone

Deletion of the glycerol backbone of 1-palmitoyl-LPA yields hexadecyl phosphate (HDP), in which the phosphate moiety is directly tethered to a C_{16} hydrocarbon chain. Compared with 1-palmitoyl-LPA, the potency of this molecule is decreased by three orders of magnitude (EC_{50} approx. 45 μ M; Table 3). HDP-induced $[\text{Ca}^{2+}]_i$ transients were inhibited when the cells were first challenged with LPA (1 μ M; results not shown). Thus the glycerol backbone appears to be an important determinant of activity.

Modifications at the phosphate group: phosphonate analogues, methyl and ethyl esters of LPA

To examine the importance of the 3-phosphate group, three different phosphonate analogues of 1-palmitoyl-LPA were prepared and assayed as potential agonists or antagonists. In these analogues, one of the phosphorous oxygens is replaced by a hydrogen (-H), a methyl (- CH_3) group, or a methylene (- CH_2 -) moiety, as illustrated in Figure 1. Both the H- and methyl-phosphonates were completely inactive in inducing $[\text{Ca}^{2+}]_i$ transients at doses up to 10 μ M (Table 3), in either the presence or the absence of fatty-acid-free BSA. At these high doses, only the methylene-phosphonate analogue elicited a small and rather variable Ca^{2+} signal, but proper dose–response analysis was impossible because of the lytic properties of the molecule. In fact, all three phosphonates induce acute membrane damage/leakiness when added at concentrations > 15 μ M, as evidenced by Trypan Blue uptake and the total quenching of Indo-1 fluorescence by Mn^{2+} (results not shown); cell lysis is not observed with natural LPA even at doses as high as 0.5 mM [7]. When assayed over the

Table 3 Potency of LPA analogues

Compound	Max. concn. tested EC ₅₀	(μ M)	Remarks
1-Oleoyl-glycerol	—*	500	
Glycerol 1-phosphate	—*	500	
PAF	—*	200	Lytic† at 50 μ M
Lyso-PAF	—*	250	Lytic† at 35 μ M
HDP	Approx. 45 μ M	100	Poorly soluble (turbid suspension)‡
Methyl-LPA	> 20 μ M	100	Lytic† at 25 μ M
Ethyl-LPA	—*	100	Lytic† at 20 μ M
H-phosphonate	—*	100	Lytic† at 25 μ M
Methyl-phosphonate	—*	50	Lytic† at 15 μ M
Methylene-phosphonate	> 15 μ M	50	Lytic† at 15 μ M
S-1-P	Approx. 6 μ M	100	Poorly soluble (turbid suspension)‡

* No effect over concentration range tested.

† Cell lysis is detected after 10–20 s, whereas Ca²⁺ transients are observed within 1–2 s.

‡ EC₅₀ values for poorly soluble analogues are of limited value. See the text for further details.

concentration range 0.1–10 μ M, none of the phosphonate analogues was capable of attenuating the response to 1-oleoyl-LPA or 1-palmitoyl-LPA.

We also tested the methyl and ethyl esters of 1-palmitoyl-LPA for possible agonist or antagonist activity. Lysophosphatidyl-methanol and lysophosphatidyl-ethanol (designated methyl-LPA and ethyl-LPA respectively) were found to be virtually inactive at all concentrations tested (Table 3), with cell lysis being observed at about 20 μ M. Again, these analogues did not desensitize the cells to subsequent addition of natural LPA. Taken together, these data emphasize the importance of the phosphate moiety for LPA receptor activation.

Sphingosine 1-phosphate (S-1-P)

The sphingolipid metabolite S-1-P has recently attracted interest as a potential signalling molecule [21,22]. S-1-P has some structural resemblance to LPA in that it contains a long hydrocarbon chain linked to a terminal phosphate group. When added exogenously to fibroblasts, S-1-P promotes cell proliferation as well as Ca²⁺ release from intracellular stores [21–23]. Furthermore, S-1-P is a potent inhibitor of tumour cell motility [24], but its mode of action is not well understood.

We found that in A431 cells S-1-P induces a rapid Ca²⁺ transient, which is not significantly attenuated in the presence of excess EGTA. Dose–response analysis indicates that S-1-P is about three orders of magnitude less potent than long-chain LPA (EC₅₀ = 6 μ M; Figure 5 and Table 3). Although precise determination of the EC₅₀ value for S-1-P is hampered by its very poor water-solubility, the S-1-P dose-dependence in A431 cells is in good agreement with that reported for S-1-P-induced Ca²⁺ mobilization in Swiss 3T3 cells (EC₅₀ = 2 μ M; [22]). Although S-1-P-induced Ca²⁺ mobilization in A431 cells is subject to homologous desensitization, there is complete lack of cross-desensitization with LPA (Figure 5). From these results we conclude that S-1-P is not an agonist of the LPA receptor.

Figure 5 Effects of S-1-P

(a) Effect of sequential addition of S-1-P and 1-oleoyl-LPA on [Ca²⁺]_i in A431 cells. Note homologous desensitization of the response to S-1-P and lack of cross-desensitization between S-1-P and LPA. (b) Reversal of the order of agonist addition, again showing lack of cross-desensitization.

DISCUSSION

There is compelling evidence that the platelet-derived lipid mediator LPA acts on its own G-protein-coupled receptor present in various cell types and tissues [1,9–11]. Although much has been learned in recent years about the receptor-mediated signalling events triggered by LPA, little is known about LPA's structure–activity relationship. Previous structural studies have examined how the acyl chain composition affects such 'late' (and perhaps indirect) responses to LPA as platelet aggregation, blood-pressure changes and fibroblast proliferation [3,12,25]. From those earlier studies it has emerged that a long hydrophobic chain in the LPA molecule is a prerequisite for biological activity. In the present study, we have tested various structural analogues of LPA for their ability to activate the Ca²⁺-mobilizing receptor in A431 human carcinoma cells. A431 cells are particularly suited for structure–activity analysis because of their high sensitivity to LPA, with EC₅₀ values for long-chain LPA in the lower nanomolar range.

Our results show that the specificity of LPA action is most critically directed towards the phosphate group of the molecule. We find that three different phosphonate analogues of 1-palmitoyl-LPA are completely inactive, even when added in micromolar doses. In these phosphonates, a single oxygen in the phosphate group is replaced by isosteric groups (methyl or methylene) or a hydrogen atom. Furthermore, neither the methyl nor the ethyl ester of 1-palmitoyl-LPA is capable of receptor activation. None of these inactive LPA analogues was capable of antagonizing the effect of natural LPA. However, at doses > 10–20 μ M, the phosphonate and esterified analogues of LPA were cytotoxic due to acute membrane damage.

Somewhat less specificity is directed towards the fatty acid chain of LPA. Although a long chain (C_{16} , C_{18} or C_{20}) is necessary for agonist activity, the molecule does tolerate certain variations in chain length, degree of unsaturation and type of bond to the glycerol moiety. At a given chain length (C_{18}), unsaturated LPA is more potent than the saturated species, but it is clear that there is no strict correlation between the number of double bonds in the acyl chain and LPA's potency. As a rule, a decrease in the length of the acyl chain leads to decreased activity. The observed rank order of potency is: 1-oleoyl-LPA (18:1) > 1-arachidonoyl-LPA (20:4) = 1-linolenoyl-LPA (18:3) > 1-linoleoyl-LPA (18:2) > 1-stearoyl-LPA (18:0) = 1-palmitoyl-LPA (16:0) > 1-myristoyl-LPA. Shorter-chain LPAs are almost devoid of activity (Table 3). This relationship between chain length and biological activity is in qualitative agreement with data of van Corven et al. [12] for LPA-induced DNA synthesis, and with limited data on platelet aggregation [25], LPA-induced cardiovascular effects in cats [26] and the induction of Cl^- inward currents in *Xenopus* oocytes [5]. The principal species of LPA produced in activated platelets are 1-palmitoyl-, 1-stearoyl-, 1-arachidonoyl- and 1-oleyl-LPA, occurring in the mass proportions of approx. 4:4:2:1, respectively [27]. These LPA species are also present in serum, although precise analysis awaits further studies [5]. A quite different LPA composition is found in 'aged' plasma, where 1-linoleoyl-LPA and 1-palmitoyl-LPA are the predominant species which are supposed to originate from lyso-PC through the action of a lysophospholipase D [28].

The activity of ether-linked LPA is of possible physiological relevance, since ether-linked LPAs represent potential metabolites of 1-alkyl-2-acyl-phospholipids (such as PAF), from which they can be derived through the sequential action of a phospholipase A_2 and a phospholipase D. In other studies, ether-linked LPAs were found to be considerably more potent than the 1-acyl-LPAs in stimulating platelet aggregation [16,29] and producing cardiovascular effects in cats [3]. In the present study, in contrast, ether-linked LPA appears several-fold less potent than the corresponding ester-linked species. The reason for this discrepancy is not known. It may suggest that human platelets and carcinoma cells possess different LPA receptors, varying in some pharmacological properties. Another possibility is that the observed differences between platelets and A431 cells are simply attributable to different assay conditions: it is obvious that platelet aggregation and vasoactive effects, unlike Ca^{2+} mobilization, represent relatively 'late' and perhaps indirect responses to LPA. Hence, differences in the rate of metabolic breakdown between ester-linked and ether-linked LPA may be the determining factor. We find that ether-linked LPA cross-desensitizes with ester-linked LPA, and thus may be regarded as a genuine receptor agonist. Although ester-linked LPA is formed and released by activated platelets and is a normal constituent of serum, it remains to be examined whether ether-linked LPA occurs naturally, either in physiological events or under pathological conditions.

It is noteworthy that both enantiomers of ether-linked LPA, which have a mirror-image conformation with respect to the C-2 glycerol carbon, are about equally potent, with both isomers showing cross-desensitization. These results are in line with the lack of stereospecificity of ether-linked LPA in inducing platelet aggregation reported by Simon et al. [16]. On the other hand, the activity of another ether-linked lipid messenger, PAF, is strongly decreased when the chirality at the C-2 glycerol carbon is reversed [30].

It has recently been suggested that the putative lipid messenger S-1-P acts through the LPA receptor [23]. Based on our cross-desensitization experiments, however, it seems safe to conclude

that S-1-P does not serve as an LPA-receptor agonist. The Ca^{2+} -mobilizing activity of exogenous S-1-P requires micromolar doses and is likely to be complex, with S-1-P acting both at the plasma membrane and at intracellular stores [22]. Regardless of the precise mode of action of S-1-P, a role for exogenous S-1-P as a physiological receptor agonist remains questionable as long as there is no evidence that S-1-P occurs extracellularly at sufficiently high concentrations.

The present results may provide some first clues as to how LPA might interact with its Ca^{2+} -mobilizing receptor in mammalian cells. Virtually nothing is understood of the binding of phospholipids and their derivatives, such as PAF and the eicosanoids, to their cognate G protein-coupled receptors. In general, G-protein-coupled receptors utilize quite diverse strategies to detect their ligands (for review see [31]). For example, adrenergic receptors and rhodopsin have their binding cavity within the hydrophobic transmembrane domains. This mode of ligand–receptor interaction is also utilized by small ligands such as histamine, 5-hydroxytryptamine and acetylcholine. In contrast, peptide ligands appear to interact with hydrophilic extracellular residues of their cognate receptor. Our findings support a model in which the long-chain fatty acid enters and binds to a hydrophobic cavity in the receptor molecule. Although this putative cavity has a marked preference for LPA with an 18:1 fatty-acyl chain, it can readily accommodate other long-chain LPAs. For example, photoreactive diazirine-undecanoyl-LPA is about equally potent as palmitoyl-LPA, and brief pretreatment of the cells with this analogue suppresses the response to natural LPA, and vice versa, but not to bradykinin. This indicates that diazirine-LPA and natural LPA act at identical sites and, furthermore, implies that there is no steric hindrance when a single aromatic ring structure, such as diazirine, replaces the terminal four to six hydrocarbons.

In view of these considerations, and given the critical importance of the 3-phosphate group, we propose that the hydrocarbon chain, together with the glycerol backbone, serve to anchor the LPA molecule in such a way that the negatively charged phosphate group is optimally aligned and in the proper orientation for direct interaction with positively charged residues in an extracellular region of the receptor. Such specific binding of the phosphate group would then trigger a conformational change that activates the receptor. Obviously, a further test of this model awaits the cDNA cloning and sequencing of the LPA receptor. It will then be possible to examine by site-directed mutagenesis how the LPA receptor detects and binds its ligand.

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