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Structure/Activity Relationships in Lysophosphatidic Acid: The 2-Hydroxyl Moiety

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SUMMARY

Although lipid phosphoric acid mediators such as lysophosphatidic acid (LPA) are now recognized widely as intercellular signaling molecules, the medicinal chemistry of these mediators is poorly developed. With the goal of achieving a better understanding of the structure activity relationships in LPA, we have synthesized and tested a series of LPA analogs that lack the 2-hydroxyl moiety. Our series consisted of compounds with 2, 3, or 4 carbon diol or amino alcohol backbones and oleoyl or palmitoleoyl acyl groups. These molecules cannot be acylated further to form phosphatidic acids, nor do they have chiral centers. The rank order potency of these compounds in mobilization of calcium in MDA MB-231 cells suggested a maximum optimal chain length of 24–25 atoms. However, high potency for the inhibition of adenylyl cyclase in these cells was achieved only by one compound that also contained a dissociable proton five bond lengths from the phosphorus atom. That compound, *N*-oleoyl-2-hydroxyethyl-1-phosphate, was nearly equipotent to 1-oleoyl LPA in both assays. The striking mimicry of LPA by the ethanolamine-based compound and the presence of fatty acid amides in tissue prompts us to propose that phosphorylated *N*-acyl ethanolamides occur naturally.

LPA elicits a variety of responses in cells and tissues. First recognized as a mediator of platelet aggregation (1, 2) and smooth muscle contraction (3, 4), LPA is known now to trigger three responses in many types of cells in culture. In fibroblasts, for example, LPA mobilizes calcium (5), inhibits adenylyl cyclase (6), and increases the formation of focal adhesions (7). There is some evidence that the former two responses proceed through pertussis toxin insensitive ($G_{\alpha q}$-type) and sensitive ($G_{\alpha i/o}$-type) heterotrimeric G-proteins (6, 8, 9), respectively. The mechanism of the latter effect is ill understood, although the small G protein *rho* has been implicated (7, 10). Numerous other cell types, including keratinocytes (11), smooth muscle cells (12), epithelial cells (13), and so forth, exhibit similar responses to LPA. As an intermediary metabolite, LPA is present in all cells at low level, but is found extracellularly in at least three fluids in pathophysiologic conditions. These fluids are serum (14–16), ascites fluid from ovarian cancer patients (17) and cerebrospinal fluid in piglets after experimental intercranial hematomas (18).

Despite this intriguing cell biology, the molecular mechanism(s) of action of LPA is largely unknown and an understanding of its physiologic (or pathophysiologic) roles has lagged. Among the reasons for this ignorance is the lack of molecular tools, specifically a nearly complete lack of metabolically stable, highly water-soluble mimetics and blockers. As part of a program to develop such reagents, we have undertaken a systematic structure-function profile of LPA. Studies before this one have outlined the basic SARs in LPA regarding calcium mobilization or platelet activation. These include the importance of the phosphate di-anion, the low tolerance for bulk in the moiety at the second carbon of the glycerol backbone (i.e., hydroxyl or methoxy is tolerated, acetyl is not) and the wide range of lengths and degree of unsaturation that are tolerated in the fatty acyl chain (19, 20).

Our focus in the present study has been the hydroxyl moiety at the 2 position in LPA. Specifically, we wanted to build mimetics lacking this hydroxyl because its carbon is chiral and compounds lacking a chiral center are simpler to synthesize and/or purify, and the 2-OH has the potential to be acylated by LPA acyl transferase. The product of this

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ABBREVIATIONS: LPA, lysophosphatidic acid (*O*-acyl-2-hydroxy-*sn*-glycero-3-phosphate); S1P, sphingosine 1-phosphate; THF, tetrahydrofuran; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, SAR, structure-activity relationship; HKRB, HEPES-Krebs-Ringer-bicarbonate.

reaction, phosphatidic acid, is a lipid mediator that elicits cell responses that overlap (e.g., mitogenic effects), but are nonidentical to those of LPA, and prevention of acylation would generate agonists that would avoid the potential confounding effects arising through conversion to a phosphatidic acid analog.

In this study, we report a facile synthetic route for the generation of lipid phosphoric acids that lack both chiral centers and esterifiable groups and describe the activities of a series of such molecules in two assays. One of our compounds, *N*-oleoyl-2-hydroxyethyl-1-phosphate (a phosphorylated fatty acid amide), is nearly indistinguishable from LPA regarding activity and potency.

Experimental Procedures

Chemical Syntheses (Overview)

The syntheses of LPA analogs **1–8** (**1**, *O*-oleoyl-2-hydroxyethyl-1-phosphate; **2**, *O*-oleoyl-3-hydroxypropyl-1-phosphate; **3**, *O*-oleoyl-4-hydroxybutyl-1-phosphate; **4**, *N*-oleoyl-2-hydroxyethyl-1-phosphate; **5**, *N*-oleoyl-3-hydroxypropyl-1-phosphate; **6**, *N*-oleoyl-4-hydroxybutyl-1-phosphate; **7**, *N*-palmitoleoyl-4-hydroxybutyl-1-phosphate; **8**, *O*-oleoyl-2-keto-3-hydroxypropyl-1-phosphate) were accomplished using solvents that were purified by filtration through alumina (activity I). All reactions were performed under inert atmospheres and all products were purified on 230–400 mesh silica gel. Each product was examined by thin layer chromatography (single spot), and subject to spectroscopic analyses including ^{1}H and ^{13}C NMR, IR, and mass spectrometry. The assigned structures of LPA analogs **1–8** were consistent with the spectral data obtained. The concentration of each phospholipid was determined by colorimetric assay for phosphate as described by Kingsley and Feigenson (21). A typical synthesis of each class of analog (*N*-acyl, 2-keto, *O*-acyl) is as follows.

Synthesis of LPA Analog 4

Condensation of ethanolamine and oleoylchloride. To 150 ml of tetrahydrofuran (THF), 3.0 g of ethanolamine (51 mmol, 1 eq) was added followed by the addition of 5.0 g of 70–80% technical grade oleoyl chloride (17 mmol, 0.33 eq). The reaction mixture was stirred overnight at ambient temperature, then poured into 100 ml of ethyl acetate, and extracted twice with 100-ml aliquots of ammonium chloride-saturated water. The organic layer was dried over magnesium sulfate and concentrated under reduced pressure. The product was purified by silica gel column chromatography using 15% acetone in chloroform (R_F = 0.19). Typical yields for this reaction were 90%.

Phosphorylation of resulting alcohol. To 25 ml of CH_2Cl_2, 0.61 g of alcohol (1.9 mmol, 1 eq), and 0.2 g of tetrazole (2.8 mmol, 2 eq) were added and stirred for 5 min followed by the addition of 0.98 g of dibenzyl diisopropylphosphoramidite (2.8 mmol, 2 eq) (22). After stirring the reaction for 1 hr, 0.34 ml of 30% hydrogen peroxide was added, and stirring was continued for an additional 2 hr. Excess hydrogen peroxide was quenched by the addition of a saturated aqueous solution of sodium metabisulfite. The resulting solution was poured into 50 ml of ethyl ether and extracted twice with 50-ml aliquots of sodium bicarbonate-saturated water. The organic layer was dried over magnesium sulfate and concentrated under reduced pressure, and the product was purified by flash chromatography using 5% acetone in chloroform (R_F = 0.25). Typical yields for this reaction were 50%.

Deprotection of resulting phosphate. To 1.0 ml of THF and 1.0 ml of cyclohexene, 0.05 g of the phospholipid with benzoyl protecting groups was added followed by 0.1 mol % of 10% palladium/carbon and one drop of acetic acid. The reaction was heated to reflux until all starting material was consumed (as judged by thin layer chromatography in 5% acetone in chloroform). The reaction was cooled to room temperature, filtered over Celite, and concentrated under reduced pressure yielding LPA analog **4** in 100% conversion and 50% recovered yield.

HPLC analysis of compound 4. To ascertain its purity, the reaction product was analyzed by normal phase HPLC [Microsorb-MV silica, 5-μm particle size, 100-Å pore size, 4.6 × 250 mm (Rainin Instruments, Woburn, MA)] and was detected with an evaporative light scattering detector (Alltech Model 500 ELSD). The reaction product (150 nmol of phosphorus) was dissolved in, and applied to, the column in a 70:30 mixture of solvent A (chloroform/methanol/28% ammonium hydroxide, 80/19.5/0.5) to solvent B (chloroform/methanol/water/28% ammonium hydroxide, 34.5/55/10/0.5). At a constant flow rate of 1 ml/min, material was eluted with the following protocol: isocratic in 70:30 (A:B) from 0–8 min, a linear gradient to 20:80 (A:B) from 8–18 min, a linear gradient to 100% solvent B from 18–23 min, and isocratic in 100% solvent B from 23–28 min. The only material that was retained on the column migrated as a single peak and eluted at 20.3 min. This material was bioactive, contained phosphorus, and exhibited the same specific activity as the starting material. Another peak of material did not adsorb to the column matrix (elution time: 2.9 min), contained no detectable phosphate, had no bioactivity, and absorbed light strongly at 270 nm. We believe this material to be the butylated hydroxytoluene present as a stabilizer in the cyclohexene.

Synthesis of LPA Analog 8

Condensation of dihydroxyacetone and oleoyl chloride. To 150 ml of THF, 2 g of dihydroxyacetone (22 mmol, 1 eq) and 2.7 ml of pyridine (33 mmol, 1.5 eq) were added followed by 3.3 g of 70–80% technical grade oleoyl chloride (11 mmol, 0.5 eq). The reaction was then heated to reflux for 6 hr, cooled to room temperature, and poured into 200 ml of ethyl acetate. After two extractions with ammonium chloride-saturated water (200 ml), the organic layer was dried over magnesium sulfate and concentrated under reduced pressure. The product was purified by flash chromatography using 15% acetone in chloroform (R_F = 0.52). Typical yields for this reaction were 50%.

Phosphorylation of resulting alcohol. To 10 ml of CH_2Cl_2, 0.25 g of alcohol (0.7 mmol, 1 eq) and 0.08 g of tetrazole (1.13 mmol, 1.6 eq) were added and stirred for 5 min followed by the addition of 0.39 g of dibenzyl diisopropylphosphoramidite (1.13 mmol, 1.6 eq) (22). After stirring the reaction for 1 hr, 0.2 ml of 30% hydrogen peroxide was added, and stirring was continued for an additional 3 hr. Excess hydrogen peroxide was quenched by the addition of a saturated aqueous solution of sodium metabisulfite. The resulting solution was poured into 50 ml of ethyl ether and extracted twice with sodium bicarbonate-saturated water (50 ml). The organic layer was dried over magnesium sulfate, concentrated under reduced pressure, and the product was purified by flash chromatography using 5% acetone in chloroform (R_F = 0.44) Typical yields for this reaction were 50%.

Deprotection of resulting phosphate. To 1.0 ml of THF and 1.0 ml of cyclohexene, 0.035 g of the phospholipid with benzoyl protecting groups was added followed by 0.1 mol % of 10% palladium/carbon and one drop of acetic acid. The reaction was heated to reflux until all starting material was consumed (as judged by thin layer chromatography in 5% acetone in chloroform). The reaction was cooled to room temperature, filtered over Celite, and concentrated under reduced pressure, yielding LPA analog **8** in 100% conversion and 50% recovered yield.

Synthesis of LPA Analog 1

Condensation of ethylene glycol and oleoyl chloride. To 300 ml of THF, 6.0 g of ethylene glycol (99 mmol, 3 eq) and 8 ml of pyridine (99 mmol, 3 eq) were added followed by 10 g of 70–80% technical grade oleoyl chloride (33 mmol, 1 eq). After stirring the reaction mixture overnight at ambient temperature, the solution was

poured into 500 ml of ethyl acetate and extracted twice with ammonium chloride-saturated water (500 ml). The organic layer was dried over magnesium sulfate and concentrated under reduced pressure, and the product was purified by flash chromatography using 30% ethyl acetate in hexanes ($R_F = 0.25$). Typical yields for this reactions were 65%.

Phosphorylation of resulting alcohol. To 6 ml of CH_2Cl_2, 0.20 g of alcohol (0.61 mmol, 1 eq) and 0.07 g of tetrazole (0.97 mmol, 1.6 eq) were added and stirred for 5 min followed by the addition of 0.36 g of dibenzyl diisopropylphosphoramidite (0.97 mmol, 1.6 eq) (22). After stirring the reaction for 1 hr, 0.2 ml of 30% hydrogen peroxide was added, and stirring was continued for an additional 3 hr. Excess hydrogen peroxide was quenched by the addition of a saturated aqueous solution of sodium metabisulfite. The resulting solution was poured into 25 ml of ethyl acetate and extracted twice with sodium bicarbonate-saturated water (25 ml). The organic layer was dried over magnesium sulfate and concentrated under reduced pressure, and the product was purified by flash chromatography using 5% acetone in chloroform ($R_F = 0.52$). Typical yields for this reaction were 50%.

Deprotection of resulting phosphate. To 1.0 ml of THF and 1.0 ml of cyclohexene, 0.05 g of the phospholipid with benzoyl protecting groups was added followed by 0.1 mol % of 10% palladium/carbon and one drop of acetic acid. The reaction was heated to reflux until all starting material was consumed (as judged by thin layer chromatography in 5% acetone in chloroform). The reaction was cooled to room temperature, filtered over Celite, and concentrated under reduced pressure, yielding LPA analog **1** in 100% conversion and 50% recovered yield.

To synthesize LPA analog **7**, we substituted analytical grade palmitoleoyl chloride for oleoyl chloride. Likewise, LPA analogs **2** and **3** were realized by substituting 1,3 dihydroxypropane and 1,4 dihydroxybutane for ethylene glycol, respectively. Finally, LPA analogs **5** and **6** were generated by substituting 1-aminopropanol and 1-aminobutanol for ethanolamine, respectively.

Preparation of phospholipids for bioassays. Phospholipids, which were stored dissolved in organic solvent (usually 1:1 chloroform/methanol) at −20° under argon, were aliquoted (0.1–2.0 μmol, 1–50 μl) into microcentrifuge tubes, dried *in vacuo*, and resuspended in water with 0.1% (w/v) fatty acid-free bovine serum albumin. After brief vortexing, the mixture was treated for 5 min in a bath sonicator. The solution was stored at room temperature for no more than 2 hr before use. Subsequent cycles of freezing/thawing did not affect activity. In some cases, phospholipids were suspended in buffer (HKRB, calcium measurements, or HEPES-buffered Dulbecco's modified Eagle's culture medium, cAMP determinations).

Bioassays

All phospholipids were tested on the metastatic human breast cancer cell line, MDA MB-231. In addition, many of the compounds were tested on the human embryonic kidney cell line, HEK 293. Several compounds were analyzed also using stage V-VI *Xenopus laevis* oocytes. Two responses were assayed for each compound, calcium mobilization and inhibition of adenylyl cyclase. These assays were performed as we described previously (23, 24).

Ca^{2+} mobilization. Briefly, cell monolayers grown in 150 cm^2 dishes (≥80% confluent) were overlaid with 10 ml of HKRB buffer (20 mM HEPES, 103 mM NaCl, 4.8 mM KCl, 0.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 15 mM glucose, pH 7.4) containing 5 μg/ml INDO-1AM. After 45–60 min at 37°, the monolayer was washed briefly with warm buffered saline, and the cells were freed from the substratum by incubation with trypsin/EDTA. After brief centrifugation, the cell pellet was resuspended in HKRB at approximately 1.5×10^6 cells/ml and kept at 37° for no more than 90 min before use. Records of free intracellular calcium were made on 2-ml aliquots of cells suspended in a quartz cuvette in a temperature-controlled fluorimeter (Aminco SLM 8000; SLM Instruments, Urbana, IL). Excitation was with light at 332 nm (slit width 4 nm), and emission wavelengths were 400 nm and 485 nm (slit widths 4 nm). Calcium responses recorded were peak responses in traces of the ratio of the emitted light. Maximum and minimum fluorescence ratios were calculated by the sequential addition of digitonin (to 75 μM) and EDTA (to 5 μM).

cAMP accumulation. Monolayers of MDA MB-231 cells were treated with trypsin, centrifuged, and suspended in HEPES (15 mM)-buffered Dulbecco's modified Eagle's medium (serum-free). Cells (5–10 × 10^4 cells in 0.2 ml) were treated with isobutylmethylxanthine (1 mM) for 15 min at room temperature and then for 15 min at 37° with forskolin (1 μM) with or without phospholipid. The assay was terminated by the addition of 0.5 ml 0.15 N hydrochloric acid. After centrifugation, the cAMP in the supernatant fluid was measured in an automated immunoassay (GammaFlow).

Oocyte recording. Chloride currents in response to applied phospholipids in manually defolliculated stage V-VI *X. laevis* oocytes were measured under two-electrode voltage clamp as we have described previously (25).

Materials

MDA MB-231 cells and HEK 293 cells were from the American Type Culture Collection (Rockville, MD). Frogs (*X. laevis*) were from *Xenopus* I (Ann Arbor, MI). Culture medium was from Life Technologies, Inc. (Bethesda, MD). The chemical reagents were from Sigma/Aldrich Chemical (St. Louis, MO), Phaltz-Bauer (Waterbury, CT), or ICN (Costa Mesa, CA). Phospholipids were purchased [1-oleoyl-LPA from Avanti Polar Lipids (Alabaster, AL) or S1P from BIOMOL (Plymouth Meeting, PA)].

Results

It was necessary to develop first a facile synthetic route to lipid phosphoric acids that would afford compounds containing mono-unsaturated acyl groups, e.g., oleic acid (18:1 Δ^9). Compounds with at least one *cis* double bond are needed because the properties of potency and solubility are intertwined, and compounds with unsaturated centers are markedly more soluble (in aqueous or organic solvents) than saturated compounds. The synthetic scheme described in Fig. 1 (condensation of a primary alcohol or amine with an acyl acid chloride, reaction of the remaining alcohol with the phosphorylating reagent and removal of the protecting groups to reveal the phosphate monoester) is analogous to that published during the course of our studies by Bittman *et al.* (26). The significant advantage of our scheme is the use of a more selective hydrogen donor (cyclohexene versus hydrogen gas) for the reduction of the triester that, although removing the protecting benzoyl groups, spares the 9,10-olefin.

The compounds generated using this synthetic route were a series of lipid phosphoric acids wherein the glycerol backbone was replaced by a 2, 3, or 4 carbon diol or amino alcohol. Thus we reacted oleoyl acid chloride (or, in some cases, palmitoleoyl acid chloride) with ethylene glycol, 1,3-propanediol, 1,4-butanediol, or dihydroxyacetone. Because the dissociable proton provided by the hydroxyl moiety of LPA might be an important functionality as well as contributing to aqueous solubility, we also used 2-aminoethan-1-ol, 3-aminopropan-1-ol, or 4-aminobutan-1-ol as starting materials. These yielded compounds with amide rather than ester functionalities (the more nucleophilic amino group reacts preferentially with the acid chloride), thus the dissociable proton absent from the diol-based compounds was reintroduced. The structures of the synthetic compounds and oleoyl-LPA as well as our designations for them are given in Fig. 2.

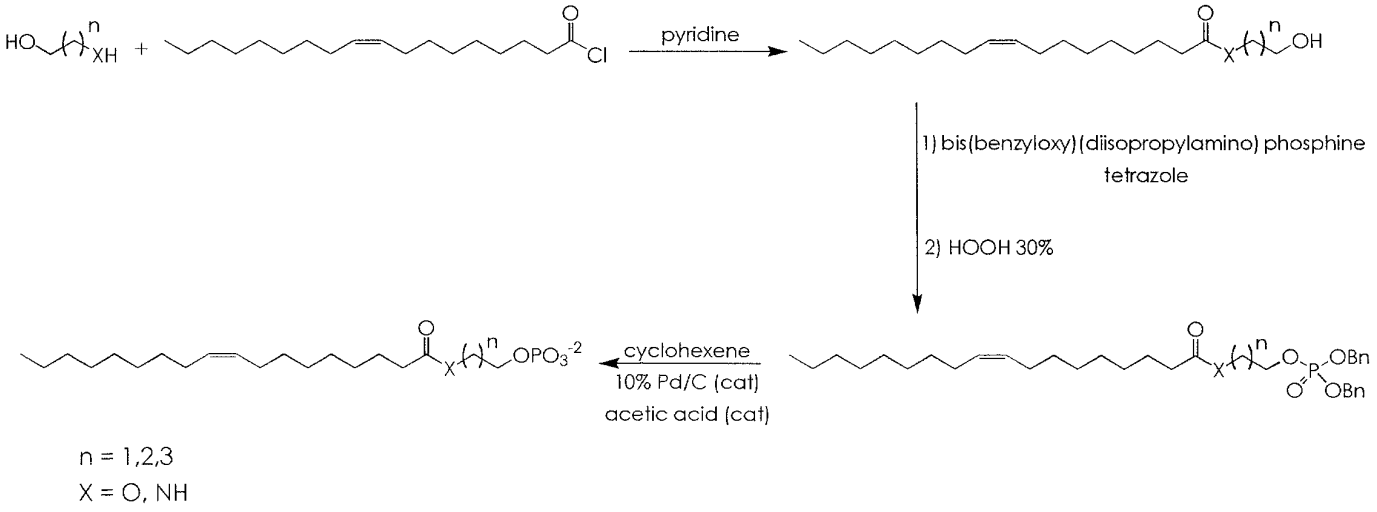


Fig. 1. Synthetic route to lipid phosphoric acids (details are given in Experimental Procedures). *10% Pd/C*, 10% palladium on carbon.

These compounds have neither a chiral center nor a hydroxyl moiety.

To determine the biologic activity of these LPA analogs, we tested them on cultured MDA MB-231 cells in two assays: calcium mobilization and inhibition of adenylyl cyclase. Calcium mobilization was measured as INDO-1 fluorescence signals from cell populations, whereas inhibition of adenylyl cyclase was gauged by measuring cAMP accumulation in cell populations treated with forskolin and IBMX. As shown in the dose response curves presented as Fig. 3, three synthetic compounds approached oleoyl-LPA's potency in mobilizing calcium. In rank order, these compounds are oleoyl-LPA, **4**, **7**, and **8** (i.e., the keto analog of LPA). The remaining four compounds were at least two log orders less potent in this assay. Unexpectedly, we found that the overall length of the compound affected potency in this assay. Compounds sharing an aminobutanol backbone but differing by two methylene groups ([**7** (palmitoleoyl) and **6** (oleoyl)] exhibited distinctly different potencies, with the longer compound being less potent. All of the diol-based compounds were low potency relative to the amino alcohol-based compounds.

The four compounds that were highest potency in the calcium mobilization assays were also the four most potent compounds at inhibiting forskolin-driven rises in cAMP levels in MDA MB-231 cells. None of the other synthetic compounds showed any significant inhibition of adenylyl cyclase activation at the maximal dose tested (25 μM). However, the dose-response curves presented in Fig. 4 show that of these four active compounds, one pair (oleoyl-LPA and **4**) was distinctly more potent than the other pair (**7** and **8**). Further, comparison of the EC_{50} values for both responses (see Fig. 2) shows that, consistent with previous reports for oleoyl-LPA (6, 20), the three most potent synthetic compounds were roughly 20-fold more potent in mobilizing calcium than in suppressing cAMP increases.

Because compound **4** is nearly as potent as oleoyl-LPA in two independent assays, we explored its properties further. This compound mobilizes calcium in other cells (e.g., HEK 293), its inhibition of adenylyl cyclase (but not calcium mobilization) in MDA MB-231 cells is blocked by pertussis toxin, and it stimulates [^{3}H]thymidine incorporation into the DNA of quiescent MDA MB-231 cells (data not shown). Further, oleoyl-LPA and **4** share the unusual property of eliciting depolarization in defolliculated *X. laevis* oocytes. All of these properties mimic well known effects of oleoyl-LPA (for a recent review of LPA's cell biology, see Ref. 27).

Although compound **4** and oleoyl-LPA have similar structures and are nearly equipotent full agonists in all of our assays, it is difficult to ascertain, absent a LPA receptor DNA clone or a radioligand binding assay, whether these two compounds are acting through the same receptor. One indication that this might be the case is the complete cross-desensitization of the calcium mobilizing response elicited in MDA MB-231 cells by this compound (Fig. 5). S1P is another structurally similar lipid phosphoric acid mediator that mimics many of LPA's actions on cells. Such similarities have prompted speculation that LPA and S1P share a receptor (28), but this suggestion is belied by the lack of cross-desensitization (23). As with LPA, the S1P and **4** responses fail to cross-desensitize (Fig. 5).

Discussion

Several aspects of our study are significant. First, we have identified a facile synthetic route to lipid phosphoric acids that is rapid and inexpensive, gives a high yield, and, most importantly, allows synthesis of compounds with mono-unsaturated acyl groups. Because the compounds with unsaturated fatty acid moieties are far more soluble than the analogous saturated compounds, the latter aspect of our synthetic route represents an advantage over the similar synthetic route reported recently by Bittman *et al.* (26).

Second, our results, although achieved with only a limited set of compounds, suggest two aspects of the SAR of lipid phosphoric acid mediators that have not been appreciated fully. One is that the overall length of the compound appears to influence potency strongly, at least regarding calcium mobilization. For example, the aminobutanol-based compound with a palmitoleoyl acyl group (**7**) is nearly equipotent (calcium mobilization) with the oleoyl-containing aminoethanol-based compound (**4**) (these compounds are positional isomers), but far more potent than the aminobutanol-based compound with an oleoyl substituent (**6**, larger by two methylene groups). The prediction that addition of two methylene

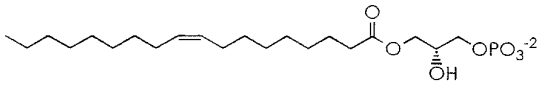
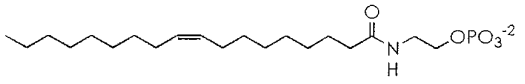
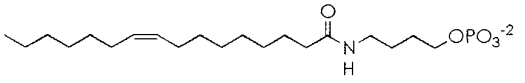
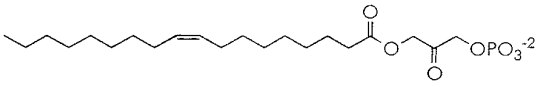
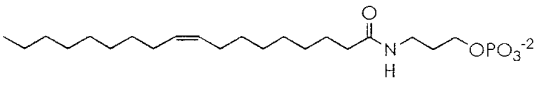
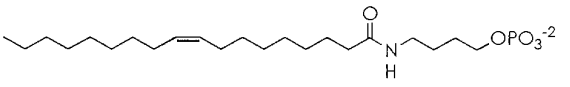
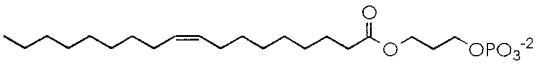
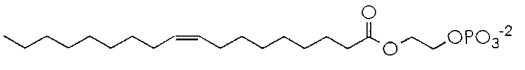
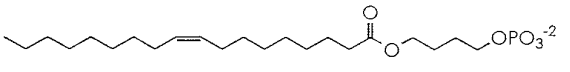
COMPOUND #	ANALOG STRUCTURE	EC50s (nM) MDA MB 231 cells Ca^{+2}	cAMP
LPA (25)		0.9	20
4 (24)		1.2	101
7 (24)		4	>1000
8 (25)		31	>1000
5 (25)		170	>1000
6 (26)		170	>1000
2 (25)		700	>1000
1 (24)		>1000	>1000
3 (26)		>1000	>1000

Fig. 2. Structure representations and relative potencies of oleoyl-LPA and seven synthetic analogs. Values were calculated from the curves presented in Figs. 3 and 4 using the curve-fitting function in Origin (MicroCal). *Values in parentheses after compound designations*, the size of each compound (the number of atoms in the molecule's backbone, phosphorus to ω carbon).

groups to the acyl chain of **4** (to yield *N*-eicosenoyl-2-hydroxyethyl-1-phosphate) would result in a less potent compound seems to be borne out by our preliminary studies with such a compound.[1] Although compounds with additional methylene groups are less water-soluble, the small loss in solubility does not, in our judgment, account for the large difference in potency.

The inhibition of adenylyl cyclase activation is a more selective assay in that only compound **4** approached the potency of oleoyl-LPA. Interestingly, compound **4** is similar to LPA regarding the position of the dissociable proton. Although its presence surely contributes to enhanced aqueous solubility, only when the dissociable proton is positioned five bond lengths from the phosphorus atom (**4**), rather than more distant (compounds **5**, **6** or **7**), is the close mimicry of LPA realized. This structural parameter might be an important functionality in lipid phosphoric acid mediators.

Compound **4** exceeds our initial criteria for an "improved" LPA: it mimics LPA entirely in all of our assays (albeit with slightly lower potency), yet it is without a chiral center and cannot function as a substrate for acyl transferases. Compound **4** is also less dependent on an albumin carrier for activity than is LPA (data not shown), which might prove advantageous in the development of a radioligand binding assay. Sugiura *et al.* (29) have reported the synthesis of an analogous compound (*N*-palmitoyl-2-hydroxyethyl-1-phosphate) and found it potent in aggregating human platelets, as would be expected of a LPA mimetic (30). Despite these advantages, an aminoethanol-based compound might remain labile due to its amide and phosphate functionalities.

The disparity between the EC_{50} values for calcium mobilization and adenylyl cyclase inhibition, which has been ob-

[1] K. R. Lynch, D. W. Hopper, S. J. Carlisle, J. G. Catalano, M. Zhang, and T. L. Macdonald, unpublished observations.

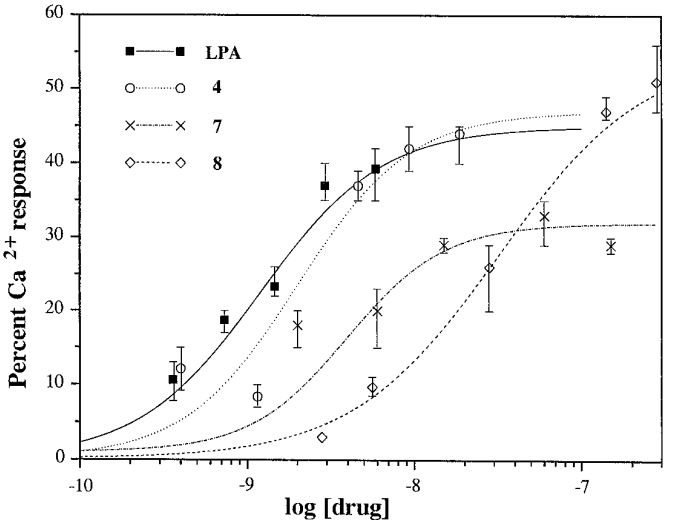


Fig. 3. Dose-response curves for calcium mobilization in MDA MB-231 cells. Peak calcium responses (n = 3) in INDO-1-loaded cell populations were measured for the indicated compounds. The 100% value is the total calcium response measured when cells were permeablilized with digitonin. To avoid desensitization, measurements were made on naive cell populations. See Fig. 2 for a list of compound structures and designations.

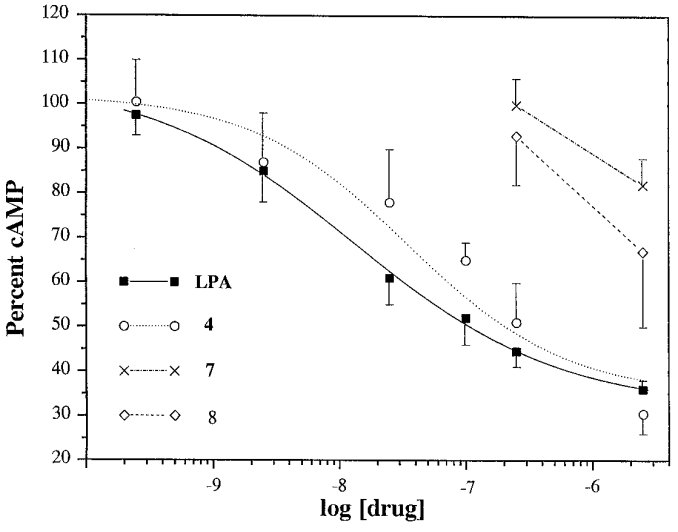


Fig. 4. Dose-response curves for inhibition of adenylyl cyclase in MDA MB-231 cells. cAMP accumulation in forskolin-treated cell populations (n = 3) were measured for the indicated compounds. All compounds were tested to a maximal dose of 25 μM; only the four compounds shown were active at this dose. The 100% value was typically 40–45 pmol of cAMP, accumulating in 2.5 $\times$ 10^5 cells after forskolin stimulation.

served by others using oleoyl-LPA (20, 31), is not observed with other receptors (e.g., angiotensin AT_1 (32) and thrombin (33)) that activate both pathways. Setting aside the obvious explanation of two receptors, the difference in the time course of these assays (2–3 sec for Ca^{2+} mobilization, 15 min for cyclase inhibition) suggests that differences in metabolic lability might underlie this discrepancy. Thus the EC_{50} values for lipid phosphoric acid mediators reflect intrinsic potency, metabolic lability, and water solubility.

Third and finally, the obvious structural relationship of compound **4** and *N*-acylethanolamides [e.g., anandamide (*N*-arachidonyl ethanolamide)] deserves comment. A single step

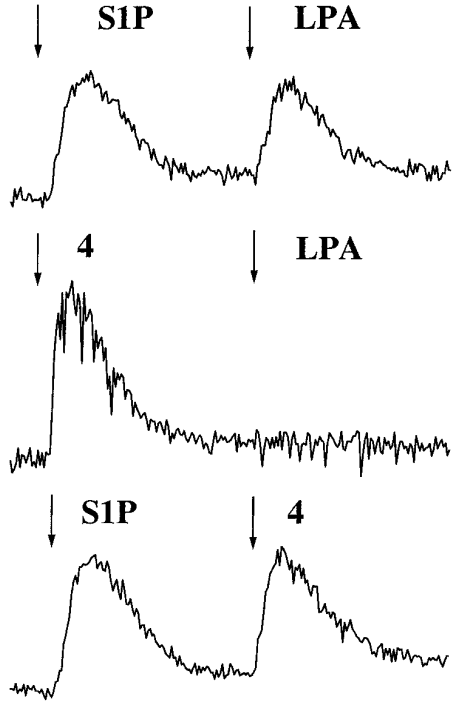


Fig. 5. Cross-desensitization of oleoyl-LPA, compound **4**, and S1P. The recordings shown represent calcium mobilization in INDO-1-loaded MDA MB-231 cells. The cell populations were challenged sequentially with maximal doses (in nM: LPA 15, **4** 100, S1P 100) of the indicated compounds. The desensitization (or lack thereof) occurred regardless of the order in which the compounds were applied.

would interconvert these two lipid mediators: anandamide being a low potency, partial agonist at the CB1 and CB2 cannabinoid receptors, whereas at least one phosphorylated *N*-acyl ethanolamide (i.e., compound **4**) is a high potency, full agonist at the putative LPA receptor. The striking mimicry of LPA by a phosphorylated fatty acid ethanolamide and the proven existence of such compounds in biologic fluids (34) leads us to hypothesize that phosphorylated *N*-acyl ethanolamides are naturally occurring lipid mediators. Analogous to the biosynthetic route suggested for anandamides (35), the phosphorylated compounds could appear as a result of phospholipase C action on membrane *N*-acyl phosphatidylethanolamine. An intriguing corollary to our hypothesis is that fatty acid ethanolamides containing oleoyl and linoleoyl substituents, which are not active at cannabinoid receptors but for which no biologic function has been ascribed (36), might be a by-product of further metabolic conversion of their phosphorylated forms.

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