Identification of a Novel Growth Factor-like Lipid, 1-O-cis-alk-1'-eny1-2-lyso-sn-glycero-3-phosphate (Alkenyl-GP) That Is Present in Commercial Sphingolipid Preparations*

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Lysophosphatidic acid, a member of the acidic phospholipid autacoid (APA) family of lipid mediators, elicits diverse cellular effects that range from mitogenesis to the prevention of programmed cell death. Sphingo- line 1-phosphate and sphingosylphosphorylcholine have also been proposed to be ligands of the APA receptors. However, key observations that provide the foundation of this hypothesis have not been universally reproducible, leading to a controversy in the field. We provide evidence that 1-O-cis-alk-1’-eny1-2-lyso-sn-glycero-3-phosphate (alkenyl-GP) is present in some commercial sphingolipid preparations and is responsible for many of their APA-like effects, which were previously attributed to sphingosylphosphorylcholine. Alkenyl-GP was generated by acidic and basic methanolation from ethanolamine lysosphasmolagen, which was present in the sphingomyelin fraction that is used to manufacture sphingosylphosphorylcholine. We present the structural identification of alkenyl-GP, using 1H and 13C NMR, Fourier transform infrared spectrometry, and mass spectrometry. Alkenyl-GP was a potent activator of the mitogen-activated protein kinases ERK1/2 and elicited a mitogenic response in Swiss 3T3 fibroblasts. In contrast, sphingosylphosphorylcholine at a concentration of 10 μM was only a weak mitogen and only weakly activated the extracellular signal-regulated protein kinases. Alkenyl-GP has recently been detected as an injury-induced component in the anterior chamber of the eye (Lilom, K., Guan, Z., Tseng, H., Desiderio, D. M., Tigi, G., and Watsky, M. (1998) Am. J. Physiol. 274, C1065–C1074), indicating that this lipid is a naturally occurring member of the APA mediator family.

The autacoids, 1-oleoyl-2-lyso-sn-glycero-3-phosphate (lysophosphatidic acid, LPA), sphingosine 1-phosphate (SPP), and sphingosylphosphorylcholine (SPC) have been the subject of intense investigation fueled by the diversity of biological responses that they elicit in a variety of cell types (for reviews see Refs. 1 and 2). Perhaps the three most interesting cellular responses elicited by these autacoids include the following: mitogenesis/antimitogenesis (3–6), effect on cell shape and motility (7–11), and anti-apoptotic action (12, 13). The somewhat overlapping spectrum of cellular responses elicited by SPP and LPA has led to the hypothesis that these mediators might activate the same receptors (14, 15). This concept is supported by other investigators, who found that SPC not only activated similar cellular responses and signal transduction pathways as LPA but that these two lipids also showed heterologous desensitization, suggesting that they activated the same receptor (16, 17). On the other hand, several reports have found no evidence for heterologous desensitization between sphingolipid mediators and glycerolipid mediators, leading to a controversy in the field (18).

We report that part of this controversy appears to be due to a glycerolipid that has been present in a commercially available SPC preparation used by many investigators. The impurity was identified as 1-O-cis-alk-1’-eny1-2-lyso-sn-glycero-3-phosphate (alkenyl-GP), an analog of LPA. Alkenyl-GP is generated in the manufacturing process of SPC from ethanolamine-containing lysophasmolagen (alkenyl-GPE), which is present in the sphingomyelin (SM) fraction, the precursor used to manufacture SPC. We provide a method of purification and the full structural elucidation of this novel LPA analog using NMR, FT, IR, and mass spectrometry (MS) and also enzymatic and chemical analytical methods. We also describe a high yield, semi-synthetic method for the production of alkenyl-GP. Alkenyl-GP, like its acyl counterpart, activates the extracellular signal-regulated protein kinases (ERKs) 1 and 2 and acts as a

References:
1. The abbreviations used are: LPA, lysophosphatidic acid; SPP, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; alkenyl-GP, 1-O-cis-alk-1’-eny1-2-lyso-sn-glycero-3-phosphate; ERK, extracellular signal-regulated protein kinase(s); BuOH, butanol; HAc, glacial acetic acid; MeOH, methanol; MAP, mitogen-activated protein; SPC, sphingosylphosphorylcholine; FTIR, Fourier transform infrared spectrometry; MS, mass spectrometry; FAB-MS, fast atom bombardment-mass spectrometry; SM, sphingomyelin; alkenyl-GPE, ethanolamine-containing lysophasmolagen; 1-O-cis-alk-1’-eny1-2-lyso-sn-glycero-3-phosphoethanolamine; MBP, myelin basic protein; HPLC, high pressure liquid chromatography; AP, alkaline phosphatase; eSPC, d-erythro-SPC.
strong mitogen in Swiss 3T3 cells. Alkenyl-GP has recently been described as an injury-induced component of the anterior chamber fluid of the eye (19), suggesting that this novel lipid is a naturally occurring member of the acidic phospholipid autocoid (APA) family.

MATERIALS AND METHODS

Lipids—SPC was obtained either from Sigma or Matreya Inc. (Pleasant Gap, PA). SM and polar brain phospholipid extracts were purchased from Sigma, Matreya, and Avanti Polar Lipids (Alabaster, AL). Oleoyl-LPA was from Avanti Polar Lipids. RO56 1-O-cis-alk-1'-enyl-2-acyl-sn-glycero-3-phosphoethanolamine, ethanalamine-containing lysoplasmalogen, alkenyl-GPE (50 μg) was obtained from Matreya Inc. or was prepared as described below. Phospholipase A2 (from Naja naja, Sigma) catalyzed removal of the fatty acid side chain from the sn-2 position was carried out in a non-aqueous medium, according to Katz (25). After a 4-h digestion at room temperature, the solvents were evaporated with a stream of N2, and the reaction products were analyzed by TLC, and the plate was developed in solvent 2.

Enzymatic Dephosphorylation of Alkenyl-GP—Alkenyl-GP (100 μg/reaction) was digested with Escherichia coli alkaline phosphatase (AP, EC 3.1.3.1, Worthington) with a specific activity of 37 units/mg, as described by Blank and Snyder (26). Control reactions received equal amounts of heat-inactivated enzyme. After 2 h, the reaction was terminated, and the lipids were extracted (37).

Hydrolysis of the Vinyl-Ether Bond by HCl Vapor—SPC (1 mg, Sigma) and alkenyl-GP (50 μg) were spotted on to a TLC plate, which was placed into a chromatography tank that contained concentrated HCl at the bottom. The HCl vapor was allowed to react with the vinyl-ether bond for 15 min. Subsequently, the lipids were scraped off, extracted with large excess of methanol, dried under a stream of N2 at 40 °C, and dissolved in 200 μl of methanol. As a control, the same lipids were processed in the same way without the exposure to HCl vapor.

Spectroscopic Analysis of Phosphatidylglycerol—Oocytes were obtained from xylazine-anesthetized adult Xenopus laevis frogs (Carolina Scientific, Burlington, NC) under aseptic conditions and were prepared for experiments as described previously (33). Lipids for bioassay were always complexed with fatty acid-free BSA (Sigma), for complexing procedure see below), diluted in frog Na+-Ringer’s solution (120 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 5 mM HEPES, pH 7.0), and were applied through superfusion to the oocyte, at a flow-rate of 5 ml/min. Membrane currents were recorded with a NIC-510 digital oscilloscope ( Nicolet, Madison, WI).

Cell Proliferation Assay—DNA synthesis induced by the different lipids was determined using [3H] thymidine (Amersham Pharmacia Biotech, 185 GBq/mmol) incorporation into confluent cultures of Swiss 3T3 fibroblasts, as described by Desai and Spiegel (6). Lipids, dissolved in methanol, were complexed with fatty acid-free BSA (Sigma) in Ca2+-free Hanks’ basal salt solution at a 1:1 molar ratio and were applied to the cells at a 10 μM final concentration. After an 18-h incubation in the presence of the lipids, 1 μCi of [3H] thymidine was added to each well; cells were harvested 6 h later to quantify the amount of acid-insoluble radioactivity (27).

Assay for ERK1/ERK2—The combined activity of the ERK1 and ERK2 was determined by a method described elsewhere (28, 29). Subconfluent cultures (75%) of Swiss 3T3 cells were serum-starved for 6 h and treated with a 1 μM concentration of each lipid for 10 min. The total protein concentration of the supernatant was determined according to Bradford (30). Total cell protein (200 μg) was used for each assay. Samples were treated with ERK1 and ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA) antiserum at a 1:100 dilution for 1 h before incubation with 20 μl of protein G-agarose (Santa Cruz Biotechnology) for 2 h at 4 °C. Beads were washed twice with 1 ml of lysis buffer and once with kinase buffer (30 mM Tris-HCl, pH 8.0, 20 mM MgCl2, and 2 mM MnCl2). The kinase reactions were started by adding 30 μl of kinase buffer that contained 10 μM ATP, 2.5 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech, 370 GBq/mmol), and 7 μg of myelin basic protein (MBP, Sigma) to each sample and were incubated for 30 min at 30 °C. MBP was separated on a 14% SDS-polyacrylamide gel (31), stained with Coomassie Blue, and dried. MBP bands were excised, and the incorporated radioactivity was determined by scintillation counting. Results represent the mean (± SEM) of three experiments.
The Novel Lipid Present in Commercial SPC Is Responsible for the LPA-like Activity—In Xenopus oocytes, APA mediators elicit Ca\(^{2+}\)-activated oscillatory Cl\(^{-}\) currents through the activation of multiple APA receptor subtypes, which couple to the inositol trisphosphate-Ca\(^{2+}\) signaling system (7, 32). To confirm its LPA-like effects, SPC was applied to oocytes that were voltage-clamped at −60 mV, and membrane currents were recorded. The SPC preparation obtained from Sigma, which is used by many of the investigators who reported its LPA-like effects (6, 11, 16, 17, 33–36), elicited oscillatory currents with a threshold concentration in the low micromolar range (Fig. 1). Because this particular brand of SPC is only 85% pure, according to the manufacturer, we also purchased SPC (nominal purity = 99%) from Matreya Inc. Surprisingly, the Matreya brand of SPC was inactive in the oocyte bioassay (Fig. 1). To eliminate batch-to-batch variations, we tested multiple batches of SPC from Sigma (Fig. 1) and Matreya. All Sigma SPC samples tested to date activated oscillatory Cl\(^{-}\) currents in the oocyte, whereas none of the Matreya brand SPCs was active up to 100 μM (data not shown). Due to the consistent differences between the two brands of SPC, HPLC was applied to test the purity of the different SPCs and to determine whether any difference in the 1-erythro- and 1-threo stereoisomer composition of the two preparations could account for the difference in their biological activity. Of the two SPC stereoisomers, we have previously shown that only the 1-erythro-SPC isomer (εSPC) activated the sphingolipid receptors in atrial myocytes, whereas the 1-threo isomer was inactive (24). As shown in Fig. 2, HPLC separation of the Sigma brand SPC, using the solvent system originally developed for the separation of SPC stereoisomers (24), showed several impurities that eluted before 1-erythro- (R\(_t\) = 32 min) and 1-threo-SPC (R\(_t\) = 41 min). Bioassays of the 1-min fractions collected during the elution showed that the compound(s) responsible for eliciting oscillatory CI\(^{-}\) currents did not co-purify with either SPC stereoisomer but did correspond to a small peak in the impurities that eluted between 10 and 13 min.

Both companies produce SPC by the acidic methanolysis of SM; this process can lead to the generation of several by-products besides SPC, depending on the conditions of the hydrolysis reaction and on the amount of water included in the reaction mixture (21). Purified SM and brain-derived phospholipids were obtained from Sigma, Matreya, and Avanti Polar Lipids and were subjected to acid semi-aqueous methanolysis using the conditions originally described by Gauer and Sweely (21) that are used to manufacture SPC by Sigma. After an 18-h aqueous acidic hydrolysis, the reaction mixtures were neutralized, and SPC was extracted according to Bligh and Dyer (37). The extracted lipids were dissolved in methanol and were tested in the oocyte bioassay for the generation of compounds capable of eliciting oscillatory Cl\(^{-}\) currents. None of the SM preparations from the different suppliers elicited oscillatory Cl\(^{-}\) currents in the oocyte. However, only the hydrolysate from the Sigma brand SM elicited oscillatory Cl\(^{-}\) currents (data not shown). Although the brain phospholipid extracts contained traces of biological activity, after 18 h of acidic hydrolysis all brain phospholipid hydrolysates were highly active and contained titers of the active compound(s) that were substantially (>10-fold) higher than that titer prior to hydrolysis. These observations suggested that a contaminating phospholipid precursor might be present in the Sigma SM, which, upon acid methanolysis, would lead to the generation of the active compound(s). To test this hypothesis, we turned our attention to a previously unidentified lipid fraction that co-elutes with SM in the manufacturing process. This lipid, which is removed by careful low pressure chromatography and cold acetone precipitation from the SM fraction in the manufacturing process by Matreya, could potentially contaminate the Sigma brand SM.

This particular fraction, designated RO56 by Matreya, migrated as a single spot (R\(_t\) 0.36) ahead of SM, when developed by TLC solvent 1, and stained positive with ninhydrin and molybdenum blue (Fig. 3A). Acid methanolysis of RO56, carried out the same way as in the manufacturing of SPC, led to the generation of an even faster migrating product (R\(_t\) 0.42) that was no longer positive with ninhydrin but remained positive with molybdenum blue (Fig. 3B). The bioassay of RO56 showed that the R\(_t\) 0.36 compound was inactive, whereas the hydrolysate was highly potent in activating oscillatory Cl\(^{-}\) currents (Fig. 3C). A major problem of this reaction was the low yield (<1%) of the active compound; this prevented the collection of a sufficient quantity of the active material for IR and NMR analysis. However, the FAB-MS spectra from the active lipid isolated from the hydrolysate of RO56 and of that lipid found in the active fraction isolated by HPLC from the Sigma SPC were both identical, which showed two deprotonated molecular ions, \((M - H)^-\), at m/z 419 and 421, respectively.

**Alkenyl-GP, a Novel LPA Analog**

FIG. 1. Sigma, but not Matreya brand, SPC elicits oscillatory Cl\(^{-}\) currents in Xenopus oocytes. LPA, applied at a 3 nM concentration, and SPC, applied at a 5 μM concentration, elicited large oscillatory currents in an oocyte voltage-clamped at −60 mV. SPC from Matreya was inactive, whereas both batches of SPC purchased from Sigma (1 and 2) were active. Downward deflection of the trace represents an inward current.

FIG. 2. HPLC purification of Sigma brand SPC. SPC (1 mg) was injected onto a 250 × 4.6-mm silica analytical column and was eluted using a gradient described by Buenemann et al. (24). This method has been used to separate 1-erythro-SPC (R\(_t\) ~32 min) from the biologically inactive stereoisomer, 1-threo-SPC (R\(_t\) ~41 min). The eluting material was monitored by an evaporative light-scattering detector through a metering valve that split the effluent in a 4:1 ratio between a fraction collector and the detector, respectively. Fractions (1 min) were collected and were tested in the oocyte bioassay at 100-fold dilution. The compound(s) that activated oscillatory Cl\(^{-}\) currents eluted with the impurities with an apparent peak retention time of ~11.5 min.

**RESULTS**

Elucidation of the Structure of RO56—The ninhydrin and
molybdenum blue staining of RO56 suggested that this lipid was a phospholipid with a free amino group, possibly with an ethanolamine moiety. Moreover, the poor yield of the acid methanolation reaction was presumed to be due to RO56 being a plasmalogen-like substance. Because we had milligram amounts of RO56, we first focused on determining its structure.

A FTIR spectrum of RO56 was obtained in CHCl₃: 732 cm⁻¹ (cis-CH=CH-), 1076 cm⁻¹ (P=O), 1222 cm⁻¹ (C=O), 1664 cm⁻¹ (C=C), 2550–2560 cm⁻¹ (CH₂, CH₃, and NH₃). ¹H NMR in CD₃OD: δ 0.89 (t, J = 6.7 Hz, 3H), 1.20–1.45 (m, 30H), 1.90–2.20 (m, 4H), 3.13–3.16 (t, J = 6.5 Hz, 2H), 3.82–3.86 (m, 4H), 4.00–4.10 (m, 2H), 4.31 (dt, J = 1.90–2.20 Hz, 4H), 3.13–3.16 (t, J = 6.2 Hz, 2H), 5.25–5.41 (m, 1H), 5.99 (dt, J₁ = 6.3 Hz; J₂ = 7.3 Hz, 1H), 5.25–5.41 (m, 1H), 5.99 (dt, J₁ = 6.2 Hz, J₂ = 1.4 Hz, 1H). ¹H NMR of RO56 in CD₃OD showed the presence of a vinyl-ether linkage, on which the two hydrogen atoms are in a cis-configuration (δ 5.99 ppm, J₁ = 6.2 Hz) since the trans-isomer would have a coupling constant greater than 10 Hz. The ¹³C NMR also showed the presence of an ethanolamine moiety (3.13–3.16 and 4.00–4.10 ppm in CD₃OD).

¹³C NMR in CD₃OD: δ 14.42, 23.73, 24.94, 28.12, 30.04, 30.33, 30.44, 30.65, 30.69, 30.79, 30.84, 30.97, 32.93, 33.07, 41.67 (d, J = 6.6 Hz), 62.91 (d, J = 5.2 Hz), 67.91 (d, J = 5.8 Hz), 70.95 (d, J = 7.6 Hz), 73.90, 108.01, 130.85, and 146.33. ¹³C NMR of RO56 in CD₂OD confirmed the presence of the vinyl-ether linkage (δ 130.85 and 146.33 ppm). A single peak at 108.01 ppm indicated the existence of a second double bond in the hydrocarbon chain, which was non-conjugated with the vinyl-ether bond. The lack of a peak above 160 ppm confirms the absence of carbonyl groups in the molecule; that conclusion is consistent with the FTIR data. ³¹P NMR of RO56 in CD₂OD appears at δ 18.17. FAB MS spectra (R = C₆H₁₇ for each compound). Negative ion mode: (M – H)⁻ at m/z 464 (Δ¹, n = 4), 462 (Δ¹⁻, n = 4), and 436 (Δ¹⁻, n = 2). Positive ion mode: (M + H)⁺ at m/z 466 (Δ¹, n = 4) and 464 (Δ¹⁻, n = 4), (M + Na)⁺ at m/z 486 (Δ¹⁻, n = 4) and 432 (Δ¹⁻, n = 0).

Based on these analytical data, the structure of RO56 was equivalent to that of alkanyl-GPE. The ¹H and ¹³C NMR of RO56 were also compared with those spectra of commercially available alkanyl-acyl-GPE and alkanyl-GPE, which completely matched those of the latter. ¹H NMR of RO56 and alkanyl-GPE both showed only one proton at δ 5.25–5.41 ppm instead of two, which represents both protons on the C=C double bond in the middle of the hydrocarbon chain. Moreover, ¹H NMR also revealed four of the six protons at δ 1.90–2.20 ppm, which are the two protons on the carbon adjacent to the vinyl-ether bond, and four others on both sides of the C=C double bond in the middle of the hydrocarbon chain. These results indicate that 50% of these molecules contain two C=C double bonds in the hydrocarbon chain (18:1), whereas the other 50% possesses only the vinyl-ether double bond (18:0).

High Yield Synthesis of the Active Compound Using Base Hydrolysis of Alkenyl-GPE—The vinyl-ether bond in alkanyl-GPE is unstable in acid but is stable in base. Treatment of alkanyl-GPE with NaOH can hydrolyze the ethanolamine moiety and generate alkanyl-GP (2a and 2b, Fig. 4). A solution of alkanyl-GPE (15 mg, 33 μmol) in CHCl₃, was dried with argon at room temperature, and the resulting material was dissolved in 10 ml of MeOH saturated with NaOH. The mixture was sealed in a pressure-resistant vial and was heated to ~100 °C in an oil bath for 2 h with stirring. The reaction mixture was allowed to cool to room temperature and was neutralized with 1 N HCl in MeOH to pH ~7.5. The precipitated NaCl was removed by filtration, and the filtrate was concentrated at 30 °C on a rotary evaporator. The residual solid was extracted (3 x) with 5 ml of MeOH, and the combined extracts were evaporated to ~0.5 ml with argon. The extract was assayed in
the oocyte bioassay and elicited oscillatory Cl⁻ currents (not shown).

Purification of the Active Compounds from the Alkenyl-GPE Hydrolysate—The crude methanol extract of the hydrolysate, dissolved in 0.5 ml of MeOH, was applied to silica gel TLC plates, and the plate was developed for 1 h with solvent 3 (see "Materials and Methods"). Primuline-staining showed two major (Rₜ 0.31 and 0.42) and five minor bands (Rₜ values 0.61, 0.66, 0.85, 0.92, and 0.97). The bands were scraped off the plates, and the lipids were extracted with three washes of 3 ml of MeOH and concentrated to ~0.5 ml. The concentrated fractions were tested in the oocyte bioassay. Only two bands, migrating close to each other with Rₜ values of 0.31 and 0.42, showed activity (not shown). The overall yield of the active compounds was ~40%, which was substantially higher than the <1% yield of the acid methanalysis reaction. Retention factors for the two active compounds in the four different TLC solvent systems are shown in Table I.

Structure Elucidation of the Active Compounds—The two active bands were separated a second time by TLC, which was ineffective in completely separating the two because both compounds showed mutual cross-contamination. Therefore, instrumental analysis was first performed with a mixture of the two active compounds. FTIR spectrum of the compounds in CHCl₃:acetone:MeOH:HAc:H₂O (10:4:3:2:1) 0.38, 0.33 (m, 4H), 3.70–3.95 (m, 5H), 4.19–4.34 (m, 1H), 5.20–5.41 (m, 1H), 6.03 (dt, J = 6.4 Hz, 3H), 1.15–1.50 (m, 24H), 1.91–2.19 (m, 4H), 3.70–3.95 (m, 5H), 4.19–4.34 (m, 1H), 5.20–5.41 (m, 1H), 5.99 (dt, J₁ = 6.2 Hz, 1/2H, 2a), 6.03 (dt, J₂ = 6.3 Hz, 1/2H, 2b), 1/2C NMR in CD₃OD: δ 14.42, 23.71, 24.89 (24.92), 28.11 (28.14), 30.02, 30.31, 30.42, 30.59, 30.63, 30.78, 30.83, 30.95, 30.98, 32.92 (33.06), 60.63, 64.25 (d), 66.99 (d), 71.59 (d), 72.23 (d), 74.22, 75.10 (d), 107.51 (107.71), 130.83 (130.86), and 146.45 (146.54). 31P NMR in CD₃OD appear at δ 72.23 (d), 74.22, 75.10 (d), 107.51 (107.71), 130.83 (130.86), and 146.45 (146.54). Table I shows the two products with different isomeric ratios. 1H NMR of the upper band (Rₜ 0.42) showed that the mixture had an isomeric molar ratio of 80:20 of compounds 2a:2b, based on the peak integration of the vinyl-ether hydrogen at 5.99 ppm for 2a and at 6.03 ppm for 2b (CD₃OD). 31H NMR of the lower band (Rₜ 0.31) showed that the mixture had an isomeric molar ratio of 30:70 of compounds 2a:2b. FAB-MS in the positive ion mode of both mixtures showed the presence of peaks at m/z 489, 487, 467, 465, and 409, respectively. These analytical data are consistent with the active compound being two regioisomers of alkenyl-GP.

Additional proof of the alkenyl-GP structure was sought by using chemical and enzymatic techniques. Because vinyl-ether bonds can be quantitatively hydrolyzed with HCl vapor (38), Sigma SPC and the mixture of two active compounds purified from the base hydrolysate of alkenyl-GPE were spotted onto TLC plates and exposed to HCl vapor for 15 min. As a control, the same lipids were spotted without exposure to HCl. Bioassay of the products showed that this brief HCl treatment destroyed the activity of the active lipids; this result provides further evidence for the presence of a vinyl-ether linkage in the active compound (Fig. 5A). We verified the presence of an unsubstituted monoesteric phosphate moiety, using the AP enzyme from E. coli, which is capable of hydrolyzing lipid monoesteric phosphates (26). Treatment of the mixture of the active compounds with this enzyme greatly diminished the activity (Fig. 5B).

An alternative method for the synthesis of alkenyl-GP is via the sequential enzymatic hydrolysis of alkenyl-acyl-GPE with PLD and PLA₂ enzymes. Alkenyl-acyl-GPE, or after treatment with PLD, was inactive in the oocyte bioassay (not shown). In contrast, the end product, following sequential treatments with PLD and PLA₂, elicited oscillatory currents in the oocyte. TLC analysis of the digest revealed a single active spot (Rₜ 0.42; solvent 3) that co-migrated with the active material found in Sigma SPC (data not shown).

Biological Activities of Alkenyl-GP—In the oocyte bioassay alkenyl-GP had an apparent EC₅₀ of 4.6 nM, whereas the apparent EC₅₀ for LPA was 16.3 nM (Fig. 6A). Heterologous desensitization analysis showed that LPA completely desensitized the alkenyl-GP response (Fig. 6B). In contrast, alkenyl-GP only partially desensitized the response to LPA (Fig. 6C) indicating that it was a receptor subtype-selective agonist.

The activation of the MAP kinases ERK1 and ERK2 was evaluated in Swiss 3T3 cells (Fig. 7A). Alkenyl-GP and LPA
were strong activators of the ERKs, whereas eSPC caused only a moderate 1.8-fold activation of the kinases, as compared with LPA and alkenyl-GP, which elicited a 4.6- and 4.1-fold increase, respectively. The combined application of 10 μM alkenyl-GP and eSPC caused a 5.8-fold activation of the ERKs.

Alkenyl-GP, eSPC, Sigma SPC, and LPA (10 μM each) were tested on the proliferation of Swiss 3T3 cells by measuring [3H]thymidine incorporation (Fig. 7B). Alkenyl-GP and LPA both increased [3H]thymidine incorporation approximately 5- and 6-fold, respectively, whereas purified eSPC elicited only a moderate 1.5-fold increase, which was less than the 2.5-fold increase observed with the impure SPC from Sigma. When eSPC was added together with alkenyl-GP, (10 μM each), a slightly higher (6.4-fold) increase in [3H]thymidine incorporation was observed.

**DISCUSSION**

The present study has elucidated the structure of a novel LPA-like autacoid, alkenyl-GP, which is present in the SPC from Sigma, a preparation used by numerous investigators who report on the overlapping biological responses between LPA and SPC. The manufacturer carefully alerts the users that this particular product is only 85% pure. Our experiments were prompted by our inability to reproduce several published reports, using high purity SPC and SPP. For example, in contrast to other investigators (14), we have not been able to detect oscillatory Cl− currents in oocytes elicited by SPP, which was produced by PLD cleavage of SPC, nor have we been able to detect these currents in response to pure SPC preparations up to 100 μM, except for the Sigma brand. Xu and co-workers (16, 17), using SPC from Sigma, showed heterologous desensitization between LPA and SPC in ovarian and breast cancer cell lines and in Jurkat T cells. These investigators, based on these cross-desensitization studies, adapted the hypothesis originally put forward by Durieux and colleagues (14) that glycerolipid and sphingolipid mediators were acting on the same receptors that activate Ca2+ mobilization in different cell types. On the other hand, we have previously reported that in cardiac myocytes SPP and SPC elicited a K+ current, with nanomolar EC50 concentrations, through a receptor that was not activated by glycerolipids, including LPA, phosphatidic acid, and lysophosphatidylserine (24). Others (18, 39) also could not verify cross-desensitization between SPP and LPA, contradicting the hypothesis that LPA and sphingolipid mediators activated the same putative receptor(s). Furthermore, recent reports on the cloning of two LPA receptors found no evidence that these receptors were activated by SPP or SPC (40–42).

HPLC analysis of the Sigma brand SPC revealed that the LPA-like biological activity responsible for eliciting oscillatory Cl− currents in the Xenopus oocyte bioassay did not co-purify with either one of the previously identified α-erythro-or α-threo-SPC compounds, but rather eluted as a distinct peak (Rt = 11.5 min). The low (5–8%) abundance of this impurity initially did not permit the isolation of sufficient quantities for instrumental chemical analysis other than FAB-MS. Nonetheless, FAB-MS revealed that the active compound contained two major deprotonated molecule anions, (M − H)−, at m/z 419 and 421, which were different from the corresponding ions and fragments of SPC and SPP. Moreover, the active impurity did not stain with ninhydrin, suggesting that it did not contain a sphingoid base.

These observations prompted us to analyze the manufacturing process in which SPC is produced by Sigma. The acidic methanolysis procedure, in the presence of dilute HCl, yielded the active compound only if we used SM from Sigma; the hydrolysates prepared from SM from two other commercial sources did not generate the active compound. Importantly,
when the acidic methanolation reaction was performed without H$_2$O, no detectable amounts of the active compound were found (data not shown). We speculate that a small amount of alkenyl-GP survives in the dilute aqueous-methanolic solution of HCl (−1 N) used in the manufacturing process, probably due to an equilibrium between the product and its precursor. Considering the 4.6 nm $EC_{50}$ of alkenyl-GP, such a trace amount, present in the reaction mixture, could easily explain the biological activity. In stronger acid, alkenyl-GP completely decomposed, and therefore, no activity was observed. This scheme also indicates that there was no trace alkyl-GP in the starting material, as it would have survived due to its stability in strong acid.

Acidic methanolation of the crude phospholipid fraction formed the active compound(s); therefore, we focused our attention on the possibility that other non-sphingolipid contaminants present in SM could give rise to the active compound(s). We analyzed the manufacturing process of SM and identified a ninhydrin-positive phospholipid that co-purifies with SM, from which it could only be removed by careful low pressure chromatography. This previously unidentified lipid, designated RO56 by Matreya researchers, migrated as a single spot in basic or acidic TLC solvents, and when exposed to acidic methanalysis, it became active in the oocyte bioassay. The very low yield of the acidic methanolation reaction, the positive staining of the RO56 precursor with ninhydrin, together with other reports on the presence of plasmalogens in the base hydrolysates of the Folch extracts used in the manufacturing of SM, suggested the possibility that RO56 might be a plasmalogen.

NMR, FTIR, and MS were used to elucidate the structure of RO56. These techniques verified our hypothesis that RO56 was alkenyl-GPE. This conclusion was based on the identification of all the functional groups characteristic of the plasmalogen structure, particularly the cis-vinyl-ether bond. We also fulfilled the requirements for the proof of the chemical structure of plasmalogens put forward by Snyder and co-workers (43), using enzymatic and chemical modifications. Establishing that RO56 was alkenyl-GPE allowed us to develop a hypothetical scheme for the reactions that could lead to the generation of alkenyl-GP through the base hydrolysis of the ethanolamine moiety (Fig. 5).

To overcome the degradation of the vinyl-ether group under acidic conditions, and in order to synthesize a sufficient amount of alkenyl-GP for structural determination, we turned to basic hydrolysis conditions. We found that the yield of the basic reaction was at least 40 times greater than that of the acidic hydrolysis. However, TLC analysis of the basic hydrolysate of alkenyl-GPE revealed the generation of a second active lipid that migrated slightly behind ($R_f$ 0.31; solvent 3) the active compound present in the acidic hydrolysate ($R_f$ 0.42, solvent 3). The FAB-MS spectra of the two compounds, which showed identical molecule ions and fragmentation patterns, suggested that these compounds were regioisomers. $^1$H and $^{13}$C NMR spectra of the partially separated active lipids verified that these compounds were regioisomers of alkenyl-GP. By using the ACD computer program to model the NMR spectra of the two regioisomers, we found that the experimental data were consistent with the 1-O-cis-alk-1'-enyl-2-lyso-sn-glycero-3-phosphatidylcholine (2a) being more abundant (~80%) in the faster migrating band with the vinyl-ether hydrogen chemical shift at 5.99 ppm. In contrast, the compound in the slower migrating TLC band showed the abundance of the 1-O-cis-alk-1'-enyl-3-lyso-sn-glycero-2-phosphatidylcholine (2b) regioisomer with the vinyl-ether hydrogen chemical shift at 6.03 ppm. The generation of regioisomers during base-catalyzed hydrolysis of phospholipids has been previously reported, and a scheme similar to the one proposed (Fig. 5) has been proposed by Hanahan et al. (44) for the base hydrolysis of platelet-activating factor. Because no significant differences were found in the biological activity of the two partially pure (~70–80%) regioisomers, we concluded that they both were biologically active, which underlines that phosphomonoesters at the sn-2 and sn-3 position both can activate APA receptors. Heterologous desensitization analysis in the oocyte indicated that alkenyl-GP activates only distinct subtypes of the APA receptors.

LPA is a potent mitogen in several cell types (45) but is antimitogenic in others (27, 46). The mitogenic response elicited by LPA has been linked to the activation of the Ras-ERK-signaling pathway (47, 48). SPC has also been shown to be mitogenic in Swiss 3T3 fibroblasts (6), and its mitogenic activity has also been linked to the activation of the ERKs (36). Alkenyl-GP, similar to LPA, activated the ERK1 and ERK2 protein kinases in Swiss 3T3 cells. Purified eSPC had only a marginal effect on the activation of ERK1 and ERK2. When added together with alkenyl-GP, eSPC showed an additive effect in activating ERKs. These results are in agreement with the report by Seufferlein and Rozengurt (36); however, these investigators used SPC from Sigma. By using peak integration for the impurity present in the Sigma SPC, we estimate that it constitutes ~5–8% of the total material. This amount of alkenyl-GP could account for the activation of ERKs reported by Seufferlein and Rozengurt (36).

LPA has generated considerable interest because of its mitogenic effect in a variety of cell types. We tested the purified, biologically active stereoisomer e-SPC, Sigma SPC, alkenyl-GP, and LPA for their effect on the proliferation of Swiss 3T3 cells, which is the cell line that has been used by other investigators who report on the LPA-like actions of SPC. Alkenyl-GP, similar to LPA, was highly mitogenic at micromolar concentrations in this cell line. Interestingly, eSPC alone was weakly mitogenic in our hands; however, when applied together with alkenyl-GP, it enhanced the mitogenic effect of alkenyl-GP. Sigma SPC, consistent with the presence of the contaminating alkenyl-GP impurity, was more potent than eSPC in causing mitogenesis.

Alkenyl-GP is a novel member of the APA family and is derived from a different group of lipids than LPA, which is the plasmalogens. Alkenyl-GP has been recently identified among a group of naturally occurring mitogenic lipids generated in response to injury (19). Plasmalogens, the precursors of alkenyl-GP, are abundant in certain tissues (49), particularly in the heart (50) and in the brain, where it constitutes over 32% of the myelin phospholipids (51). Enzymes capable of specifically cleaving the ethanolamine and choline substituents of the phosphate moiety of plasmalogens have also been identified in the brain (52) and other tissues (53). A lysophospholipase D (EC 3.3.2.2 and 3.3.2.5), specifically catalyzing the hydrolysis of the vinyl-ether bond of plasmalogens, has also been described in the brain (54). However, preliminary data indicate that alkenyl-GP is not cleaved by this enzyme; the possibility that it could have a sufficiently long half-life in biological fluids is supported by its $\sim 5\mu M$ in vivo steady-state concentration in the anterior chamber fluid of the eye (19). A more detailed analysis of the synthesis and degradation of alkenyl-GP will be necessary in forthcoming studies.

Our data underline the utmost importance for the use of analytically pure reagents, and thus it appears necessary to re-evaluate the previous reports on the different actions of SPC because of the likely presence of alkenyl-GP, which could have led to mixed biological actions. For these experiments, chemical...
and regiosomer pure compounds must be used. At this stage of the research, we believe that the best method to resolve the controversy of whether APA receptors are activated by sphingolipids is to use the heterologous expression of the already cloned LPA receptor subtypes in a host cell that does not express endogenous receptors. Thus far, there is only one system that fulfills this criterion, the yeast expression system described by Erickson and colleagues (42). In this system SPP did not activate the Edg-2 receptor (42). However, this system has two potential shortfalls. It requires high micromolar concentrations of LPA to activate a reporter gene, which is much higher than the nanomolar concentration required for mammalian cells; and it only works for those LPA receptor types that can efficiently couple to the yeast mating pheromone pathway. A more detailed characterization of the receptor subtype-specific action of alkenyl-GP is provided elsewhere (55).

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