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Naturally Occurring Analogs of Lysophosphatidic Acid Elicit Different Cellular Responses through Selective Activation of Multiple Receptor Subtypes

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ABSTRACT

Lysophosphatidic acid (LPA), plasmalogen-glycerophosphate (alkenyl-GP) and, cyclic-phosphatidic acid (cyclic-PA) are naturally occurring phospholipid growth factors (PLGFs). PLGFs elicit diverse biological effects via the activation of G protein-coupled receptors in a variety of cell types. In NIH3T3 fibroblasts, LPA and alkenyl-GP both induced proliferation, whereas cyclic-PA was antiproliferative. LPA and alkenyl-GP decreased cAMP in a pertussis toxin-sensitive manner, whereas cyclic-PA caused cAMP to increase. LPA and alkenyl-GP both stimulated the activity of the mitogen-actived protein kinases extracellular signal regulated kinases 1 and 2 and c-*Jun* NH_2-terminal kinase, whereas cyclic-PA did not. All three PLGFs induced the formation of stress fibers in NIH3T3 fibroblasts. To determine whether these lipids activated the same or different receptors, heterologous desensitization patterns were established among the three PLGFs by monitoring changes in intracellular Ca^{2+} in NIH3T3 fibroblasts. LPA cross-desensitized both the alkenyl-GP and cyclic-PA responses. Alkenyl-GP cross-desensitized the cyclic-PA response, but only partially desensitized the LPA response. Cyclic-PA only partially desensitized both the alkenyl-GP and LPA responses. We propose that pharmacologically distinct subsets of PLGF receptors exist that distinguish between cyclic-PA and alkenyl-GP, but are all activated by LPA. We provide evidence that the PSP24 receptor is selective for LPA and not activated by the other two PLGFs. RT-PCR and Northern blot analysis indicate the co-expression of mRNAs encoding the EDG-2, EDG-4, and PSP24 receptors in a variety of cell lines and tissues. However, the lack of mRNA expression for these three receptors in the LPA-responsive Rat-1 and Sp2-O-Ag14 cells suggests that a number of PLGF receptor subtypes remain unidentified.

LPA is a lipid mediator with diverse biological properties (for reviews, see Tokumura, 1995; Moolenaar *et al.*, 1997). LPA is released from platelets and is found naturally among a group of lipids bound to serum albumin (Gerrard and Robinson, 1989; Tigyi and Miledi, 1992). The cellular responses elicited by LPA vary widely and can arbitrarily be grouped into four categories: 1) effects on the cell cycle that are either mitogenic (van Corven *et al.*, 1989) or antimitogenic (Tigyi *et al.*, 1994); 2) effects on Ca^{2+} homeostasis that can lead to the induction of inward currents in oocytes (Tigyi and Miledi, 1992), the contraction of smooth muscle cells (Tokumura *et al.*, 1980), and neurotransmitter release (Shiono *et al.*, 1993); 3) effects on the cytoskeleton that can lead to changes in cell shape and motility, including the retraction of neurites (Tigyi and Miledi, 1992), induction of stress fibers (Gohla *et al.*, 1998), stimulation of chemotaxis

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ABBREVIATIONS: LPA, 1-acyl-2-lyso-*sn*-glycero-3-phosphate; alkenyl-GP, 1-O-*cis*-alk-1′-enyl-2-lyso-*sn*-glycero-3-phosphate; AM, acetoxymethyl ester; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular calcium concentration; cyclic-PA, 1-acyl-2,3-cyclic-*sn*-glycero-3-phosphate; DMEM, Dulbecco's minimal essential medium; ERK, extracellular signal regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; $InsP_3$, inositol trisphosphate; JNK, *Jun* NH_2-terminal kinase; MAP, mitogen-activated protein; PCR, polymerase chain reaction; PLGF, phospholipid growth factor; RT, reverse transcriptase; SPC, sphingosylphosphorylcholine.

(Jalink *et al.*, 1993) and migration (Zhou *et al.*, 1995), and tumor cell invasiveness (Imamura *et al.*, 1993); and 4) effects on apoptosis and differentiation, including the prevention of programmed cell death (Umansky *et al.*, 1997) and the inhibition of neuronal differentiation (Tigyi and Miledi, 1992). LPA activates signaling pathways through the heterotrimeric G proteins $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ (for a review, see Moolenaar *et al.*, 1997). Recent evidence suggests that LPA signaling to the MAP kinase and Rho GTPase pathways involves the transactivation of the epidermal growth factor receptor (Cunnick *et al.*, 1998; Gohla *et al.*, 1998).

Cyclic-PA [previously referred to as cLPA (Liliom *et al.*, 1996)], which was first isolated from *Physarum polycephalum* (Murakami-Murofushi *et al.*, 1992), has been found among the serum and brain lipids at micromolar concentrations. It is also a stable intermediate of the phospholipase D-catalyzed hydrolysis of phospholipids (Friedman *et al.*, 1996). Depending on the cell type, the effects of cyclic-PA and LPA have been found to be either similar or opposing. Cyclic-PA has an effect on cell proliferation that is opposite that of LPA, in that it is antiproliferative in TIG-3 fibroblasts (Murakami-Murofushi *et al.*, 1993). Cyclic-PA also elevates cAMP (Murakami-Murofushi *et al.*, 1993), as opposed to LPA, which inhibits cAMP. In contrast, LPA has similar effects to cyclic-PA in *Xenopus laevis* oocytes, where both PLGFs activate Cl^- currents. In Sp2-O-Ag14 myeloma cells (Tigyi *et al.*, 1994) and in mesangial cells (Inoue *et al.*, 1995), LPA elevates cAMP levels. LPA is also antiproliferative in Sp2-O-Ag14 cells (Tigyi *et al.*, 1994), similar to the effect of cyclic-PA on fibroblasts. The novel alkenyl-GP is generated in response to corneal injury in the aqueous humor (Liliom *et al.*, 1998b) and elicits proliferation in keratocytes (Liliom *et al.*, 1998b) and Swiss 3T3 fibroblasts (Liliom *et al.*, 1998a).

One way to explain the diverse and sometimes opposing effects elicited by PLGFs is through the presence of multiple receptors with distinct ligand and signal transduction properties that are expressed in a cell-type-specific manner. This hypothesis is supported by the cloning of four genes that encode LPA receptors (Guo *et al.*, 1996; Hecht *et al.*, 1996; An *et al.*, 1998; Lee *et al.*, 1998). Additionally, structure-activity relationships have been demonstrated in different cell types and tissues that are suggestive of different receptor subtypes (Tokumura, 1995).

Using heterologous desensitization, we previously reported that cyclic-PA and LPA activate partially overlapping receptors in *X. laevis* oocytes (Liliom *et al.*, 1996). Xu (1995b) reported heterologous desensitization among LPA, SPC, and lysophosphatidylserine, proposing that the cross-desensitization among these lipids indicated that they act on the same receptor. These authors also noted that although LPA was mitogenic, lysophosphatidylserine was antimitogenic in Jurkat, human ovarian, and breast cancer cell lines (Xu *et al.*, 1995a, 1995b). We have since determined that alkenyl-GP [an impurity that is present in the Sigma brand SPC, which was the SPC used by Xu *et al.* (1995b)] is responsible for the LPA-like effects (Liliom *et al.*, 1998a). Moreover, alkenyl-GP showed only partial cross-desensitization with LPA in *X. laevis* oocytes, which suggests that it, too, is a receptor subtype-selective ligand for PLGF receptors (Liliom *et al.*, 1998a).

In the present study, LPA, cyclic-PA, and alkenyl-GP, three naturally-occurring members of the PLGF family, were used to establish that they activate pharmacologically distinct receptor subtypes and different signal transduction mechanisms in NIH3T3 cells. Our data indicate that subsets of PLGF receptors exist that distinguish between cyclic-PA and alkenyl-GP but are all activated by LPA. Furthermore, we provide evidence that the PSP24 receptor (Guo *et al.*, 1996) is selectively activated by LPA, and not by alkenyl-GP or cyclic-PA. These data support the hypothesis that distinct PLGF mediators elicit distinct biological responses through the selective activation of multiple PLGF receptors. RT-PCR and Northern blot analysis indicates the co-expression of mRNAs encoding the EDG-2, EDG-4, and PSP24 receptors in a variety of cell lines and tissues. However, the lack of mRNA expression for these three LPA receptors in the LPA-responsive Rat-1, HEK 293, and Sp2-O-Ag14 cells, and in liver tissue, suggests that there are more PLGF receptor subtypes yet to be identified.

Experimental Procedures

Materials. LPA (oleoyl) was purchased from Avanti Polar Lipids (Alabaster, AL), cyclic-PA (oleoyl, palmitoyl) was synthesized as described previously (Kobayashi *et al.*, 1993), and alkenyl-GP was prepared by base hydrolysis from ethanolamine-containing lysoplasmalogen, as reported previously (Liliom *et al.*, 1998a). All three PLGFs were 99% pure as tested by mass spectrometry (data not shown). PTX was obtained from List Biologicals (Campbell, CA). Fura-2 AM, BAPTA-AM, and rhodamine phalloidin were purchased from Molecular Probes (Eugene, OR). ^{125}I-cAMP and [γ-^{32}P]ATP were from Amersham (Arlington Heights, IL). Rabbit anti-cAMP antibody was provided by Dr. Helena Parfenova (University of Tennessee, Memphis, TN). All other chemicals were purchased from Sigma (St. Louis, MO). Lipids were complexed with an equal concentration of fatty acid-free BSA dissolved in Ca^{2+}-free Hanks' balanced salt solution.

Cells. NIH3T3 fibroblasts (American Type Culture Collection, Rockville, MD) were maintained in DMEM that contained 10% FBS (Summit Biotechnology, Ft. Collins, CO). Oocytes were obtained from adult *X. laevis* frogs as described previously (Tigyi *et al.*, 1998).

Molecular cloning of the PLGF receptor cDNAs. Isolation of the cDNA encoding the PSP24 receptor (GenBank Database, Accession No. U76385) was reported elsewhere (Guo *et al.*, 1996). The vzg-1 cDNA (GenBank Database, Accession No. U70622), which is the mouse homologue of the EDG-2 orphan receptor (GenBank Database, Accession No. U80811), was cloned from mouse NIH3T3 cells by RT-PCR. Template RNA was isolated with the Trizol Reagent (Life Technologies, Gaithersburg, MD) and reverse-transcribed with the Access RT-PCR System from (Promega, Madison, WI). The cDNA was amplified with *Taq* polymerase (Promega) using the forward primer: 5′-ACCGGGATCCGATCAGCCAACCCG-3′, which included a restriction site for *Bam*HI (underlined), and the reverse primer: 5′-AAAGCGGCCGCAGTAAGTAGGTATTATT-3′, including a site for *Not*I (underlined). The PCR product was subcloned into the pBluescript SK(−) (Stratagene, LaJolla, CA) plasmid under the control of the T7 promoter for cRNA synthesis. The sequence was verified by automated sequencing with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Cetus, Norwalk, CT).

RT-PCR and Northern blot analysis of PSP24, EDG-2, and EDG-4 PLGF receptor expression. The RT-PCR protocol used for the identification of the PLGFR mRNAs has been reported elsewhere (Tigyi *et al.*, 1998), using the following primers: PSP24 (mouse) forward primer, 5′-^{1}ATGGCCTGTAACAGCACA, and reverse primer, 5′-GGACGAGGGATCTG^{1180}; EDG-2 (mouse) forward primer, 5′-^{229}AACCGCCGCTTCCAT, and reverse primer, 5′-GTAGACACTATAGCTA^{580}, and EDG-4 (human) forward primer, 5′-

^{147}CAATCTGCTGGTCATAGCAG, and reverse primer 5′-ACTACTGTTGTCAGAACTGG^{737}. Northern blots were prepared with the NorthernMax kit from Ambion (Austin, TX) using 2 μg of poly(A)$^+$ mRNA isolated from rat tissues with the Trizol reagent and hybridized with [α-^{32}P]dCTP-labeled (Ready-To-Go DNA labeling kit; Pharmacia, Piscataway, NJ) full length receptor cDNA probes. The GAPDH probe was used as a control. Blots were stripped and reprobed with the different receptor cDNAs using the Strip-EZ kit (Ambion).

Synthesis and testing of receptor cRNAs. Full-length PSP24 and vzg-1 cRNA was synthesized with the mCAP RNA Capping Kit (Stratagene). The cRNAs (1 μg) were tested for translation in rabbit reticulocyte lysates (Promega) *in vitro*, and the synthesized proteins were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Electrophysiological recording and expression of PLGF receptors in *X. laevis* oocytes. Oscillatory Cl^- currents elicited by the PLGF ligands were recorded using a two-electrode voltage clamp system (Tigyi *et al*., 1998). Collagenase-treated and defolliculated oocytes were injected with 0.2–2 ng of *in vitro*-transcribed cRNA (Guo *et al*., 1996), and electrophysiological recording was performed 2–3 days after injection.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator Fura-2 AM as described previously (Tigyi *et al*., 1998). NIH3T3 cells were plated onto glass coverslips at a density of 2–3 $\times$ 10^5 cells/coverslip and used at 70–80% confluency. The cells were serum-starved in DMEM for a period of 18–24 hr before monitoring, unless otherwise specified.

Cell proliferation assays. NIH3T3 fibroblasts were plated at a density of 10^4 cells/cm^2 in 24-well plates in DMEM that contained 10% FBS. The following day, the cells were changed to DMEM that contained 2% FBS and the lipids were added to each well. Cells were counted daily with a Coulter Counter (Model Z_f; Coulter Electronics, Hialeah, FL). For dose-response assays, NIH3T3 fibroblasts were cultured in the presence of a 0–40 μM concentration of the lipids under the same conditions and the cells were counted after 3 days.

cAMP measurements. cAMP determinations were made by radioimmunoassay in subconfluent PLGF-stimulated cultures. Cells were plated in 60-mm dishes in DMEM that contained 10% FBS, and changed to serum-free DMEM the next day. The next day, the cells were incubated with 1 mM 3-isobutyl-1-methylxanthine for 10 min before treatment with a 10 μM concentration of the lipids in the presence or absence of Ca^{2+}. Nominally Ca^{2+}-free conditions were achieved by replacing the 1.8 mM $CaCl_2$ with 0.89 mM $MgCl_2$ and 1 mM EGTA, and including 5 μM BAPTA-AM, for 30 min before the addition of the PLGFs.

Immune complex MAP kinase assays. Subconfluent cultures of NIH3T3 cells were serum-starved for 18 hr, treated with a 1 μM concentration of the PLGFs for 10 min, and the combined ERK 1/2 activities were determined as described previously (Liliom *et al*., 1998a). JNK activity was measured by the method of Cadwallader *et al*. (Cadwallader *et al*., 1997), using GST-Jun as a substrate after a 45 min exposure to a 1 μM concentration of each PLGF.

Staining of F-actin with rhodamine phalloidin. NIH3T3 cells were plated on glass coverslips at a density of 2.5 $\times$ 10^4 cells/ml and serum-fasted for 18 hr before treatment with 1 or 10 μM concentrations of the PLGFs. After 15- and 30-min treatments, the cells were fixed and permeabilized for 10 min at room temperature in Dulbecco's PBS that contained 3.7% paraformaldehyde and 0.1% Triton X-100. The cells were incubated for 1 hr with 5 units/ml of rhodamine phalloidin. After 1 hr, the cells were rinsed 3 times with PBS and mounted with Aquamount. The distribution of F-actin was visualized with a Laser Sharp MRC-1024 LaserScanning Confocal Imaging System (Bio-Rad Laboratories, Richmond, CA).

Statistical analysis was performed using Student's *t*-test, and the results were considered significant at $p < 0.05$.

Results

LPA and alkenyl-GP stimulate the proliferation of NIH3T3 cells, whereas cyclic-PA is antiproliferative. NIH3T3 cells were treated with a 20 μM concentration of each lipid in the presence of 2% FBS and the cells were counted daily, for 3 days (Fig. 1A). The inclusion of 2% FBS was required to maintain a basal rate of proliferation to demonstrate the antiproliferative effect of cyclic-PA. Control cultures (20 μM BSA) increased in number by 2.7-fold. LPA caused an additional 2.4-fold increase in cell number over control that equaled a 6.5-fold increase over the starting density, whereas alkenyl-GP caused a 2-fold increase over control, representing a 5.3-fold overall increase in cell number. In contrast, cyclic-PA caused an antiproliferative effect by maintaining the cells at their starting density and inhibited the 2.5-fold increase seen with the control cells, without decreasing the viability of the cells as determined by trypan blue dye exclusion. When NIH3T3 cells were incubated with

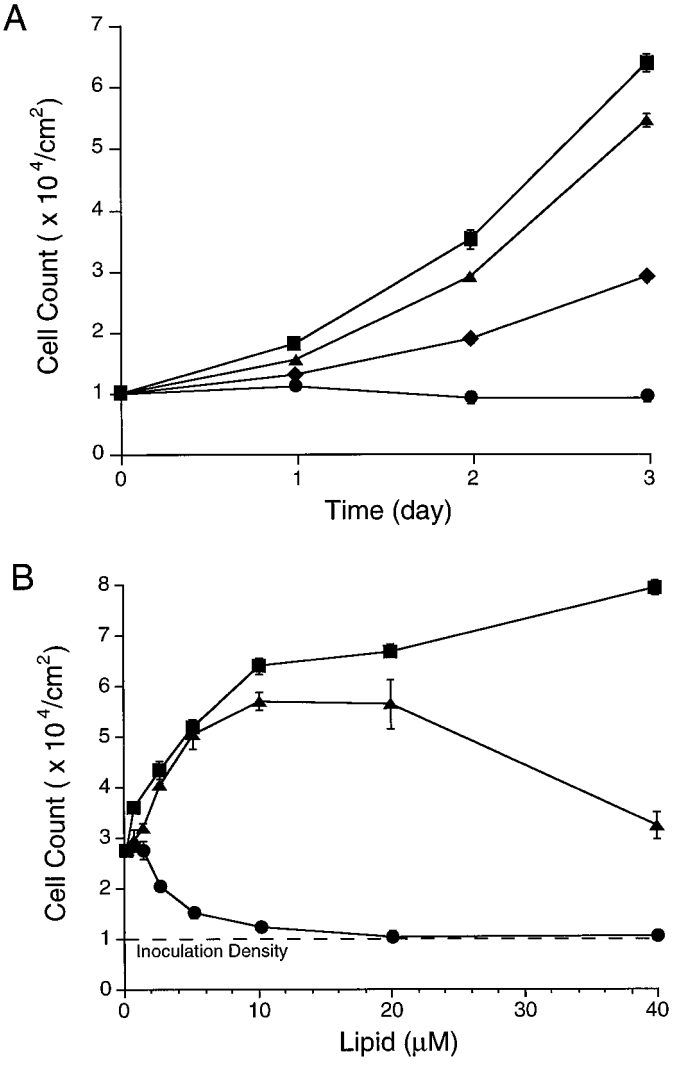

Fig. 1. Effect of PLGFs on cell proliferation. A, NIH3T3 cells were treated with a 20 μM concentration of LPA (■), alkenyl-GP (▲), or cyclic-PA (●) in the presence of 2% FBS (♦), for up to 3 days. B, Cells were treated with the designated concentrations of LPA (■), alkenyl-GP (▲), or cyclic-PA (●), in the presence of 2% FBS. After 3 days, the cells were counted. *Data points*, mean cell count obtained from quadruplet cultures ± standard deviation.

100 ng/ml of PTX for 18 hr before the addition of the lipids, the proliferative effect of LPA and alkenyl-GP was completely inhibited, whereas the antiproliferative effect of cyclic-PA was unaffected (data not shown).

Similar proliferative effects were observed with LPA and alkenyl-GP under defined, serum-free conditions [data not shown (see Liliom *et al.*, 1998a)]. In these experiments, after a 2-day culture in the presence of a 10 μM concentration of each lipid, LPA caused a 2.4-fold increase and alkenyl-GP caused a 2-fold increase in cell number, whereas cyclic-PA caused no change in cell number.

NIH3T3 cells were grown in the presence of 2% FBS and increasing amounts of the PLGFs for 3 days, at which point the cells were counted (Fig. 1B). The proliferative effect of LPA and alkenyl-GP, and the antiproliferative effect of cyclic-PA were dose-dependent, with apparent EC_{50} values of 1.7 μM for LPA and 1.5 μM for alkenyl-GP and an apparent IC_{50} value of 1.6 μM for cyclic-PA.

LPA and alkenyl-GP decrease, whereas cyclic-PA elevates cAMP. Fig. 2A shows the changes in basal cAMP levels that were elicited by a 10 μM concentration of each of the three PLGFs, or control (BSA), in subconfluent cultures of NIH3T3 cells. After a 5-min treatment, LPA and alkenyl-GP decreased basal cAMP levels significantly ($p < 0.05$), whereas cyclic-PA increased cAMP 1.6-fold ($p < 0.01$). In parallel cultures that were treated for 18 hr with 100 ng/ml of PTX, LPA and alkenyl-GP no longer decreased cAMP but showed a slight increase over control levels, which was significant for alkenyl-GP ($p < 0.05$; Fig. 2A). In contrast, in cells treated with PTX, cyclic-PA still caused a 1.8-fold increase in cAMP, which was statistically significant compared with control ($p < 0.02$).

Several types of adenylyl cyclases are stimulated by Ca^{2+} (Cooper *et al.*, 1995). Because cyclic-PA was found to elevate $[Ca^{2+}]_i$ (see Fig. 5), we investigated the potential role of Ca^{2+} in the elevation of cAMP. When Ca^{2+} was removed from the extracellular medium and BAPTA-AM was used to prevent increases in $[Ca^{2+}]_i$, the elevation of cAMP stimulated by cyclic-PA was abolished (Fig. 2B). In contrast, after the depletion of Ca^{2+}, no effect was seen on the inhibition of cAMP accumulation by LPA or alkenyl-GP (data not shown).

The cyclic-PA-elicited rise in cAMP is inhibited by LPA and alkenyl-GP. We examined the interaction of LPA and cyclic-PA on cAMP signaling. Stimulation of NIH3T3 cells with a 10 μM concentration of cyclic-PA every 5 min caused a steady rise in cAMP over a 15-min period (Fig. 2C). Ten minutes after stimulation with cyclic-PA, the addition of 10 μM LPA caused a rapid decrease in cAMP. Addition of alkenyl-GP or LPA along with cyclic-PA, prevented the rise in cAMP after a 5-min treatment.

LPA and alkenyl-GP, but not cyclic-PA, stimulate the MAP kinases ERK and JNK. LPA and alkenyl-GP both stimulated a significant increase in the ERK 1/2 activity, after a 10-min treatment, with 2.7- and 2.5-fold increases over control levels, respectively (Fig. 3A). In contrast, cyclic-PA caused a slight, statistically insignificant inhibition of the basal ERK 1/2 activity. Similarly, LPA and alkenyl-GP both caused a significant increase in the JNK activity, with 1.38- and 1.34-fold increases over control levels, respectively (Fig. 3B). In contrast, cyclic-PA caused an apparent yet insignificant decrease in the basal activity of JNK.

All three PLGFs induce stress fiber formation in NIH3T3 fibroblasts. Subconfluent, serum-starved cultures of NIH3T3 cells were exposed to each PLGF and stained with rhodamine phalloidin to localize filamentous actin. Whereas the control cells remained devoid of stress fibers (Fig. 4), all three PLGFs induced the formation of stress fibers. The effect of cyclic-PA seemed to last the longest. Stress fibers induced by LPA and alkenyl-GP were less abundant at 30 min (data not shown) than at 15 min by visual observation. In contrast, the stress fibers that were induced by cyclic-PA at 30 min seemed to be more abundant than those seen at 15 min.

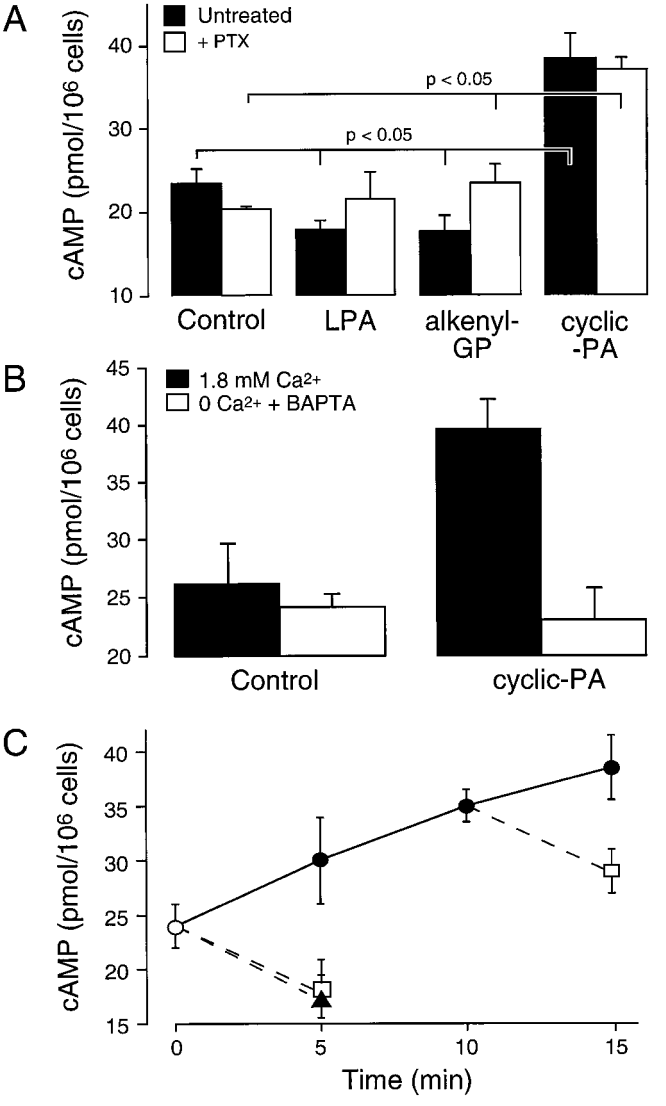

Fig. 2. Effect of PLGFs on cAMP. A, NIH3T3 cells were treated with a 10 μM concentration of LPA, alkenyl-GP, or cyclic-PA for 5 min (■). Identical cultures were pretreated for 18 hr with 100 ng/ml of pertussis toxin (*PTX*), and similarly treated with the lipids (□). After treatment with the lipids, the cells were lysed and cAMP levels were measured by radioimmunoassay. B, Cells were treated with cyclic-PA (10 μM) for 5 min in the presence of 1.8 mM Ca^{2+} (■) or nominal absence of Ca^{2+} + 5 μM BAPTA (□). *Bars*, mean ± standard deviation of triplicate samples. C, NIH3T3 cells were treated with a 10 μM concentration of cyclic-PA (●) at 5-min intervals, and incubated for 5, 10, and 15 min. After 10 min of cyclic-PA treatment, 10 μM LPA (□) was added for an additional 5 min. Cells were likewise treated simultaneously with 10 μM cyclic-PA and 10 μM LPA, or alkenyl-GP (▲), and incubated for 5 min. Basal cAMP levels (○) were measured from untreated cells.

LPA, alkenyl-GP, and cyclic-PA partially cross-desensitize. Using heterologous desensitization between LPA and cyclic-PA, we previously demonstrated the existence of at least two distinct LPA receptors in *X. laevis* oocytes (Liliom *et al*., 1996). We have now expanded these experiments to NIH3T3 cells and included the novel PLGF, alkenyl-GP (Liliom *et al*., 1998a). All three PLGFs elicited a $[Ca^{2+}]_i$ response in a dose-dependent manner (Fig. 5) with apparent EC_{50} values of 96 nM, 570 nM, and 3.6 μM for LPA, alkenyl-GP, and cyclic-PA, respectively. The size of the responses elicited by a maximally effective dose of 1-, 3-, and 10 μM for LPA, alkenyl-GP, and cyclic-PA, respectively, were quantified by measuring the area under the Ca^{2+} traces. The response size was the largest for LPA, followed by alkenyl-GP and then cyclic-PA (Table 1).

Repeated application of the same PLGF caused the cells to become desensitized to that lipid. After desensitization, the other two PLGFs were applied to test the responsiveness of the cells to the heterologous lipids. To maintain the desensitized state of the cells, the cells were washed with the desensitizing PLGF between application of the other two PLGFs. When the cells were desensitized with LPA, they were no longer responsive to cyclic-PA or alkenyl-GP (Fig. 6A). Even though the cells were no longer responsive to alkenyl-GP and cyclic-PA after desensitization with LPA, they were still responsive to ATP or bradykinin (Table 1). Cells that were desensitized with alkenyl-GP were unresponsive to cyclic-PA but were partially responsive to LPA (Fig. 6B). Cells desensitized with cyclic-PA remained partially responsive to both alkenyl-GP and LPA (Fig. 6C). The quantified results of these desensitization studies are summarized in Table 1. The reduction in the size of the Ca^{2+} transients for each PLGF was paralleled by a similar reduction in the generation of $InsP_3$ (data not shown).

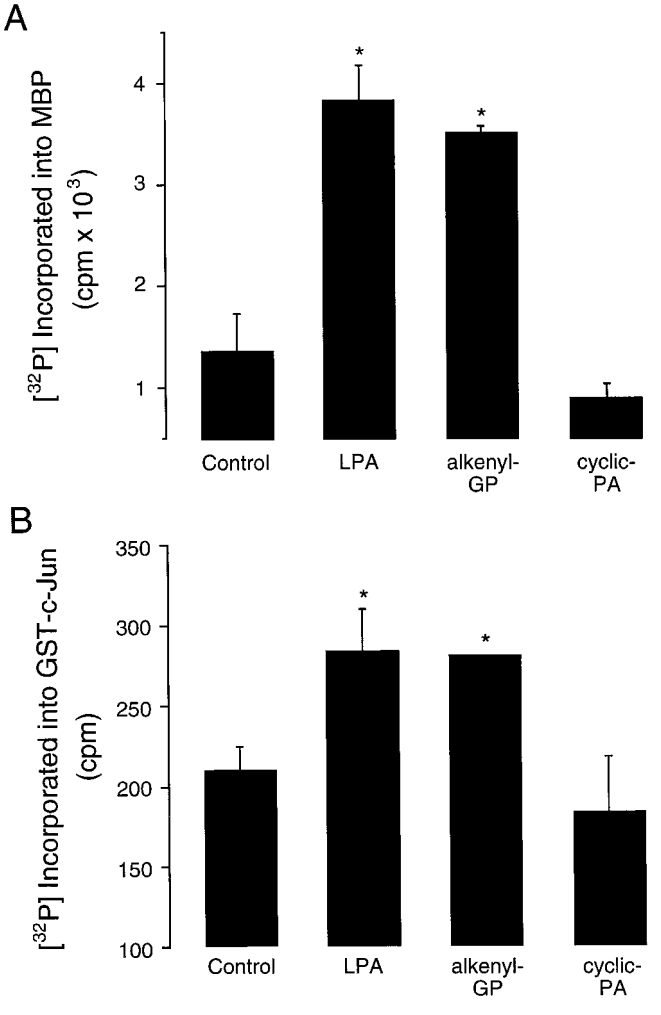

Fig. 3. Effect of PLGFs on the MAP kinases ERK and JNK. A, NIH3T3 cells were treated with a 1 μM concentration of the PLGFs for 10 min, after which the ERK 1/2 activity was measured. The activation of ERK 1/2 by LPA and alkenyl-GP was found to be significant (*) compared with control ($p < 0.04$), whereas the inhibition by cyclic-PA was not statistically significant. B, NIH3T3 cells were treated with a 1 μM concentration of the PLGFs for 45 min and the JNK activity was measured. Similar to the ERKs, LPA and alkenyl-GP caused a significant activation of JNK (*, $p < 0.03$). Enzyme activities represent the mean of triplicate samples and are representative of two other experiments.

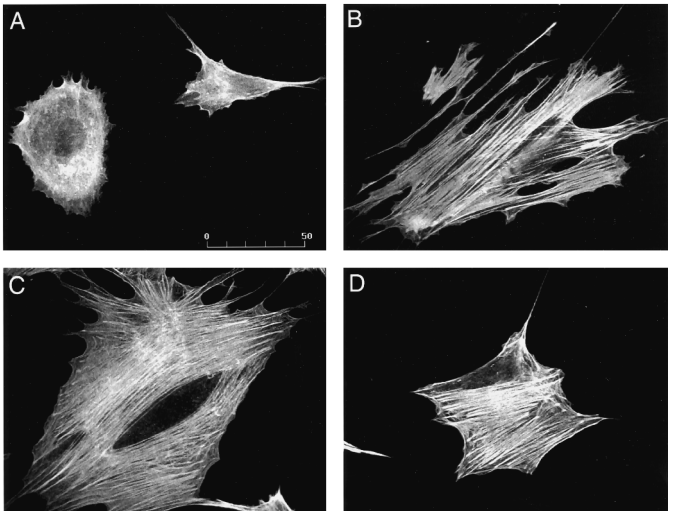

Fig. 4. PLGFs induce the formation of stress fibers. NIH3T3 cells were treated with a 10 μM concentration of BSA (A), LPA (B), alkenyl-GP (C), or cyclic-PA (D) for 15 min. After treatment, the cells were fixed, stained with rhodamine phalloidin, and imaged by laser confocal microscopy (calibration bar = 50 μm).

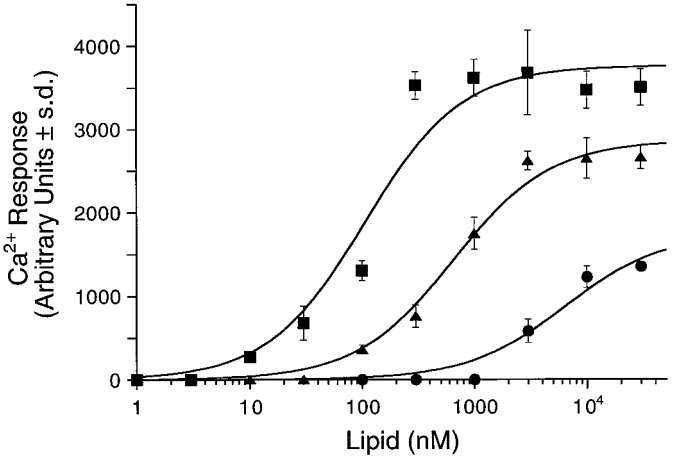

Fig. 5. The PLGF-elicited mobilization of Ca^{2+} is dose dependent. NIH3T3 cells were serum starved for 18–24 hr, loaded with Fura-2, and monitored for changes in $[Ca^{2+}]_i$ after treatment with the indicated concentrations of LPA (■), alkenyl-GP (▲), or cyclic-PA (●). *Data points*, area measured under the peak of each response, reported as mean values ± standard deviation of at least three measurements.

PLGF-elicited Ca^{2+} responses show differences in resensitization depending on the length of serum-starvation. NIH3T3 cells were serum-starved for increasing amounts of time and tested for their responsiveness to PLGFs by monitoring calcium mobilization in Fura-2-loaded cells. Cells that were not serum-starved (0 hr) were completely unresponsive to all three lipids (Fig. 7). At 2 hr, the cells became responsive to alkenyl-GP and LPA, but not to cyclic-PA. Both the LPA and alkenyl-GP responses increased in size up to 12 hr, after which time the response to both lipids remained at maximum levels. The time course of the appearance of the alkenyl-GP response was significantly different from that of LPA response. In contrast, the cyclic-PA response was not present until 12 hr of serum withdrawal, at which time it reached its maximum level.

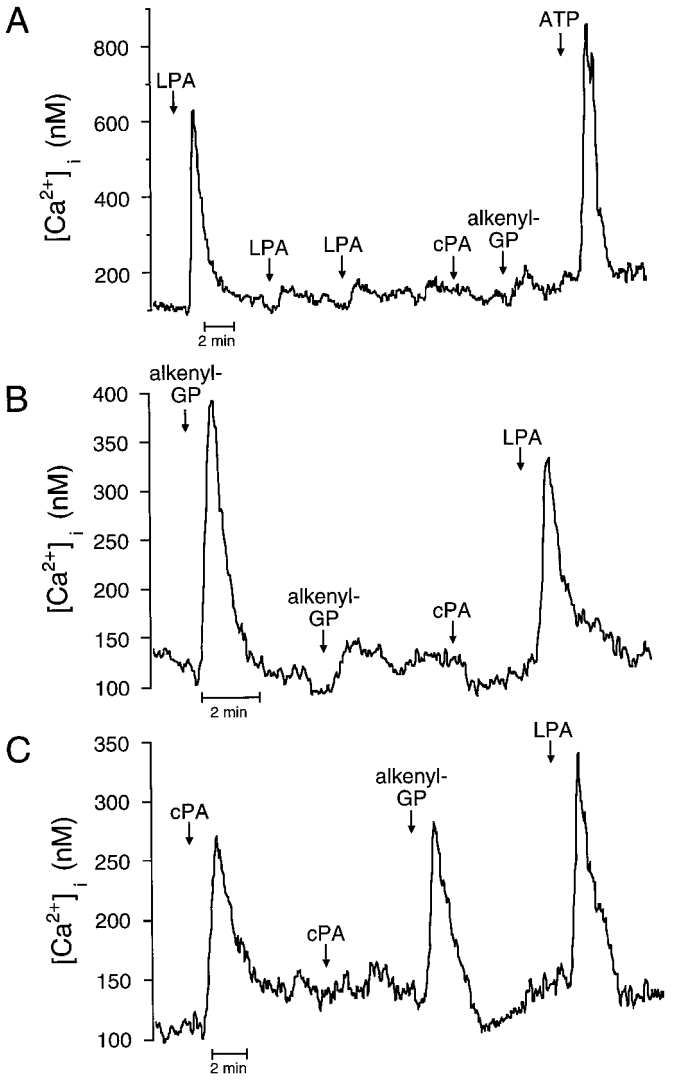

Fig. 6. LPA, alkenyl-GP, and cyclic-PA partially cross-desensitize in NIH3T3 cells. Cells that had been serum starved for 18–24 hr were desensitized with repeated applications of either 1 μM LPA (A), 3 μM alkenyl-GP (B), or 10 μM cyclic-PA (C), and tested for their responsiveness to the other two lipids. After desensitization with LPA, the cells were no longer responsive to 10 μM cyclic-PA or 3 μM alkenyl-GP, but were still responsive to 10 μM ATP (A). After desensitization with alkenyl-GP, the cells were no longer responsive to 10 μM cyclic-PA, and only partially responsive to 1 μM LPA (B). Cells desensitized with cyclic-PA were partially responsive to both 3 μM alkenyl-GP and 1 μM LPA (C). For quantified results, see Table 1.

The PSP24 gene encodes a receptor that is not activated by alkenyl-GP or cyclic-PA in *X. laevis* oocytes. The PSP24 gene from *X. laevis* (Guo *et al.*, 1996), and its mouse homologue (Kawasawa *et al.*, 1998), has recently been identified as a functional LPA receptor that couples to phosphoinositide-Ca^{2+} signaling. We have expanded the characterization of this receptor to include alkenyl-GP along with LPA and cyclic-PA (Fig. 8). To test the ligand selectivity of this receptor, without the possible differences in the processing and coupling of the receptor that may occur in a heterologous expression system, we turned to homologous overexpression in *X. laevis* oocytes. Oocytes were injected either with buffer (sham-injected) or cRNAs encoding either the PSP24 or the vzg-1/EDG-2 receptors (Fig. 8A). Oocytes endogenously express the PSP24 receptor mRNA but do not express the vzg-1/EDG-2 mRNA (Fig. 9). *In vitro* translation of both cRNAs showed that they were efficiently transcribed in reticulocyte lysates (not shown). Oocytes that overexpressed the PSP24 cRNA showed a 2.5-fold increase over control in response to 10 nM LPA; however, the response to 10 nM alkenyl-GP and 1 μM cyclic-PA did not change significantly. The oscillatory Cl^- current response to the PLGFs remained at control levels in oocytes injected with the vzg-1/EDG-2 cRNA (Fig. 8, A and B). When oocytes were co-injected with a mixture of PSP24 and vzg-1/EDG-2 cRNA, the increase in

TABLE 1
Quantification of the LPA, alkenyl-GP, and cyclic-PA responses after cross-desensitization in NIH3T3 cells.
Numbers represent peak areas of Ca^{2+} transients in arbitrary units ± standard deviation. Numbers in parentheses represent percent of maximum response.

Ca^{2+} response	LPA	Alkenyl-GP	Cyclic-PA	BK
Maximum	3951 ± 115	2715 ± 148	1666 ± 84	1375 ± 24
After desensitization with				
LPA	Desensitized	0	0	1160 ± 13
Alkenyl-GP	2233 ± 108 (56.5)	Desensitized	0	
Cyclic-PA	2919 ± 130 (73.9)	1467 ± 80 (54.0)	Desensitized	

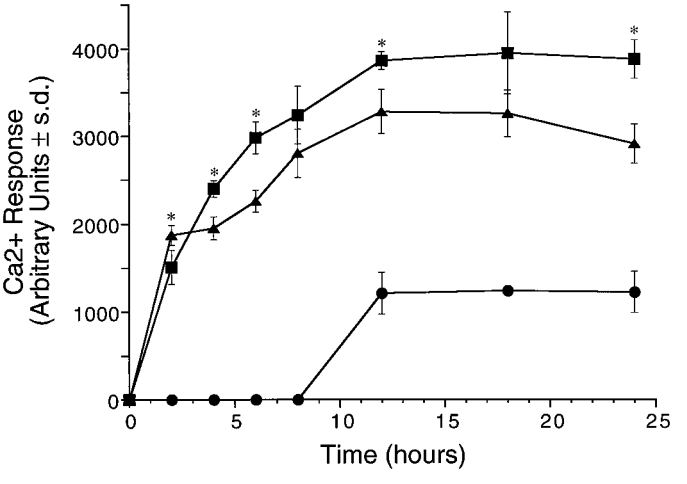

Fig. 7. PLGF elicited Ca^{2+} responses show differences in resensitization depending on the length of serum-starvation. NIH3T3 cells were serum-starved for the indicated amount of time, loaded with Fura-2, and monitored for their responsiveness to 1 μM LPA, 3 μM alkenyl-GP, and 10 μM cyclic-PA. *Data points*, area measured under the peak of each response, reported as mean ± standard deviation of at least three measurements. *, LPA and alkenyl-GP data points were significantly different ($p < 0.05$).

the LPA response was similar to that found in oocytes injected with the PSP24 cRNA alone (Fig. 8C).

Co-expression of multiple PLGF receptor mRNAs in cells and tissues. RT-PCR analysis of the PSP24, EDG-2, and EDG-4 receptors in a variety of LPA-responsive and -nonresponsive cell lines, and in various rat tissues, showed the simultaneous co-expression of at least two of these receptors (Fig. 9A). *X. laevis* oocytes and PC12 cells expressed only the PSP24 mRNA. Rat brain tissue, NIH3T3 cells, and Swiss 3T3 cells co-expressed PSP24 and EDG-2. HeLa cells co-expressed EDG-4 and EDG-2. Interestingly, the LPA responsive Rat-1, HEK 293, and Sp2-O-Ag14 cells did not show any detectable expression of the three PLGF receptor mRNAs. The LPA-nonresponsive Hep G2 cells (Tigyi *et al.*, 1998) showed no detectable expression of any of the three PLGF receptors. The authenticity of the RT-PCR products was verified by Southern analysis using probes to the full-length coding sequence of each gene (Fig. 9B).

Northern blot analysis using poly(A)$^+$ mRNA from eight different rat tissues confirmed the co-expression of PSP24 and EDG-2 in all but liver tissue. Interestingly, the liver showed no detectable expression of any of the three receptors. Surprisingly, we did not detect the expression of EDG-4 in any of our samples, with the exception of HeLa cells (Fig. 9A and B). The lack of EDG-4 expression was not caused by an impaired quality of the mRNA, in that the blot was simultaneously probed with the GAPDH probe, which gave a strong positive signal.

Discussion

With the ever-growing list of biological activities associated with the PLGF family of lipid mediators, there has been a great deal of interest in determining how these ligands elicit such diverse biological responses in almost every type of cell, spanning the phylogenetic spectrum from slime mold to man (Tokumura, 1995). In addition to LPA, alkenyl-GP, and cyclic-PA, at least nine other LPA-like phospholipids have been found in serum (Tigyi and Miledi, 1992). The diverse cellular effects and natural occurrence of multiple PLGFs led to our working hypothesis that their distinct cellular effects might be elicited by the selective activation of multiple receptor subtypes.

To further characterize and compare the three PLGFs, the biological and signal transduction properties were investigated in NIH3T3 cells. LPA and alkenyl-GP both induced cell proliferation, whereas cyclic-PA was antiproliferative. The mitogenic action of LPA in fibroblasts has recently been shown to be mediated via the transactivation of the epidermal growth factor receptor (Cunnick *et al.*, 1998; Gohla *et al.*, 1998) and the Ras-Raf-ERK pathway, which involves the $\beta\gamma$ subunits of a PTX-sensitive $G_{i/o}$ heterotrimeric G protein (Howe and Marshall, 1993). LPA and alkenyl-GP both stimulated the activity of the ERK 1/2 and JNK MAP kinases. In contrast, cyclic-PA slightly inhibited the basal activity of both kinases. As expected, given the $G_{i/o}$-mediated signaling, LPA and alkenyl-GP decreased cAMP levels in a PTX-sensitive manner. In contrast, cyclic-PA caused an increase in cAMP, which was completely abolished when the rise in $[Ca^{2+}]_i$ was blocked, demonstrating that a Ca^{2+}-activated adenylyl cyclase(s) was involved. The elevation of cAMP has been shown to abrogate the activation of the ERKs by the PKA-mediated phosphorylation of Raf-1 in Rat-1 fibroblasts (Howe and Marshall, 1993; Hordijk *et al.*, 1994), which could be the underlying mechanism of the inhibitory effect of cyclic-PA. In cyclic-PA stimulated cells, LPA and alkenyl-GP both abolished the increase in cAMP. This inhibition indicates that the effects of the receptor subtypes that are activated by

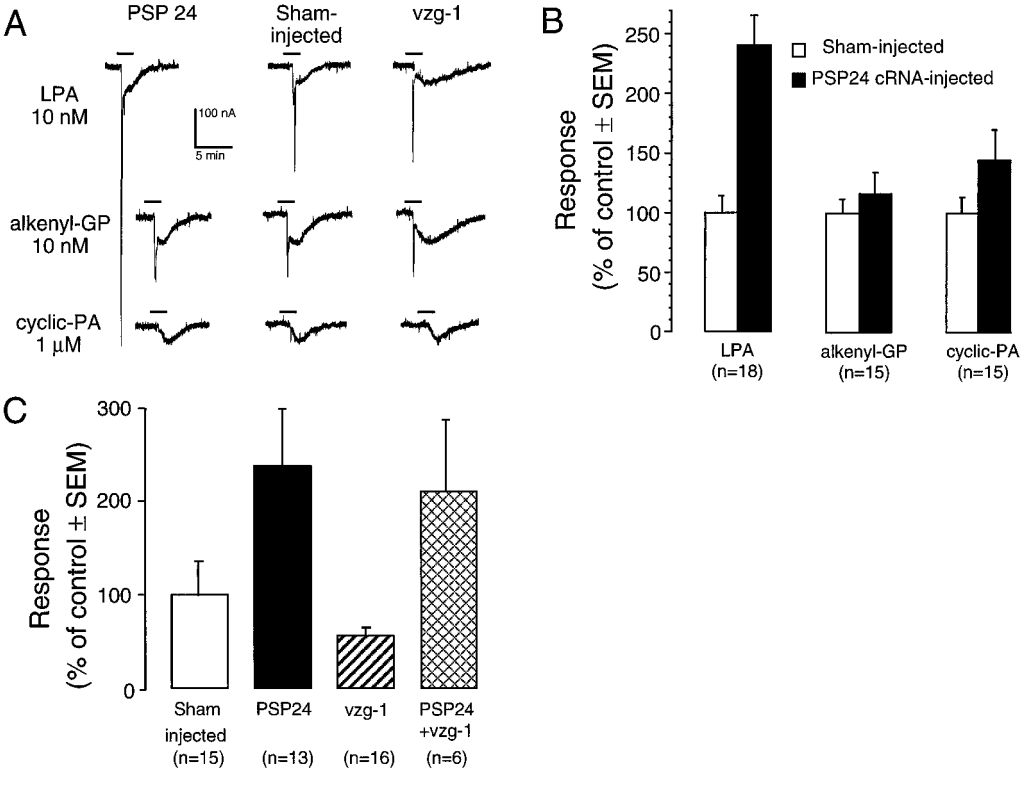

Fig. 8. Ligand specificity of the PSP24 and vzg-1/EDG-2 LPA receptors expressed in *X. laevis* oocytes. A, PLGF receptor cRNAs were microinjected into oocytes (0.2 ng/oocyte), and oscillatory Cl^- current responses were measured 2–3 days later. Sham-injected oocytes received 50 nl of buffer. The oocytes that were injected with the PSP24 cRNA showed an increase in the response that was elicited by LPA but not by alkenyl-GP or cyclic-PA. Oocytes that were injected with the vzg-1 cRNA did not show an increase in the response to any of the PLGFs. B, Statistical evaluation of the responses to the three PLGFs in oocytes that overexpress the PSP24 cRNA. Note the 2.5-fold increase in the response to LPA (10 nM), whereas the responses to alkenyl-GP (10 nM) and cyclic-PA (1 μM) remained at the control level (n = the number of oocytes tested). C, When PS24 cRNA was injected along with the vzg-1/EDG-2 cRNA (0.2 ng each), the increase in the LPA (10 nM) response was the same as in oocytes injected with PSP24 cRNA alone. *Bars*, mean of the change in response size over sham-injected control cells ± standard error.

LPA and alkenyl-GP play a predominant role in mediating the proliferative effect through $G_{i/o}$ and are capable of overriding the rise in cAMP and the ensuing antiproliferative effect. In assessing the effects of cyclic-PA, one also has to take into consideration the different dose-response properties of this ligand as well as the delayed expression of the cyclic-PA response after serum starvation. The long-lasting absence of the cyclic-PA response during the first 12 hr of serum starvation might not only be caused by a slow recovery from desensitization caused by the PLGFs present in serum, but also may represent the *de novo* expression of a particular form(s) of receptor. Note also that cells cultured in serum showed no detectable Ca^{2+} mobilization in response to the PLGFs, which suggests that these receptors are likely to be either completely desensitized or not constitutively expressed, a situation that may occur *in vivo*.

LPA has been shown to induce stress fiber formation, which is mediated through the G_{13} heterotrimeric G protein and the small-molecular-weight G protein, Rho (Gohla *et al*., 1998). All three PLGFs stimulated the formation of stress fibers, indicating that they activate the Rho pathway. LPA, alkenyl-GP, and cyclic-PA all elicited Ca^{2+} transients; however, the maximum cellular response and the apparent EC_{50} values were characteristically different for each agonist. Furthermore, the responses to each PLGF appeared with different time courses after serum starvation. The lack of the cyclic-PA response during the first 12 hr of serum starvation provided reassurance that the cyclic-PA was free of contaminating LPA, in that the cells were responsive to LPA after only 2 hr of serum-starvation.

Ca^{2+} mobilization, a common signaling event between the three PLGFs, was selected to evaluate whether the PLGFs activate the same or different receptors using heterologous desensitization. Upon repeated exposure to the same PLGF, the cells became unresponsive to subsequent applications, which is indicative of homologous desensitization of G protein-coupled receptors. After homologous desensitization, the other two PLGFs were applied to determine whether the three PLGFs heterologously desensitize each other. The lack of responsiveness to a heterologous ligand indicates that the two ligands share the same receptor, whereas if the cells were responsive to the heterologous ligand, then the two ligands must activate different receptors. The complex pattern of heterologous desensitization (Fig. 6, Table 1) indicated the expression of multiple PLGF receptors in the NIH3T3 cells, which were selectively activated by the different ligands. Our data show that alkenyl-GP and LPA are promiscuous ligands that activate more than a single subset of receptors. To explain this pattern of desensitization, we propose a model to distinguish PLGF receptors coupled to the $InsP_3$-Ca^{2+} second messenger system based on their pharmacological selectivity for the three PLGFs. We propose to designate Type I receptors that are specific for LPA and are not activated by cyclic-PA and alkenyl-GP. Type II receptors are activated by LPA and alkenyl-GP, but they are not activated by cyclic-PA. Type III receptors are selectively activated by cyclic-PA and are also nonselectively activated by alkenyl-GP and LPA.

Using pharmacological (Liliom *et al*., 1996) and genetic approaches (Guo *et al*., 1996), we have previously demonstrated the existence in *X. laevis* oocytes of at least one other LPA receptor subtype that was selectively activated by cyclic-PA. Recently, four genes, PSP24 (Guo *et al*., 1996), EDG-1 (Lee *et al*., 1998), vzg-1 [(Hecht *et al*., 1996); also known as EDG-2 (An *et al*., 1997)], and EDG-4 (An *et al*., 1998) have been cloned that encode functional receptors activated by LPA. Although these receptors must be further characterized in cells that lack endogenous LPA receptors, the predicted

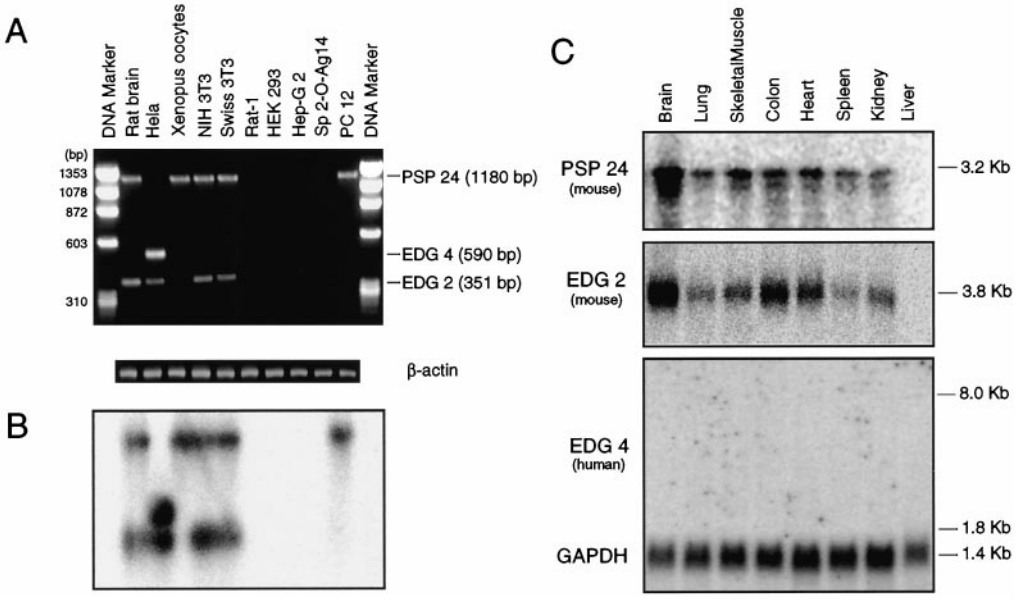

Fig. 9. RT-PCR and Northern analysis reveal the co-expression of multiple PLGF receptors. A, RT-PCR was performed with mRNA isolated from rat brain and nine cell types using specific primers to the PSP24 (mouse), EDG-2 (mouse), and EDG-4 (human) receptors and β-actin as control. *Numbers in parentheses*, size of the predicted amplification products. The PCR products were transferred to a Zeta-probe membrane and hybridized with ^{32}P-labeled cDNA probes encompassing the full coding sequence of the different receptors. B, Southern analysis confirmed the identity of the PCR products. C, Northern blot analysis was performed with poly(A)$^+$ mRNA isolated from nine different rat tissues using cDNA probes encompassing the full coding sequence of the PSP24 (mouse), EDG-2 (mouse), and EDG-4 (human) receptors and GAPDH as a control. The blot was stripped and rehybridized sequentially with different probes using the Strip-EZ kit from Ambion and imaged by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

amino acid sequences of the three cDNAs are divergent. This divergence suggests that they might encode subtypes of LPA receptors that differ in their ligand selectivity and signaling. The ligand specificity of the PSP24 receptor (Fig. 8) is in agreement with that of a type I receptor because it is only activated by LPA and not by either cyclic-PA or alkenyl-GP. We have not been able to show functional coupling between the vzg-1/EDG-2 receptor and the $InsP_3$-Ca^{2+} second messenger system in *X. laevis* oocytes, confirming a recent report by Hooks *et al*. (1998). Similarly, no coupling was found between the heterologously expressed vzg-1/EDG-2 receptor and the $G_{q/11}$ like G proteins in Sf9 insect cells (Zondag *et al*., 1998). The apparent lack of coupling might indicate that the signaling properties of PLGF receptors vary from cell type to cell type and/or that some LPA receptors do not signal through this pathway, and therefore cannot be assessed using the heterologous desensitization technique that relies on Ca^{2+} monitoring. Recently, we have reported the functional expression of the vzg-1/EDG-2 receptor in yeast cells and have shown that it was activated by all three PLGFs (Erickson *et al*., 1998). This ligand selectivity is in agreement with that of a Type III receptor. However, cyclic-PA, a type-III selective agonist, elicits Ca^{2+} mobilization and does not inhibit cAMP signaling through a PTX-sensitive mechanism, unlike vzg-1/EDG-2 (Hecht *et al*., 1996; An *et al*., 1997). Although this observation does not detract from the validity of the agonist selectivity of the PLGF receptor subtypes, it reinforces the idea that the signaling properties may not predict the ligand selectivity of a receptor.

The ligand selectivity of the receptors should not be confused with partial agonist properties of the ligands. The possibility still exists that LPA and alkenyl-GP are partial agonists of the type II and III receptors. This possibility cannot be eliminated until the different LPA receptors are identified and heterologously expressed in LPA-nonresponsive hosts and proper pharmacological binding studies are conducted. Note that after serum starvation, the alkenyl-GP response initially was significantly greater than the LPA response (Fig. 7); this suggests that LPA was a less effective ligand of the type II receptor(s) than alkenyl-GP, which is contrary to what would be expected if alkenyl-GP were a partial agonist of this same receptor subtype.

We also provide evidence for the co-expression of multiple PLGF receptor mRNAs in LPA-responsive cell lines and tissues. It is equally important to recognize the lack of expression of the three PLGF receptors in several LPA-responsive cell lines (Rat-1, HEK 293, Sp2-O-Ag14) and tissues [liver (Im *et al*., 1997)], indicating that other PLGF receptors exist and will have to be identified. Our data also confirm the biological significance of previous reports on multiple endogenous lipid mediators with LPA-like biological activities [for a review, see Tokumura (1995)] and expand these reports with the novel concept that these mediators selectively activate multiple receptors that are often co-expressed in the same cell. We also demonstrate some of the individual differences in the signal transduction mechanisms activated and the cellular responses elicited by the different PLGFs.

It is important to recognize that a cellular response may originate from the activation of multiple receptor subtypes, a possibility often overlooked by researchers studying LPA signaling via the activation of protein kinase cascades. Although we recognize that the classification of the receptors proposed above is incomplete, the concept of receptor subtype-selective PLGFs should enhance the current research and offers a more comprehensive explanation for the diverse and cell type-specific responses activated by LPA. Clearly, more research is necessary to refine this model in two important ways. First, more of the endogenous and synthetic receptor-specific ligands must be identified; second, more PLGF receptor genes must be cloned.

Acknowledgments

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