

Short-Chain Phosphatidates Are Subtype-Selective Antagonists of Lysophosphatidic Acid Receptors

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ABSTRACT

Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are members of the phospholipid growth factor family. A major limitation in the field to date has been a lack of receptor subtype-specific agonists and antagonists. Here, we report that dioctylglycerol pyrophosphate and dioctylphosphatidic acid are selective antagonists of the LPA₁ and LPA₃ receptors, but prefer LPA₃ by an order of magnitude. Neither molecule had an agonistic or antagonistic effect on LPA₂ receptor. Consistent with this receptor subtype selectivity, dioctylglycerol pyrophosphate inhibited cellular responses to LPA in NIH3T3 fibroblasts,

HEY ovarian cancer cells, PC12 pheochromocytoma cells, and *Xenopus laevis* oocytes. Responses elicited by S1P in these cell lines that endogenously express S1P₁, S1P₂, S1P₃, and S1P₅ receptors were unaffected by dioctylglycerol pyrophosphate. Responses evoked by the G protein-coupled receptor ligands acetylcholine, serotonin, ATP, and thrombin receptor-activating peptide were similarly unaffected, suggesting that the short-chain phosphatidates are receptor subtype-specific lysophosphatidate antagonists.

Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are members of the phospholipid growth factor (PLGF) family. The responses elicited by PLGFs are pleiotropic and include effects on cell proliferation, survival, morphology, adherence, chemotaxis, contraction, and activation of ionic conductance (for reviews, see Goetzl et al., 2000; Tigyi, 2001). Although the cellular responses elicited by PLGFs have been well described in vitro, the physiological function of these mediators is not clearly understood. LPA has been implicated in a number of disease and injury states, due to elevated levels of LPA in fluids surrounding the tissues involved, including corneal injury, lung disease, atherosclerosis, ovarian cancer, and wound healing (for reviews, see Goetzl et al., 2000; Tigyi, 2001).

G protein-coupled receptors of the endothelial differentiation gene (EDG) family and PSP24 transduce the biological effects of LPA (for review, see Contos et al., 2000). The EDG family consists of eight members, which are further divided into two subfamilies based on their specificity for LPA or S1P. LPA₁ (EDG-2), LPA₂ (EDG-4), and LPA₃ (EDG-7) are

activated by LPA, whereas S1P₁ (EDG-1), S1P₂ (EDG-5/AGR16, H128), S1P₃ (EDG-3), S1P₄ (EDG-6), and S1P₅ (EDG-8) are activated by S1P (for review, see Contos et al., 2000). The PSP24 receptor for LPA has been found to augment Ca²⁺ responses in *X. laevis* oocytes (Guo et al., 1996; Fischer et al., 1998). An as-yet-unidentified LPA receptor has recently been implicated as a mediator of cell proliferation and platelet aggregation (Hooks et al., 2001).

The expression and signaling by the PLGF receptors have been well characterized (Hecht et al., 1996; An et al., 1998; Graler et al., 1998; Bandoh et al., 1999; Im et al., 2000a). Each receptor has a distinct coupling pattern to various G proteins (An et al., 1998; Windh et al., 1999; Im et al., 2000a,b). Because most cells are responsive to PLGF and simultaneously express multiple PLGF receptors (Fischer et al., 1998), studying the individual receptors in endogenous systems has proven to be difficult. To understand the role of PLGF in vivo, as well as to explore receptor pharmacophores for drug discovery, selective agonists and antagonists of individual PLGF receptors must be identified.

Few studies have reported on the development of novel PLGF agonists and antagonists. Local and general anesthetics have been shown to inhibit PLGF receptors by uncoupling from the G protein in *X. laevis* oocytes (Chan and Durieux, 1997; Tigyi et al., 1997). *N*-Acyl serine phosphoric acid and

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ABBREVIATIONS: LPA, lysophosphatidic acid; S1P, sphingosine-1-phosphate; PLGF, phospholipid growth factor; EDG, endothelial differentiation gene; DGPP, diacylglycerol pyrophosphate; PA, phosphatidic acid; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcription-polymerase chain reaction; DAG, diacylglycerol.

N-acyl tyrosine phosphoric acid have been shown to inhibit LPA-activated platelet aggregation, oscillatory Cl[−] currents, and neutrophil adhesion to endothelial cells (Sugiura et al., 1994; Liliom et al., 1996; Hooks et al., 1998; Siess et al., 1999). In contrast, *N*-acyl serine phosphoric acid is a potent agonist in MDA MB231 cells (Hooks et al., 1998) and weak agonist in Jurkat T cells heterologously expressing LPA₁ and LPA₄ (An et al., 1998). A phosphorothioate analog of LPA was shown to inhibit the interaction between leukocytes and the endothelium; however, the receptor(s) targeted by the molecule and the mechanism of inhibition have not been elucidated (Scalia et al., 2000). Cyclic phosphatidic acid has been found to inhibit LPA-elicited platelet aggregation (Gueguen et al., 1999). However, cyclic phosphatidic acid is an agonist for the PLGF receptors and shows partial cross-desensitization with LPA (An et al., 1998; Fischer et al., 1998), thus it is unclear what receptor(s) mediates its effect.

Recently, our laboratory reported a validated computational model of the S1P₁ (EDG-1) receptor (Parrill et al., 2000). This model identified three amino acids that ion-pair with S1P. Mutating any of the three amino acids to a non-charged residue abolished ligand binding. These studies established that the ionic interaction between the S1P₁ receptor and the PLGF is a key component of the pharmacophore. Based on this model, we hypothesized that a second interaction takes place between the receptor and the hydrophobic tail of the PLGF, which leads to the activation of the receptor. With this in mind, we investigated the effects of short-chain phosphatidates on the LPA-induced activation of the LPA₁, LPA₂, and LPA₃ receptors.

Here, we report that diacylglycerol pyrophosphate (DGPP) 8:0 and dioctyl-phosphatidic acid (PA) 8:0 are selective antagonists of the LPA₁ and LPA₃ receptors, with an order of magnitude preference for LPA₃. Neither molecule had an effect on LPA₂. Consistent with the receptor subtype selectivity, DGPP 8:0 inhibited cellular responses elicited by LPA in NIH3T3 fibroblasts, HEY ovarian cancer cells, PC12 pheochromocytoma cells, and *X. laevis* oocytes. Responses elicited by S1P in these same cell lines endogenously expressing S1P₁, S1P₂, S1P₃, or S1P₅ receptors were unaffected by dioctylglycerol pyrophosphate. Responses evoked by G protein-coupled receptor ligands acetylcholine, serotonin, ATP, and thrombin peptide were similarly unaffected, suggesting that the short-chain phosphatidates are receptor subtype-specific, lysophosphatidate antagonists.

Experimental Procedures

Materials. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL); other chemicals were obtained from Sigma Chemical (St. Louis, MO). Cytofectene transfection reagent was from Bio-Rad (Hercules, CA). Fura-2 AM was from Molecular Probes (Eugene, OR). Culture media, fetal bovine serum (FBS), and G418 were obtained from Cellgro (Herndon, VA). Flag-tagged cDNAs encoding human LPA₂ and LPA₃ subcloned into the pCDNA3 expression plasmid (Invitrogen, Carlsbad, CA) were a generous gift from Dr. Junken Aoki (University of Tokyo, Tokyo, Japan).

Cells and Cell Culture. The B103 cell line was provided by Dr. Jerold Chun (University of California, San Diego, San Diego, CA). RH7777 cells, stably expressing human LPA₂ (EDG-4), were provided by Dr. Kevin Lynch (University of Virginia, Charlottesville, VA). NIH3T3, RH7777, and PC12 cell lines were obtained from the American Type Culture Collection (Manassas, VA). HEY cells were

provided by Dr. Lisa Jennings (University of Tennessee, Memphis, TN). The PC12 cell line was maintained and differentiated as described previously (Tigyi et al., 1996). All other cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 2 mM glutamine. Oocytes were obtained from adult *X. laevis* frogs as described previously (Tigyi et al., 1999).

Lipid Preparation. Before use, LPA was complexed, at a 1:1 M ratio, with 1 mM bovine serum albumin dissolved in Ca²⁺-free Hanks' balanced salt solution containing 1 mM EGTA, and further diluted in the appropriate assay medium or buffer. Aliquots of all the other lipids were dissolved in MeOH and mixed with diluted LPA before application, or as otherwise indicated.

Stable Transfection. RH7777 cells were transfected with the cDNA constructs encoding the human orthologs of LPA_{1–3} receptors by using Cytofectene (Bio-Rad). Transfected cells were selected in DMEM containing 10% FBS and 1 mg/ml Geneticin. Resistant cells were collected and subcloned by limiting dilution. The resulting clones were then screened using functional assays and RT-PCR analysis.

Transient Transfection. RH7777 cells were plated on poly-L-lysine-coated glass coverslips (Bellco, Vineland, NJ) 1 day before transfection. The following day, cells were transfected overnight (16–18 h) with 1 μg of plasmid DNA mixed with 6 μl of Cytofectene. The cells were then rinsed twice with DMEM and cultured in DMEM containing 10% FBS. The next day, the cells were rinsed with DMEM and serum was withdrawn for a minimum of 2 h before monitoring intracellular Ca²⁺.

Measurement of Intracellular Ca²⁺. Changes in intracellular Ca²⁺ were monitored using the fluorescent Ca²⁺ indicator Fura-2 AM as described previously (Tigyi et al., 1999).

Electrophysiological Recording in *X. laevis* Oocytes. Oscillatory Cl[−] currents, elicited by LPA, were recorded using a two-electrode voltage-clamp system as described previously (Tigyi et al., 1999).

RT-PCR Analysis. The identification of EDG and PSP24 receptor mRNA by RT-PCR was performed as described previously (Tigyi et al., 1999). The oligonucleotide primers selected from the coding sequences of the individual receptors were as follows (lowercase numbers indicate the nucleotide number in the open reading frame, numbers in parentheses denote the length of the product): forward human S1P₁ (EDG-1) (269) 5'-₈₁TCATCGTCCGGCATTACAAC-TA-3', mouse (243) 5'-₅₉₂ACCACAAGCACTATATTCT-3', reverse human 5'-GAGTGAGCTTGTAGGTGGT₃₅₁-3', mouse 5'-CTAACAGTAGTAGGATGAAG₈₃₅-3'; human LPA₁ (EDG-2) (337) forward 5'-₆₅AGATCTGACCAGCCGACTCAC-3', mouse (317) forward 5'-₂₂₉AACCGCCGCTTCCATTTTCT-3', human reverse 5'-GT-TGGCCATCAAGTAATAA₄₂₂-3', mouse reverse 5'-ATACAGTTCCAGCCCACT₅₄₆-3'; S1P₃ (EDG-3) human (469) forward 5'-₁₃₇CTTGTCATCTGCAGCTTCATC-3', mouse (337) forward 5'-₄₃₈ATATGATGCCAACAAGAAGC-3', reverse human 5'-TGCTGATGCAGAAGGCAATGTA₅₉₇-3', mouse reverse 5'-GAGGAAGAGGATAAAAAGT₇₇₅-3'; LPA₂ (EDG-4) forward human (574) 5'-₆₃₄CTGCTCAGCCGCTCCTATTTG-3', forward mouse (287) 5'-₄₂₆CCGTGTGGTCCACTCATCGT-3', reverse human 5'-AG-GAGCACCACAAAGTCATCAG₁₁₈₅-3', mouse 5'-TGCGGCGCATCTCAGCATCTCG₈₉₃-3'; S1P₂ (EDG-5) forward human (741) 5'-₁₁ATGGGCAGCTTGTACTCGGAG-3', mouse (287) 5'-₅₅₁CCTC-TATGCTAAGCACTATGTG-3', reverse human 5'-CAGCCAGCA-GACGATAAAGAC₇₂₀-3', mouse 5'-GATTGACGAGTGAGTTAAGGG₈₃₄-3'; S1P₄ (EDG-6) forward human (511) 5'-₂₈₀TGAACATCACGCTGAGT-GACCT-3', mouse (293) 5'-₆₁₇GCTATGTGCTCTTTTGTGTGGT-3', reverse human 5'-GATCATCAGCACCGTCTTCAGC₇₉₀-3', mouse 5'-GTAGATGAGAGGATTAATGGCT₉₁₀-3'; LPA₃ (EDG-7) forward human (354) 5'-₉₁AGCAACACTGATACTGTTCATG-3', mouse (711) 5'-₃₈₀ACACATGTCAATCATGAGGATG-3', reverse human 5'-GCATCCTCATGATTGACATGTG₄₄₆-3', mouse 5'-CTGATGCT-GTCCTCCAGGTACT₁₀₉₁-3'; S1P₅ (EDG-8) forward human (322) 5'-₈₈ATCTGTGCGCTCTATGCAAGGA-3', rat (322) 5'-₂₇GCCGGT-

GAGCGAGGTCATCGTC-3', reverse human 5'-GGTGTAGATGATAG-GATTCAGCA₁₁₆₁-3', rat 5'-TAGGCCTGGCGTAGAGCGGCA₅₅₈; PSP24 forward human (341) 5'-₃₂₀CTGCATCATCGTGTACCAGAG-3', mouse (288) 5'-₇₂₅GTGCACTACCAGTCGGACAAGCT-3', reverse human 5'-ACGAAGCTCTATGCAGGCCTCGC₁₁₈₄-3', mouse 5'-CGAA-CTCTATGCAGGCCTCGCG₁₀₁₃; β -actin forward 5'-₅₇TCACCATG-GATGATGATATCGC-3', reverse primer 5'-CGTGCTCGATGGGG-TACTTCA₂₈₁-3'.

Cell Proliferation Assay. Proliferation of NIH3T3 cells was assessed by direct cell counting as described previously (Tigyi et al., 1999). NIH3T3 cells were plated in 24-well plates at a density of 10,000 cells/well, in DMEM containing 10% FBS. The following day, the cells were rinsed and serum-starved in DMEM for 6 h. Lipids were then added for 24 h. Cell numbers were determined by counting in a Coulter counter (Beckman Coulter, Inc., Fullerton, CA). The incorporation of [³H]thymidine into RH7777 cells was determined as described previously (Tigyi et al., 1999).

Neurite Retraction Assay. LPA- and S1P-elicited neurite retraction were performed as described previously (Tigyi et al. 1996) using PC12 cell cultures after 2 days of differentiation. Cell were exposed to 100 nM S1P or ligand and the change in neurite length was measured after 15 min for a population of at least 50 cells/experiment.

Data Analysis. Data points from the intracellular Ca²⁺ measurements represent the total peak area of the Ca²⁺ transients elicited, as determined by the FLWinLab software (PerkinElmer, Wellesley, MA). Data points represent the average of at least three measurements \pm standard deviation. The significance of the data points was determined using the Student's *t* test and values were considered significant at *p* < 0.05.

Results

Preparation and Characterization of Stable Cell Lines Expressing LPA₁, LPA₂, and LPA₃. In an effort to develop selective antagonists to the LPA₁, LPA₂, LPA₃ receptors, we first had to establish a system for screening potential compounds. RH7777 cells were chosen as a model system because they have been reported to be nonresponsive to LPA or S1P in a variety of cellular assays and were found to be devoid of mRNA for any of the known PLGF receptors (Fukushima et al., 1998; Hooks et al., 2001). Stable cell lines transfected with the PLGF receptors, as well as control cell lines transfected with empty vector, were established in RH7777 cells. The resulting clones were screened by monitoring intracellular Ca²⁺ transients, and by RT-PCR (data not shown). This screening process led to the identification of at least three positive cell lines expressing LPA₁ and LPA₃, whereas no positive cell lines expressing LPA₂ could be identified. Vector-transfected cells were also found to be nonresponsive to LPA (data not shown). Although we were unable to isolate stable clones expressing LPA₂, the transient expression of LPA₂ resulted in the LPA-mediated activation of intracellular Ca²⁺ transients, demonstrating that the construct was functionally active in these cells. The stable LPA₂ cell line used in these experiments was isolated and characterized by Im et al. (2000b) who kindly provided us this clone.

The cell lines were further characterized in an effort to identify a suitable assay for screening potential antagonists. LPA-elicited activation of extracellular signal receptor-activated kinase 1/2 was seen in stable LPA₁- and transient LPA₂-expressing cells, whereas extracellular signal receptor-activated kinase 1/2 was not activated in stable LPA₃-expressing cells. LPA 18:1 elicited Ca²⁺ transients in all stable cell lines expressing LPA₁, LPA₂, and LPA₃. Dose-response

curves revealed EC₅₀ values of 378 \pm 53, 998 \pm 67, and 214 \pm 26 nM for LPA₁, LPA₂, and LPA₃-expressing cells, respectively (Fig. 1, A–C). The EC₅₀ value determined for the stable RH7777 LPA₂ clone was different from the 18 \pm 2.5 nM previously reported by An et al. (1998) in HTC4 hepatoma cells using a reporter gene assay. Therefore, a dose-response curve was also established for cells transiently expressing LPA₂ (Fig. 1B), which yielded an EC₅₀ value of 186 \pm 39 nM.

The ability of LPA to stimulate DNA synthesis in the stable cell lines was examined by measuring the incorporation of [³H]thymidine. Neither wild-type nor the vector-transfected RH7777 cells showed an increase in [³H]thymidine incorporation after a 24-h incubation with 10 μ M LPA (data not shown), which is in contrast to a previous report that LPA is mitogenic in these cells (Hooks et al., 2001). LPA₁-expressing cells showed a 1.8-fold increase in [³H]thymidine incorporation, whereas, LPA₂- and LPA₃-expressing cells did not show an increase in [³H]thymidine incorporation, compared with control cells.

Short-Chain Phosphatidates Inhibit Ca²⁺ Responses Mediated by LPA₁ and LPA₃. Because Ca²⁺ transients

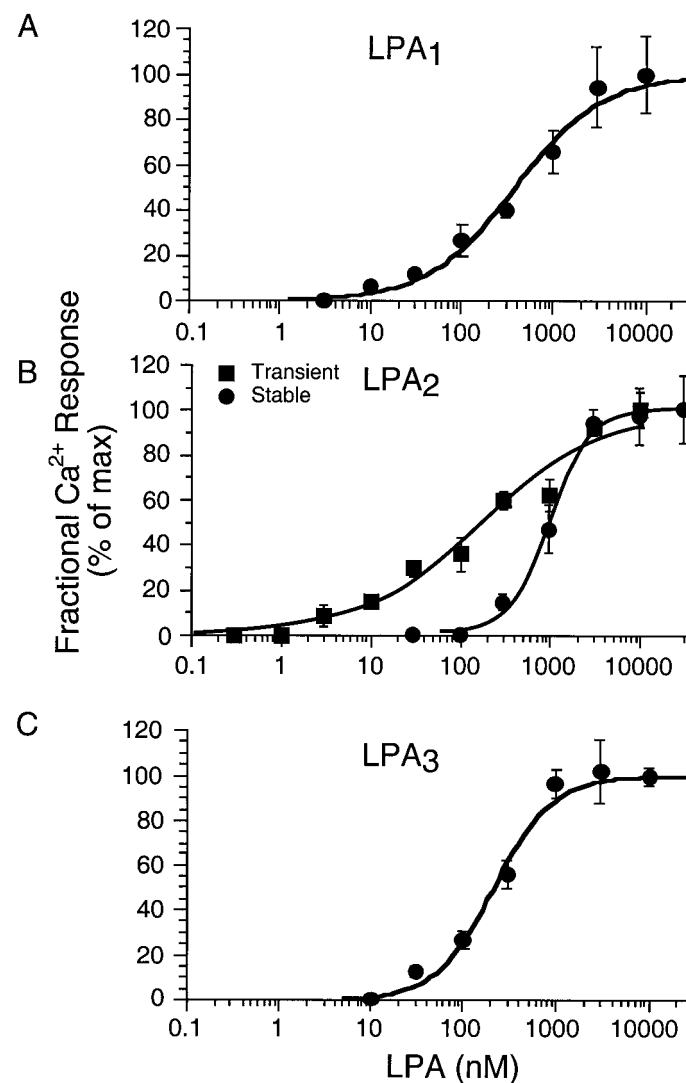


Fig. 1. Dose-response relationship for Ca²⁺ responses in RH7777 cells heterologously expressing LPA₁ (A), LPA₂ (B), or LPA₃ (C). Each data point represents the average of at least three measurements \pm S.D.

were elicited in stable cell lines expressing LPA₁, LPA₂, and LPA₃ (Fig. 1), we chose to use this assay for screening potential antagonists. In an effort to identify selective antagonists for the LPA-activated PLGF receptors, we relied on the structural features of the LPA pharmacophore as a starting point. It has been shown that the biological activity of LPA decreases with shorter chain lengths (Jalink et al., 1995; Bando et al., 2000). In agreement with this, LPA 8:0 was unable to elicit Ca²⁺ responses in any of the cells, up to concentrations as high as 10 μ M. Next, we tested whether short-chain (8:0) LPA was an inhibitor of LPA₁, LPA₂, and LPA₃. When the cells were challenged with a mixture of LPA 8:0 and LPA 18:1, the Ca²⁺ responses were not affected in any of the three stable cell lines (Figs. 2A, 3A, and 4A).

We hypothesized that a modification of the LPA pharmacophore, which sterically restricted the mobility of the fatty acid chain, might also effect its ligand properties. For this reason, we tested compounds with a second short-chain fatty acid at the *sn*-2 position. Such short-chain phosphatidates have increased hydrophobicity over the corresponding short-chain LPA, which could exert constraints on their interaction with the ligand-binding pocket of the receptor. PA and DGPP are naturally occurring lipids, which share some key chemical properties with the LPA pharmacophore, having an ionic phosphate group(s) and fatty acid chains, whereas neither is an agonist of the PLGF receptors (see below). With this similarity in mind, short-chain DGPP was tested as an inhibitor of LPA₁, LPA₂, and LPA₃.

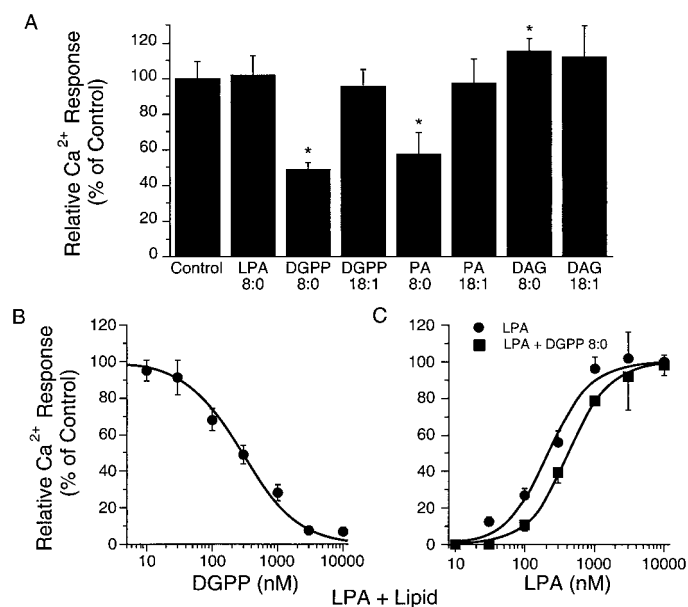


Fig. 2. Pharmacological characterization of the inhibition of the LPA response in LPA₃ (EDG-7) cells by short- and long-chain phosphatidates and related lipids (A). LPA (250 nM) was mixed with a 1 μ M concentration of the test lipids and Ca²⁺ responses were measured. The peak areas of the Ca²⁺ responses are represented as the average values of a minimum of three measurements \pm S.D. DGPP inhibits LPA₃-mediated Ca²⁺ responses in a dose-dependent manner (B). Cells were exposed to a 250 nM concentration of LPA 18:1 mixed with increasing concentrations of DGPP 8:0, and the peak area of the resulting Ca²⁺ responses was measured. DGPP shifts the LPA dose-response curve to the right (C). LPA₃ cells were exposed to increasing concentrations of LPA 18:1 either alone or mixed with a 500 nM concentration of DGPP 8:0. The peak areas of the Ca²⁺ responses are represented as the average values of a minimum of three measurements \pm S.D.

DGPP 8:0 Is a Competitive Inhibitor of LPA₁ and LPA₃. LPA-elicited Ca²⁺ responses were inhibited by DGPP 8:0 and PA 8:0 in cells expressing LPA₁ and LPA₃ but not LPA₂ (Figs. 2–4). Inhibition curves were determined in cells expressing LPA₁ and LPA₃ by using increasing concentrations of DGPP 8:0, whereas the concentration of LPA was kept constant at the EC₅₀ value relative to the receptor studied. K_i values of 106 \pm 28 nM for LPA₃ (Fig. 2B) and 6.6 \pm 0.68 μ M for LPA₁ (Fig. 3B) were determined from the curves. Using a constant amount of DGPP 8:0 near to its IC₅₀ value (250 nM for LPA₃, 3 μ M for LPA₁), the dose-response curves for LPA₃ (Fig. 2C) and LPA₁ (Fig. 3C), as expected, were shifted by a factor of 2 to the right, indicating a competitive mechanism of inhibition.

To better define the structure-activity relationship for DGPP, short- (8:0) and long-chain (18:1) species of LPA, DGPP, PA, and DAG were tested on LPA₁- and LPA₃-expressing cell lines (Figs. 2A and 3A). Figure 2A shows the effect of these lipids on the Ca²⁺ responses in LPA₃-expressing cells when exposed to a combination of LPA 18:1 and each of these lipids. For these experiments, the concentration of LPA was chosen to be 250 nM, a value near the EC₅₀ value, whereas test lipids were applied at a concentration 5 times the K_i value of DGPP 8:0 on LPA₃. LPA 8:0 had no effect on LPA₃, whereas both DGPP 8:0 and PA 8:0 significantly inhibited the Ca²⁺ responses by 50 and 56%, respectively. In contrast, DAG 8:0 significantly increased the Ca²⁺ responses. When the chain length of DGPP and PA was in-

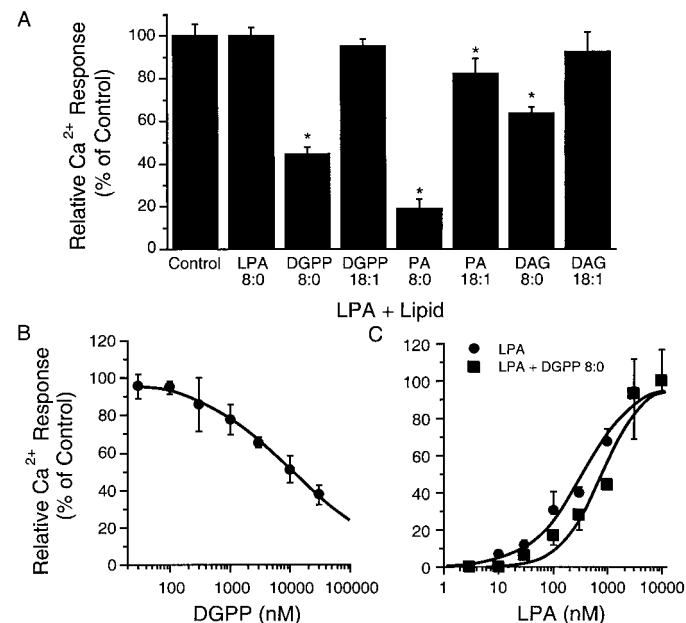


Fig. 3. Pharmacological characterization of the inhibition of the LPA response in LPA₁ cells by short- and long-chain phosphatidates and related lipids (A). LPA (250 nM) was mixed with a 10 μ M concentration of the test lipids and Ca²⁺ responses were measured. The peak areas of the Ca²⁺ responses are represented as the average values of a minimum of three measurements \pm S.D. DGPP inhibits LPA₁-mediated Ca²⁺ responses in a dose-dependent manner (B). Cells were exposed to a 250 nM concentration of LPA 18:1 mixed with increasing concentrations of DGPP 8:0, and the peak area of the resulting Ca²⁺ responses was measured. DGPP shifts the LPA dose-response curve to the right (C). LPA₁ cells were exposed to increasing concentrations of LPA 18:1 either alone or mixed with a 10 μ M concentration of DGPP 8:0. The peak areas of the Ca²⁺ responses are represented as the average values of a minimum of three measurements \pm S.D.

creased to 18:1, these analogs were no longer inhibitors of LPA₃ (Fig. 2A). DAG 18:1, likewise, did not have an inhibitory effect on LPA₃.

The same set of lipids was tested on LPA₁-expressing cells (Fig. 3A). Octyl chain-length analogs of DGPP, PA, and DAG, when used at a high 10 μ M concentration, all decreased the responses to 50, 19, and 64% of control, respectively. When the chain length was increased to 18:1, DGPP and DAG no longer had an inhibitory effect, whereas PA 18:1 maintained a modest inhibitory effect, decreasing the Ca²⁺ response by 18%.

The panel of lipids was also tested on LPA₂-expressing cells (Fig. 4, A and B). Neither long- nor short-chain species of PA, when applied alone, elicited a response at concentrations up to 10 μ M, in cells stably or transiently expressing LPA₂ (data not shown). When these lipids were assayed in the stable cell line expressing LPA₂, none of the short- or long-chain lipids had an inhibitory effect, whereas both PA 8:0 and 18:1 significantly increased the Ca²⁺ responses, to 162 and 137% of control, respectively. To confirm the results obtained from the stable clone, the lipid panel was tested on cells transiently expressing LPA₂ (Fig. 4B). Again, neither the short- nor the long-chain species of DGPP or PA had an inhibitory effect on the Ca²⁺ response. In contrast to the stable LPA₂ clone, neither PA analog enhanced the Ca²⁺ response in cells with transient expression of LPA₂.

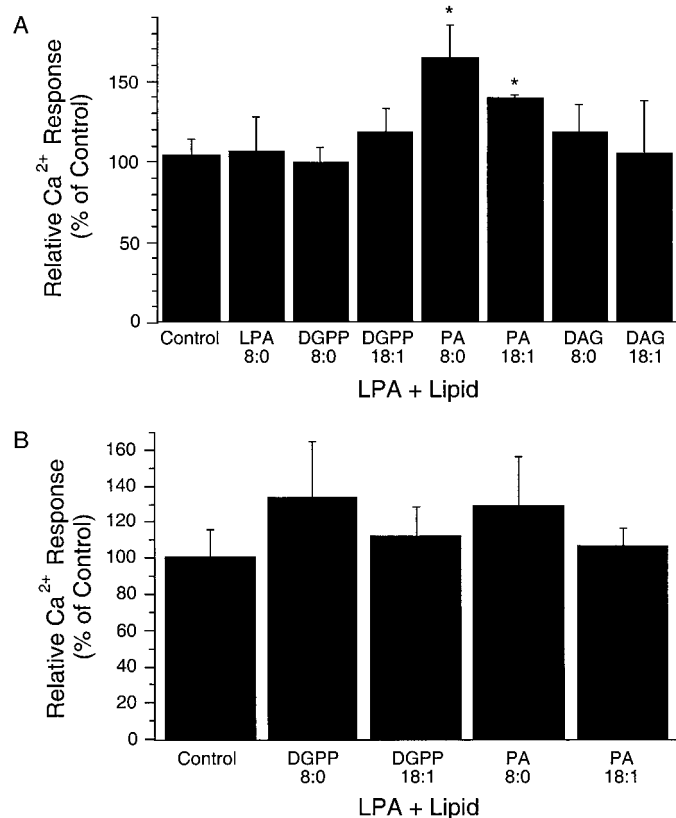


Fig. 4. Pharmacological characterization of the inhibition of the LPA response in stable LPA₂ cells by short- and long-chain phosphatidates and related lipids (A). Cells were exposed to a 500 nM concentration of LPA 18:1 mixed with a 5 μ M concentration of the indicated lipids. Cells transiently expressing LPA₂ cells were exposed to a 100 nM concentration of LPA 18:1 mixed with a 1 μ M concentration of the indicated lipids (B). The peak areas of the Ca²⁺ responses were measured and are represented as the average values of a minimum of three measurements \pm S.D.

DGPP 8:0 Is a Competitive Inhibitor of LPA-Elicited Responses in *X. laevis* Oocytes. In oocytes, the EC₅₀ value for LPA 18:1 eliciting oscillatory inward currents was 56 nM (data not shown). DGPP 8:0 inhibited the Ca²⁺-mediated, inward Cl⁻ currents elicited by LPA in *X. laevis* oocytes with a K_i value of 52 \pm 21 nM (Fig. 5A). In the presence of a 200 nM concentration of DGPP 8:0, the dose-response curve for LPA 18:1 was shifted to the right, indicating a competitive mechanism of action (Fig. 5B). To examine whether DGPP 8:0 acts through an intracellular, or extracellular mechanism, DGPP 8:0 was injected intracellularly and the oocyte was exposed to LPA 18:1. Figure 5C shows that after the intracellular injection of DGPP 8:0, estimated to reach a concentration >300 nM, the extracellular application of 5 nM LPA 18:1 elicited a response equal in size to that of the control response before intracellular injection of DGPP. In comparison, the response normally elicited by LPA 18:1 was completely inhibited when DGPP 8:0 was applied extracellularly (Fig. 5C). The inhibitory effect of DGPP 8:0 was reversible, because after a 10-min wash, the response recovered to control level (Fig. 5C).

To show the specificity of DGPP 8:0 for the PSP24 and LPA₂ receptors expressed in the oocyte, the expression of neurotransmitter receptors was induced by the injection of poly A⁺ mRNA from rat brain. This resulted in the expression of several types of G protein-coupled receptors for serotonin and acetylcholine, which are not expressed in noninjected oocytes. These neurotransmitters activate the same inositol trisphosphate-Ca²⁺ signaling pathway that is activated by LPA. In such oocytes, DGPP 8:0 and PA 8:0 did not inhibit either the serotonin- or the carbachol-elicited responses (data not shown).

DGPP 8:0 Inhibits the LPA- but not S1P-, ATP-, and Thrombin-Activated Ca²⁺ Responses in NIH3T3, B103, HEY, and PC12 Cells. The effect of DGPP 8:0 on LPA-elicited responses was examined in mammalian systems that endogenously express LPA receptors. NIH3T3 cells were screened by RT-PCR for the expression of mRNA for LPA and S1P receptors. Figure 6A shows that in NIH3T3 cells mRNA transcripts for LPA₁ and LPA₂ receptors were abundant, whereas only a small amount of PSP24 mRNA was detected. To show that DGPP 8:0 specifically inhibited LPA-elicited but not S1P-elicited Ca²⁺ responses, NIH3T3 cells were exposed to 100 nM LPA or S1P in the presence of 10 μ M DGPP 8:0. As shown in Fig. 6B, DGPP 8:0 significantly inhibited but did not fully block the LPA-elicited Ca²⁺ responses, whereas the S1P-elicited response was not affected. To further characterize the specificity of inhibition for the LPA receptors, we tested the effect of DGPP 8:0 on Ca²⁺ responses elicited by 1 μ M ATP and 1 μ M thrombin receptor-activating peptide, which were near the EC₅₀ value observed in our NIH3T3 cells. The response elicited by ATP, another polyphosphate-containing ligand activating the P2Y receptor, in the presence of 10 μ M DGPP 8:0 was 112 \pm 17% (n = 3) of that elicited by ATP alone in the vehicle, whereas it was 117 \pm 17% (n = 3) of that elicited by the thrombin receptor-activating peptide alone.

The S1P₂ receptor has been shown to mediate Ca²⁺ responses (An et al., 1999). To further evaluate the selectivity of DGPP 8:0 for the LPA-specific members of the EDG family, we tested its effect on S1P-activated Ca²⁺ mobilization in B103 cells (Fukushima et al., 1998). These cells were chosen

because they express S1P₂ and no other S1P-specific members of the EDG family and do not respond to LPA (Fukushima et al., 1998). B103 cells did not respond to 10 μ M LPA, or 1 μ M DGPP, whereas they showed a response to S1P as low as 10 nM. In B103 cells exposed to 100 nM S1P, a concentration near its apparent EC₅₀ value in these cells, the Ca²⁺ response in the presence of a 10-fold excess of DGPP was unaffected and remained 106 \pm 11% of the S1P control response (n = 3).

LPA has been shown to be generated from and play a role in ovarian cancer cells (Xu et al., 1995). Using RT-PCR, we found that the HEY ovarian cancer cell line expresses mRNA transcripts for S1P₁, S1P₂, LPA₁, and LPA₃ receptors (Fig. 6C). We therefore tested DGPP 8:0 on HEY ovarian cancer cells to determine whether it had an effect on a therapeutically relevant target. Figure 6D shows that a 1 μ M concentration of DGPP 8:0 inhibited the LPA-elicited Ca²⁺ response by 88%, whereas DGPP 18:1 had no effect. Likewise, PA 8:0 inhibited the Ca²⁺ response by 94%, whereas PA 18:1 had no effect.

PC12 cells were found to express LPA₁, LPA₂, S1P₃, and S1P₅ transcripts (data not shown). LPA and S1P both elicit neurite retraction in differentiated PC12 cells (Fig. 7A). Differentiated PC12 cells, when exposed to 10 μ M DGPP alone, continued to extend neurites. A 15-min exposure to 100 nM LPA or S1P both elicited a decrease of nearly 50% in total neurite length. When a 10 μ M concentration of DGPP 8:0 was added to LPA, it blocked neurite retraction, whereas it was completely ineffective in preventing S1P-induced retraction (Fig. 7A).

The hallmark effect of a growth factor is its ability to elicit cell proliferation. Because LPA has been shown to stimulate the proliferation of a variety of different cell types, the ability of short-chain phosphatidates to inhibit cell proliferation was

examined in NIH3T3 cells. Figure 7B shows that DGPP 8:0 significantly inhibited the LPA-induced proliferation of NIH3T3 cells, reducing cell number to control levels, whereas it had no effect on solvent-treated control cells. Interestingly, PA 8:0 had no significant inhibition in this assay. To define the structure-activity relationship for the inhibitory effect of DGPP 8:0, the short- and long-chain species of DGPP, PA, and DAG were also tested. As shown in Fig. 7B, none of these lipids alone had a significant inhibitory or stimulatory effect on the proliferation of solvent-treated control cells. Only DGPP 8:0 inhibited LPA-induced proliferation, whereas DGPP 18:1, or long- and short-chain PA, and DAG had no effect on the LPA-induced proliferation.

Discussion

A major limitation in the PLGF field has been a lack of receptor subtype-specific agonists and antagonists. In the present study, RH7777 cells were used for heterologous expression of LPA₁, LPA₂, and LPA₃ receptors to screen potential antagonists. We have identified the short-chain phosphatidate DGPP 8:0 as a selective, competitive antagonist of LPA₃, with a K_i value of 106 \pm 28 nM. The same molecule was found to be a poor antagonist of LPA₁, with a K_i value of 6.6 \pm 0.68 μ M, whereas it did not inhibit LPA₂.

We have now shown that LPA 8:0 was neither an agonist nor an antagonist of LPA₁, LPA₂, or LPA₃ in a mammalian expression system. Our results with the short-chain phosphatidates confirm those of Bandoh et al. (2000) who demonstrated that LPAs, with acyl chains shorter than 12 carbons, were not agonists in insect cells expressing LPA₁, LPA₂, or LPA₃ (Bandoh et al., 2000). The same investigators showed that LPA₃ has a 10-fold preference for LPA with the fatty acid chain esterified to the *sn*-2, versus the *sn*-1 position.

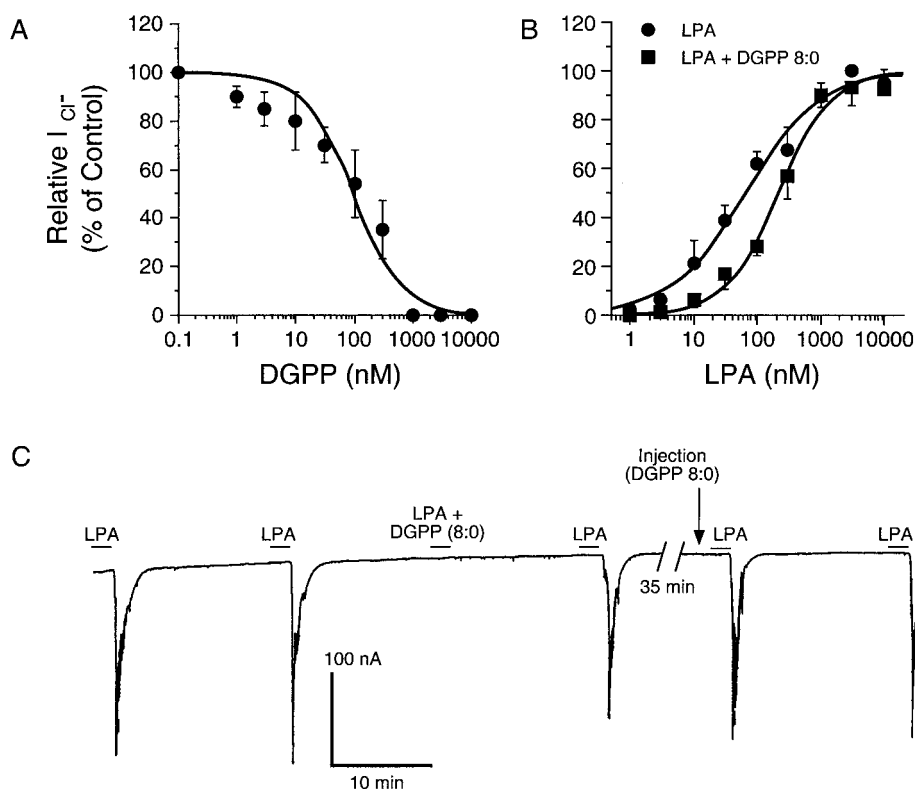


Fig. 5. Pharmacological characterization of DGPP 8:0 on the LPA-elicited Cl⁻ currents in *X. laevis* oocytes. The EC₅₀ value for LPA 18:1 was 56 nM (data not shown). Oocytes were exposed to a 5 nM concentration of LPA 18:1 mixed with increasing concentrations of DGPP 8:0, and the peak amplitude of the resulting oscillatory Cl⁻ currents was measured (A). Oocytes were exposed to increasing concentrations of LPA 18:1 mixed with a 200 nM concentration of DGPP 8:0 (B). Data points represent the average values of a minimum of three measurements \pm S.D. C, oocytes were treated with 5 nM LPA 18:1, or a mixture of 5 nM LPA 18:1 and 1 μ M DGPP 8:0 as indicated. The intracellular injection of 1 μ M DGPP 8:0 is indicated by the arrow.

Therefore, the position of the hydrocarbon chain relative to the phosphate moiety does not abolish ligand activation. LPA₃ also shows a preference for long-chain, unsaturated fatty acids over their saturated counterparts and the presence of an ether linkage or vinyl-ether side chain also decreased the EC₅₀ value by 2 orders of magnitude (Bando et al., 2000). Moreover, there is an optimal hydrocarbon chain length of 18 carbons, and shorter or longer chain analogs were weaker agonists. These pharmacological properties of LPA₃ suggest that receptor activation is dependent upon chain length, as well as the mobility of the side chain (ester versus ether linkage).

We propose a multipoint contact model for the interaction between the LPA receptors and LPA, which recognizes that a combination of interactions, involving both the ionic anchor and the hydrophobic tail, is required for agonist activation (Parrill et al., 2000). In support of this hypothesis, we found that the short-chain LPA 8:0 was not able to activate LPA₁, LPA₂, or LPA₃, emphasizing the importance of the interaction between the hydrophobic tail and the ligand binding pocket. Consequently, we propose the hydrophobic tail as the "switch" region of the PLGF pharmacophore. Because of the relative tolerance of the *sn*-1 and *sn*-2 substitution of the fatty acids by these receptors, we focused on short-chain phosphatidates. We speculated these compounds would not be able to activate the receptors due to their truncated hydrocarbon chains. We also explored the effects of a bisphosphate moiety, which does not change the negatively charged character of the anchoring region, but rather increases the charge.

This conceptual drug design was tested on clonal cell lines expressing the LPA₁, LPA₂, and LPA₃ receptors. The pharmacological properties of DGPP 8:0 and PA 8:0 were found to

be substantially different between the three receptors. Both molecules were effective at inhibiting LPA₃, whereas they were more than an order of magnitude less effective on LPA₁. Neither molecule was effective on LPA₂. DGPP 8:0 was also found to be a competitive inhibitor of LPA₁ and LPA₃, displacing the dose-response curves to the right for LPA on both receptors. The observed selectivity provokes consideration that these antagonists may interact with the extracellular loops, which exhibit approximately 40% sequence homology among LPA₁, LPA₂, and LPA₃. This sequence homology is markedly lower, and more likely to promote specificity, than the approximately 60% homology between the transmembrane domains of these three receptors. The lack of agonist activity of the corresponding long-chain species of PA and DGPP highlights the constraints that prevail in the binding pocket. The importance of the ionic anchor in docking the ligand in the binding pocket is supported by the lack of inhibition by DAG 8:0, although its cellular effects are probably confounded by its intracellular actions on other molecular targets, such as protein kinase C.

PA and DGPP are naturally occurring phospholipids identified in bacteria, yeast, and plants, but the latter is not found in mammalian cells. DGPP was discovered in 1993 as a novel lipid in plants and is a product of the phosphorylation of PA by phosphatidate kinase (Wissing and Behrbohm, 1993; Munnik et al., 1996). A recent study has shown that DGPP stimulates prostaglandin production in the P388D1/MAB cell line through the activation of cytosolic phospholipase A₂ (Balboa et al., 1999). These authors suggested that although DGPP activated a putative receptor, its effects were not mediated through the activation of LPA receptors (Balboa et al., 1999). Our results with the long-chain DGPP and PA analogs confirmed this notion, because these compounds

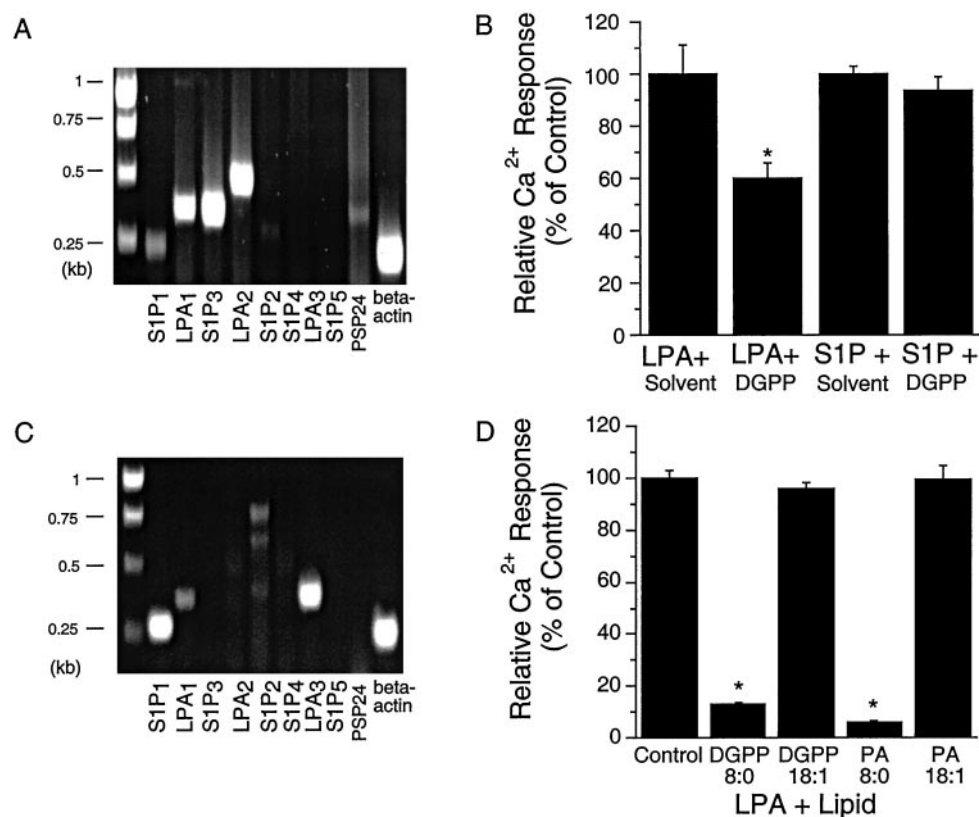


Fig. 6. DGPP 8:0 inhibits the LPA-elicited Ca²⁺ responses in NIH3T3 fibroblasts and HEY ovarian cancer cells. A, RT-PCR analysis of NIH3T3 cells for PLGF receptor transcripts. B, NIH3T3 cells were exposed to a 100 nM concentration of LPA 18:1, or S1P, mixed with a 10 μ M concentration of DGPP 8:0. C, RT-PCR analysis of HEY cells for the presence of the PLGF receptor transcripts. D, HEY cells were exposed to a 100 nM concentration of LPA 18:1, or S1P, mixed with a 1 μ M concentration of DGPP 8:0. The peak areas of the resulting Ca²⁺ responses were measured and are represented as the average of a minimum of three measurements \pm S.D.

did not possess agonist properties in the LPA receptor-expressing cell lines at concentrations up to 10 μ M. In light of the present data, the mechanism leading to prostaglandin production by DGPP 8:0 should be reinvestigated to determine whether this effect is due to the agonist or antagonist properties of this compound and a putative receptor should be identified. Although we did not find any agonist action of DGPP 8:0 in the mammalian cell lines included in the present study, we reported previously that high micromolar concentrations of DGPP 18:1 activated oscillatory Cl⁻ currents in *X. laevis* oocytes (Tigyi et al., 2000).

The effect of short-chain phosphatidates was also examined on LPA receptors expressed endogenously in four different cell types. First, DGPP 8:0 and PA 8:0, at nanomolar concentrations, were effective inhibitors of LPA-elicited Cl⁻ currents in *X. laevis* oocytes. To determine the site of action, DGPP 8:0 was injected into oocytes followed by an extracellular application of LPA. DGPP 8:0 was only effective at inhibiting the LPA-elicited Cl⁻ currents when applied extracellularly, demonstrating that it exerts its antagonist effect

on the cell surface. We reported previously that the PSP24 receptor is one of the receptors that mediates oscillatory Cl⁻ current responses to LPA in the oocyte (Guo et al., 1996; Fischer et al., 1998). In a recent report, Kimura et al. (2001) isolated two *X. laevis* orthologs of LPA₁, which they suggested were the only EDG family LPA receptors detectable in the oocyte. The present results indicate that DGPP 8:0 is a very effective inhibitor of the LPA response in the oocyte system with a K_i value of 52 ± 21 nM. PA 8:0 showed similar inhibitory properties (data not shown). This is in sharp contrast with mammalian ortholog of LPA₁, which showed a K_i value of 6.6 μ M. Thus, further experiments will be needed to determine which LPA receptor is responsible for the high sensitivity to short-chain phosphatidates in *X. laevis* oocytes.

To establish the selectivity of short-chain phosphatidates we turned to cell lines that endogenously express different combinations of the EDG family receptors. RT-PCR analysis revealed that LPA₁, LPA₂, and not LPA₃ are expressed in NIH3T3 cells. In these cells, DGPP 8:0, at a high 100-fold excess, only modestly inhibited the Ca²⁺ responses by 40%. This degree of inhibition parallels that seen in the stable cell line expressing LPA₁, where it was also a weak antagonist. Both short-chain phosphatidates were evaluated for their ability to block the LPA-induced proliferation of NIH3T3 cells. DGPP 8:0 effectively inhibited the LPA-induced proliferation, whereas long-chain DGPP did not. Although PA 8:0 was effective at inhibiting the Ca²⁺ responses, it was not effective at inhibiting cell proliferation. We speculate that the lack of inhibition by PA 8:0 might be due to its more rapid metabolism compared with DGPP in this long-term assay. Our results are in agreement with van Corven et al. (1992) who previously reported that PA (12:0) did not inhibit the mitogenic effect of PA 18:1. The fact that both PA and DAG failed to inhibit the proliferation suggests that DGPP 8:0 is likely to be more stable for the duration of this assay. The stability of DGPP has also been demonstrated by Balboa et al. (1999) who reported that DGPP was not metabolized during the 30-min course of their experiments.

RT-PCR revealed predominant expression of LPA₃ mRNA in HEY cells, with only a trace of LPA₁ mRNA detected, whereas LPA₂ transcripts were not found. When short-chain DGPP and PA were evaluated in HEY ovarian cancer cells, at a 10-fold excess over LPA, both were effective inhibitors, whereas neither long-chain molecule had any effect. This degree of inhibition parallels that seen in the stable cell line expressing LPA₃, where both DGPP 8:0 and PA 8:0 were effective inhibitors. The distance between the ionic anchor identified in the S1P₁ receptor model (Parrill et al., 2000) and the farthest point of the extracellular loops is not sufficient to accommodate long-chain acyl groups. The ineffectiveness of the long-chain molecules provides support for our hypothetical binding model involving the interaction between the antagonists and the extracellular loops. This hypothesis, however, will have to be tested in experiments with site-directed mutants of the key positions.

The specificity of DGPP 8:0 for LPA receptors was demonstrated in oocytes, B103 neuroblastoma cells, NIH3T3 fibroblasts, and PC12 cells. In these cells, DGPP 8:0 was only effective at inhibiting the LPA-elicited Ca²⁺ responses and not the responses elicited by S1P, ATP, thrombin, acetylcholine, or serotonin. RT-PCR analysis of the EDG family S1P receptors showed that S1P₁ and S1P₃ were expressed in

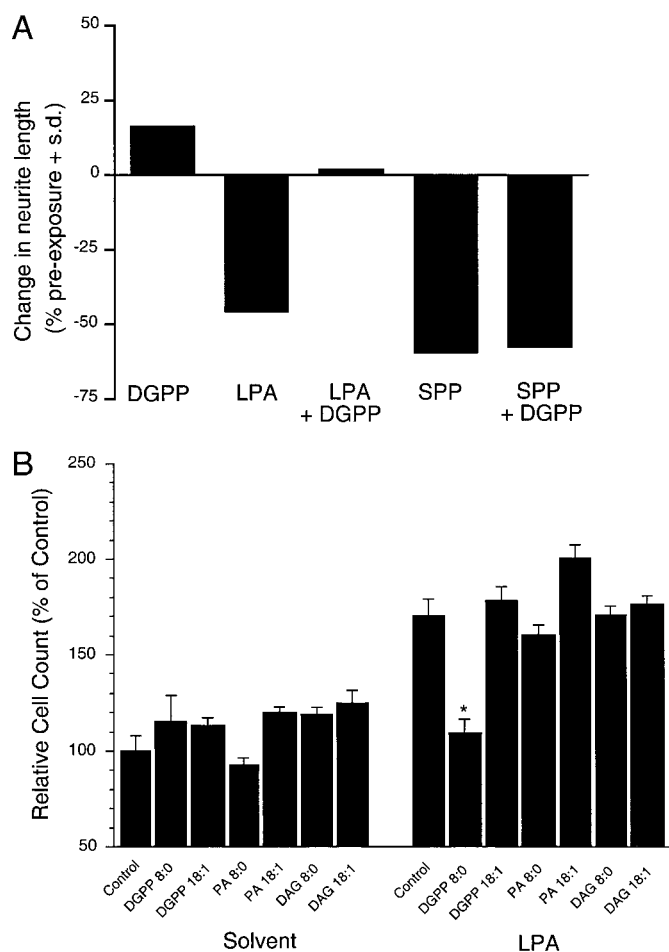


Fig. 7. DGPP inhibits LPA- but not S1P-induced neurite retraction in PC12 cells (A). Cells were exposed to 100 nM LPA or S1P with or without 10 μ M DGPP 8:0 for 15 min. The total neurite length was determined for microscopic field containing at least 50 cells. DGPP alone did not cause retraction and was effective in blocking the effect of LPA. DGPP 8:0 inhibits the LPA-elicited proliferation of NIH3T3 cells (B). NIH3T3 cells were serum-starved for 6 h and exposed to a 5 μ M concentration of LPA 18:1 mixed with a 10 μ M concentration of the indicated lipids. Control cells received solvent (bovine serum albumin) in place of LPA 18:1. The cells were incubated for 24 h with the lipids and counted. Data are representative of three experiments.

NIH3T3 cells, S1P₂ transcripts were present in B103 cells, whereas S1P₃ and S1P₅ receptors were expressed in PC12 cells. Taken together, these results indicate that DGPP does not affect the responses mediated through these S1P receptors. We did not find a cell line that expressed predominantly S1P₄, thus this receptor was not included in the present study. We reported recently that DGPP 8:0 blocks LPA-induced shape change in human platelets (Rother et al., 2001) but not the response elicited by platelet-activating factor, ADP, collagen, thrombin, or thromboxane. Thus, there is growing evidence that short-chain phosphatidates are selective blockers of LPA receptors. Taken together, short-chain phosphatidates provide an important new and commercially available tool for the field in studying not only the LPA receptors but also other PLGF receptors.

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