WITHDRAWN:
SPHINGOSYLPHOSPHORYLCHOLINE AND
LYSOPHOSPHATIDYLCHOLINE ARE LIGANDS FOR THE G PROTEIN
COUPLED RECEPTOR GPR4*

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WITHDRAWN
SUMMARY

Sphingosylphosphorylcholine (SPC¹) and lysophosphatidylcholine (LPC) are bioactive lipid molecules involved in numerous biological processes. We have recently identified ovarian cancer G protein coupled receptor 1 (OGR1) as a specific and high affinity receptor for SPC, and G2A as a receptor with high-affinity for LPC, but low affinity for SPC. Among G protein coupled receptors (GPCRs), GPR4 shares highest sequence homology with OGR1 (51%). In this work, we have identified GPR4 as not only another high affinity receptor for SPC, but also a receptor for LPC, albeit of lower affinity.

Both SPC and LPC induce increases in intracellular calcium concentration in GPR4-, but not vector-transfected, MCF10A cells. These effects are insensitive to treatment with BN52021, WEB-2170 and WEB-2086 [specific platelet activating factor (PAF) receptor antagonists], suggesting that they are not mediated through an endogenous PAF receptor. SPC and LPC bind to GPR4 in GPR4-transfected CHO cells with Kd/SPC=36 nM, and Kd/LPC=159 nM, respectively. Competitive binding is elicited only by SPC and LPC. Both SPC and LPC activate GPR4-dependent activation of serum response element (SRE) reporter and receptor internalization. Swiss 3T3 cells expressing GPR4 respond to both SPC and LPC, but not sphingosine-1-phosphate (S1P), PAF, psychosine (Psy), glucosyl-β1’1-

¹ The abbreviations used are: ERK: extracellular signal-regulated kinase; CHO, Chinese hamster ovary; Glu-Sph, Glucosyl-β1’1-sphingosine; Gal-Cer, galactosyl-β1’1-ceramide; Lac-Cer: lactosyl-β1’1-ceramide; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; GPCR, G protein coupled receptor; OGR1, ovarian cancer G protein coupled receptor 1; ox-LDL: oxidized low-density lipoprotein; PAF, platelet activating factor; Psy, psychosine; S1P, sphingosine-1-phosphate; SM, sphingomyelin; SRE, serum response element; SPC, sphingosylphosphorylcholine; SLE, systemic lupus erythematosus; TDAG8, T cell death-associated gene 8.
sphingosine (Glu-Sph), galactosyl-β1’1-ceramide (Gal-Cer), or lactosyl-β1’1-ceramide (Lac-Cer) to activate extracellular signal-regulated kinase (ERK) MAP kinase in a concentration- and time-dependent manner. SPC and LPC stimulate DNA synthesis in GPR4-expressing Swiss 3T3 cells. Both ERK activation and DNA synthesis stimulated by SPC and LPC are pertussis toxin (PTX)-sensitive, suggesting the involvement of a Gi-heterotrimeric G protein. In addition, GPR4 expression confers chemotactic responses to both SPC and LPC in Swiss 3T3 cells. Taken together, our data indicate that GPR4 is a receptor with high affinity to SPC and low affinity to LPC, and that multiple cellular functions can be transduced via this receptor.

INTRODUCTION

SPC is a bioactive lipid molecule involved in numerous biological processes, where it acts as a signaling molecule (1). We have recently identified a GPCR, OGR1, as the first specific high affinity receptor for SPC (2). OGR1 shares homology with several other GPCRs, including GPR4, G2A, T cell death associated GPCR8 (TDAG8), and the PAF receptor (3-10). We have postulated that these receptors belong to a subfamily and their ligands may be lysolipids containing the phosphorylcholine moiety shared by SPC and PAF (2). Other than SPC and PAF, there are two naturally occurring phosphorylcholine-containing lysolipids: LPC and lyso-PAF. LPC is an important lipid mediator involved in many cellular processes. In particular, LPC is believed to play an important role in atherosclerosis and inflammatory diseases by altering various functions of a variety of cell types, including endothelial cells, smooth muscle cells, monocytes, macrophages and T cells (11-13). However, the reported signaling mechanisms of LPC are variable and the initial interaction of LPC with cell membranes is poorly understood. We have recently identified G2A as the first receptor for LPC (14). G2A is also a low-affinity receptor for SPC.
In the present study, we sought to identify the ligand(s) for GPR4. We tested SPC, LPC, PAF, lyso-PAF and psychosine [Psy; a recently identified glycosphingolipid ligand of TDAG8 (15)] as potential ligands for GPR4. GPR4 exhibits the highest homology with OGR1 (51% identity and 64% similarity in amino acid sequence) (2). Similarly to OGR1, GPR4 responded to SPC, but also responded to LPC, mediating an increase in intracellular calcium concentration, SRE activation, receptor internalization, ERK activation, and stimulation of cell migration. LPC bound to GPR4, albeit with lower affinity compared to SPC, and competed with SPC for specific binding to GPR4. GPR4 did not bind or respond to PAF, lyso-PAF, Psy, Glu-Sph, Gal-Cer, or Lac-Cer. Our results indicate that SPC is a high-affinity and LPC is a lower-affinity ligand for GPR4, and its activation by SPC and LPC mediates biological functions.

EXPERIMENTAL PROCEDURES

Materials—LPCs (14:0, 16:0, 18:0, and 18:1), lysophosphatidylinositol (LPI; from liver, 80% 18:0), 18:1-LPA, 16:0-PAF, 16:0-lysoPAF, psychosine, glucosyl-β1’1-sphingosine, galactosyl-1’1-C8-ceramide, and lactosyl-β1’1-C8-ceramide were from Avanti Polar Lipids, Inc. (Alabaster, AL). Sphingomyelin (SM; bovine brain, mainly 18:0), C6-ceramide, sphingosine-1-phosphate (S1P) and SPC were from Toronto Research Chemicals (Toronto, ON) or Matreya, Inc. (Pleasant Gap, PA). D-erythro- and L-threo-SPC were from Matreya, Inc. (Pleasant Gap, PA). pcDNA1-C3 (encoding the C3-exoenzyme), was a kind gift from Dr. A. Wolfman, Cleveland Clinic Foundation. The PAF receptor antagonist, BN52021, was from Biomol (Plymouth Meeting, PA). WEB-2170 and WEB-2086 were from Boehringer Ingelheim (Ridgefield, CT). [3H]SPC or [3H]18:0-LPC were custom synthesized by Amersham Pharmacia Biotech, Buckinghamshire, England (68 Ci/mm, 1 mCi/ml for [3H]SPC and 102 Ci/mm, 1 mCi/ml for [3H]18:0-LPC). [3H]16:0-LPC (60 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO).
**Cell culture**—MCF10A cells (passage 34) were purchased from the Barbara Ann Karmanos Cancer Institute (Detroit, MI) and cultured as recommended by the provider. Experiments were performed using MCF10A cells from passage 40-46. Other cells were obtained from ATCC and were cultured either in RPMI1640 with 10% FBS or DMEM with 5% FBS (CHO and Swiss 3T3 cells).

**Human RNA Master Blot Probed with GPR4**—Human RNA Master Blot (Clontech, Palo Alto, CA) was probed with radiolabeled full-length GPR4. Briefly, the full-length GPR4 was gel purified and 25 ng was used for the synthesis of a StripAble DNA α-32P-labeled probe (Ambion, Austin, TX), as per the manufacturer’s instructions. The radiolabeled probe (20 ng, 20 X 10^6 CPM) was hybridized to the Master Blot in ExpressHyb hybridization solution (Clontech) overnight with continuous agitation at 65°C. The following day, the Master Blot was washed following the manufacturer’s instructions and exposed to a Phospho Screen (Molecular Dynamics, Sunnyvale, CA).

**Real-time Quantitative PCR of GPR4**—Total RNA was extracted from cells using the SV Total RNA Isolation System (Promega, Madison, WI). One to five micrograms of total RNA were reverse transcribed using Superscript II RT (Gibco BRL, Rockville, MD). Eight nanograms of derived cDNA were used as a template for real-time quantitative SYBR Green I PCR. Primers for human GPR4 (Genbank accession number U21051) were 5’-TAATGCTAGCGAACCACACGTGGGAG and 5’-TCCAGTTGTCGTGGTGCAG, yielding a 230 bp product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in a separate tube as a housekeeping gene with primers 5’-GAAGGTGAAGGTCGGAGT and 5’-GAAGATGGTGATGGGATTTC, yielding a 226 bp product. Primers for mouse GPR4 were 5’-CTACCTGGCTGTGGCTCAT and 5’-CAAAGACGCGGTATAGATTCA, yielding a 222 bp product. Mouse GAPDH was amplified with primers 5’-TGATGGGTGTAACCAAGACA and 5’-CCAGTGGATCAGGGGATGAT. All SYBR Green I core reagents, including AmpliTaq Gold DNA polymerase, were from PE Applied Biosystems.
(Foster City, CA). The thermal cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C, 15 seconds and 60°C, 1 minute. PCR reactions and product detection were carried out in an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The amplified product was detected by measurement of SYBR Green I, which was added to the initial reaction mixture. The threshold cycle (C_T) values obtained through the experiments indicate the fractional cycle numbers at which the amount of amplified target reach a fixed threshold. The C_T values of both target and internal reference (GAPDH) were measured from the same samples, and the expression of the target gene relative to that of GAPDH was calculated using the comparative C_T method. This method normalizes the expression levels and allows calculation of the relative efficiency of the target and reference amplification.

Cloning—A GPR4 PCR fragment (nucleotides #1175-1535) (4) was obtained by PCR amplification using cDNA from HEY ovarian cancer cells as the template. This PCR fragment was used to screen a human genomic library (Clontech, Palo Alto, CA) to obtain the full-length clone of GPR4. GPR4 was subsequently cloned into mammalian expression vectors using PCR amplifications with the high fidelity Advantage cDNA polymerase (Clontech). The PCR reactions were conducted for fewer than 20 cycles and the sequence of the products was confirmed by sequencing. The primers: 5’-CAGGAATTCTCGGCAACCACACGTGGGAGG, and 5’-CGCTCTAGAGCCACTCGGGGTTCATTGTG were used to generate full length GPR4, which was digested with EcoR I and Xba I and cloned into the pBs3HA vector (pBluescript II KS+ vector with three HA-tags inserted; a kind gift from Dr. J. DiDonato, Cleveland Clinic Foundation). The resulting 3HA-GPR4 was subsequently cloned into the mammalian expression vector pIRES-hygro (Clontech) to generate pIRES-hyg-GPR4, using primer 5’-CAGATGCATAAACGCTCAACTTTGG and the T7 primer (inserted into the Nsi I and Not I sites of pIRES-hygro). pGPR4-GFP was generated using the T3 primer and 5’-GTCGGTACCTGTGCTGGCGGCAGCATC (stop codon was deleted and the resulting GPR4 was
cloned into Hind III and Kpn I sites of pEGFP-N1; Clontech). pSRE-Luc was purchased from Stratagene (La Jolla, CA). MCF10A cells were transiently transfected with pGPR4-GFP and used for calcium assays. CHO cells were transfected with pIREShyg-GPR4 (LipofectAMINE reagent; Life Technologies, Rockville, MD) and stable clones were selected with 200 µg/ml hygromycin in DMEM/F12 plus 5% FBS. HEK293 cells were transfected with pGPR4-GFP and stable clones were selected with 400 µg/ml G418 in RPMI 1640 plus 10%FBS. Swiss 3T3 cells expressing GPR4 were derived by infection with retroviruses encoding receptor (MSCV GPR4 ires-GFP) followed by FACS sorting of GFP positive cells (16).

**Calcium assays**—Measurement of [Ca^{2+}]_i, was performed as described previously (2). Briefly, pGPR4-GFP-transfected MCF10A cells were grown in specialized glass-bottom dishes (Bioptech, Inc., Butler, PA) and loaded with fura-2 in HEPES buffered saline. Using a dual-wavelength spectrofluorometer (RFK-6002, Photon Technology Int'l, So. Brunswick, NJ) coupled to an inverted fluorescence microscope (Olympus, IX-70, Lake Success, NY), GFP-positive cells were identified using an excitation wavelength of 488 nm, a dichroic 505 nm long-pass filter and an emitter filter at bandpass of 535 nm (Chroma Technology, Brattleboro, VT). Measurements of [Ca^{2+}]_i were performed on individual GPR4-GFP positive cells at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Conversion of the 340/380-ratio value into [Ca^{2+}]_i in nM was estimated by comparing the cellular fluorescence ratio with ratios acquired using fura-2 (free acid) in buffers containing known Ca^{2+} concentrations. [Ca^{2+}]_i was then calculated as described by Grynkiewicz et al. (17). All calcium assays were performed in the presence of 1 mM EGTA in the assay buffers. Therefore, intracellular calcium release, not calcium influx, was analyzed.

**Internalization**—pGPR4-GFP stably expressing HEK293 cells were cultured in 6 cm tissue culture dishes in RPMI1640 with 10% FBS. After 16-24 h serum starvation, cells were treated with different lipids
at 37°C for 2 h. Cells were washed with cold PBS and fixed with 4% paraformaldehyde in PBS. The subcellular localization of GPR4-GFP protein was visualized under a Leica TV confocal fluorescence microscope with a 63x oil immersion lens (Wetzler, Heidelberg, Germany). The excitation and emission wavelengths were 488 nm and 515-540 nm, respectively.

**Binding assays**—CHO cells were chosen for GPR4 binding assays, since HEK293 cells express relatively high levels of endogenous GPR4. CHO cells stably transfected with empty vector or GPR4 were serum starved for 20 h, then collected after exposure to 2 mM EDTA in PBS. The pelleted cells were stored at -80°C until use. Binding assays were performed essentially as described previously (2), except binding was performed at 4°C. Briefly, frozen cells (10⁶ cells/ml) were homogenized in a binding buffer (2). Assays were performed in 96-well plates in triplicate with 100µl cell homogenate (equivalent to 10⁵ cells/well). Different amounts of [³H] SPC or [³H] 16:0-LPC were added to the cell homogenates in 50 µl of binding buffer, in the presence or absence of cold SPC or 16:0-LPC, or other competitors. The plates were incubated at 4°C for 120 min, unless otherwise indicated. Cell-bound [³H] SPC or [³H] LPC was collected onto a filter (Printed Filtermat A, Wallac, Gaithersburg, MD) using an automated cell harvester (HARVESTER 96, Tomtec, Orange, CT). Specific binding was calculated by subtraction of nonspecific binding (binding detected in the presence of 100-fold excess unlabeled SPC or 16:0-LPC) from the total binding.

**Reporter (SRE) assays**—The SRE reporter system (pSRE-Luc) was a gift from Dr. Songzhu An (UCSF), or purchased from Stratagene (La Jolla, CA). Both systems gave identical results. HEK293 and HEK293-GPR4 cells were cultured in RPMI1640 with 10%FBS in 10 cm dishes to ~85% confluence. To the cells in each dish, pSRE-Luc (10 µg) was transfected in the presence of 60 µl LipofectAMINE reagent. Cells were seeded in 96-well plates 16 h after transfection, incubated for another 24 h in RPMI1640 with 10% FBS, and starved in serum-free medium for 16 h. SPC (dissolved in PBS to 10 mM) and other lipids
(LPCs were dissolved in 70% ethanol. Other lipids were dissolved in PBS, 70-95% ethanol, or 100% MeOH) were diluted in serum free RPMI 1640 and added to the cells, followed by a 10 h incubation. Luciferase activity was measured in Microlite™ 1 plates (DYNEX Technologies, Inc., Virginia) using 60 µl of cell lysate and 20 µl luciferase substrate. PTX (100 ng/ml) was added during the 16 h serum starvation period and pcDNA1-C3 (encoding the C3-exoenzyme, 2 µg) was co-transfected with pSRE-Luc (10 µg).

**ERK activation assays**—Swiss 3T3 cells were infected with MSCV GPR4-ires-GFP or MSCV ires.GFP, and subsequently cells sorted by FACS for positive expression of GFP as described previously (16). Cells were plated in 6-well plates in DMEM containing 5% FBS, serum-starved overnight, and then treated lipids in DMEM for the indicated times. Cells were lysed on ice in RIPA buffer containing 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 2mM EGTA, 25 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 x protease inhibitors (Sigma P8340). Lysates containing equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Antibodies against phosphorylated ERK1/2 (Cell Signaling Technologies, Beverly, MA) were used to probe the membrane and the ECL system (Amersham) was used for detection. To normalize the amounts of protein loaded in each lane, membranes were stripped and re-probed with antibodies against total ERK (Cell Signaling Technologies). In some experiments, cells were pretreated with 100 ng/ml PTX for 12-16 h prior to SPC and LPC stimulation.

**DNA synthesis assay**—The effect of SPC and LPC on DNA synthesis was measured using [³H] thymidine incorporation. Briefly, GPR4-ires-GFP- and GFP-Swiss 3T3 cells were plated in 96-well plates, serum-starved for 24 h, and treated with SPC, LPC, or other lipids in serum-free DMEM for 24 h. Cells were incubated with 0.75 µCi/ml [³H] thymidine in serum-free DMEM for the last 18 h. Cells were harvested onto filter papers presoaked in 1% polyethylenimine using the automated cell harvester
HARVEST 96. Incorporated [3H] thymidine was counted in a 1450 Microbeta Trilux Liquid Scintillation and Luminescence Counter (Perkin-Elmer-Wallac, Inc.)

Cell Migration Assay—Chemotaxis was measured in a modified Boyden chamber assay. Briefly, different lipids were added to the lower chambers. GPR4-ires-GFP- and GFP-Swiss 3T3 cells were serum starved for 4 h, trypsinized, and seeded in the upper chambers of Boyden-transwell plates (Corning Inc., Corning, NY). The chambers were incubated for 6 to 8 h. The number of cells that migrated to the lower face of the membrane was counted in 4 random fields. Data are represented as the average ± SD of three independent experiments. For the chemokinetic assay, the same concentrations of lipids were added to both the upper and lower chambers. For Rho inhibition studies, C3-exoenzyme was transiently transfected into Swiss 3T3 cells and cell migration assays were performed 48 hours later.

RESULTS

Human RNA Master Blot Probed with GPR4—GPR4 has been shown to be expressed in many human tissues (18). For a wider analysis of GPR4 expression in human tissues, we probed the Human RNA Master Blot (Clontech) containing RNAs from 50 different human tissues with the full length human GPR4 clone labeled with [32P]dCTP (Experimental Procedures). GPR4 showed the highest expression in ovary, liver, lung, kidney, lymph node, and sub-thalamic nucleus (Fig. 1). Other areas of the brain had a lower expression of GPR4, as did the aorta, placenta, bone marrow, skeletal muscle, spinal cord, prostate, small intestine, and some fetal tissues. GPR4 was also expressed at a detectable level in appendix, trachea, testis, spleen, thymus, pituitary gland, adrenal gland, thyroid gland, and heart, but not in other tissues including some areas of the brain, colon, bladder, uterus, stomach, pancreas, salivary gland, mammary gland, peripheral blood leukocytes, fetal brain, and fetal heart (Fig. 1).
Both SPC and 16:0-LPC induced transient increases in intracellular calcium concentration ([Ca^{2+}]_i) in GPR4-transfected MCF10A cells—We have shown that OGR1 is a high-affinity receptor for SPC (2). To test whether GPR4, which shares 51% sequence homology with OGR1, is also a receptor for SPC, MCF10A cells were transiently transfected with pGPR4-GFP. MCF10A cells were chosen since these cells do not respond to either SPC or 16:0-LPC in calcium assays and they express very low levels of endogenous GPR4 among many human cell lines tested (Fig. 2).

The GFP receptor fusion was used to identify positively transfected cells, and single-cell calcium assays were performed as described in our previous studies (2). SPC (1 µM) stimulated an increase in [Ca^{2+}]_i in GPR4-, but not vector-transfected MCF10A cells (Fig. 3A, 1st and the 2nd panels), suggesting that GPR4 is a receptor for SPC. This is further confirmed by the stereo selectivity of GPR4 favoring D-erythro-SPC (the bioactive form of SPC) vs. L-threo-SPC (Fig. 3A, 3rd panel). Interestingly, unlike OGR1, which is specific for SPC as its ligand (2), GPR4-transfected cells were stimulated to produce increased [Ca^{2+}]_i by an additional phosphorylcholine-containing lysolipid, 16:0-LPC (Fig. 3A, 4th panel).

To assess the affinities and potencies of SPC and 16:0-LPC, concentrations of each were varied and calcium mobilization was measured (Fig. 3B). SPC appeared to have a higher efficiency (EC_{50}=105 nM) than LPC (EC_{50}=1.1 µM), although the [Ca^{2+}]_i responses to LPC in GPR4-transfected cells were higher than those of SPC at greater concentrations of LPC (up to 10 µM) (Fig. 3B).

LPC has been shown to activate cellular responses in a PAF receptor-dependent manner (19-21). However, LPC and SPC were not able to induce an increase in calcium through the endogenous PAF receptor in parental cells (Fig. 3A, upper panel). Therefore, it is unlikely that the increase in calcium induced by LPC was mediated by a PAF receptor. Nevertheless, to confirm that LPC and/or SPC did not activate the endogenous PAF receptor in GPR4-transfected cells, three specific PAF receptor antagonists,
BN52021, WEB-2170, and WEB-2086, were used. Both BN52021 (200 µM) and WEB-2086 (2 µM) completely abolished the calcium signal induced by PAF (100 nM) (Fig. 3C and 3D). However, the cellular calcium response to LPC or SPC was not affected, indicating that calcium increases induced by SPC and LPC were not mediated through an endogenous PAF receptor. Another PAF antagonist, WEB-2170 (2 µM), also completely blocked the action of PAF, but did not affect the increase in calcium induced by either LPC or SPC (data not shown). In addition, LPC and SPC showed not only homologous, but also heterologous, desensitization to each other (Fig. 3E), suggesting that these two lipids activated the same receptor.

To determine which G protein is involved in the increased [Ca^{2+}] response to SPC and LPC in GPR4-transfected cells, the sensitivity of this activity to PTX was tested. The increase in [Ca^{2+}] response to both SPC and LPC, as well as to stimulation of endogenous LPA receptor(s), but not PAF or ATP receptors, was completely abolished by PTX (100 ng/ml, 16 h pretreatment) (Fig. 3F), suggesting the involvement of a Gᵢ pathway.

In plasma, LPC is mainly present in albumin- and lipoprotein-bound forms (22). To determine whether BSA-bound SPC and LPC are able to induce increases in [Ca^{2+}], we pre-incubated SPC (1 µM) and LPC (1 µM) with a molar excess of BSA [0.5% fatty acid-free BSA (Sigma)], for a lipid:BSA molar ratio of approximately 1:75. At this molar ratio, BSA blocked more than 50% and 95% of the increases in [Ca^{2+}], induced by SPC and LPC, respectively (Fig. 3G). These results suggest that albumin-bound LPC may not be able to activate this receptor, and support the concept of multiple LPC compartmentalization (e.g. bound and free) (23).

Recently, Im et al have identified Psy as a ligand for TDAG8. TDAG8 shares approximately 38% homology with OGR1, GPR4 and G2A (15). To determine whether Psy is a ligand for GPR4, and to
delineate the structural specificity of ligands for GPR4, we tested the effect of Psy, Glu-Sph, Gal-Cer, and Lac-Cer to increase \([\text{Ca}^{2+}]_i\) in MCF10A cells. We found that at 1 \(\mu\text{M}\), Psy, Glu-Sph, and Lac-Cer did not stimulate increases in \([\text{Ca}^{2+}]_i\) in either MCF10A parental or GPR4-expressing cells (Fig. 3H). Gal-Cer (1\(\mu\text{M}\)) induced the same level of increased \([\text{Ca}^{2+}]_i\), in both parental and GPR4-expressing MCF10A cells (Fig. 3H). These data suggest that these glycosphingolipids are unlikely to be ligands of GPR4.

**SPC and LPC bind to GPR4**—To characterize the binding of SPC and LPC to GPR4, we conducted radioligand binding assays, using a method similar to that developed for OGR1 as described previously (2). Cell homogenates were used for binding assays. Binding was conducted at 4°C for 120 min or as indicated. \([^3\text{H}]\) SPC and \([^3\text{H}] 16:0\)-LPC specifically bound to cell homogenates from GPR4-transfected CHO cells in a time-dependent manner and reached equilibrium after 60 min incubation at 4°C (Fig. 4A and 4B). Both CHO cells and CHO cells transfected with empty vector displayed low background binding of SPC and LPC (Fig. 4A and 4B). SPC and 16:0-LPC bindings were saturable and Scatchard analyses indicated dissociation constants (K_d) of 36 nM for SPC and 159 nM for LPC. The maximum binding capacities for SPC and 16:0-LPC were 996 fmole/10^5 cells for SPC and 1,528 fmole/10^5 cells for 16:0-LPC (Fig. 4C and 4D). SPC (p<0.001) and various LPC species (16:0, 18:0 and 18:1; p values 0.001-0.01), but not LPA (18:1), LPI (18:0), S1P, SM (18:0), 16:0-PAF or 16:0-lyso-PAF (p values >0.05), successfully competed for binding (Fig. 4E and 4F). Binding assays using \([^3\text{H}]\) 18:0-LPC gave similar results (data not shown). We also tested the four glycosphingolipids, Psy, Glu-Sph, Gal-Cer, and Lac-Cer, for their ability to compete for the binding of \([^3\text{H}]\) SPC and \([^3\text{H}] 16:0\)-LPC to GPR4. None of these glycosphingolipids competed successfully (Data not shown). Thus, GPR4 was able to specifically bind both SPC and LPC (16:0, 18:0 and 18:1), with a higher affinity for SPC than LPC.

**Internalization of GPR4 induced by SPC and LPC**—G protein coupled receptors undergo agonist-dependent desensitization and internalization (24-26). When HEK293 cells were transfected with
the pEGFP-N1 vector, GFP protein was expressed in the cytosol of the cells (2). The GPR4-GFP fusion protein, on the other hand, was expressed only on the plasma membrane (Fig. 5A). One micromolar concentrations of SPC and 16:0-LPC, but not 16:0-PAF, induced internalization of GPR4 at 37°C (Fig. 5B, C and F). The PAF receptor-specific antagonist BN52021 did not block the internalization of GPR4 induced by either SPC or 16:0-LPC (Fig. 5D and E). Similarly, WEB-2170 and WEB-2086 did not affect the internalization of GPR4 induced by either SPC or 16:0-LPC (data not shown).

**LPC and SPC activated the SRE reporter system in HEK293 cells**—The serum-response element (SRE) reporter system is a sensitive assay for receptors of lipid factors (27,28). Using the luciferase assay, vector-transfected HEK293 cells transfected with the SRE reporter system responded to SPC (1 µM), but not 16:0-LPC, with less than 1.5-fold activation (Fig. 6A). Activation was increased 3.1- and 4-fold, respectively, in response to 16:0-LPC (1 µM) and SPC (1 µM) in GPR4-transfected HEK293 cells that were also transfected with the SRE reported system (Fig. 6B). These increases were statistically significant (p<0.001) when compared to the responses in vector-transfected cells (Fig. 6A). In contrast, although LPA and S1P induced significant transcriptional activation of SRE in vector-transfected HEK293 cells, this activation was not altered by GPR4 transfection. In addition, we tested other phosphorylcholine-containing lipids, including 16:0-PAF, 16:0-lyso-PAF and 18:0-SM, and found that none of them induced significant transcriptional activation of SRE (Fig. 6A).

The SRE transcriptional activity in response to SPC, but not LPC, in parental HEK293 cells (Fig. 6A and 6B), can be explained by the endogenous expression of GPR4 in HEK 293 cells and the relatively lower affinity of GPR4 for LPC compared to SPC. GPR4 transfection enhanced the activation of SRE reporter by both SPC and LPC (Fig. 6A and 6B). EC<sub>50</sub> values for the activation of SRE were 63 nM for SPC and 160 nM for 16:0-LPC. The differences in EC<sub>50</sub> values obtained using SRE activation from those
using the calcium assay (105 nM and 1.1 µM for SPC and LPC, respectively) are possibly derived from different coupling efficiencies of distinct signaling pathways and/or different cellular environments.

To determine which G protein and other signaling intermediates might be involved in the activation of SRE by SPC and 16:0-LPC, we pretreated cells with PTX (100 ng/ml) for 16 h, or co-transfected the specific Rho inhibitor, C3-exoenzyme (1.5 µg pcDNA3-C3), with the reporter system. Both PTX and C3-exoenzyme partially inhibited SRE-reporter activation (Fig. 6C). When the two inhibitors were added together, SRE-reporter activation in response to either SPC or 16:0-LPC was almost completely blocked, suggesting that G_i and Rho signaling pathways were involved in SRE activation through GPR4.

**SPC and LPC activated ERK MAP kinase in a GPR4-dependent manner**—MAP kinases are key signaling intermediates of DNA synthesis and cell proliferation. To determine whether GPR4 mediates ERK MAP kinase activation in response to SPC and LPC, we conducted Western blot analyses of GPR4-ires-GFP-Swiss 3T3 and GFP-Swiss 3T3 cells treated with SPC, 16:0-LPC, and a number of other lipids. The parental and GFP infected Swiss 3T3 cells showed a basal level of ERK activation, as detected by anti-phospho-ERK antibody (Fig. 7A). SPC (100 nM) increased this level of activation (Fig. 7A). In GPR4-ires-GFP-infected Swiss 3T3 cells, both SPC (100 nM) and LPC (100 nM) enhanced ERK activation, and SPC was more potent than LPC (Fig. 7A). A number of other lipids, including S1P, Lac-Cer and PAF, also activated ERK in Swiss 3T3 cells, but activation was independent of GPR4 expression (Fig. 7A).

Lipid stock solutions, dissolved in ethanol or MeOH, were greater than 10 mM. Since the highest final concentration of lipids used in this study was 10 µM, the solvent content was less than 0.1% in any experiment. We routinely performed solvent controls and found that at final solvent concentrations of less than 0.1%, 70-100% ethanol and 100% methanol did not alter any parameters tested.
The higher potency of SPC over LPC was further reflected in the concentration- and time-
dependent ERK activation (Fig. 7B and 7C). ERK activation induced by SPC compared to that by LPC
was evident at a lower concentration (approximately 10 nM vs. 100 nM), at earlier time points (1 min vs. 5
min), and was maintained for a longer time. These results strengthen the notion that both SPC and LPC are
ligands for GPR4, but SPC has a higher affinity than LPC for GPR4.

In GPR4-infected Swiss 3T3 cells, SPC-induced ERK activation was sensitive to PTX, suggesting
involvement of G\textsubscript{i} signaling (Fig. 7D). This is in contrast to our previous studies where SPC induced ERK
activation via a PTX-insensitive pathway in OGR1-transfected HEK293 cells (2). To determine whether
this difference was due to receptor subtype or different cell lines used, we tested the PTX-sensitivity of
SPC-induced ERK activation in OGR1-infected Swiss 3T3 cells. Our results showed that in Swiss 3T3
cells, SPC-induced ERK activation via OGR1 was PTX-insensitive (Fig. 7D). Thus, although GPR4 and
OGR1 are highly homologous, the same high-affinity ligand (SPC) induces activation of ERK through a
different G protein pathway for each receptor.

**SPC stimulated DNA synthesis in GPR4-infected Swiss 3T3 cells**—To determine whether SPC
and LPC affect DNA synthesis in a GPR4-dependent fashion, we measured \([^3H]\) thymidine incorporation
into GPR4-ires-GFP-Swiss 3T3 and GFP-Swiss 3T3 cells. SPC stimulated DNA synthesis in both
parental and GFP-infected cells (approximately 6.3-fold increase with 3 \(\mu\)M SPC). These results are
qualitatively consistent with observations by Desai et al (29). This stimulation was further enhanced by the
expression of GPR4 (1.8- to 2-fold increase over GFP-infected Swiss 3T3 cells; Fig. 8A). In both GFP-
and GPR4-GFP expressing cells, DNA synthesis stimulated by SPC was inhibited by PTX (Fig. 8A),
suggesting G\textsubscript{i} signaling was required for this activity. GFP-expressing cells did not respond significantly to
16:0-LPC, whereas \([^3H]\) thymidine incorporation increased 1.6-fold in GPR4-infected Swiss 3T3 cells in
response to 3 µM 16:0-LPC (Fig. 8B). Higher concentration of lipids did not further increase \[^{3}H\]
thymidine incorporation stimulated by SPC or LPC (data not shown).

**SPC and LPC induced cell migration in a GPR4-dependent manner**—As a major component
of oxidized low-density lipoprotein (ox-LDL), LPC has been proposed to play a role in atherosclerotic
lesion development (30,31). One of the roles of LPC potentially related to atherosclerosis is as a
chemoattractant for monocytes, T lymphocytes, and smooth muscle cells (32-34). We used Swiss 3T3
cells infected with GFP or GPR4-ires-GFP as a model system to compare the effects of SPC and 16:0-
LPC on cell migration. GPR4 overexpression in Swiss 3T3 fibroblasts increased cell migration in response
to SPC (100 nM; lower chamber only) and 16:0-LPC (100 nM; lower chamber only) 2.0-fold and 1.7-
fold, respectively, over that observed in GFP-Swiss 3T3 cells (Fig. 9A). Other lipids (18:1-LPA, S1P, or
16:0-PAF) did not alter cell migration in GPR4- vs. vector-transfected cells (Fig. 9A). Cell migration
stimulated by both SPC and LPC was inhibited by C3-exoenzyme expression, suggesting that Rho is
involved in this process.

Concentration response studies (Fig. 9B) indicate that SPC and LPC were effective at inducing cell
migration in the 1-100 nM concentration range. To determine whether this effect was chemotactic or
chemokinetic, we measured cells that migrated from the upper to the lower chambers in Boyden chamber
assays, conducted with lipids (at 100 nM) in both upper and lower chambers. SPC or 16:0-LPC did not
significantly change cell motility when compared to controls (without lipid in either chamber) in either GFP
or GFP-GPR4 expressing Swiss 3T3 cells (Fig. 9C). S1P slightly inhibited, PAF slightly enhanced, and
LPA did not show a significant effect on cell migration in treated vs. untreated GFP or GFP-GPR4
expressing cells (Fig. 9C). These results suggest that the effect of SPC and 16:0-LPC on cell migration was
chemotactic, not chemokinetic, and that the chemotactic effect was mediated through GPR4.

Withdrawn
DISCUSSION

GPR4 shares ~50% homology with OGR1. We therefore speculated that these two receptors may have overlapping ligand specificity. Indeed, the results presented here show that GPR4 is a second high-affinity receptor for SPC. OGR1 and GPR4 may play both overlapping and distinct physiological and pathological roles. We have shown that OGR1 and GPR4 bind SPC with similar affinities (33 nM and 36 nM, respectively) and both receptors mediate SPC-induced increases in intracellular calcium and ERK activation. However, GPR4- and OGR1-mediated ERK activation is PTX-sensitive and –insensitive, respectively (Fig. 7A), suggesting that GPR4 and OGR1 couple to different G proteins to activate ERK. More importantly, these differential couplings appear to lead to differential effects on cell proliferation. On the other hand, while OGR1 mediates PTX-insensitive growth inhibition by SPC in a number of cells tested (2), GPR4 mediates PTX-sensitive DNA synthesis by SPC in Swiss 3T3 cells. Together, these data suggested that the endogenous receptor(s) for SPC in Swiss 3T3 cells was GPR4-like, rather than OGR1-like, since parental Swiss 3T3 cells respond to SPC to activate ERK and increase DNA synthesis through a PTX-sensitive pathway (Fig. 8A). The expression of GPR4 in these cells has been confirmed by quantitative PCR analysis (Fig. 2).

GPR4 and OGR1 have different tissue distributions, which may be related to their physiological and pathological roles. Both OGR1 and GPR4 are highly expressed in the lung. However, OGR1 is expressed at high levels in the placenta, spleen, testis, small intestine and peripheral leukocytes (8,18), whereas GPR4 is not expressed, or is expressed at relatively low levels, in these tissues. While GPR4 is expressed at high levels, in the liver, kidney, and ovary (Fig. 1), OGR1 is not expressed in these tissues (8,18). The physiological and pathological roles of these receptors remain to be further investigated.

Another significant finding from this study is the identification of GPR4 as the second G protein coupled receptor for LPC [the first LPC receptor, G2A, was recently described (14)]. GPR4 binds to
LPC (in addition to SPC), but not PAF or lyso-PAF, to mediate an increase in intracellular calcium, receptor internalization, SRE activation, MAP kinase activation, DNA synthesis, and cell migration. Although effects of LPC on transmembrane signal transduction have been widely reported, a specific receptor recognizing LPC had not been identified previously (32). LPC lyses cells at high concentrations (>30 μM) (35) and many of the cellular effects previously reported for LPC were observed at high concentrations. Therefore, it is possible that some of the LPC effects in vivo are not receptor mediated.

On the other hand, evidence has been accumulating to support the notion that, at low concentrations, LPC acts through membrane receptors: a) at relatively low concentrations (<10 μM), LPC exerts cell-specific effects; b) LPC increases intracellular Ca\(^{2+}\) concentration in association with production of inositolphosphates; and c) these actions of LPC are markedly inhibited by treatment of the cells with PTX and U73122 (36). Some LPC effects are believed to be mediated through the PAF receptor in various cell types, reflected by their partial sensitivity to PAF receptor antagonists (WEB-2170, WEB-2086, and CV-6209) (21,22,37,38). We have shown in the present study, however, that intracellular calcium increase and receptor internalization induced by LPC are dependent on the expression of GPR4 and are insensitive to the PAF receptor antagonists, BN52021, WEB-2071 and WEB-2086. These results clearly show that LPC does not activate these signaling pathways through PAF receptors. We have identified G2A as the first LPC receptor (14). The expression of G2A is restricted to lymphoid tissues (39), while GPR4 is more ubiquitously expressed (Fig. 1). This, together with the different affinities of these two receptors for LPC, may reflect distinct physiological functions for G2A and GPR4.

Physiological concentrations of LPC in body fluids, including blood and ascites, are very high (5-180 μM), when compared to other signaling lipid molecules, such as LPA, S1P and SPC (22, 36,40,41 and our unpublished results). All receptors would be saturated, down regulated, and/or desensitized at these concentrations of LPC if it were all in a form available to its receptors. However, different
concentrations of LPC present in various cellular and tissue systems (i.e. different LPC compartments) may regulate cellular functions differentially (23). LPC in plasma is present mainly in albumin- and lipoprotein-bound forms (22). These forms may be active in some non-receptor-mediated functions of LPC, such as delivery of fatty acids and choline (22), but may be in a form unavailable for receptor activation. It has been shown that some of the effects of LPC are decreased in the presence of albumin (42). Thus, the functionally available concentration of LPC in vivo, and the activation of LPC receptors may be controlled by the lower concentrations of free LPC. Although this issue remains to be further addressed, our results shown in Fig. 3G appear to support this notion. The presence of a 75-fold molar excess of BSA greatly diminished the ability of LPC to elicit an increase in [Ca\textsuperscript{2+}], through the GPR4 receptor. Perhaps physiologically relevant concentrations of LPC in vivo that pertain to LPC’s interactions with GPR4 will be better understood when estimates of unbound LPC concentrations in specific tissues can be reliably made. In vivo the molar ratio of albumin (approximately 3-5% in plasma) to LPC can theoretically be from 3- to 100-fold in plasma. In extravascular sites where albumin concentration is less than in plasma, the ratio of albumin to LPC can be lower.

TDAG8, which shares approximately 38% homology with OGR1 and GPR4, has recently been shown to be a Psy receptor (15). Treatment of cultured cells expressing this receptor with Psy or structurally related glycosphingolipids results in the formation of globoid, multinuclear cells (15). We have tested the effect of Psy and related glycosphingolipids in calcium mobilization, competition of ligand binding, and MAP kinase activation assays and found no evidence that these lipids interact with GPR4. The questions of whether Psy is also a ligand for GPR4 and whether TDAG8 is a lysophospholipid receptor require further investigation.

It appears that ligands of GPR4 induced cell shape changes (Fig. 5), suggesting that SPC and LPC may affect the cellular cytoskeleton. Both LPA and S1P are able to affect cytoskeleton through Rho
SPC and LPC are also able to activate Rho, as evidenced by C3-exoenzyme sensitivity of SRE reporter activity (Fig. 6) and cell migration (Fig. 9) induced by SPC/LPC. It remains to be determined whether the cell shape change induced by SPC/LPC is a Rho-mediated effect and which cellular proteins are involved in these processes.

Different cell lines (MCF10A, HEK293, CHO, and Swiss 3T3 cells) were used in our studies. As shown in Fig. 2, MCF10A cells expressed the lowest level of endogenous GPR4 among cell lines tested. This cell line does not respond to either SPC or LPC in calcium assays (2). Therefore, calcium assays described here were performed in these cells. Because the transfection efficiency of MCF10A cells is very low (2), we were unable to establish stably expressing lines for conducting other assays. Despite their relatively high level of GPR4 expression, HEK293 cells were chosen for the internalization and SRE reporter assays, mainly because they are human in origin, and also yielded a high transfection efficiency (Fig. 1). The internalization assays utilized transfected receptor-GFP fusion proteins and the transcriptional responses in SRE reporter assays were compared to those in parental or vector-transfected cells. Therefore, the effects of the exogenous GPR4 receptor were readily separable from those of the endogenous receptor(s). CHO cells were chosen for binding assays, because they exhibit low responses to SPC and LPC in calcium assays and are readily transfected. We detected SPC- and LPC-induced MAP kinase activation through GPR4 in Swiss 3T3, but not HEK293 and CHO cells (Fig. 7 and data not shown). Hence, Swiss 3T3 cells were chosen for MAP kinase activation and mitogenic studies. It is well known that receptor mediated signaling transduction is dependent on multiple cellular factors. The molecular basis for the differential activation of GPR4 in different cells remains to be further explored.

In summary, our results indicate that SPC is a high-affinity, and LPC a lower-affinity, ligand for GPR4. This conclusion is directly derived from the results of ligand binding assays (K_d values of 36 vs. 159 nM for SPC and 16:0-LPC, respectively). This is also supported by results from assays of different
signaling pathways activated by SPC and LPC, including increases in calcium, transcriptional activation of SRE, ERK activation, and stimulation of DNA synthesis and cell migration. In recent decades, many reports have described a significant elevation of LPC levels in cells and tissues in different diseases (32, 41, 45). Numerous lines of evidence suggest that LPC, which is a major lipid component of ox-LDL, and which accumulates in atherosclerotic lesions (11), plays pathological roles in the development of atherosclerosis and other chronic inflammatory diseases (11,12). LPC also plays other important biological roles. For example, LPC functions as a fatty acid and choline carrier and delivers fatty acids more specifically to brain than other tissues (22). The identification of GPR4 as a receptor for LPC and SPC solidifies the assignment of a new lysophospholipid receptor subfamily (OGR1, GPR4, and G2A). Further studies should address possible functional redundancy amongst these receptors and add important information to our understanding of inflammatory diseases.

Acknowledgement—We thank Dr. Bryan Williams and Dr. Guy Chisolm for their critical reading of this manuscript.

REFERENCES


FIGURE LEGENDS

Fig 1. **GPR4 expression in different human tissues.** The human RNA Master Blot (Clontech) was probed with $^{32}$P-labeled GPR4 (Experimental Procedures).

Fig. 2. **Expression of GPR4 in human cell lines.** Real-time Quantitative PCR was utilized to determine relative expression levels of GPR4 expressed in cells, as described in “Materials and Methods”. All PCR reactions were performed in triplicate. The comparative $C_T$ method was used to calculate the relative expression levels of GPR4 in different cell lines as described in Experimental procedures. HEY, OCC1, NIH:Ovca3, SKOV3, Ovca429, Ovca432, and Ovca433 are ovarian cancer cells. MCF7 is a breast cancer cell line. MCF10A is an immortalized breast cell line. HeLa is a cervical cell line. All cell lines shown, except Swiss 3T3, are human cell lines.

Fig. 3. **SPC- and LPC- induce transient increases in $[Ca^{2+}]_i$ in GPR4-transfected MCF10A cells.**

A, upper panel: the effect of SPC (1 µM), 16:0-LPC (1 µM), 18:1-LPA (1µM), 16:0-PAF (0.1 µM), and ATP (20 µM) on $[Ca^{2+}]_i$ in pEGFP-N1-transfected MCF10A cells. The 2nd to the 4th panels: MCF10A cells were transiently transfected with pGPR4-GFP, and treated with SPC, L- and D-SPC, LPC, LPA, PAF, or ATP. B, SPC and 16:0-LPC concentration response curves in pEGFP-GPR4-transfected MCF10A cells. C, The effect of BN52021 on increased $[Ca^{2+}]_i$ induced by agonists. D, The effect of WEB-2086 on increased $[Ca^{2+}]_i$ induced by agonists. E, Homologous and heterologous desensitization of GPR4 by SPC and 16:0-LPC. F, The sensitivity of SPC- and 16:0-LPC-induced calcium increases to PTX pretreatment. G, The effect of BSA (0.5%) on the ability of SPC and LPC to induce an increase in $[Ca^{2+}]_i$. SPC (1µM) and LPC(1µM) were incubated with 0.5% fatty acid-free BSA for 30 min at room
temperature and the mixtures were used to stimulate MCF10A cells transfected with pGPR4-GFP. H, Gal-Cer (1 µM)-stimulated increase in [Ca\(^{2+}\)]\(_i\) in parental and pGPR4-GFP expressing MCF10A cells. All calcium measurements were performed in EGTA-containing, calcium-free buffer. The data are representative of at least five independent experiments.

Fig. 4. Binding of SPC and 16:0-LPC to GPR4. A and B, Time dependence of specific [\(^3\)H] SPC and [\(^3\)H] LPC binding. Cell homogenates (100 µL, equivalent to 10\(^5\) cells) from vector- or GPR4 stably-transfected CHO cells were incubated with [\(^3\)H] SPC (1 nM) or [\(^3\)H] 16:0-LPC (1 nM) for the indicated times. Specific binding is shown. C and D, Saturation isotherm of specific binding of [\(^3\)H]SPC and [\(^3\)H]16:0-LPC to GPR4-transfected CHO cells. Cell homogenates (100 µL) were incubated with the indicated concentrations of [\(^3\)H] SPC or [\(^3\)H] 16:0-LPC in the presence or absence of unlabeled SPC (100-fold excess) or unlabeled 16:0-LPC (100-fold excess). Specific binding is presented. E and F, Structural specificity of binding of [\(^3\)H] SPC and [\(^3\)H]16:0-LPC to GPR4. GPR4-transfected CHO cells were incubated with [\(^3\)H] SPC (1 nM), or [\(^3\)H]16:0-LPC (1 nM) in the presence or absence of 100 nM of different unlabeled lipids. Total binding is presented. All binding experiments were performed in triplicate in 96-well plates. Data are means ± SD from three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001; as compared to the control (Student’s t test).

Fig. 5. Internalization of GPR4 induced by SPC and LPC. A, HEK293 cells stably expressing pGPR4-GFP. B, pGPR4-GFP stably expressing cells was treated with SPC (1 µM) at 37°C for 2h. C, pGPR4-GFP expressing cells were treated with 16:0-LPC (1 µM) at 37°C for 2 h. D and E, as in B, and C, except cells were pretreated with BN52021 (200 µM) for 5 min. F, pGPR4-GFP-expressing cells were
treated with PAF (1 µM). All experiments were repeated at least three times. Representative data are shown.

Fig. 6. **SPC and LPC activate SRE in a GPR4-dependent manner.** A, The SRE-luciferase responses to different lipids in vector- and GPR4-transfected HEK293 cells. 18:1-LPA, 16:0-LPC, SPC, S1P, 18:0-SM, 16:0-PAF, and 16:0-lyso-PAF (1 µM of each) were used. The experiments were conducted as described in Experimental Procedures and Methods. B, Concentration-dependent SRE-luciferase activity induced by SPC and 16:0-LPC in vector- and GPR4-transfected cells. C, Inhibition of SPC- and 16:0-LPC-induced SRE activity by PTX and C3 exoenzyme. All experiments were performed in quadruplicate and were repeated at least three times. Representative data are shown. Cont.: control; *, p < 0.05; **, p < 0.001; as compared to the control. #, p<0.001 when compared to SPC- or 16:0-LPC-induced activity in vector-transfected cells. The Student's t test was performed using the GraphPad Instat software (San Diego, CA). p < 0.05 was considered to be statistically significant.

Fig. 7. **Activation of ERK MAP kinase by SPC and LPC in GFP-, and GPR4-ires-GFP-expressing Swiss 3T3 cells.** ERK MAP kinase assays were performed as described in Experimental Procedures. A, Structural specificity of lipid-induced ERK activation via GPR4 in Swiss 3T3 cells. Cells were treated with 1 µM Psy, Gal-Sph, Lac-Cer), sphingosine-1-phosphate (S1P), 16:0-LPC, SPC and 16:0-PAF for 5 min. B, Concentration-dependence of ERK activation stimulated by SPC and 16:0-LPC. Cells were treated with 1, 10, 100 and 1000 nM of 16:0-LPC or SPC for 5 min. C, Time-dependence of ERK activation stimulated by SPC and 16:0-LPC. Cells were treated with SPC (100 nM) or LPC (100 nM) for the indicated time. D, GPR4-ires-GFP-, and OGR1-ires-GFP-Swiss 3T3 cells were untreated or treated with
16:0-LPC (100 nM) or SPC (100 nM) for 5 min in the absence or presence of PTX (100 ng/ml, 16 h pre-treatment.

Fig. 8. **DNA synthesis stimulated by SPC and 16:0-LPC in GPR4-overexpressing cells.** DNA synthesis was measured by [H] thymidine incorporation as described in Experimental Procedures in both GFP- and GPR4-ires-GFP-Swiss 3T3 cells. PTX was added to selected groups at 100 ng/ml for 16 h prior to lipid treatment. The data shown represent the means ± SD from three independent experiments.

Fig. 9. **SPC and LPC stimulate cell migration in GPR4-overexpressing Swiss 3T3 cells.** Cell migration was measured in a modified Boyden chamber assay as described in Experimental Procedures. The cell numbers on the lower faces of the membranes were determined and are presented as the means ± SD of three independent experiments. **, p < 0.01; ***, p < 0.001, compared to the control. Student's t test was performed using the GraphPad Instat software (San Diego, CA). p < 0.05 was considered to be statistically significant.
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GPR4 mRNA Abundance (relative to HEY cells)

- HEY
- OCC1
- NIH:Ovcar3
- SKOV-3
- Ovca429
- Ovca432
- Ovca433
- MCF-7
- MCF-10A
- HEK293
- HeLa
- Swiss 3T3
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Swiss 3T3 GFP

Swiss 3T3 GPR4 ires GFP

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- Total ERK1/2

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- pERK1/2
- Total ERK1/2

Swiss 3T3 GPR4 ires GFP

Swiss 3T3 OGR1 ires GFP
Sphingosylphosphorylcholine (SPC) and lysophosphatidylcholine (LPC) are ligands for GPR4
Kui Zhu, Linnea M. Baudhuin, Guiying Hong, Freager S. Williams, Kelly L. Cristina, Janusz H.S. Kabarowski, Owen N. Witte and Yan Xu

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