The G Protein-Coupled Receptor GPR4 Suppresses ERK Activation in a Ligand-Independent Manner†

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ABSTRACT: The lysophospholipids, lysophosphatidic acid, sphingosine-1-phosphate, and sphingosylphosphorylcholine (SPC), are bioactive lipid molecules that regulate diverse biological processes. Although the specific G protein-coupled receptors for lysophosphatidic acid and sphingosine-1-phosphate have been well-characterized, much less is known of the SPC receptors. It has been reported that ovarian cancer G protein-coupled receptor 1 (OGR1) is a high affinity receptor for SPC, and its closely related homologue GPR4 is a high affinity receptor for SPC with low affinity for lysophosphatidylcholine (LPC). However, in a functional assay to examine the specificity of ligand binding, we found that neither SPC nor LPC, or other related lysophospholipids, induced internalization of GPR4 from the plasma membrane. In agreement, these lysolipids also did not induce translocation of β-arrestin2-GFP from the cytosol to the plasma membrane in GPR4 expressing cells. However, when these cells were cotransfected with G protein-coupled receptor kinase 2, in the absence of added ligands, β-arrestin2-GFP accumulated in cytoplasmic vesicles, reminiscent of vesicular labeling usually observed after agonist stimulation of GPCRs. In addition, neither SPC nor LPC stimulated the binding of GTPγS to membranes prepared from GPR4 expressing cells and did not activate ERK1/2. Surprisingly, enforced expression of GPR4 inhibited activation of ERK1/2 induced by several stimuli, including SPC, sphingosine-1-phosphate, and even EGF. Collectively, our results suggest that SPC and LPC are not the ligands for GPR4 and that this receptor may constitutively inhibit ERK1/2 activation.

The lysophospholipids, lysophosphatidic acid (LPA),[1] sphingosine-1-phosphate (S1P), and sphingosylphosphorylcholine (lysosphingomyelin, SPC), are bioactive serum-borne lipid mediators that have recently emerged as modulators of important biological processes including cell growth, survival, differentiation, motility, tumorigenesis, and angiogenesis (*1*−*6*). Interest in these lipids has increased recently with the discovery that they are ligands for specific G protein-coupled receptors (GPCRs), although the evidence is much stronger for the existence of S1P and LPA receptors (*1*−*4*). S1P and LPA are the ligands of GPCRs previously known as the endothelial differentiation gene-1 (EDG-1) family and more recently renamed as S1P receptors (S1PRs) and LPA receptors. To date, five members of the S1PR family ($S1P_1$/EDG-1, $S1P_2$/EDG-5, $S1P_3$/EDG-3, $S1P_4$/EDG-6, and $S1P_5$/EDG-8) bind S1P and dihydro-S1P with high affinity and specificity, and there are three specific LPA receptors (LPA_1/EDG-2, LPA_2/EDG-4, LPA_3/EDG-7).[2]

SPC has been shown to be a very effective modulator of several important processes in the cardiovascular system, including calcium sensitization of coronary artery contraction (*7*) and regulation of heart beat rate (*5*). SPC also stimulates the growth of diverse types of cells (*8*−*10*), is upregulated in the stratum corneum of patients with atopic dermatitis (*11*), and is a potent wound healing agent (*12*). Other studies indicate that SPC acts as an extracellular signaling molecule

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[1] Abbreviations: DTT, dithiothreitol; EDG-1, endothelial differentiation gene-1; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal regulated kinase; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK2, G protein-coupled receptor kinase 2; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; OGR1, ovarian cancer G protein-coupled receptor 1; PMSF, phenylmethylsulfonyl fluoride; SHP-1, Src homology 2 domain-containing protein tyrosine phosphatase-1; S1P, sphingosine-1-phosphate; SPC, sphingosylphosphorylcholine; TDAG8, T cell death associated gene 8.

[2] The IUPHAR subcommittee on lysophospholipid receptor nomenclature has recommended that henceforth the colloquial Edg nomenclature be replaced with $S1P_{\text{subscript number}}$, where the number indicates the order of molecular cloning. Chun, J., Goetzl, E. J., Hla, T., Igarashi, Y., Lynch, K. R., Moolenaar, W., Pyne, S., and Tigyi, G. (2002) *International Union of Pharmacology, XXXIV, Lysophospholipid Receptor Nomenclature, Pharmacol. Rev. 54,* 265−269.

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(*13*, *14*). Although in some cells SPC can bind to S1PRs, albeit with much lower affinity than S1P (*15*−*17*), others have found that SPC does not bind to S1PRs (*18*, *19*). More recently, a new subfamily of orphan GPCRs was identified as receptors for SPC, LPC, and the glycolipid, psychosine (galactosylsphingosine) (*20*−*23*). Ovarian cancer G protein-coupled receptor 1 (OGR1), initially isolated from ovarian cancer cells but later shown to have a broader tissue distribution, is a high affinity receptor for SPC that transiently increases intracellular calcium, activates ERK1/2, and inhibits cellular proliferation (*20*).

Several other orphan receptors have high homology to OGR1. One of these, T cell death associated gene 8 (TDAG8), was originally cloned from T cells (*24*). Expression of TDAG8 is upregulated during apoptosis upon T cell receptor engagement, which is a necessary event to eliminate self-reactive immature thymocytes (*24*, *25*). Recently, psychosine was identified as a specific ligand for TDAG8, and it was proposed to be involved in globoid cell leukodystrophy, a disorder where psychosine accumulates in the brain due to the lack of the degradative enzyme, galactosylceramidase (*21*). Another closely related receptor, G2A, initially isolated as a gene whose expression was induced at the G2/M transition to delay mitosis in order for the cell to repair damaged DNA (*26*), was recently identified as the first receptor for LPC and may also be a low affinity receptor for SPC (*23*). G2A could have an important role in autoimmune disease because its deletion resulted in a phenotype similar to the human syndrome of systemic lupus erythematosus (*27*), and it also is potentially important in cancer because of its oncogenic properties (*28*, *29*).

Of all the lysolipid GPCRs identified to date, GPR4 has the highest sequence homology to OGR1, with 51% identity and 64% similarity (*30*, *31*). Recently, it was reported that the binding of SPC and LPC to GPR4 mediated increases in intracellular calcium concentration, SRE activation, receptor internalization, ERK activation, and stimulation of cell migration (*22*). GPR4 binds LPC, albeit with a lower affinity than SPC, and it was suggested that GPR4 mediates their biological functions (*22*). However, a recent study showed that G2A ligand independently stimulates accumulation of inositol phosphates and induces apoptosis (*32*). We have now reinvestigated the functional interactions of SPC and LPC with GPR4. We found that neither SPC nor LPC or other related lysolipids activated GPR4, as measured by receptor internalization, β-arrestin translocation, GTPγS binding, and ERK1/2 activation. Surprisingly, in the absence of ligand, the expression of GPR4 reduced the activation of ERK induced by GPCR and EGF receptor tyrosine kinase signaling.

EXPERIMENTAL PROCEDURES

Materials. S1P, sphingosine, and ceramide-1-phosphate were from Biomol (Plymouth Meeting, PA). Glucocerebrosides, galactocerebroside, L-α-lysophosphatidylinositol, DL-α-lysophosphatidylcholine, and DL-α-lysophosphatidylcholine-γ-*O*-hexadecyl were from Sigma (St. Louis, MO). All other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). Poly-D-lysine was from Boehringer Mannheim (Indianapolis, IN). EGF was purchased from Life Technologies (Gaithersburg, MD). [^{3}H]SPC (79 Ci/mmol) was obtained from Amersham Biosciences (Piscataway, NJ).

Cloning. A full-length GPR4 clone was obtained by PCR amplification from human lung reverse transcribed RNA (Clontech, Palo Alto, CA) using the 5′-AAGTGCCCAC-CATGGGCAACCACA and 5′-TTGTGCTGGCGGCAG-CATCTTCAG primers. GPR4 was subsequently cloned into a mammalian expression vector in frame with a V5 epitope using a mammalian TOPO cloning kit (Invitrogen, Gaithersburg, MD). HindIII and EcoR1 restriction sites were added by PCR, and pGPR4 was cloned into pEGFP-N2 (Clontech) to generate a GFP-tagged GPR4 construct fused in frame at the C-terminus. Insert sequences were confirmed by sequencing.

Cell Culture and Transfection. HEK 293 cells were cultured in DMEM (Biofluids) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. Cells were seeded in 6-well plates at 2×10^5 cells/well 1 day prior to transfection. DNA (5 μg) was complexed with calcium phosphate and added to cells for 12−18 h as described (*33*). Cells were then washed three times with phosphate buffered saline (PBS, Biofluids). In some experiments, cells were transfected using Lipofectamine Plus (Life Technologies) according to the manufacturer's instructions. The transfection efficiency was typically 70%. HEK 293 cells stably transfected with pGPR4 were selected with 1.3 mg/mL G418 in DMEM plus 10% FBS. CHO cells were cultured in F12 (Biofluids) supplemented with 10% FBS and 2 mM glutamine and transfected with Lipofectamine 2000 (Life Technologies).

Confocal Microscopy. HEK 293 cells were grown on poly-D-lysine coated coverslips at a density of 2×10^5 cells per well in a 6-well dish and transfected with vector alone, GPR4 with a C-terminal V5 epitope tag, or GPR4-GFP. Prior to internalization experiments, cells were serum starved for 24 h. Lipids were dried under nitrogen and added to the cells as BSA complexes (4 mg/mL) and incubated for 2 h at 37 °C. In some experiments, SPC and LPC were added without BSA. Green fluorescence was examined by confocal fluorescence microscopy. In other experiments, cells were washed with PBS and fixed in 3% paraformaldehyde/PBS containing 0.1% Triton X-100 for 8 min. Cells were then washed three times with PBS and blocked with PBS containing 5% goat serum for 1 h at room temperature. To visualize transfected cells, coverslips were incubated with anti-V5 antibody (1:500, Invitrogen) for 1.5 h in blocking buffer and after washing with PBS, stained with anti-mouse-conjugated Texas red as a secondary antibody (1:50, Molecular Probes, Eugene, OR). Coverslips were mounted on glass slides using an Antifade kit (Molecular Probes) and examined by confocal microscopy. Images were collected with an Olympus IX70 confocal laser scanning microscope equipped with a krypton/argon laser and a 60X oil immersion lens.

Visualization of β-Arrestin2-GFP Trafficking. HEK 293 cells were cotransfected with 2.5−5 μg of GPR4-pCDNA3 and 1.5 μg of β-arrestin2-pGFP (S65T) as previously described (*34*). Cells were plated on 35 mm plastic dishes containing a glass-bottomed well in DMEM buffered with 20 mM HEPES, pH 7.4. Cells were then washed three times with serum-free medium, treated with vehicle, SPC, LPC, or other lipids at 37 °C as described in the figure legends, and viewed on a Zeiss laser scanning confocal microscope. In some experiments, HEK 293 cells permanently expressing

GPR4 were cotransfected with β-arrestin2-GFP and G protein-coupled receptor kinase 2 (GRK2) tagged with a FLAG-epitope at its C-terminus and were used to evaluate the effects of increased expression of GRK2 on GPR4 internalization (*34*). Cells were cultured for several days either in medium containing 10% FBS or in Hanks Balanced Salt Solution containing 1 mg/mL glucose, 5 mM $CaCl_2$, and 2.5 mM $MgCl_2$.

ERK1/2 Activation. Cells were seeded in 60 mm plates and transfected the following day with GPR4 and HA-tagged ERK2 (at a 1:2 ratio). After 2 days, cells were treated with the indicated lipids and lysed by the addition of 0.5 mL of lysis buffer containing 25 mM HEPES (pH 7.4), 0.3 M NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM dithiothreitol (DTT), 20 mM β-glycerophosphate, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/mL leupeptin for 10 min on ice. Lysates were centrifuged for 10 min at 4 °C. Anti-HA (2 μg, Santa Cruz Biotechnology, Santa Cruz, CA) was then added to lysates (800 μg of protein) and incubated for 2 h at 4 °C with rocking. Protein A/G sepharose beads (20 μL, Santa Cruz Biotechnology) were added, and the incubation was continued for an additional hour. The beads were pelleted and washed three times in lysis buffer and twice in kinase buffer (12.5 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 0.5 mM DTT, 12.5 mM β-glycerophosphate, 0.5 mM NaF, 0.5 mM Na_3VO_4). The kinase assay was initiated by resuspending the beads in 50 μL of kinase buffer containing 50 μM ATP, 0.5 mg/mL MBP, and 5000 dpm/pmol [γ-^{32}P]-ATP and incubated 20 min at 30 °C. Reactions were stopped by the addition of 12 μL of 6X Laemmli sample buffer, and the samples were boiled 5 min. Proteins were separated on 12% SDS−PAGE and transferred to nitrocellulose. Nitrocellulose membranes were stained with Ponceau S (Sigma) to visualize protein bands and then exposed to the phosphoimager plate or film for autoradiography. In other experiments, after stimulation, cells were harvested in the following buffer: 50 mM HEPES (pH 7.4), 150 mM NaCl, 20 μM orthovanadate, 10 mM pyrophosphate, 100 mM NaF, 10 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 μg/mL pepstatin A, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. After centrifugation at 10,000*g* for 5 min at 4 °C, supernatant proteins were quantitated by a Bradford assay (Biorad Laboratories, Hercules, CA), and 40 μg of protein were separated by 10% SDS−PAGE. Proteins were transferred to a nitrocellulose membrane and probed with specific antibodies as follows: phospho-ERK1/2 antibodies (1:5000, New England Biolabs) and anti-ERK2 (1:10,000, Santa Cruz Biotechnology) as a loading control. Immunocomplexes were visualized by enhanced chemiluminescence (*35*).

[γ-^{35}S]GTP Binding. HEK 293 cells were cotransfected with GPR4 or $S1P_1$ mixed with DNA encoding human Gαi1 and bovine Gβ2 and Gγ1. After 48 h, cells were harvested, microsomes were prepared and aliquoted, and [γ-^{35}S]GTP binding was performed as described previously (*36*). Briefly, membranes (5 μg) were incubated in 0.1 mL of binding buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 5 μg of saponin, 10 μM GDP, 0.1 nM [γ-^{35}S]GTP (1200 Ci/mmol)) in the absence and presence of ligand. After 30 min at 30 °C, bound [γ-^{35}S]GTP was separated from free [γ-^{35}S]GTP by filtration through Whatman GF/C paper using a Brandel Cell Harvester (Gaithersburg, MD).

[^{3}H]SPC Binding. Binding was measured essentially as described (*22*). CHO cells transfected with empty vector or GPR4 were serum starved for 20 h and then homogenized with a Dounce homogenizer in binding buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 15 mM NaF, 0.2 mM PMSF, 1 mM $MgCl_2$, and 4 mg/mL fatty acid-free BSA (*20*). Cell homogenates from 10^5 cells were incubated for 2 h at 4 °C in a total volume of 200 μL of binding buffer with [^{3}H]SPC (4×10^6 dpm, 200 nM). Binding was measured with a Brandel Cell Harvester. Specific binding was calculated by subtracting the nonspecific binding (binding detected in the presence of 20 μM unlabeled SPC) from the total binding.

RESULTS

Receptor Internalization. GPR4 shares high sequence homology with OGR1, and both have been identified as specific SPC receptors (*20*, *22*). Similar to many other GPCR agonists, which induce the internalization of the receptor, it has been demonstrated that SPC or 16:0-LPC induces the internalization of GPR4-GFP overexpressed in HEK 293 cells (*22*). Thus, we used this system to investigate the mechanism whereby SPC regulates the internalization of GPR4. In GFP vector transfected cells, GFP fluorescence was primarily distributed in the cytosol, whereas in contrast, GPR4-GFP appeared to be expressed in two distinct cellular locations (Figure 1A). Fluorescence signals were localized on the plasma membrane, and a significant green fluorescence was also observed in a perinuclear distribution (Figure 1A). However, in contrast to a previous study (*22*), even after 2 h at 37 °C, treatment of cells with SPC did not induce a significant internalization of GPR4-GFP (Figure 1B). Moreover, treatment with LPC, another suggested ligand of GPR4, did not induce detectable receptor internalization (Figure 1C). We also examined the effects of other structurally related lipids, including ceramide, ceramide-1-phosphate, sphingosine, S1P, and psychosine. However, none of these lipids induced detectable GPR4-GFP internalization or a significant redistribution of GPR4-GFP into intracellular vesicles (Figure 1D−F). In contrast, receptor internalization was readily observed within 15 min after the addition of S1P to cells overexpressing $S1P_1$-GFP (Figure 1G,H), in agreement with previous results (*19*).

Although the GFP tagging of GPCRs has been extensively used to study receptor internalization, to exclude the possibility that the GFP protein might interfere with the processing or trafficking of GPR4 in response to its ligand, we also examined the internalization of GPR4 tagged with the small V5 epitope. As expected, immunostaining revealed that GPR4 was predominantly expressed on the plasma membrane of unstimulated cells (Figure 2B). However, even 2 h after stimulation with SPC (100 nM), most of the GPR4 was still on the plasma membrane, and no significant increase in internalization could be observed (Figure 2C). A much higher concentration of SPC (1 μM) also did not cause noticeable internalization (Figure 2D). Nonetheless, in agreement with a previous study (*20*), 1 μM SPC induced internalization of OGR1 (Figure 2E,F).

β-Arrestin Recruitment. β-Arrestins are adapter proteins that bind with high affinity to agonist-activated, phospho-

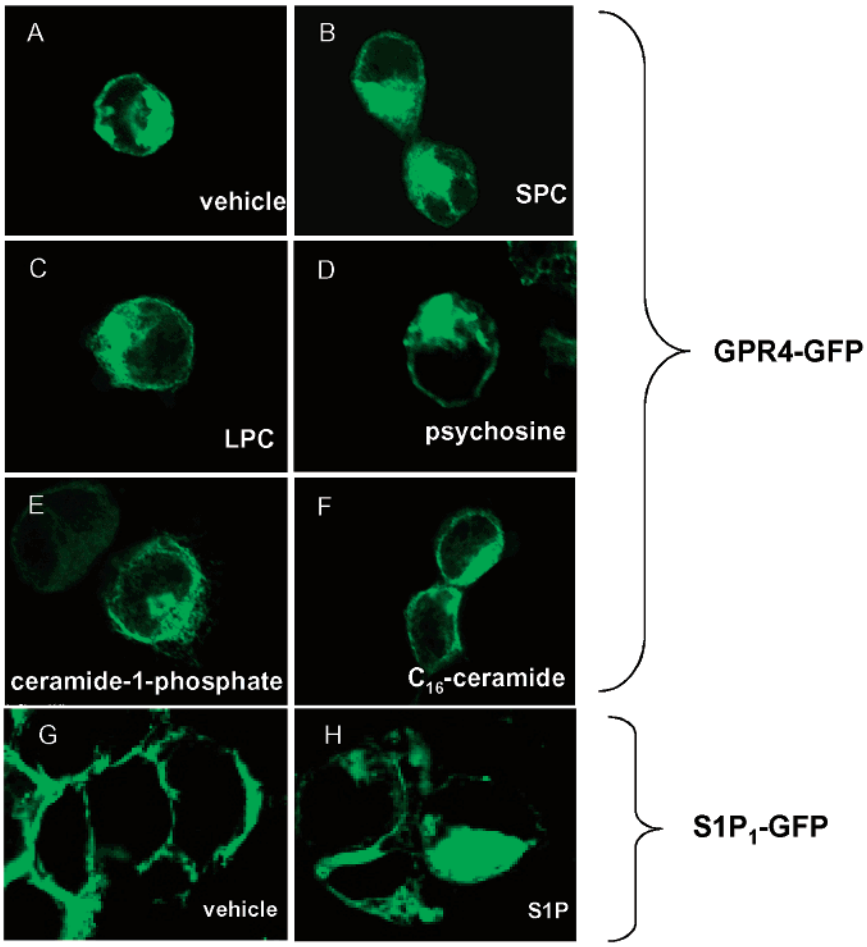


FIGURE 1: Effect of SPC and related lipids on internalization of GPR4-GFP. (A−F) HEK 293 cells were plated on poly-D-lysine coated coverslips and transfected with GPR4-GFP. After serum starvation for 24 h, cells were treated with the indicated lipid (1 μM) for 2 h, fixed, and visualized by confocal fluorescence microscopy. (G and H) S1P induces internalization of $S1P_1$-GFP. HEK 293 cells transfected with $S1P_1$-GFP were serum starved for 24 h and treated with vehicle (G) or with 100 nM S1P for 15 min (H) and visualized by confocal microscopy. Representative results from more than 50 cells examined.

rylated GPCRs to terminate receptor G protein coupling, clustering the receptors in clathrin coated pits from which they can be endocytosed (*37*, *38*) and initiating a second wave of signaling (*39*, *40*). Two β-arrestins have been identified outside the visual system, and their translocation to either agonist-activated or constitutively activated GPCRs appears to be a regulatory mechanism common to this class of receptors (*34*, *38*, *41*, *42*). β-arrestin2-GFP translocation to the plasma membrane can be used to identify ligands of orphan receptors (*43*). In agreement with our previous study of $S1P_1$ in HEK 293 cells (*44*), in the absence of stimulation, β-arrestin2-GFP was uniformly distributed in the cytoplasm with no evidence of plasma membrane accumulation or vesicular labeling (Figure 3A). This distribution was identical in cells stably expressing vector or GPR4. Importantly, SPC or LPC did not induce translocation of β-arrestin2-GFP to the plasma membrane, even at a concentration as high as 40 μM (Figure 3B−D). As these results suggest that SPC and LPC might not be ligands for GPR4 as previously suggested (*22*), we examined the effects of a number of other structurally related lipids. However, none of the lipids examined, including S1P, sphingosine, galactosylsphingosine (psychosine), ceramide, ceramide-1-phosphate, glucocerebroside, galactocerebroside, L-α-lysophosphatidylinositol, DL-α-lysophosphatidylcholine, or DL-α-lysophosphatidylcholine-γ-*O*-hexadecyl, had a significant effect on the redistribution of β-arrestin2-GFP (Figure 3 table). In sharp contrast, and in agreement with the identification of $S1P_2$ as a receptor for S1P, the addition of S1P rapidly induced the translocation of β-arrestin2-GFP from the cytosol to the plasma membrane in $S1P_2$ transfectants (Figure 3E,F).

GTPγS Binding. To examine further whether SPC or LPC are ligands for GPR4, we used the more sensitive and quantitative [γ-^{35}S]GTPS binding assay. Membranes from HEK 293 cells transfected with GPR4 together with human $G\alpha_{i1}$, cow β2, and cow γ1 exhibited very little [γ-^{35}S]GTPS binding that was not stimulated by SPC, LPC, LPA, S1P, or psychosine (Figure 4A). In contrast, cotransfection of these cells with DNAs encoding $S1P_1$ and the G proteins resulted in robust [γ-^{35}S]GTPS binding in response to S1P, while LPC, LPA, and psychosine, even at concentrations as high as 10 μM, did not stimulate binding (Figure 4B). SPC also stimulated [γ-^{35}S]GTPS binding to membranes from $S1P_1$ transfected cells, albeit with reduced potency and efficacy (Figure 4B). This is consistent with the finding that SPC is a partial agonist for $S1P_1$ (*15*, *16*) and indicates that if SPC were indeed a ligand for GPR4, this method could detect it.

[^{3}H]SPC Binding. In another attempt to confirm previous results showing that SPC binds to GPR4, we measured the binding of [^{3}H]SPC to CHO cells transfected with vector or GPR4 exactly as previously described (*22*). CHO cells, rather than HEK 293 cells, were used for GPR4 binding assays

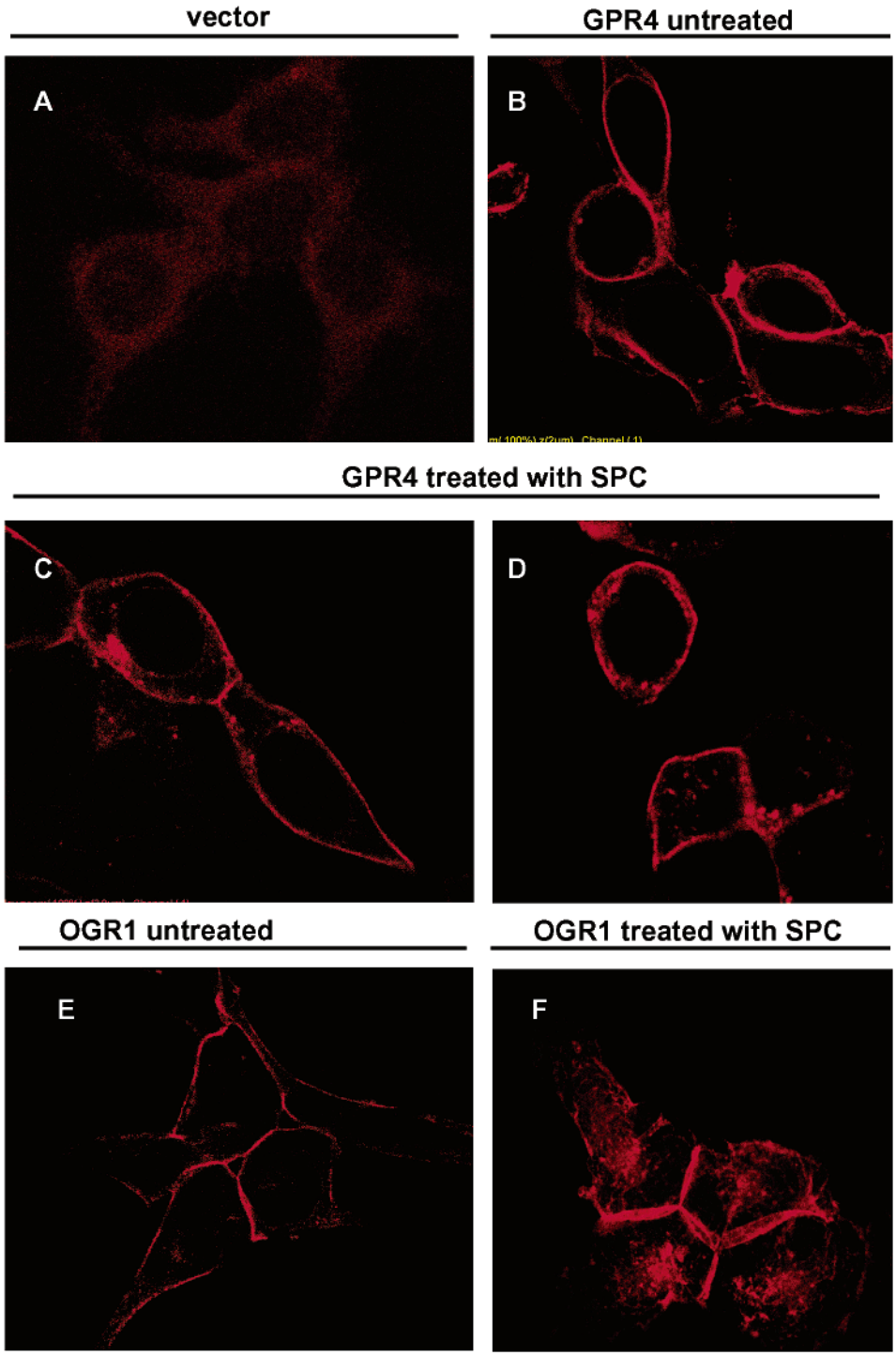
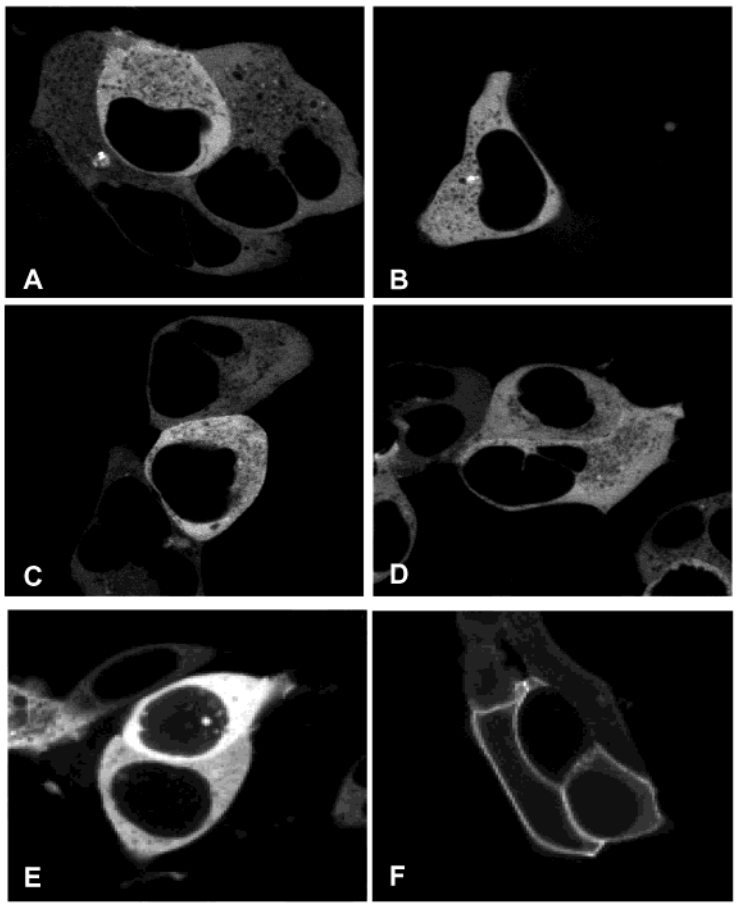


FIGURE 2: SPC does not induce internalization of GPR4. HEK 293 cells transfected with vector (A) or V5 epitope-tagged GPR4 (B−D) were cultured on poly-D-lysine coated coverslips. After 24 h in serum-free medium, cells were treated for 2 h with vehicle (B), 100 nM (C), or 1 μM SPC (D), fixed, stained with V5 antibody, and examined by confocal microscopy as described under Experimental Procedures. Representative results from more than 50 cells examined. For comparison, HEK 293 cells were transfected with V5 epitope-tagged OGR1 and treated for 2 h with vehicle (E) or 1 μM SPC (F).

because CHO cells that express relatively low levels of endogenous GPR4 were previously used for these assays (*22*). Although [^{3}H]SPC was specifically bound to cell homogenates from vector transfected CHO cells, no increase in specific binding was detected in cells overexpressing GPR4 (Figure 4C). This specific binding could be due to intracellular binding sites as whole homogenates were used for these assays. Moreover, when the binding of [^{3}H]SPC was measured by the method developed for determining the binding of S1P to its receptors expressed on intact cells (*17*, *18*), no specific binding of [^{3}H]SPC to either vector or GPR4 expressing cells was detected (data not shown). In contrast, there was more than a 10-fold increased specific binding of [^{3}H]S1P to $S1P_1$ expressing CHO cells as compared to vector cells.

Endogenous Ligand for GPR4? To examine the possibility that vanishingly small concentrations of endogenous ligands of GPR4 were present, we prepared stable cell lines coexpressing GPR4, β-arrestin2-GFP, and GRK2. Remarkably, in the absence of any agonist, this combination resulted



β-arrestin recruitment

Lipid	Receptor
	GPR4
S1P	-
sphingosine	-
ceramide-1-phosphate	-
SPC	-
Lyso-PI	-
Lyso-PC	-
gamma-O-hexadecyl-Lyso-PC	-
Lyso-PA	-
PA	-
galactocerebrosides	-
glucocerebrosides	-
psychosine	-
S1P	$S1P_1$ +
S1P	$S1P_3$ +

FIGURE 3: SPC does not induce β-arrestin2-GFP redistribution. HEK 293 cells were cotransfected with expression plasmids for β-arrestin2-GFP together with GPR4 (A−D) or $S1P_2$ (E and F). β-arrestin2-GFP fluorescence was visualized by confocal microscopy in cells treated with vehicle (A,E), 40 μM 3-*sn*-lysophosphatidylcholine-1-hexadecyl for 30 min (B), 40 μM 3-*sn*-lysophosphatidylcholine for 60 min (C), SPC for 30 min (D), or S1P for 60 min (F). As shown in the table, HEK 293 cells were cotransfected with expression plasmids for β-arrestin2-GFP together with GPR4, $S1P_1$, or $S1P_3$. β-Arrestin2-GFP fluorescence was visualized by confocal microscopy. The indicated lipids were tested for their ability to recruit β-arrestin to the plasma membrane.

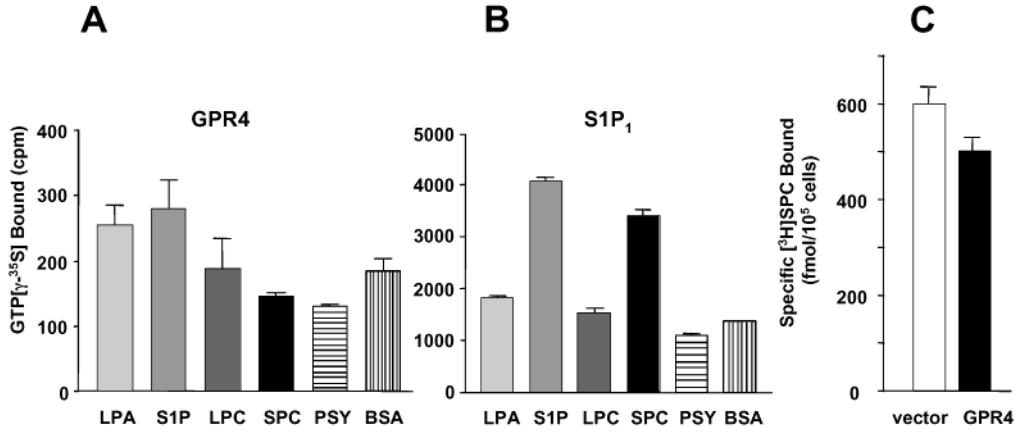


FIGURE 4: [γ-^{35}S]GTP binding to HEK 293T cell membranes in response to lysophospholipids. Membranes prepared from HEK 293 cells expressing (A) GPR4 or (B) $S1P_1$ and G proteins were incubated with 0.1 nM [γ-^{35}S]GTP and 10 μM GDP, in the presence of vehicle (0.1% BSA) or the indicated lysolipids (10 μM), and bound GTPγS was determined. Data shown represent the means ±SE. (C) Binding of [^{3}H]SPC. Cell homogenates (equivalent to 10^5 cells) from vector or GPR4 transfected CHO cells were incubated with [^{3}H]SPC (200 nM) in the absence or presence of 20 μM unlabeled SPC, and specific binding was determined.

in the accumulation of β-arrestin2-GFP in cytoplasmic vesicles (Figure 5B) and at the plasma membrane after prolonged incubation (Figure 5C). This labeling by β-arrestin2-GFP is similar to that observed with other GPCRs, such as the substance P and vasopressin receptors, in the presence of their respective agonists, and for a constitutively desensitized vasopressin receptor mutant in the absence of agonist (*41*, *45*). Because these cells were cultured in serum, it was possible that a ligand of GPR4 was present in serum, which has been shown to contain large amounts of SPC, S1P, and other lysophospholipids (*5*, *46*), and contributed to the translocation of β-arrestin2-GFP. To examine this possibility, the cells were extensively washed in phosphate buffered saline to remove media and serum and then incubated for 3

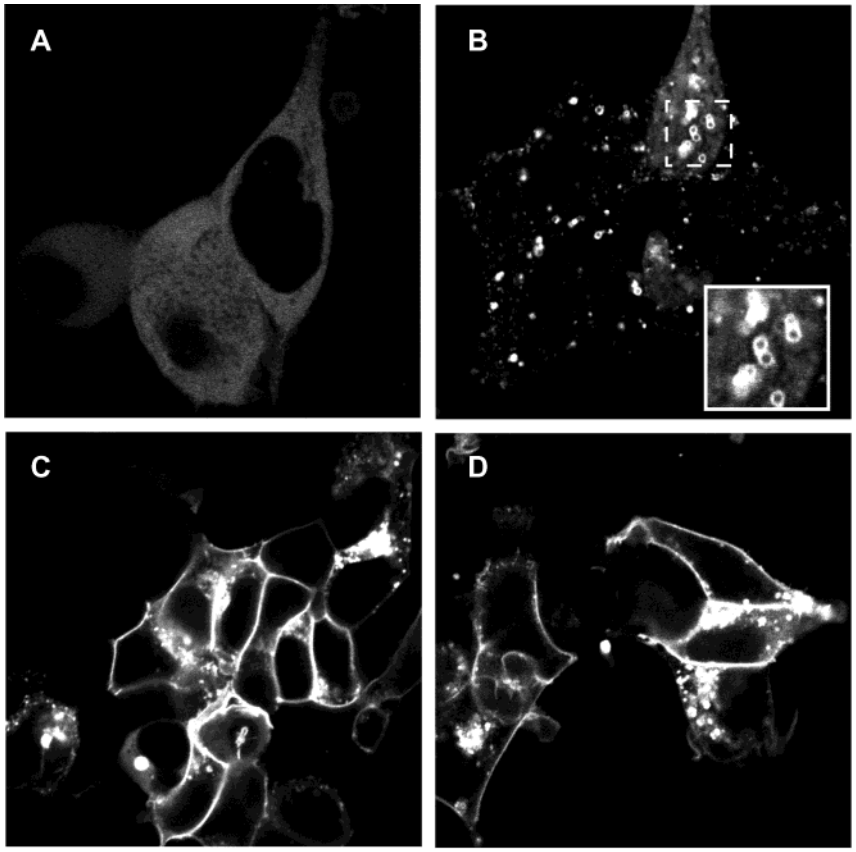


FIGURE 5: GRK2 induces β-arrestin2 translocation in HEK 293 cells expressing GPR4. HEK 293 cells stably expressing GPR4 were transfected with β-arrestin2-GFP without (A) or with GRK2 (B). The cells were then grown in minimal essential medium with 10% fetal bovine serum for an additional 3 days (C) or were extensively washed in phosphate buffered saline to remove media and serum and then incubated for 3 days in Hanks Balanced Salt Solution containing 1 mg/1 mL glucose, 5 mM $CaCl_2$, and 2.5 mM $MgCl_2$ (D).

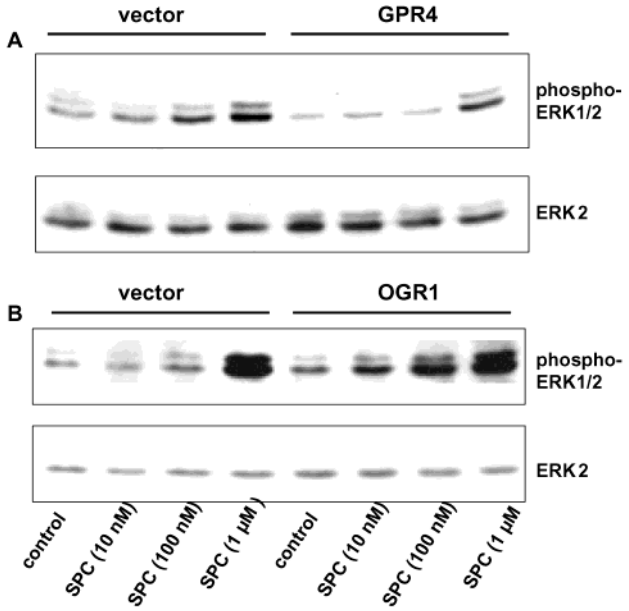


FIGURE 6: Overexpression of GPR4 reduces SPC-induced ERK1/2 activation. HEK 293 cells transfected with vector, GPR4 (A), or OGR1 (B) were serum-starved for 24 h. After treatment with the indicated concentrations of SPC for 10 min, cells were lysed, and proteins (40 μg) were separated by 10% SDS−PAGE, transferred to nitrocellulose, and probed with phospho-specific ERK1/2 antibody. For loading controls, blots were stripped and reprobed with ERK2 antibody.

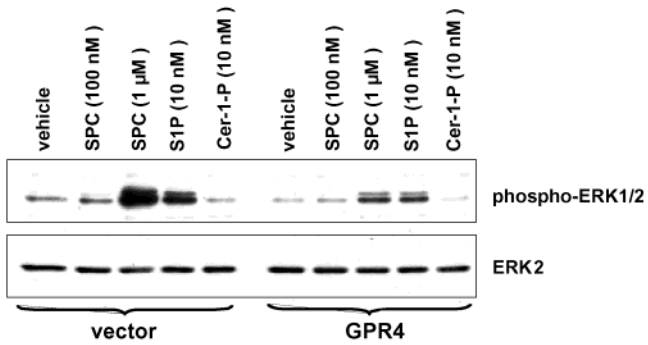


FIGURE 7: Effect of various lipids on ERK1/2 activation in GPR4 expressing cells. HEK 293 cells stably expressing GPR4 or vector were treated with 100 nM or 1 μM SPC, 10 nM S1P, or 10 nM ceramide-1-phosphate for 10 min, and ERK1/2 activation was determined with a phospho-specific ERK antibody. Blots were stripped and reprobed with the ERK2 antibody as a loading control.

days in chemically defined medium containing glucose. However, in both serum and serum-free conditions, there was a similar translocation of β-arrestin2-GFP from the cytosol and accumulation mainly at the plasma membrane and in intracellular vesicles (Figure 5C,D). To exclude the possibility that β-arrestin2 recruitment to the plasma membrane was caused by endogenous GPR4 (*22*) or by other GPCRs, control experiments were carried out where only β-arrestin2 and GRK2 were coexpressed in HEK 293 cells. We found that the presence of excess GRK2 alone was not sufficient to cause β-arrestin2 translocation or internalization, suggesting that the expression of GPR4 was responsible for this translocation.

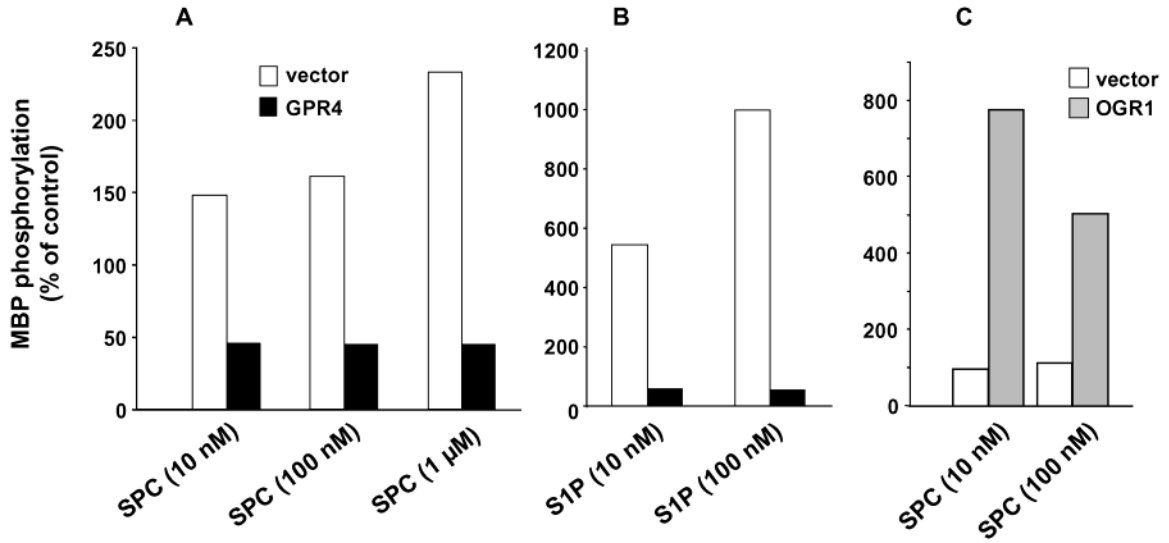


FIGURE 8: Expression of GPR4 markedly reduces SPC- and S1P-induced ERK activation. HEK 293 cells were transiently cotransfected with HA-tagged ERK2 and empty vector (open bars) or GPR4 (A, B) or OGR1 (C). Cells were treated with vehicle, SPC (A and C), or S1P (B) at the indicated concentrations for 10 min. HA-ERK2 was immunoprecipitated from whole cell lysates and assayed for kinase activity using myelin basic protein (MBP) as the substrate as described in the Experimental Procedures. Results are typical of two independent experiments. ^{32}P incorporation into MBP was detected and quantitated by a phosphoimager. Data are expressed as a percent of untreated control.

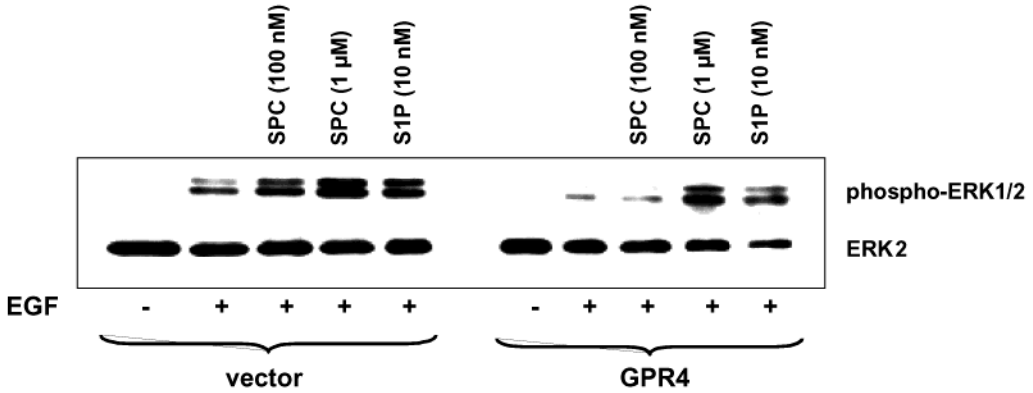


FIGURE 9: Expression of GPR4 inhibits EGF-induced ERK1/2 activation. HEK 293 cells stably expressing GPR4 or vector were first stimulated without (−) or with (+) 100 nM EGF for 10 min and then treated without or with the indicated concentrations of SPC or S1P for an additional 10 min. Cells were lysed, and proteins were separated on 10% SDS−PAGE, transferred to nitrocellulose, and probed with phospho-specific ERK1/2 antibody. For loading controls, blots were stripped and reprobed with the ERK2 antibody.

GPR4 Suppresses Activation of ERK1/2. Because β-arrestins also contribute to GPCR signaling by acting as scaffolds for components of the ERK mitogen-activated protein kinase cascade (*47*, *48*), we next examined the role of GPR4 in ERK1/2 activation. In contrast to previous studies indicating that SPC does not activate ERK in HEK 293 cells (*20*), we found that SPC induced a marked phosphorylation of ERK1/2 in parental (data not shown) or vector HEK 293 cells in a dose-dependent manner (Figure 6A). A significant effect was observed at a concentration as low as 100 nM, and robust activation was noted at 1 μM SPC (Figure 6A). Surprisingly, expression of GPR4 suppressed, rather than enhanced, SPC-induced activation of ERK1/2. In contrast, overexpression of OGR1, the closely related GPR4 homologue, enhanced SPC induced activation of ERK1/2 (Figure 6B) and shifted the dose response to the left. These results support the notion that SPC is a ligand for OGR1 (*20*).

It was of interest to determine whether the inhibitory effect of ectopic expression of GPR4 on SPC-induced ERK1/2 activation was specific for this lipid. While ceramide-1-phosphate, psychosine, or LPC had no effects on ERK1/2 phosphorylation in these cells (Figure 7, data not shown), S1P potently stimulated ERK in agreement with previous reports (*17*, *18*, *49*). Overexpression of GPR4 also markedly reduced S1P-stimulated phosphorylation of ERK1/2 (Figure 7).

These experiments were carried out with pooled clones of cells stably transfected with GPR4. To avoid potential problems associated with clonal selection and to further substantiate these unexpected findings, HEK 293 cells were transiently cotransfected with GPR4 and HA-tagged ERK2, and ERK activation was examined by an in vitro kinase assay. Both S1P and SPC markedly enhanced myelin basic protein phosphorylation in anti-HA-ERK2 immunoprecipitates from vector transfected HEK 293 cells (Figure 8). Notably, GPR4 transfection drastically repressed SPC- and S1P-stimulated activation of ERK (Figure 8A,B). However, overexpression of OGR1 enhanced SPC induced activation of ERK1/2 (Figure 8C).

As GPCRs can transactivate the EGF receptor leading to ERK signaling (*48*, *50*, *51*), we next examined whether ERK1/2 activation by EGF was inhibited by GPR4 and whether this could be due to the binding of SPC to GPR4.

In agreement with previous studies (*49*, *52*), EGF stimulated phosphorylation of ERK1/2 in HEK 293 cells. Overexpression of GPR4 reduced this activation (Figure 9). Moreover, treatment with SPC did not suppress EGF-induced activation of ERK in vector cells but rather acted additively with EGF (Figure 9). Likewise, S1P also enhanced EGF-induced phosphorylation of ERK1/2. Once more, GPR4 overexpression also decreased EGF-induced ERK1/2 phosphorylation, in the absence or presence of SPC or S1P (Figure 9).

DISCUSSION

If SPC or LPC are indeed agonists for GPR4, then their addition should facilitate phosphorylation of the receptor, β-arrestin binding to the phosphorylated receptor, and internalization. However, our data demonstrate that SPC and LPC do not induce GPR4 internalization, β-arrestin translocation to the plasma membrane, or enhance GTPγS binding in cells overexpressing GPR4. In addition, we were unable to detect increased specific binding of SPC to cells overexpressing GPR4. Taken together, these results suggest that SPC and LPC are not ligands for GPR4. It is not clear why these results differ from the report of Zhu et al. showing that SPC and LPC are ligands for GPR4 (*22*). However, it is possible that in the earlier studies, SPC signaled through a pathway other than that mediated by GPR4. Moreover, the previous conclusions were mainly based on the responses of cells expressing GPR4 fused to GFP (*22*), which might have influenced the processing or trafficking/translocation of GPR4 to induce sensitivity to SPC and LPC. Several other studies also indicate that SPC might not function through GPR4. For example, another orphan GPCR, GPR12, that has no homology with GPR4, has high stereospecific affinity for natural SPC and does not bind LPC or LPA (*53*). Moreover, embryonal cerebral cortical neurons and the hippocampal cell line HT22, which both express GPR12, but not GPR4, responded to SPC with an increase in synaptic contacts and proliferation, respectively (*53*). A further complication is that SPC has recently been shown to induce contraction of the middle cerebral artery by calcium sensitization that was GTP independent, excluding the requirement for activation of GPCRs and suggesting an intracellular action of SPC (*54*). Taken together with our results, this suggests that SPC and LPC are not ligands for GPR4, and it should be reorphaned. Interestingly, a similar controversy surfaced with another closely related GPCR, G2A, which has been proposed to be a receptor for LPC with a low affinity for SPC (*23*). However, there is evidence that G2A couples functionally and SPC-independently to $G\alpha_{13}$ (*29*, *32*, *55*).

Our data with translocation of β-arrestin in cells expressing GRK2 suggests that these cells produce an unidentified endogenous ligand that stimulates GPR4 in an autocrine or paracrine fashion or that it might be partially constitutively activated. Transient overexpression of GPR4 could be sufficient to increase the number of conformationally active (R*) forms of GPR4 even in the absence of agonist (*56*), which then in the presence of overexpressed GRK2 will lead to the internalization of β-arrestin-GPR4 complexes. In addition, an endogenous ligand could induce stabilization of a constitutively active but inherently unstable protein, as was previously described for a mutated constitutively active form of the β2-adrenergic receptor (*57*).

Mutation in a GPCR resulting in G protein activation in the absence of an agonist was described more than a decade ago (*56*). Constitutive activity mostly arises from mutations clustered in the cytoplasmic extensions of TMs 3 and 6 as well as within TMs 2, 3, 6, and 7. Similarly, mutations in the junction region between the i3 loop to the TM6 XBBXXB motif (B, basic amino acid; X, nonbasic amino acid) or its variants within this region have been shown to lead to constitutive activation of many GPCRs (*58*). This region has been suggested to be involved in constraining GPCRs in inactive conformations, and mutations abolishing the constraints result in constitutive activation of the receptors. Thus, the T6.34(279)K mutation of the μ opioid receptor results in ligand independent activation (*58*). Modeling based on the crystallographic analysis of rhodopsin (*59*) substantiates that conformational changes produced by mutations at the T6.34 locus will affect interaction between the cytoplasmic ends of TM3 and TM6 that involves the highly conserved arginine at position 3.50 and possibly arginine at position 6.35. Interestingly, GPR4 has a motif rich in basic amino acids (KAPIKR) at the junction of the third intracellular loop and TM6. Of note, GPR3, another orphan receptor originally named human adenylate cyclase constitutive activator since it conferred constitutive signaling via $G\alpha_s$ (*60*), also has a similar basic motif that loosens interactions proposed to keep the receptor in an inactive state (*58*, *59*).

The majority of GPCRs studied to date stimulate ERK1/2 via diverse signaling pathways (*48*, *50*, *51*, *61*−*63*). The finding that β-arrestins bind to ERK has provided yet another mechanism of GPCR-mediated ERK regulation (*47*). The ability of GPR4 to repress ERK1/2 activity, while unique, is not unprecedented as it has been demonstrated that angiotensin II type 2 receptor (*64*, *65*) and $S1P_5$ (*66*) can also link to inhibition of ERK activation. A recent study suggests that the inhibition of ERK activity through the $S1P_5$ receptor is constitutive and insensitive to the ligand, S1P (*67*). Intriguingly, S1P is also a ligand that is formed by cells and could induce constitutive activation of its S1P receptors, especially when overexpressed. Depending on the cell type, several pathways have been implicated in this inhibition of ERK1/2 activity by GPCRs, including Src homology 2 domain-containing protein tyrosine phosphatase-1 (SHP-1) (*65*), serine-threonine protein phosphatase PP2A (*64*), and MAPK phosphatase-1 (*68*). It has also been shown that AT_2 inhibits ERK activity by inhibiting transactivation of the EGFR due to increased association with SHP-1 (*69*). This effect may be relevant as GPR4 inhibited EGF-induced ERK activation. Recent studies suggest that the stability of the interaction between GPCR and β-arrestin determines the mechanism and functional consequence of ERK activation (*70*). The C-terminal tail of GPR4 may control the extent of β-arrestin-bound ERK activation and influences both the subcellular localization of activated ERK and the physiologic consequences of ERK activation. Because ERK1/2 plays an important role in growth and suppression of apoptosis downstream of GPCRs, it is possible that the ability of GPR4 to inhibit ERK in response to diverse stimuli might be related to its biological function.

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