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Phytosphingosine-1-phosphate stimulates chemotactic migration of L2071 mouse fibroblasts *via* pertussis toxin-sensitive G-proteins

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Abbreviations: BAPTA/AM, 1,2-bis (Aminophenoxy) ethane-N,N,N',N'-tetraacetoxymethyl ester; $[Ca^{2+}]_i$, intracellular calcium concentration; GPCRs, G-protein coupled receptors; IP3, inositol-1,4,5-trisphosphate; LPA, lysophosphatidic acid; PD98059, 2'-amino-3'-methoxyflavone; PhS1P, phytosphingosine-1-phosphate; PI3K, phosphatidylinositol-3-kinase; PTX, pertussis toxin; LY294002, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; S1P, sphingosine-1-phosphate; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; U-73122, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino) hexyl]-1H-pyrrole-2,5-dione; U-73343, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione

Abstract

Phytosphingosine-1-phosphate (PhS1P) was found to stimulate an intracellular calcium increase via phospholipase C but not pertussis toxin (PTX)-sensitive G-proteins in L2071 mouse fibroblasts. PhS1P also activated ERK and p38 kinase, and these activations by PhS1P were inhibited by PTX. Moreover, PhS1P stimulated the chemotactic migration of L2071 cells via PTX-sensitive G_i protein(s). In addition, the PhS1P-induced chemotactic migration of L2071 cells was also dramatically inhibited by LY294002 and SB203580 (inhibitors of phosphoinositide 3-kinase and p38 kinase, respectively). L2071 cells are known to express four S1P receptors, i.e., $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$, and pretreatment with an $S1P_1$ and $S1P_3$ antagonist (VPC 23019) did not affect on PhS1P-induced chemotaxis. This study demonstrates that PhS1P stimulates at least two different signaling cascades, one is a PTX-insensitive but phospholipase C dependent intracellular calcium increase, and the other is a PTX-sensitive chemotactic migration mediated by phosphoinositide 3-kinase and p38 kinase.

Keywords: calcium signaling; chemotaxis; fibroblasts; GTP-binding proteins; pertussis toxin; phytosphingosine-1-phosphate

Introduction

Many reports have demonstrated the involvement of lipid factors in cellular responses. In particular, sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are important lipid mediators, which modulate a wide range of physiological activities (van Corven *et al.,* 1989; English *et al*., 1999; Wang *et al*., 1999; Fishman *et al.,* 2001; Cummings *et al.,* 2002; Idzko *et al.,* 2002; Kim *et al.,* 2004). For example, S1P has been reported to induce chemotactic migration and angiogenesis in human umbilical vein endothelial cells (English *et al.,* 1999; Wang *et al.,* 1999), and to induce cellular chemotaxis and modulate cytokine release in mature human dendritic cells during Th2 immune responses (Idzko *et al.,* 2002) and in human bronchial epithelial cells (Cummings *et al.,* 2002). Moreover, LPA has been reported to regulate the cellular migration of ovarian cancer cells (Fishman *et al.,* 2001), and to regulate the proliferation of several cell types, including fibroblasts and amniotic cells (van Corven *et al.,* 1989; Kim *et al.,* 2004). Although several previous reports have demonstrated the pivotal roles of S1P and LPA in the modulation of several biological responses via their specific receptors, the role of other lipid mediators should clearly be considered. Phytosphingosine-1-phosphate (PhS1P) can be generated by the phosphorylation of phytosphingosine, and is one of the most widely distributed natural sphingoid bases which is found abundantly in fungi and plants, but which is also found in animals including man (Shurer *et al.,* 1991; Dickson, 1998). Moreover, PhS1P is structurally similar to S1P, i.e., PhS1P has a hydroxyl group at C-4 of the sphingoid long-chain base, whereas S1P has a *trans*-double bond be-

tween C-4 and C-5. Although PhS1P is present in animals as a minor component of cell membranes, the physiological role of PhS1P in the modulation of cellular activities has not been previously studied.

In terms of S1P cell surface receptors, a family of G-protein coupled receptors (GPCRs) has been shown to contain specific S1P sites (An *et al.,* 1997, 2000; Okamoto *et al.,* 1998; Yamazaki *et al.,* 2000). This family includes $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ (An *et al.,* 1997, 2000; Okamoto *et al.,* 1998; Yamazaki *et al.,* 2000), and previous reports have demonstrated that PhS1P also acts on cell surface bound GPCRs, $S1P_4$ and $S1P_1$ (Candelore *et al.,* 2002; Inagaki *et al.,* 2005). Moreover, even though PhS1P has been known to bind $S1P_4$ or $S1P_1$, its effect on cellular functioning has not been previously investigated. In this study, we investigated PhS1P-induced cell migration and the signaling pathways involved in L2071 mouse fibroblasts. We found that two separate signaling pathways are involved together with pertussis toxin (PTX)-sensitive trimeric G proteins.

Materials and Methods

Reagents and cell culture

PhS1P, S1P, and VPC 23019 were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The reverse transcription-PCR kit was purchased from Invitrogen Corporation (Carlsbad, CA), and FBS from Hyclone (Logan, UT). Enhanced chemiluminescence reagents were from Amersham Biosciences (Piscataway, NJ), and phospho-ERK1/2, phospho-p38 kinase, and ERK2 antibodies from New England Biolabs (Beverly, MA). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), 1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U-73122), 1-[6-((17β-3-Methoxyestra-1,3, 5(10)-trien-17-yl)amino) hexyl]-2,5-pyrrolidinedione (U-73343), 1,2-bis (Aminophenoxy) ethane-N,N,N', N'-tetraacetoxymethyl ester (BAPTA/AM), 2'-Amino-3'-methoxyflavone (PD98059), and 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole (SB203580) were obtained from Calbiochem (San Diego, CA) and were dissolved in DMSO before being added to cell culture. The final concentrations of DMSO in culture were 0.1% or less. L2071 mouse fibroblasts were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium with 10% FBS, 1% sodium bicarbonate buffer.

Ca^{2+} measurement

Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by Grynkiewicz's method using fura-2/AM (Grynkiewicz *et al.,* 1985). Briefly, prepared cells were incubated with 3 μM fura-2/AM at 37°C for 50 min in fresh serum free RPMI 1640 medium with continuous stirring. 2×10^6 cells were aliquoted for each assay into Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES, pH 7.3, 10 mM glucose, 2.2 mM $CaCl_2$, and 0.2 mM EGTA). Fluorescence was measured at 500 nm at excitation wavelengths of 340 nm and 380 nm.

Stimulation of cells with PhS1P for Western blot analysis

Cultured cells (2×10^6) were stimulated with the indicated concentrations of PhS1P for the predetermined lengths of time. After stimulation, the cells were washed with serum free RPMI 1640 medium and lysed in lysis buffer (20 mM Hepes, pH 7.2, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Detergent insoluble materials were pelleted by centrifugation (12,000 × *g*, 15 min, at 4°C), and the soluble supernatant fraction was removed and stored at either -80°C or used immediately. Protein concentrations in the lysates were determined using Bradford protein assay reagent.

Electrophoresis and immunoblot analysis

Protein samples were prepared for electrophoresis then separated using a 10% SDS-polyacrylamide gel and the buffer system described previously (Bae *et al.,* 2003). Following the electrophoresis, the proteins were blotted onto nitrocellulose membrane, which was blocked by incubating with TBST (Tris-buffered saline, 0.05% Tween-20) containing 5% non-fat dried milk. The membranes were then incubated with anti-phospho-ERK antibody, anti-phospho-p38 kinase antibody or anti-ERK antibody and washed with TBST. Antigen-antibody complexes were visualized after incubating the membrane with 1:5,000 diluted goat anti-rabbit IgG or goat anti-mouse IgG antibody coupled to horseradish peroxidase using the enhanced chemiluminescence detection system.

Chemotaxis assay

Chemotaxis assays were performed using multiwell chambers (Neuroprobe Inc., Gaithersburg, MD) as described previously (Bae *et al.,* 2003). Briefly, polycarbonate filters (8 μm pore size) were precoated with 20 μg/ml of fibronectin in HEPES-buffered RPMI 1640 medium. A dry coated filter was placed on a 96-well chamber containing different concentrations of PhS1P. L2071 cells were suspended in

RPMI 1640 medium at a concentration of 1×10^6 cells/ml, and 25 μl of the cell suspension were placed onto the upper well of the chamber. After incubation for 4 h at 37°C, non-migrating cells were removed by scraping, and cells that migrated across the filter were dehydrated, fixed, and stained with hematoxylin (Sigma, St. Louis, MO). The stained cells in three randomly chosen high power field (400×) were then counted for each well.

Reverse transcription-PCR analysis

mRNA was isolated by using a QIAshredder and an RNeasy kit (Qiagen, Hilden, Germany). mRNA, M-MLV reverse transcriptase, and $pd(N)_6$ primers (Invitrogen Corporation, Carlsbad, CA) were used to obtain cDNA. The sequences of the primer used were as follows; $S1P_1$ receptor (277 bp product): forward, 5'-ACCTTTGGCACTTTTATTGA-3'; reverse, 5'-AATACCTAGTGACAGCCGAA-3'. $S1P_2$ receptor (298 bp product): forward, 5'- GTTAACCAAGCCACAGAGAG-3'; reverse, 5'-GTAGAGAGGCCCTAAAAAGG-3'. $S1P_3$ receptor (245 bp product): forward, 5'-AAACCAGTGACACCAGAGAC-3'; reverse, 5'-GATTGGGCATCAAATGTAGT-3'. $S1P_4$ receptor (192 bp product); forward, 5'-ATACAGTTGGAACAGTTGGG-3'; reverse, 5'-GCAACTGTGGGTATGACTCT-3'. We ran 30 PCR cycles at 94°C (denaturation, 1 min), 62°C (annealing, 1 min), and 72°C (extension, 1 min). PCR products were electrophoresed on

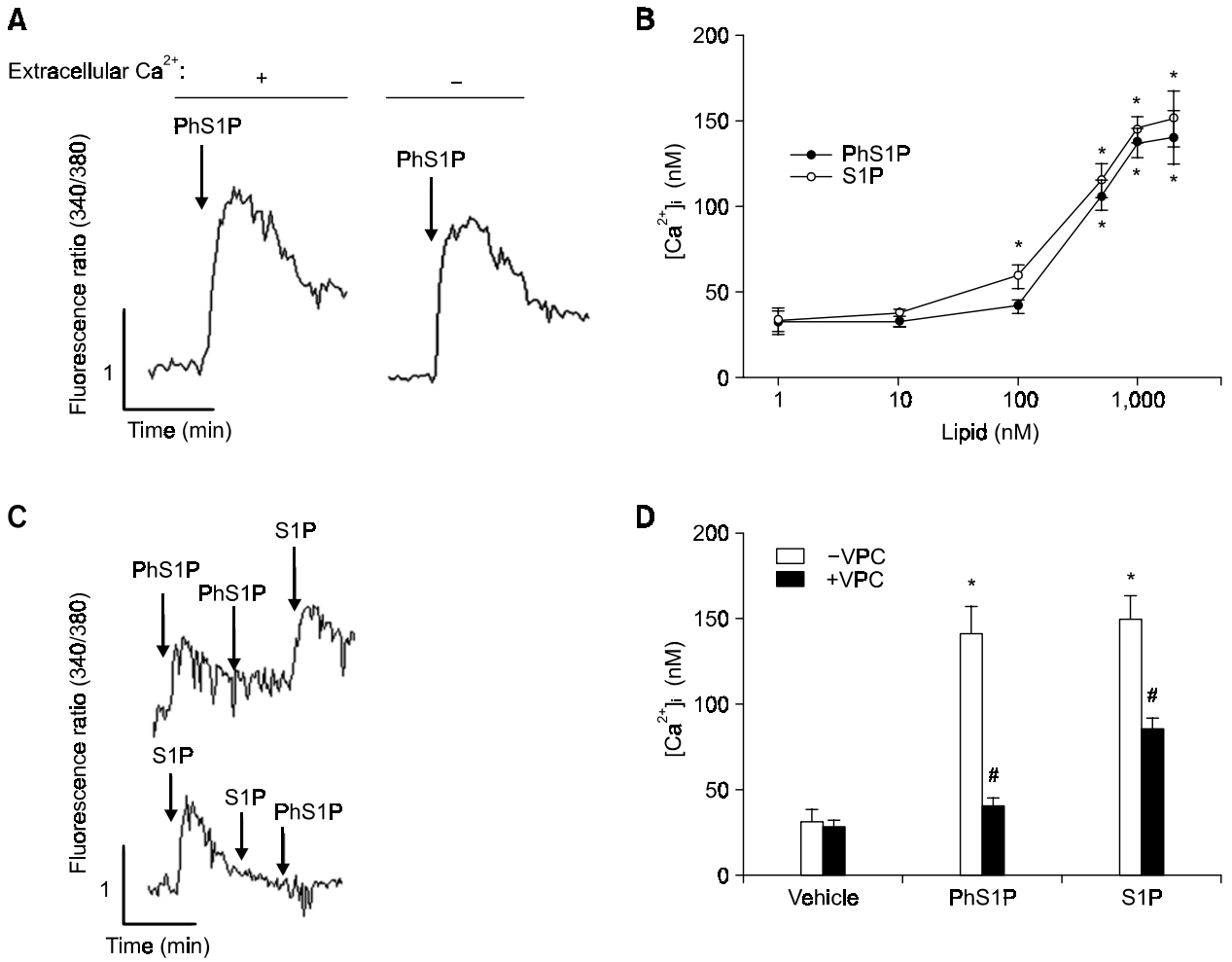

Figure 1. Effect of PhS1P on $[Ca^{2+}]_i$ in L2071 cells. L2071 cells were stimulated with 2 μM PhS1P in the absence or presence of extracellular calcium, and peak $[Ca^{2+}]_i$ levels were determined fluorometrically using fura-2/AM (A). L2071 cells were stimulated with various concentrations of PhS1P, and again peak $[Ca^{2+}]_i$ levels were recorded. L2071 cells were challenged with 2 μM PhS1P or 2 μM S1P at the time indicated by the arrow (C). L2071 cells were pretreated with 10 μM of VPC 23019 or DMSO for 3 min prior to 2 μM of PhS1P, 2 μM of S1P, or vehicle (ethanol), and $[Ca^{2+}]_i$ was determined (D). Results are presented as mean ± SEM of three independent experiments performed in duplicate (B, D). * indicates results significantly different from the control (-PhS1P or -S1P) at the $P < 0.05$ level. # indicates results significantly different from the control (-VPC) at the $P < 0.05$ level.

a 2% agarose gel and visualized by ethidium bromide staining.

Transient transfection of $S1P_4$

Human $S1P_4$ cDNA was kindly provided from D. S. Im (Pusan National University, Korea). Transfections were performed using LipofectAMINE reagents (Invitrogen Corporation) according to the manufacturer's instructions. HEK293 cells were plated in six-well plates at a density of 5×10^5 cells/well and grown overnight. Cells were transfected with 2 μg of each plasmid construct for 6 h by the Lipofectamine method. After transfection, HEK293 cells were cultured in 10% FBS containing RPMI 1640 medium for 48 h.

Statistics

The results are expressed as mean ± SEM of the number of determinations indicated. Statistical significance of differences was determined by ANOVA. Significance was accepted when $P < 0.05$.

Results

PhS1P stimulates calcium mobilization in L2071 cells

Activation of some GPCRs by lysolipids, such as, $S1P_2$ or LPA_3 leads to PLC activation and the subsequent production of inositol-1,4,5-trisphosphate (IP_3) and $[Ca^{2+}]_i$ increase (An *et al.,* 1998; Kon *et al.,* 1999). To examine whether L2071 cells express such GPCR(s) for PhS1P, we examined the effect of PhS1P on $[Ca^{2+}]_i$ in L2071 cells. As shown in Figure 1A, the stimulation of L2071 cells with 1 μM PhS1P caused a $[Ca^{2+}]_i$ increase in the presence or absence of extracellular calcium. The concentration-dependency of PhS1P-induced $[Ca^{2+}]_i$ increase was also investigated, and an increase in $[Ca^{2+}]_i$ was apparent at a PhS1P concentration of 500 nM and maximal $[Ca^{2+}]_i$ activity was observed at a concentration of 1-2 μM (Figure 1B). We also measured the effect of various concentrations of S1P, which has a similar chemical structure with PhS1P, on $[Ca^{2+}]_i$ increase in these cells. Moreover, S1P also induced $[Ca^{2+}]_i$ increase, showing similar concentration-dependency with that of PhS1P (Figure 1B).

S1P is a well-known lysophospholipid that stimulates $[Ca^{2+}]_i$ rise in fibroblasts (Mattie *et al.,* 1994). Thus, we suspected a possibility that PhS1P utilizes GPCRs for S1P to elicit Ca^{2+} response, and applied desensitization experiments. As shown in Figure 1C, stimulation of L2071 cells with PhS1P desensitized cells, resulting in no response to the second PhS1P stimulation, meaning homologous desensitization. However, PhS1P-desensitized L2017 cells responded to S1P (Figure 1C). The homologous desensitization was also observed with S1P (Figure 1C). In the reverse cases, S1P-desensitized L2071 cells were not responding to PhS1P, meaning heterologous desensitization (Figure 1C). Therefore, these results suggest that PhS1P may share the same receptor(s) with those of S1P.

In order to determine whether PhS1P induces calcium signaling in L2017 cells via S1P receptors, we utilized the S1P receptor-selective antagonist, VPC 23019 which is an antagonist of $S1P_1$ and $S1P_3$ (Davis *et al.,* 2005). As shown in Figure 1D, preincubation of L2071 cells with 10 μM of VPC 23019 partially inhibited S1P-induced calcium increase. However, PhS1P-induced calcium increase was

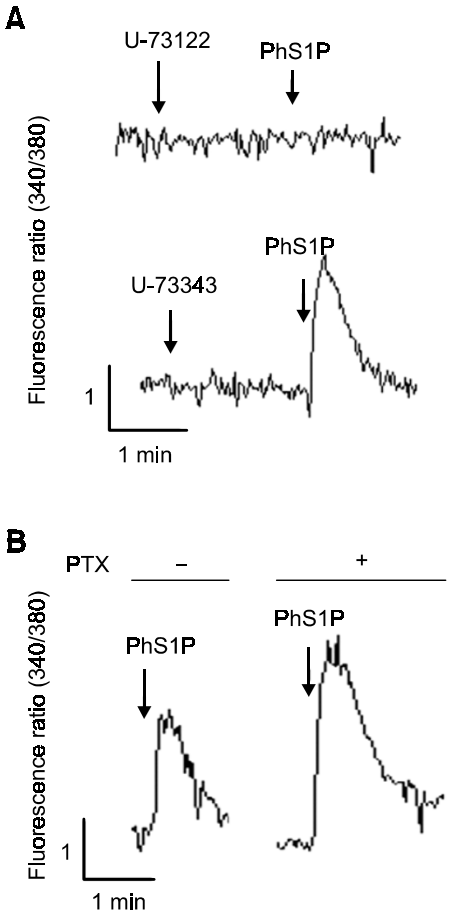

Figure 2. PhS1P-induced Ca^{2+} signaling is U-73122-sensitive but PTX-insensitive in L2071 cells. L2071 cells were pretreated with 5 μM of U73122 or 5 μM of U73343 prior to 2 μM of PhS1P, and $[Ca^{2+}]_i$ levels were determined (A). L2071 cells were pretreated in the absence or presence of 100 ng/ml of PTX for 24 h. Cells were then loaded with fura-2/AM and peak $[Ca^{2+}]_i$ levels were determined fluorometrically after stimulation with 2 μM of PhS1P. Data are representative of four independent experiments (A, B).

completely inhibited by preincubating L2071 cells with 10 μM of VPC 23019 (Figure 1D).

PhS1P-stimulated calcium mobilization is U-73122-sensitive but PTX-insensitive

PLC-dependent IP_3-mediated response is one of the most well known mechanisms of $[Ca^{2+}]_i$ increase in the absence of extracellular calcium (Noh *et al.,* 1995). To determine the role of PLC on PhS1P-induced $[Ca^{2+}]_i$ increase, we pretreated L2071 cells with a specific PLC inhibitor, U-73122, or with its inactive analogue, U-73343. Figure 2A shows that U-73122, but not U-73343, completely inhibited PhS1P-induced $[Ca^{2+}]_i$ increases, which indicates that PhS1P induces $[Ca^{2+}]_i$ increases via PLC activation in L2071 cells.

In addition, we investigated the role of PTX-sensitive G-proteins on PhS1P-induced $[Ca^{2+}]_i$ increase. Cultured L2071 cells were preincubated with 100 ng/ml PTX prior to being stimulated with 1 μM PhS1P. It was found that pretreatment with PTX did not block $[Ca^{2+}]_i$ increase by PhS1P (Figure 2B), demonstrating that PhS1P induces $[Ca^{2+}]_i$ in a PTX-insensitive manner.

PhS1P stimulates ERK and p38 kinase in L2071 cells in a PTX-sensitive manner

MAPK has been reported to mediate extracellular signals to the nucleus in various cell types (Johnson and Lapadat, 2002). In this study, we examined whether PhS1P stimulates MAPKs by Western blotting using anti-phospho-specific antibodies against each enzyme. When L2071 cells were stimulated with 1 μM PhS1P for several lengths of time, ERK phosphorylation levels were transiently increased, and peaked within 2-10 min of stimulation, and

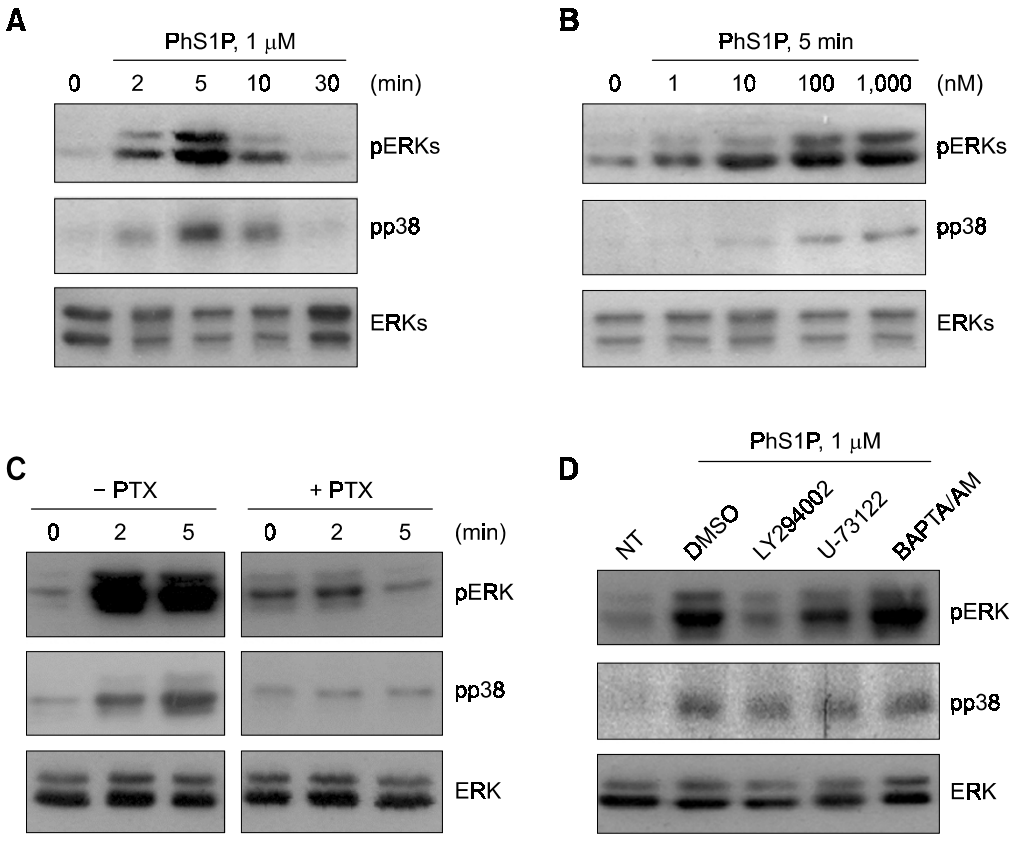

Figure 3. PhS1P stimulates MAPK phosphorylation in a PTX-sensitive manner in L2071 cells. L2071 cells were stimulated with 2 μM of PhS1P for different times (A). Cells were then stimulated with various concentrations of PhS1P for 5 min (B). L2071 cells pretreated with/without 100 ng/ml PTX for 24 h were stimulated with 2 μM PhS1P for 0, 2, or 5 min (C). Cells were pretreated with LY294002 (50 μM), U-73122 (5 μM), or BAPTA/AM (10 μM) for 15 min prior to adding 2 μM PhS1P for 5 min (D). Protein samples (30 μg) were subjected to 10% SDS-PAGE, and phosphorylated ERK and p38 kinase levels were determined by immunoblotting using anti-phospho-ERK antibody or anti-phospho-p38 kinase antibody. The results shown are representative of at least three independent experiments (A-D).

returned to baseline 30 min after stimulation (Figure 3A). Another important MAPK, p38 kinase was also transiently activated by PhS1P stimulation with kinetics that resembled those of ERK activation (Figure 3A). We also examined the concentration-dependencies of PhS1P-induced ERK and p38 kinase activations. When L2071 cells were stimulated with various concentrations of PhS1P, ERK and p38 kinase were concentration- dependently activated (Figure 3B). In terms of ERK activation, PhS1P caused significant activation at 10 nM and maximal activation at 0.1-1 μM (Figure 3B). p38 kinase activation was also induced by PhS1P at *ca.* 10 nM and maximally induced by PhS1P at 1 μM (Figure 3B).

We also examined the effect of PTX, a specific inhibitor of G_i type G proteins, on PhS1P-induced MAPK phosphorylation. When L2071 cells were preincubated with 100 ng/ml PTX prior to being stimulated with 1 μM PhS1P, PhS1P-induced ERK and p38 kinase phosphorylations were almost completely inhibited (Figure 3C). These results indicate that PhS1P stimulates the activations of ERK and p38 kinase via PTX-sensitive G-proteins. In addition, we examined the signaling pathway of PhS1P-induced MAPK phosphorylation by stimulating L2071 cells with PhS1P in the presence of several signaling molecule inhibitors. LY294002 and U-73122 are selective inhibitors of phosphatidylinositol-3-kinase (PI3K) and PLC, respectively (Smith *et al.,* 1990; Vlahos *et al.,* 1994), and BAPTA/AM is a Ca^{2+} chelator (Tsien, 1980). As shown in Figure 3D, LY294002 completely inhibited PhS1P-induced ERK phosphorylation, but BAPTA/AM did not (Figure 3D). U-73122 also partially inhibited PhS1P-induced ERK phosphorylation (Figure 3D). These results indicate that PhS1P stimulates ERK phosphorylation via a PI3K- and PLC-mediated pathway. In addition, PhS1P-induced p38 kinase phosphorylation was also inhibited by LY294002 or U-73122 but not by BAPTA/AM (Figure 3D), suggesting that PhS1P-induced p38 kinase activation is PI3K- and PLC-dependent.

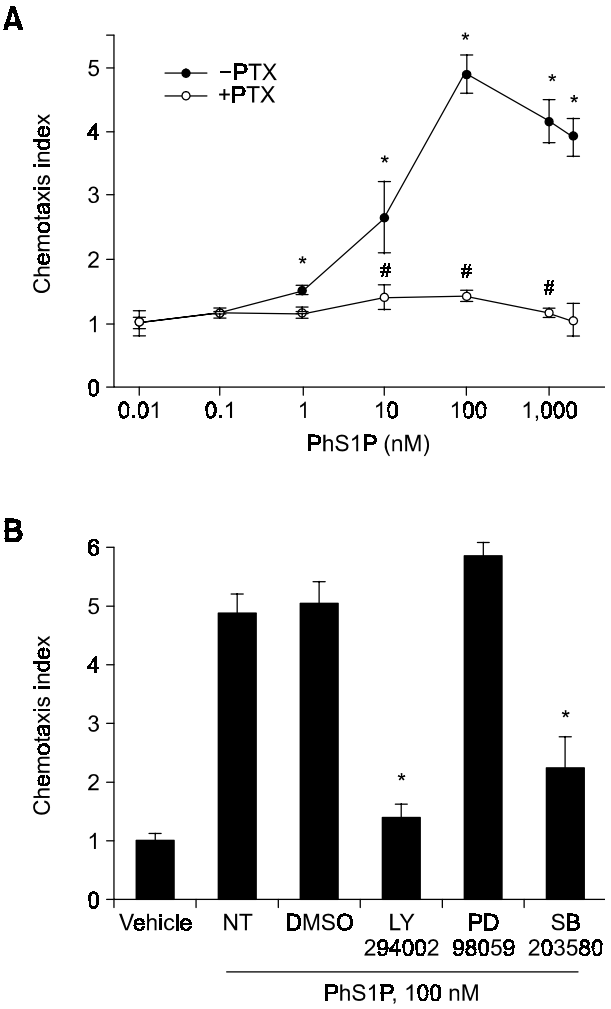

Figure 4. PhS1P induced L2071 chemotaxis via PTX-sensitive G-protein and PI3K and p38 kinase-mediated signaling. Chemotaxis assays were performed using a modified Boyden chamber assay, as described in Materials and Methods. L2071 cells, which had been pretreated with/without 100 ng/ml PTX for 24 h, were subjected to chemotaxis assays after being treated with different concentrations of PhS1P (0.01, 0.1, 1, 10, 100, 1,000, and 2,000 nM) (A). Untreated L2071 cells were pretreated with vehicle (DMSO), PD98059 (50 μM), SB203580 (20 μM), or LY294002 (50 μM) for 15 min, and then subjected to chemotaxis assays with 100 nM PhS1P for 4 h (B). The numbers of cells that migrated were counted in three high power fields (400×). Vehicle is ethanol which was used to dissolve PhS1P. NT means not treated (neither DMSO nor other inhibitors were treated). Data are presented as the mean ± SEM of three independent experiments performed in duplicate (A, B). * indicates results significantly different from the control (-PhS1P in A, DMSO+PhS1P in B) at the $P < 0.05$ level. # indicates results significantly different from the control (-PTX) at the $P < 0.05$ level.

PhS1P induces mouse fibroblast chemotaxis via PTX-sensitive G-proteins, p38 kinase, and PI3K-dependent signaling

We examined the effect of PhS1P on the migration of mouse fibroblasts. Since intracellular signaling via several chemoattractant receptors results in the activation of several integrins that are involved in leukocyte adhesion and migration (Wang *et al.,* 2002), we investigated the effect of PhS1P on fibroblast migration on specific extracellular matrices. PhS1P was found to induce the chemotactic migration of mouse fibroblasts on fibronectin but not on fibrinogen or BSA (data not shown). Figure 4A shows the concentration-responsive curve of PhS1P-induced mouse fibroblast migration, which showing

maximal activity at 100 nM. These findings imply that PhS1P induces the chemotaxis of mouse fibroblasts. Moreover, since PhS1P-induced MAPK phosphorylations were inhibited by PTX in L2071 cells, we examined the effects of PTX on PhS1P-induced mouse fibroblast chemotaxis. When L2071 cells were pretreated with 100 ng/ml PTX prior to chemotaxis assays, the number of cells migrating toward PhS1P was reduced by around 90% versus cells not treated with PTX (Figure 4A), thus strongly suggesting the involvement of PTX-sensitive G protein(s) in L2071 chemotaxis.

Several reports show that many chemoattractants stimulate PI3K-mediated Akt activity and that a PI3K pathway is involved in the chemotaxis of leukocytes stimulated by these chemoattractants (Hirsch *et al.,* 2000; Wang *et al.,* 2002). Since we observed that the stimulation of cells with PhS1P led to a rapid increase in the phosphorylation of Akt (data not shown), we examined whether the PI3K pathway is required for PhS1P-induced L2071 cell chemotaxis, and we found that pretreatment of cells with 50 μM LY294002 (a well-known PI3K inhibitor) for 15 min at 37°C prior to stimulation with PhS1P did indeed affect cellular chemotaxis (Figure 4B). These results indicate that PhS1P activates the PI3K pathway and that this signaling is required for the PhS1P-induced chemotaxis of L2071 mouse fibroblast cells.

We also examined the roles of ERK and p38 kinase on PhS1P-induced L2071 chemotaxis. PD98059 and SB203580 are selective inhibitors of ERK and p38 kinase, respectively (Kim *et al.,* 2006; Lee *et al.,* 2006). When L2071 cells were preincubated with PD98059 or SB203580 prior to chemotaxis assays, PhS1P-induced L2071 chemotaxis was significantly blunted by SB203580, but PhS1P-induced chemotaxis was not inhibited by PD98059 (Figure 4B), which implies that p38 kinase-mediated signaling is involved in PhS1P-induced L2071 chemotaxis.

PhS1P stimulates L2071 chemotaxis independently of $S1P_1$, $S1P_3$ and $S1P_4$

To determine whether L2071 cells express receptors for PhS1P, we analyzed the mRNA expressions of $S1P_{1-4}$ by semi-quantitative RT-PCR. As shown in Fig. 5A, L2071 cells expressed several forms of S1P receptors, namely, $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$, and the expression levels of $S1P_2$ and $S1P_3$ were higher than those of $S1P_1$ and $S1P_4$.

In order to determine whether PhS1P induces L2071 chemotaxis via S1P receptors, we utilized VPC 23019 which is an antagonist of $S1P_1$ and $S1P_3$ (Davis *et al.,* 2005). As shown in Figure 5B, S1P-induced L2071 chemotaxis was completely inhibited by pretreating L2071 cells with 10 μM VPC 23019.

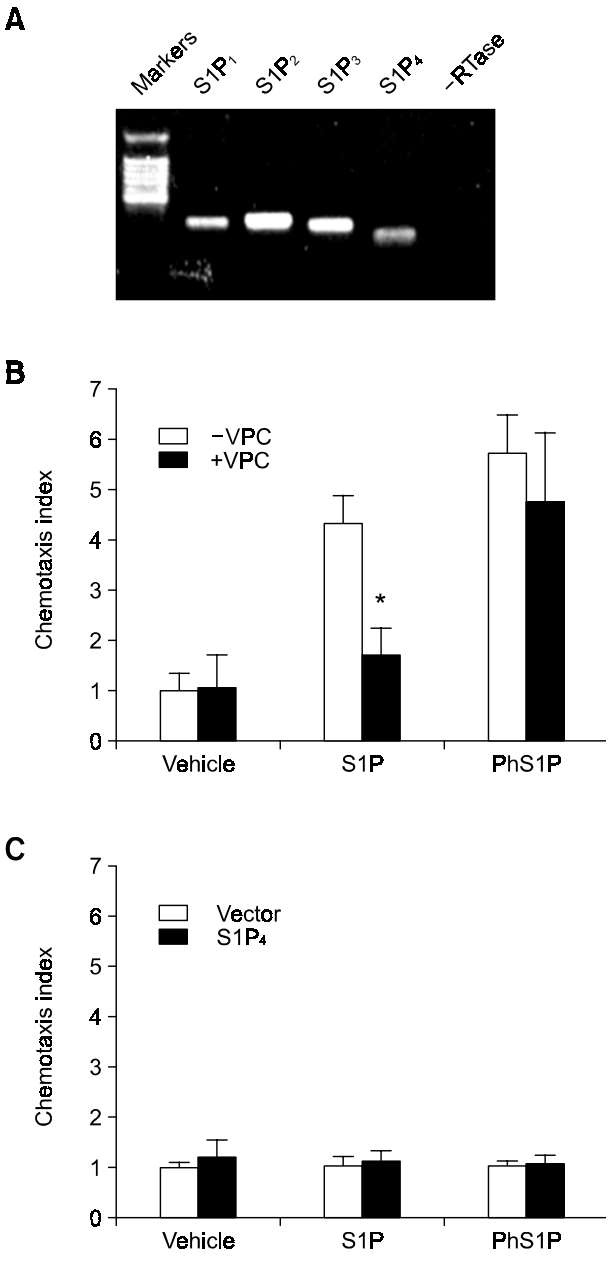

Figure 5. PhS1P stimulated L2071 chemotaxis independently of $S1P_1$, $S1P_3$, and $S1P_4$. RT-PCR analysis was performed on mRNA isolated from cultured L2071 mouse fibroblasts. The data presented are representative of three independent experiments. RTase indicates Maloney murine leukemia virus reverse transcriptase (A). L2071 cells were treated with vehicle (DMSO) or VPC 23019 (10 μM) for 15 min, and then subjected to chemotaxis assays in the presence of 1 μM S1P or 100 nM PhS1P for 4 h (B). HEK293 cells were transfected with vector or $S1P_4$ for 48 h, and then subjected to chemotaxis assays in the presence of 1 μM S1P or 100 nM PhS1P for 4 h (C). Migrated cell numbers were determined by counting in three high power fields (400×). Data are presented as the mean ± SEM of three independent experiments performed in duplicate (B, C). *Statistically significant from the control (-VPC) at the $P < 0.05$ level.

However, PhS1P-induced L2071 chemotaxis was unaffected by pretreating with this material (Figure 5B). These results suggest that PhS1P stimulates L2071 chemotaxis independently of $S1P_1$ and $S1P_3$.

We further examined the effect of S1P or PhS1P on cell migration in vector- or $S1P_4$-transfected HEK293 cells. Cell migration by PhS1P was not significantly increased in $S1P_4$-transfected HEK293 cells against vector-transfected HEK293 cells (Figure 5C).

Discussion

Fibroblast migration is required for the remodeling of the provisional extracellular matrix, as fibroblasts provide a fibrous attachment for growing tissue and wound healing (Wilberding *et al.,* 2001; Hotary *et al.,* 2002), and thus, the modulation of chemotactic migration is an important issue in fibroblast biology. Several groups have reported that various extracellular stimuli are involved in the regulation of fibroblast chemotactic migration (Kundra *et al.,* 1994; Hama *et al.,* 2004). However, the role of PhS1P in fibroblast chemotaxis has not been studied. In the present study, we found that PhS1P stimulates the chemotactic migration of mouse fibroblasts L2071 cells, which suggests that PhS1P has a potential role in tissue remodeling, wound healing, and various functional issues related to fibroblast migration.

Previous reports have suggested that $S1P_1$ and $S1P_4$, types of S1P receptors, are specific cell surface receptors of PhS1P (Candelore *et al.,* 2002; Inagaki *et al.,* 2005). Here, we also found that PhS1P increased $[Ca^{2+}]_i$ in L2071 cells, and that this PhS1P-induced Ca^{2+} response was desensitized by pretreating S1P (Figure 1C), which suggests that PhS1P acts on a S1P receptor in mouse fibroblasts. To further support our notion that PhS1P stimulates L2071 cells via its specific receptor, we examined the effect of the S1P receptor antagonist (VPC 23019) on the PhS1P-induced chemotactic migration of L2071 mouse fibroblast cells. Since PhS1P-induced migration was not inhibited by VPC 23019, an antagonist of $S1P_1$ and $S1P_3$ (Davis *et al.,* 2005), and PhS1P-induced migration was not increased in $S1P_4$-transfected HEK293 cells (Figure 5), we suggest that PhS1P stimulates L2071 mouse fibroblasts chemotaxis independently of known its receptors, $S1P_1$ and $S1P_4$. Previous reports have demonstrated that S1P receptor family members have various roles on chemotactic migration (Matloubian *et al.,* 2004; Goparaju *et al.,* 2005; Wang *et al.,* 2005). Matloubian *et al.* (2004) found that $S1P_1$ is essential for lymphocyte recruitment, whereas Goparaju *et al.* (2005) reported that $S1P_2$ regulates cell migration in an inhibitory manner. Recently Goetzl and colleagues demonstrated that the S1P- $S1P_4$ T-cell axis does not induce cellular migration, but rather suppresses T cell proliferation and the generation of several cytokines, e.g., IL-2, IL-4, and IFN-γ (Wang *et al.,* 2005). Because previous reports on the receptors for PhS1P are very limited to $S1P_1$ and $S1P_4$, it should be considered the possibility that PhS1P can bind to other receptors besides the known S1P receptors, resulting in chemotactic migration. The findings of the present study suggest that other receptor except $S1P_1$ and $S1P_4$ may be involved in PhS1P-induced L2071 mouse fibroblast chemotactic migration.

In this study, we also investigated the effect of PTX, which specifically inactivates $G_{i/o}$-mediated signaling pathways, on PhS1P-induced signaling. When L2071 cells were pretreated with 100 ng/ml of PTX for 24 h prior to PhS1P stimulation, the PhS1P-induced $[Ca^{2+}]_i$ increase was not inhibited (Figure 2B). However, the activations of ERK and p38 kinase and the chemotactic migration induced by PhS1P were completely inhibited by pretreating PTX, as shown in Figures 3 and 4. These results also imply that PhS1P utilizes PTX-sensitive GPCR. Our investigation of signals triggering PhS1P-induced chemotaxis in L2071 cells, based on the use of the specific inhibitors PD98059, SB203580, and LY-294002 and Western blotting, identified that p38 kinase and PI3K have critical roles in this process. Taken together, it is viewed that PhS1P stimulates at least two different G-protein-coupled signals; one which involves PTX-insensitive G-protein-mediated PLC activation and a $[Ca^{2+}]_i$ increase, and which involves PTX-sensitive G-protein-mediated chemotactic migration via p38 kinase and PI3K. Moreover, as calcium signaling regulates various kinds of cellular physiologies and PhS1P dramatically stimulates a PLC-mediated $[Ca^{2+}]_i$ increase, it would be interesting to examine the functional roles of PhS1P in mouse fibroblasts in terms of calcium signaling-dependent processes, as it is felt that this type of work would probably unearth additional important lipid-mediating roles for PhS1P.

In conclusion, in this study, we found that PhS1P induces the chemotactic migration of L2071 mouse fibroblasts by modulating the activities of several intracellular signaling molecules, like p38 kinase and PI3K, and transmembrane signaling molecules, like PTX-sensitive trimeric G proteins and PLC. Since this study represents the only report issued to date on the role of PhS1P on mouse fibroblast chemotaxis, further studies on the pathologic and physiologic roles of PhS1P, and on the roles of PhS1P specific cell surface receptor(s) in L2071 cells are

required.

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