

Sphingosine-1-Phosphate Inhibits PDGF-induced Chemotaxis of Human Arterial Smooth Muscle Cells: Spatial and Temporal Modulation of PDGF Chemotactic Signal Transduction

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Abstract. Activation of the PDGF receptor on human arterial smooth muscle cells (SMC) induces migration and proliferation via separable signal transduction pathways. Sphingosine-1-phosphate (Sph-1-P) can be formed following PDGF receptor activation and therefore may be implicated in PDGF-receptor signal transduction. Here we show that Sph-1-P does not significantly affect PDGF-induced DNA synthesis, proliferation, or activation of mitogenic signal transduction pathways, such as the mitogen-activated protein (MAP) kinase cascade and PI 3-kinase, in human arterial SMC. On the other hand, Sph-1-P strongly mimics PDGF receptor-induced chemotactic signal transduction favoring actin filament disassembly. Although Sph-1-P mimics PDGF, exogenously added Sph-1-P induces more prolonged and quantitatively greater PIP₂ hydrolysis compared to PDGF-BB, a

markedly stronger calcium mobilization and a subsequent increase in cyclic AMP levels and activation of cAMP-dependent protein kinase. This excessive and prolonged signaling favors actin filament disassembly by Sph-1-P, and results in inhibition of actin nucleation, actin filament assembly and formation of focal adhesion sites. Sph-1-P-induced interference with the dynamics of PDGF-stimulated actin filament disassembly and assembly results in a marked inhibition of cell spreading, of extension of the leading lamellae toward PDGF, and of chemotaxis toward PDGF. The results suggest that spatial and temporal changes in phosphatidylinositol turnover, calcium mobilization and actin filament disassembly may be critical to PDGF-induced chemotaxis and suggest a possible role for endogenous Sph-1-P in the regulation of PDGF receptor chemotactic signal transduction.

THE accumulation of smooth muscle cells (SMC)¹ during formation and progression of atherosclerotic lesions and in restenosis after angioplasty is due to a combination of proliferation and directed migration of the cells from the media into and within the intimal layer of the artery wall (for review see Ross, 1993). Initiation of both these events is most likely mediated by a number of regulatory polypeptides that are present in the lesion, such as

PDGF (Ross et al., 1990; Ferns et al., 1991; Jawien et al., 1992). Both proliferation and directed migration (chemotaxis) of arterial SMC are markedly stimulated by PDGF B-chain homodimer (PDGF-BB), and inhibition of PDGF in vivo partially blocks SMC accumulation following balloon injury of a normal vessel (Grotendorst et al., 1981; Ferns et al., 1990; Raines et al., 1990).

After binding of PDGF-BB to its cell surface receptors, the receptor dimerizes and becomes autophosphorylated intracellularly on a number of tyrosine residues that act as docking sites for molecules containing SH2 (src-homology 2 domains). These include enzymes such as phospholipase C γ (PLC γ), phosphatidylinositol 3-kinase (PI 3-kinase), rasGTPase activating protein, the tyrosine phosphatase Syp, and members of the Src family, as well as linker molecules such as growth factor-receptor bound protein 2 and Src homology and collagen protein (Shc; for review see Claesson-Welsh, 1994). A number of different signal transduction pathways are thus induced after PDGF receptor activation. The direct impact of individual signal

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1. *Abbreviations used in this paper:* DMS, *N,N*-dimethyl sphingosine; FAK, focal adhesion kinase; IBMX, 3-isobutyl-1-methylxanthine; IP₁, inositol monophosphate; MAP kinase, mitogen-activated protein kinase; PDGF-BB, PDGF B-chain homodimer; PDS, plasma-derived serum; PI, phosphatidylinositol; PIP₂, phosphatidylinositol bisphosphate; PKA, cAMP-dependent protein kinase; PLC γ , phospholipase C γ ; SMC, smooth muscle cell; Sph-1-P, sphingosine-1-phosphate.

transduction pathways on specific biological functions are, in most cases, not well understood.

Emerging results indicate that the intracellular signaling pathways from the PDGF receptors leading to directed migration may be different from those leading to proliferation (Rönstrand et al., 1992; Kashishian and Cooper, 1993; Bornfeldt et al., 1994). In human arterial SMC, PDGF-stimulated phosphatidylinositol (PI)-turnover (mediated through activation of PLC γ) correlates with directed migration (Bornfeldt et al., 1994). Migration of SMC on type I collagen toward PDGF-BB also requires functional α 2 β 1 integrins (Skinner et al., 1994). Activation of the MAP kinase cascade through growth factor-receptor bound protein 2 and ras-GTP formation, on the other hand, correlates with the magnitude of proliferation, and does not appear to be required for migration of SMC (Bornfeldt et al., 1994).

Recently, a role for sphingolipids in growth factor signal transduction has been proposed (for review see Hakomori, 1990; Kolesnick and Golde, 1994). One of the sphingolipid metabolites that levels can be altered after growth factor receptor stimulation is sphingosine-1-phosphate (Sph-1-P). Sph-1-P can be formed in vivo by phosphorylation of sphingosine by sphingosine kinase (for review see Hakomori, 1990), and is degraded mainly by sphingosine-phosphate lyase, which cleaves Sph-1-P to a fatty aldehyde and ethanolamine-phosphate (for review see van Veldhoven and Mannaerts, 1993). Sph-1-P has recently been suggested as a PDGF receptor signaling molecule (Olivera and Spiegel, 1993), and yet exogenously added Sph-1-P inhibits migration of some cell types through an unknown mechanism (Sadahira et al., 1992). This apparent discrepancy prompted us to further study the effect of Sph-1-P on PDGF-induced signal transduction pathways involved in proliferation versus migration of human SMCs.

Here we show that Sph-1-P does not share the effects of PDGF-BB on mitogenic signal transduction. Instead, Sph-1-P selectively mimics PDGF receptor signaling events favoring actin filament disassembly, thereby inhibiting actin filament assembly, formation of focal adhesion sites and PDGF-induced migration and chemotaxis. Spatial and temporal differences between PDGF and Sph-1-P signaling may be critical to its interference with PDGF-induced migration.

Materials and Methods

Cell Cultures

Human newborn (13 d) arterial smooth muscle cells were obtained from the thoracic aorta of an infant after death due to congenital heart defects. The cells were isolated by the explant method and cultured as described previously (Ross and Kariya, 1980). Cells were used in passages 2–10, and were characterized as smooth muscle by morphologic criteria and by expression of smooth muscle α -actin. The cells were negative in mycoplasma assays and had a normal chromosome number. Subconfluent cell cultures were kept in DME/1% human plasma-derived serum (PDS) for 2 d before experiments. For degradation of the catalytic subunit of cAMP-dependent protein kinase (PKA), the cells were incubated in the presence of 25 μ M forskolin and 500 μ M 3-isobutyl-1-methylxanthine (IBMX), both from Calbiochem-Novabiochem (La Jolla, CA), 24 h before experiments as previously described (Richardson et al., 1990). This treatment resulted in a complete loss of PKA catalytic activity, and a marked reduction of PKA catalytic subunits from the cytosolic and the membrane fraction as judged by Western blot analysis (L. M. Graves, unpublished results).

Growth Factors, Peptides, and Other Compounds

D-Erythro-sphingosine-1-phosphate (the naturally occurring isomer) was chemically synthesized as previously described (Ruan et al., 1992), and *N,N*-dimethylsphingosine (DMS); another metabolite of sphingosine with structural similarities to Sph-1-P) was synthesized as described by Igarashi et al. (1989). Sph-1-P and DMS were not cytotoxic at any concentration or incubation time used, as judged by LDH (lactate dehydrogenase) release from cells. Exogenously added Sph-1-P (1 μ M) was rapidly taken up by the SMC to a level of approximately fourfold the level of endogenous Sph-1-P. Sph-1-P was not significantly converted to sphingosine during a 30-min incubation in the SMC. Human recombinant PDGF-BB was kindly provided by Zymogenetics Inc. (Seattle, WA). PKI peptide (a peptide inhibitor of PKA) and Kemptide were synthesized at the peptide synthesis facility, Howard Hughes Medical Institute (Seattle, WA). Recombinant rat Erk-2 was a gift from Dr. M. Cobb (University of Texas, Austin, TX). Monoclonal antibodies, derived from mouse, directed against β 1 (P4C10) and α 2 (P1H6) integrin subunits were kindly supplied by Dr. W. G. Carter (Fred Hutchinson Cancer Research Center, Seattle, WA).

Measurement of Sphingolipids in Human Arterial SMCs

Approximately 30 million cells were trypsinized (0.01% trypsin/0.11 mM EDTA) as previously described (Bornfeldt et al., 1994), washed twice in DME/0.25% BSA and the cell pellets were stored at -80°C until characterization of glycosphingolipids was undertaken. Sphingosine and Sph-1-P were quantified by acylation with radioactive acetic anhydride (Ohta et al., 1994; Yatomi et al., 1995.) after precipitation from \sim 8 million cells with 4 ml ice-cold methanol. Lipid extraction from the cells was performed as described previously by Bligh and Dyer (1959). The sphingolipids were separated using thin-layer chromatography as previously described (Sadahira et al., 1992) and compared to standard lipids.

PDGF Receptor Autophosphorylation

Cells in 6-well plates were preincubated for 30 min with 1 μ M Sph-1-P or DMS, and then were stimulated with PDGF-BB for 7 or 10 min at 37°C . The stimulation was terminated by three washes with ice-cold PBS followed by solubilization of the cells in 1% Triton X-100, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mM Na_2VO_4 . The samples were either directly separated on a 6% SDS-polyacrylamide gel (10 μ g protein/lane) or separated following immunoprecipitation over night with a PDGF receptor β subunit-specific antibody (10 μ g pR7212, kindly provided by Dr. D. Bowen-Pope, University of Washington) and blotted onto nitrocellulose membranes. The membranes were blocked in TBS containing 5% BSA at 45°C for 2 h and then incubated with 0.1 μ g/ml phosphotyrosine antibody PY20 (ICN ImmunoBiomedicals, Costa Mesa, CA) in TBS/0.05% Tween-20 and 5% BSA for 1 h at room temperature. The bands were visualized using the ECL detection kit (Amersham Inc., Amersham, UK).

Measurement of DNA Synthesis and Proliferation

Cells were grown in 24-well trays, and when they reached \sim 80% confluency, were changed to DME/1% human PDS for 48 h. Then, PDGF-BB and Sph-1-P or DMS were added, and the cells were incubated for an additional 18 h and subsequently labeled with 2 μ Ci/ml [^3H]thymidine (New England Nuclear, Boston, MA) for 2 h. [^3H]thymidine incorporation into DNA was measured as trichloroacetic acid insoluble radioactivity.

Proliferation was measured by determining cell number. Cells were plated in 12-well plates in DME/10% FCS with a density of 30,000 cells/well (day 0). The following day (day 1) the medium was changed to DME/1% PDS and PDGF-BB (1 nM) or 10 mM acetic acid/0.25% BSA (vehicle) were added to the cells on day 1 and 3. In one set of experiments, the cells were added Sph-1-P (1 μ M) or the same volume of 50% ethanol (vehicle) at the same time as PDGF-BB. In another set of experiments, Sph-1-P or vehicle were added to the cells daily. The cells were trypsinized, fixed in Holley's fixative (3.7% formaldehyde, 86 mM NaCl, 106 mM Na_2SO_4) and counted 7 d after plating.

Measurement of MAP-Kinase, MAP-Kinase Kinase and PKA Activity

Enzyme activities were measured as previously described (Bornfeldt et

al., 1994). Briefly, cells in 100-mm dishes (~3 million cells) were stimulated with PDGF-BB for 5 min (the time giving maximal activation) or Sph-1-P at indicated times. Immediately after stimulation, the cells were scraped and sonicated in buffer H containing 50 mM β -glycerophosphate (pH 7.4), 1.5 mM EGTA, 0.1 mM Na_2VO_4 , 1 mM DTT, 10 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ pepstatin, 20 $\mu\text{g}/\text{ml}$ leupeptin, and 1 mM benzamide.

PKA was assayed by measuring phosphorylation of Kemptide (0.17 mM) in the presence or absence of PKI peptide (15 μM) as described (Graves et al., 1993). PKA activity was calculated as the amount of Kemptide phosphorylated in the absence of PKI peptide minus that phosphorylated in the presence of PKI peptide.

Measurement of PDGF-induced Phosphatidylinositol 3-Kinase Activity

Phosphatidylinositol 3-kinase (PI 3-kinase) activity was measured following a 10 min preincubation of cells in 100-mm dishes with 1 μM Sph-1-P or 10 nM Wortmannin (a PI 3-kinase inhibitor; Sigma, St. Louis, MO) and a subsequent stimulation with 1 nM PDGF-BB for 10 min at 37°C. The cells were washed once in ice-cold PBS, then twice in Buffer A (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM MgCl_2 , 1 mM CaCl_2 and 100 μM Na_2VO_4). Thereafter, the cells were solubilized in Buffer A containing 1% NP-40 and 10% glycerol and centrifuged at 13,000 g for 10 min at 4°C. The PDGF receptor β subunit was immunoprecipitated at 4°C overnight using 10 $\mu\text{g}/\text{ml}$ PDGF receptor β subunit polyclonal IgG1 antibody (pR7212). PI 3-kinase activity in immunoprecipitates was measured according to Myers et al. (1993). The spots comigrating with the phosphatidylinositol 4-P standard (Sigma) were scraped off the plate, dissolved in Ecolume™ (ICN Biomedical Inc., Irvine, CA), and the radioactivity of each sample was determined.

Migration Assays

The migration and chemotaxis of cells were quantified using a 48-well micro-Boyden chamber apparatus (NeuroProbe Inc., Cabin John, MD) as previously described (Bornfeldt et al., 1994) or a Transwell™ system (Costar, Cambridge, MA). Briefly, for the micro-Boyden chamber, PDGF-BB or vehicle (10 mM acetic acid with 0.25% BSA) with and without Sph-1-P, DMS or ethanol (diluent) were diluted in DME with 0.25% BSA and loaded into the lower wells of the chamber in triplicates. The wells were subsequently covered with a PVP-free filter with 8- μm pores (Nucleopore Corp., Pleasanton, CA) coated with type I collagen (Vitrogen; Collagen Corp., Palo Alto, CA). The cells were trypsinized (0.01% trypsin/ 0.11 mM EDTA), washed twice in DME/0.25% BSA, and resuspended at a density of 1 or 2 million cells/ml. Cells (25,000 or 50,000 cells in 50 μl) in the presence of PDGF-BB and/or Sph-1-P or DMS were loaded into the upper wells of the Boyden chamber. In the transwell assay, 100,000 cells in 100 μl were allowed to adhere to the collagen type I-coated membrane for 2 h at 37°C before addition of Sph-1-P (30-min preincubation) and PDGF-BB. The chambers were incubated for 4 h at 37°C in an atmosphere of 95% air/5% CO_2 . At the end of the incubation time, the cells attached to the filter were fixed and stained in Dif Quick stain (American Hospital Supply Corp., McGaw Park, IL). The migrated cells on the lower side of the filter were counted manually. Chemotaxis was calculated as the difference between the number of migrated cells in the presence (i.e., total migration) or absence (i.e., chemokinesis) of a concentration gradient of the chemoattractant.

Scanning Electron Microscopy

The cells were trypsinized and plated in a Transwell chamber as described above. Following 2-h incubation to allow adherence of the cells to the membrane, Sph-1-P (1 μM) was added to the upper and lower well for 30 min. Migration was stimulated by the addition of PDGF-BB (1 nM) to the lower chamber for 2 h. The cells were subsequently fixed in 3% glutaraldehyde in PBS for 1 h at 4°C, rinsed twice in PBS, and postfixed in 1% OsO_4 in PBS for 1 h at room temperature. After rinses in PBS and distilled water, the cells were dehydrated in a series of graded ethanol solutions to 70% ethanol and stained in 3% uranyl acetate in 70% ethanol for 1 h. Critical point drying was performed after dehydration in 100% ethanol, and the cells were then gold-palladium sputter coated (resulting in an ~10 nM coat) and studied using a scanning electron microscope at 15 kV (JEOL 35C; Jeol Ltd., Tokyo, Japan).

Attachment and Spreading of SMC

Cells were trypsinized in a similar manner as for the chemotaxis assay and plated (50,000 cells/well) onto collagen type I-coated 96-well trays in the presence or absence of DMS or Sph-1-P at the indicated concentrations. After 15 min to 4 h the unattached cells were washed off twice in PBS, the attached cells were fixed in 3.7% formaldehyde for 1 h at room temperature. The cells were stained with 0.5% toluidine blue in 3.7% formaldehyde for 1 h and then solubilized in 2% SDS. Attachment was measured as absorbance at 650 nm using a kinetic microplate reader (Molecular Devices, Menlo Park, CA).

The time-course of cell spreading on type I collagen was examined with cells plated onto glass cover-slips coated with collagen type I in presence of 1 μM Sph-1-P. 10 min, 30 min, 2 h, and 4 h after plating the cells were washed in PBS, fixed in 2% formalin/PBS for 30 min at room temperature, washed again, and were photographed using a phase contrast microscope (Zeiss Axiovert 100; Carl Zeiss, Inc., Thornwood, NY).

Measurement of Inositol Phosphates

Levels of inositol monophosphate (IP_1) and inositol trisphosphate (IP_3) were measured after PDGF-BB, Sph-1-P, or DMS stimulation as previously described (Bornfeldt et al., 1994). Cells in 6-well plates were labeled with 2 $\mu\text{Ci}/\text{ml}$ myo- $[\text{}^3\text{H}]$ inositol (Amersham Corp., Arlington Heights, IL) for 24 h at 37°C and incubated for 30 min in the presence of 20 mM LiCl and then stimulated with 1 nM PDGF-BB and/or the indicated concentrations of Sph-1-P or DMS for different times. IP_1 was eluted following separation and elution of glycerol-derivates on Bio Rad 1X8 AG columns, with 0.2 M ammonium formate and 0.1 M formic acid. IP_3 was eluted with 0.8 M ammonium formate and 0.1 M formic acid after elution of IP_2 with 0.4 M ammonium formate and 0.1 M formic acid.

Measurement of Intracellular Calcium Levels

Intracellular Ca^{2+} levels were measured according to Grynkiewicz et al. (1985). SMC were grown on chambered cover glass slides (Island Scientific). The cells were washed twice in DME, and then incubated in DME with 2 μM Fura 2-acetoxymethylester (Calbiochem-Novabiochem) for 30 min at room temperature, and subsequently washed and kept for 20 min in the dark in 0.5 ml calcium imaging buffer with the following composition: 140 mM NaCl, 10 mM Hepes, 5 mM KCl, 0.5 mM MgCl_2 , 1.5 mM CaCl_2 and 10 mM glucose (pH 7.4). The buffer was made with ultra pure reagents (Sigma) and sterile H_2O , using tissue culture glassware. Fluorescence in single cells was measured as a 340/380 nm ratio (R) with a Nikon fluorescence microscope following PDGF-BB, Sph-1-P or DMS stimulation. Some experiments were performed in the presence of 5 mM EGTA or after depletion of intracellular calcium stores with 1.5 μM thapsigargin (Calbiochem-Novabiochem). Random areas in single cells (excluding the nucleus) were selected and analyzed using "Image 1" (Universal Image Corp., Westchester, PA). For calibration, R_{min} and free fura fluorescence were estimated by equilibrating the cells in calcium-free imaging buffer with 2 mM EGTA and 2 μM A23187, and R_{max} and bound fura fluorescence was estimated by raising the calcium concentration to 60 mM.

Measurement of Cyclic AMP

SMC in 100-mm dishes were stimulated with Sph-1-P, DMS or vehicle for different periods of time. The plates were washed with ice-cold PBS, and proteins were subsequently precipitated with 1 ml 70% ice-cold ethanol. After scraping of the plates, the suspension was centrifuged at 13,000 g for 20 min at 4°C, the supernatant collected and 0.5 ml was dried in a speedvac centrifuge. Levels of cAMP were determined by a cAMP ELISA kit in prototypic stage, generously provided by GIBCO BRL (Gaithersburg, MD).

Actin Nucleation Assay

Human SMC were trypsinized and plasma membranes were subsequently isolated. The cells were homogenized by 50 strokes in a Dounce homogenizer at 4°C in 20 mM MOPS, 0.2 M sucrose, 10 mM EDTA, and 10 mM EGTA (pH 7.0). The suspension was centrifuged at 500 g for 10 min at 4°C and then the supernatant was centrifuged for 10 min at 7,000 g . The supernatant was centrifuged at 100,000 g for 1 h at 4°C, and the pellet resuspended in 50 mM Tris-HCl (pH 7.0). The membranes (2–4 $\mu\text{g}/\text{ml}$) were incubated in 50 mM Tris-HCl (pH 7.0) with pyrene-labeled actin (0.8 μM G-pyrenyl actin) and 8 μM unlabeled actin. To label actin with *N*-(1-

pyrene)iodoacetamide (NPI; Molecular Probes, Inc., Eugene, OR), actin from rabbit skeletal muscle (Sigma) was homogenized in a Dounce homogenizer (15 strokes) in 0.1 mM CaCl₂, 0.2 mM ATP, 1 mM NaHCO₃, 0.02% NaN₃, and 1 mM DTT. The solution was then adjusted by the addition of MgCl₂ (final concentration: 2 mM), KCl (final concentration: 105 mM), and NPI at a molecular ratio of actin/NPI at 1:7.5, and then stirred at room temperature for 24 h. The solution was centrifuged at 180,000 g at 4°C for 2 h, and the pellet (polymerized actin) was collected. The pellet was homogenized in 0.2 mM CaCl₂, 0.4 mM ATP, 1 mM DTT, 2 mM Tris-HCl (pH 8.0) and 0.02% NaN₃ and dialyzed against the same buffer for 2 days (molecular weight cut-off 3,500). After centrifugation at 180,000 g for 30 min at 4°C, the supernatant (pyrene-labeled actin) was used for actin nucleation assays within 2–4 d. Actin nucleation was measured as increase in fluorescence at 407 nm (excitation 365 nm) as described previously (Kouyama and Mihashi, 1981), and expressed as nucleating activity (arbitrary fluorescence units).

Immunocytochemistry and Phalloidin Staining of Actin Filaments

SMC were plated on glass cover slips coated with collagen type I (Vitrogen) in presence or absence of 1 nM PDGF-BB, 1 μM Sph-1-P, or DMS. The cells were fixed in 2% formalin for 30 min at room temperature, permeabilized in 0.2% Triton X-100 in 50 mM Tris-HCl (pH 7.5) for 2 min, and incubated with a monoclonal vinculin antibody at a dilution of 1:20 in PBS/1% BSA (Calbiochem-Novabiochem), monoclonal β1 integrin antibody or a monoclonal α2 integrin antibody (1:5 dilution of monoclonal supernatant) for 1 h at room temperature. The cells were subsequently incubated with biotin-labeled anti-mouse IgG (Vector Labs., Inc., Burlingame, CA). Actin filaments were visualized with 0.2 μM FITC-labeled phalloidin (Sigma) following the fixation and permeabilization methods described above.

Results

Sph-1-P Does Not Affect PDGF-stimulated Proliferation or Its Associated Signal Transduction Pathways

Since glycosphingolipids and their metabolites have been implicated in various signal transduction pathways, the glycosphingolipids expressed by the human arterial SMC used in this study were characterized. The glycosphingolipid profile is similar to that observed in fibroblasts that express the gangliosides GM₂ and GM₃, with the major neutral glycosphingolipid being ceramide mono-hexose (data not shown). SMC under basal conditions show high levels of sphingosine and Sph-1-P (both 40–80 pmol/10⁶ cells) compared to other cell types, such as Swiss 3T3 cells (16 pmol/10⁶ cells; Olivera and Spiegel, 1993). In our experiments, PDGF-BB does not markedly alter the levels of sphingosine at any time up to 60 min after stimulation. Small (up to 50%) and transient increases in levels of Sph-1-P were observed following stimulation of SMC with 1 nM PDGF-BB (data not shown).

Exogenous addition of 1 μM Sph-1-P to the human SMC does not alter PDGF-BB-induced DNA synthesis (Fig. 1). Alone, Sph-1-P increases basal DNA synthesis by 73.4 ± 9.8%, while the same concentration of DMS (another sphingosine metabolite with structural similarities with Sph-1-P, used as a control) tends to decrease basal DNA synthesis (23.4 ± 2.4% decrease). The concentration of PDGF-BB required to give half-maximal stimulation (EC₅₀) is similar in the presence or absence of 1 μM Sph-1-P or DMS (50.0 pM in vehicle-treated cells, 58.5 pM in Sph-1-P treated cells and 63.1 pM in DMS-treated cells). Daily additions of 1 μM Sph-1-P do not significantly affect

PDGF-induced increases in cell number after 6 d. Addition of 1 nM PDGF-BB results in a 69% increase in cell number 6 d after addition (222,600 ± 20,500 cells/well compared to 131,400 ± 6,900 cells/well in vehicle-treated cells, mean ± of triplicate samples), and daily addition of 1 μM Sph-1-P does not markedly alter the effect of PDGF-BB (193,800 ± 16,900 cells/well in presence of Sph-1-P). Further, daily addition of 1 μM Sph-1-P to the SMCs does not increase cell number (131,400 ± 6,900 cells/well in vehicle-treated cells compared to 108,700 ± 6,900 cells/well in Sph-1-P-treated cells) in spite of the 73% increase in DNA synthesis. Similar results were obtained in three independent experiments, all performed in triplicate.

The MAP kinase cascade has been implicated in PDGF-induced mitogenic responses of human SMC (Bornfeldt et al., 1994). Preincubation in the presence of 1 μM Sph-1-P or DMS for 10 min show that neither Sph-1-P nor DMS alter maximal activation of MAP kinase kinase or MAP kinase by PDGF-BB at a time (5 min) when activation is maximal in these cells (Fig. 2, A and B) although Sph-1-P tends to shift the PDGF-BB MAP kinase kinase dose-response curve to the right approximately twofold (Fig. 2 A). Thus, the EC₅₀-values of PDGF-BB-induced MAP kinase kinase activation is estimated to be 447 pM in Sph-1-P treated cells and 178 pM in vehicle and DMS-treated cells. As shown in Fig. 2 A, a concentration of PDGF close to the EC₅₀-value (300 pM), results in an ~23-fold activation of the MAP kinase kinase in the presence of vehicle or DMS but only an ~11-fold activation in the presence of Sph-1-P. This effect of Sph-1-P does not seem to be attributable to an inhibition of PDGF receptor function, since neither Sph-1-P, nor DMS (both at 1 μM) affects PDGF β-receptor autophosphorylation in human arterial SMC (data not shown). However, at higher concentrations of

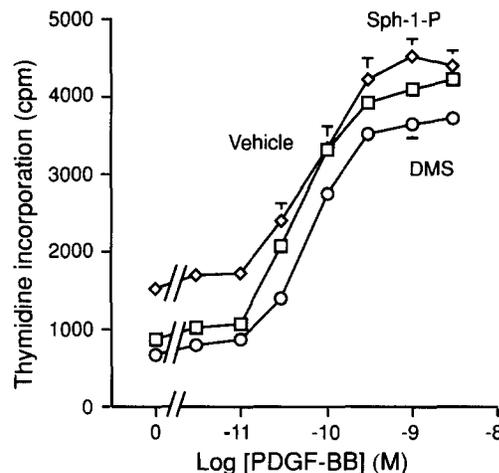


Figure 1. Sph-1-P does not affect PDGF-BB-induced DNA synthesis. To measure DNA synthesis human SMC were stimulated with PDGF-BB at the indicated concentrations in the absence or presence of 1 μM Sph-1-P or DMS for 18 h at 37°C. [³H]thymidine incorporation into DNA was measured as trichloroacetic acid (5%) insoluble radioactivity after an additional incubation period of 2 h in the presence of 2 μCi/ml [³H]thymidine. The results are presented as mean cpm/well ± SD (n = 3). The basal [³H]thymidine incorporation was 487 ± 2 cpm/mg protein.

PDGF-BB similar maximal activities of MAP kinase kinase are observed in the presence and absence of Sph-1-P.

Alone, Sph-1-P (1 μ M) does not significantly activate MAP kinase (see Fig. 9) and activation of MAP kinase kinase was never more than 10% of that observed with 1 nM PDGF-BB. However, stimulation of the cells with 20 μ M Sph-1-P results in a consistent and measurable activation of MAP kinase kinase (\sim 30% of that stimulated by 1 nM PDGF-BB) and MAP kinase (\sim 50% of the activation seen with 1 nM PDGF-BB; data not shown).

PI 3-kinase activation by PDGF-BB has also been implicated in the signaling pathways required for mitogenic responses (Fantl et al., 1992). In the SMC used in the present study PDGF receptor-associated PI 3-kinase activity is markedly increased 10 min after stimulation with 1 nM PDGF-BB. Incubation of the cells for 10 min in 1 μ M Sph-1-P has a slight inhibitory effect on PDGF-BB-induced PI 3-kinase activation (Table I). The inhibition by the PI 3-kinase inhibitor Wortmannin (10 nM) is, however, more significant (\sim 70% inhibition; data not shown).

Sph-1-P Inhibits PDGF-BB-induced Migration and Chemotaxis

Despite the lack of effect of Sph-1-P on SMC proliferation, Sph-1-P markedly inhibits basal migration of human arterial SMC, as well as PDGF-BB-induced migration and chemotaxis in the Boyden chamber assay (Fig. 3). The concentration of Sph-1-P required to give half-maximal inhibition (IC_{50}) of total migration is estimated to be 16 nM and is \sim 20 nM for chemotaxis, whereas DMS does not inhibit migration or chemotaxis even at 1 μ M (Fig. 3). When SMC are allowed to attach and spread in the absence of Sph-1-P for 2 h in the Transwell assay, and then are sub-

Table I. Sph-1-P and PDGF-BB-Induced PI 3-Kinase Activation

Treatment	PI 3-Kinase activity cpm
Vehicle	75 \pm 14
1 μ M Sph-1-P	88 \pm 36
Vehicle + 1 nM PDGF-BB	4963 \pm 725
1 μ M Sph-1-P + 1 nM PDGF-BB	3494 \pm 416

Human SMC in 100-mm dishes were preincubated with 1 μ M Sph-1-P for 10 min at 37°C, and then stimulated with PDGF-BB (1 nM) for an additional 10 min. The cells were washed, scraped and PI 3-kinase associated with the PDGF receptor β subunit was immunoprecipitated with the PDGF receptor β subunit monoclonal antibody pR7212. PI 3-kinase activity was measured according to Myers et al. (1993), and the spots comigrating with the phosphatidylinositol 4-P standard were scraped and the radioactivity quantitated. The results are shown as mean of triplicate dishes \pm SD of a representative experiment.

jected to Sph-1-P-treatment for 30 min and a gradient of PDGF-BB for 4 h, it becomes clear that cells in the presence of Sph-1-P do not extend leading lamellae (Fig. 4 B), whereas vehicle-treated cells extend long processes towards a higher concentration of PDGF-BB (Fig. 4 A). In fact, most of the Sph-1-P-treated cells are still found within the pores of the filter (Fig. 4 B, arrowhead). Total migration towards 1 nM PDGF-BB in the Transwell assay was increased by 120 \pm 13% (mean \pm SD, $n = 3$) compared to basal migration, and 46 \pm 14% in the presence of 1 μ M Sph-1-P (data not shown).

The inability of SMC to migrate towards PDGF-BB in the presence of Sph-1-P is also manifested by inhibition of cell spreading in response to PDGF-BB. 10 min after plating on collagen type I, DMS-treated (1 μ M) cells are still round, whereas cells plated in presence of 1 nM PDGF-BB and DMS (or vehicle) show extensive spreading (compare Fig. 5, A and B). In contrast, cells plated in presence of 1 μ M Sph-1-P and 1 nM PDGF-BB are not spread at all at this point in time, but show characteristic blebbing (Fig. 5 C). 2 h after plating, DMS-treated cells have extended long processes and are more elongated compared to the Sph-1-P-treated cells. The differences between DMS-treated cells and Sph-1-P-treated cells are less obvious 4 h after plating (data not shown). Thus, cells plated in presence of 1 μ M Sph-1-P spread slower (an \sim 2-h delay is generally seen), but the effects of Sph-1-P are completely reversible. Despite the marked effects of Sph-1-P on spreading and migration of SMC, there is no difference in attachment of SMC to type I collagen in presence or absence of concentrations of Sph-1-P or DMS up to 10 μ M when measured at 15 min to 4 h after plating (data not shown).

Sph-1-P Increases Phosphatidylinositol Turnover and Calcium Mobilization, but with Temporal and Spatial Differences as Compared with PDGF-stimulation

Formation of IP_1 and IP_3 is markedly induced by Sph-1-P in human SMC, but not by DMS. The effect of 1 μ M Sph-1-P is threefold greater than the maximal effect (1 nM) of PDGF-BB (Fig. 6 A). Furthermore, the kinetics of the effect of Sph-1-P and PDGF-BB on IP_3 formation are different. The induction of IP_3 by Sph-1-P is observed earlier (1 min after stimulation) and is still marked as late as 60 min after stimulation (Fig. 6 B). PDGF-BB stimulation of IP_3 formation is more transient, with a peak at 5 min after

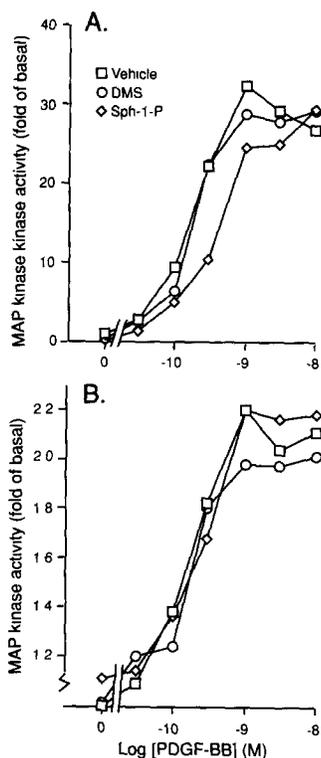


Figure 2. Sph-1-P does not alter maximal PDGF-BB-induced MAP kinase signaling in human SMC. SMC in 100-mm dishes were incubated in DME/1% human PDS for 2 d, preincubated for 10 min with vehicle (ethanol/water 1:1), 1 μ M DMS, or 1 μ M Sph-1-P, and then stimulated with PDGF-BB for 5 min at 37°C. For measurement of MAP kinase kinase activity (A) cell lysates were precleared on a DE-52 mini column, and phosphorylation of myelin basic protein by activated recombinant Erk-2 was measured. MAP kinase activity (B) was measured as phosphorylation of myelin basic protein during a 15-min incubation at 30°C. The results are expressed as mean fold increase over basal levels in vehicle-treated cells of three different experiments, each performed in triplicate.

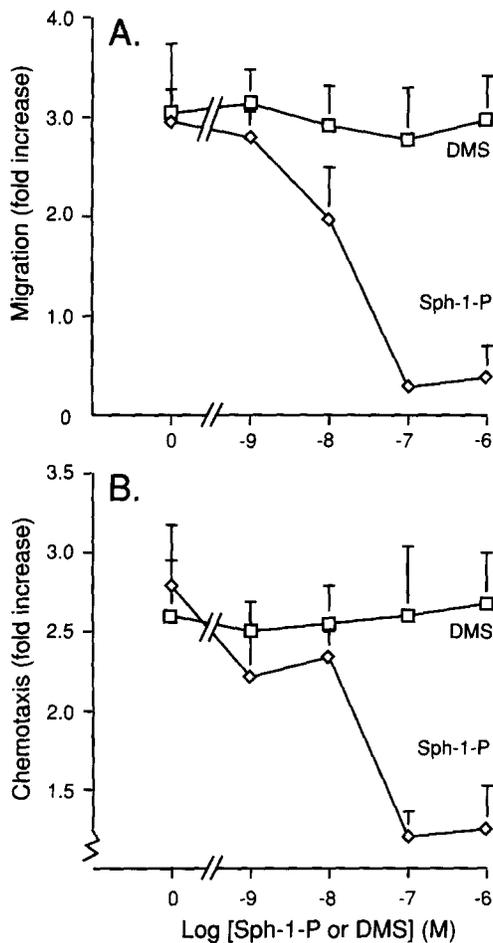


Figure 3. Sph-1-P inhibits PDGF-induced migration and chemotaxis of human SMC. Human SMC were plated in a Boyden chamber coated with collagen type I, and Sph-1-P or DMS was added in both the upper and lower well simultaneously with PDGF-BB. Total migration (A) and chemotaxis towards PDGF-BB (B) was measured after a 4-h incubation, at which time the migrated cells on the lower side of the filter were fixed, stained, and counted. The results are presented as mean \pm SD ($n = 3$). Basal migration was 39 ± 8 cells/400XHPF.

stimulation and a normalization after 30 min (Fig. 6 B). The effects of Sph-1-P and PDGF-BB on IP_1 and IP_3 are additive at all time-points. The dose-response curve of Sph-1-P on IP_1 formation shows an EC_{50} -value of ~ 200 nM Sph-1-P, whereas DMS is without effects up to $10 \mu M$ (Fig. 6 C).

Studies of calcium fluxes in single cells reveal that Sph-1-P gives a rapid, transient, and concentration-dependent increase in intracellular calcium levels. Sph-1-P at 1–100 nM increases intracellular calcium without affecting the subsequent response to 1 nM PDGF-BB (Fig. 7 A). Addition of $1 \mu M$ Sph-1-P results in a calcium mobilization about two to three times (intracellular calcium levels from 30 nM up to $\sim 2 \mu M$) that of a maximal concentration of PDGF-BB (Fig. 7 B). This concentration of Sph-1-P inhibits the subsequent effect of PDGF-BB in approximately half of the cells in a representative field. The maximal calcium mobilization following stimulation with 100 nM Sph-1-P (or 1

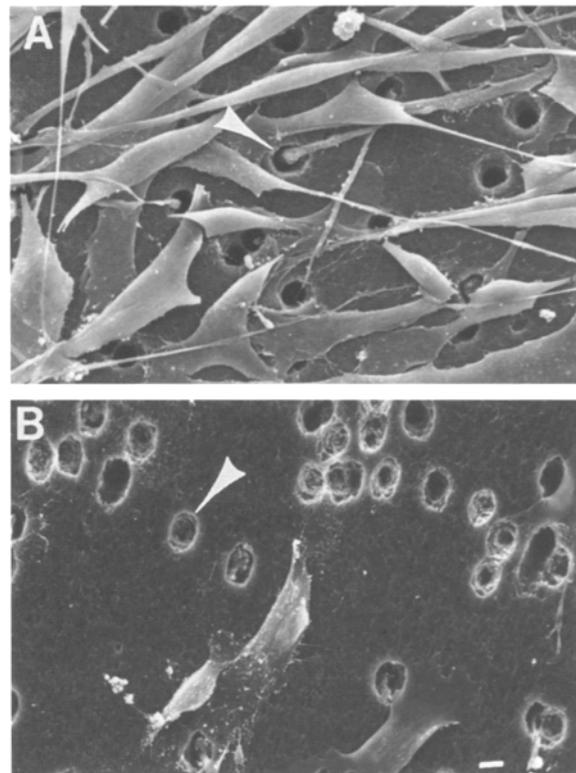


Figure 4. SMC in the presence of Sph-1-P do not extend leading lamellae in a concentration gradient of PDGF-BB. In the Transwell assay, the cells were allowed to adhere for 2 h, and subsequently preincubated with Sph-1-P for 30 min. 4 h after addition of PDGF-BB in the lower well, the cells were fixed in glutaraldehyde and the lower side of the filter processed for electron microscopy. Vehicle (ethanol)-treated cells (A), $1 \mu M$ Sph-1-P-treated cells (B). Note the extended processes in control cells and the number of Sph-1-P-treated cells remaining in the pores of the membrane (arrowhead). Bar, $10 \mu M$.

μM Sph-1-P) propagates throughout the entire cytosol (compare Fig. 8, C with D), whereas PDGF-BB induces increased calcium levels in localized areas of the cytosol (compare Fig. 8, A with B). DMS ($1 \mu M$) does not affect basal or PDGF-induced calcium mobilization. Sph-1-P ($1 \mu M$) is capable of generating calcium mobilization in presence of 5 mM EGTA (which reduces extracellular calcium levels to a concentration similar to the resting intracellular calcium level). However, exhaustion of the intracellular calcium stores with thapsigargin markedly reduced the effect of $1 \mu M$ Sph-1-P (data not shown). Together, these results suggest that Sph-1-P generates an extensive calcium mobilization from intracellular stores in human SMC.

Sph-1-P Elevates cAMP and Activates PKA through a Calcium-dependent Mechanism

Although Sph-1-P has not previously been shown to elevate cAMP levels, the effect of Sph-1-P on cAMP levels in human SMC is marked (Table II). The time-course of cAMP elevation by Sph-1-P is rapid and peaks 2–5 min after stimulation, with a sharp (~ 3 min) decline to basal levels (data not shown). The effect of Sph-1-P on levels of

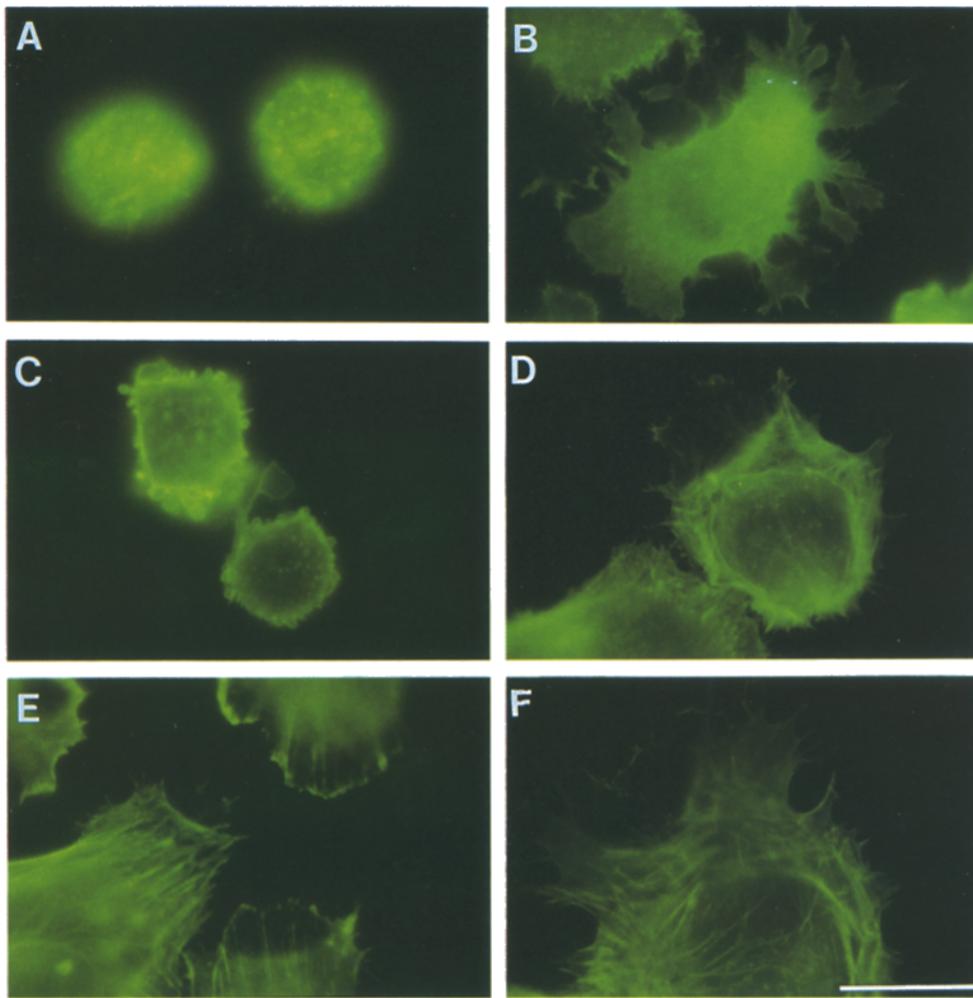


Figure 5. Sph-1-P inhibits spreading of SMC. Human SMC were plated onto collagen type I-coated glass coverslips (100,000 cells/ml) in the presence of 1 μ M DMS or 1 μ M Sph-1-P and 1 nM PDGF-BB, and were fixed with 2% formalin. After 30 min fixation at room temperature, the cells were permeabilized in 0.2% Triton X-100 and incubated for 1 h at room temperature with FITC-labeled phalloidin. In some cases the cells were depleted of PKA catalytic activity as described in the methods. The ventral plasma membrane is shown. The cells were incubated for (A) 10 min with DMS, (B) 10 min with DMS + PDGF-BB, (C) 10 min with Sph-1-P + PDGF-BB, (D) 10 min with Sph-1-P + PDGF-BB in PKA-deficient cells, (E) 30 min with DMS + PDGF-BB, (F) 30 min with Sph-1-P + PDGF-BB in PKA-deficient cells. The experiment was repeated several times with similar results. Bar, 20 μ M.

cAMP is dependent on the increase in intracellular calcium, since the stimulation of cAMP by Sph-1-P could be completely inhibited by depleting internal calcium stores for 1 h with thapsigargin. Thapsigargin-treatment did not elevate LDH release during the incubation time, showing that it is nontoxic.

Consistent with the elevation of levels of cAMP, stimulation of human SMC with 1 μ M Sph-1-P results in a rapid and transient activation of PKA (Fig. 9). The effect of Sph-1-P on levels of cAMP and PKA activation (not shown) are additive to those of 1 nM PDGF-BB (PDGF-BB stimulates PKA in human SMCs; Graves and Born-

feldt, manuscript in preparation). The activation of PKA by 1 μ M Sph-1-P is comparable to that of \sim 300 nM forskolin and 1 nM PDGF-BB.

Sph-1-P Promotes Actin Filament Disassembly and Inhibits Formation of Focal Adhesion Sites

To investigate the effect of Sph-1-P on membrane-dependent actin nucleation, an *in vitro* actin nucleation assay was performed (Fig. 10). Sph-1-P specifically inhibited actin nucleation while DMS had no effect during a 15-min measurement. Actin nucleation in the presence of 1 μ M DMS was $128.3 \pm 2.9\%$ of control, in the presence of 0.1 μ M Sph-1-P was $78.9 \pm 14.8\%$ of control, and in the presence of 1 μ M Sph-1-P was $39.6 \pm 6.1\%$ of control (mean \pm SD, $n = 3$, data not shown). Actin nucleation to plasma membranes from SMC is also inhibited by cytochalasin D (the actin nucleation in presence of 10 μ M cytochalasin D for 15 min was $55.8 \pm 15\%$ of that in control experiments; $n = 4$).

Formation of actin filaments in the presence and absence of Sph-1-P in living cells was studied by visualization of filamentous actin with FITC-labeled phalloidin, and focal adhesion site assembly was examined by immunostaining of vinculin and the $\alpha 2$ and $\beta 1$ integrin subunits. 30 min after plating of SMC on collagen type I, more than 90% of

Table II. Sph-1-P Increases cAMP Levels in Human SMC

	Levels of cAMP	
	pmol/ml	
0.1 μ M DMS	7	
1 μ M DMS	10	
0.1 μ M Sph-1-P	95	
1 μ M Sph-1-P	570	

Human SMC in 100 mm dishes (\sim 2 million cells) were incubated with DMS or Sph-1-P for 5 min at 37°C, and then were washed with ice-cold PBS, precipitated with 1 ml 70% ethanol on ice and scraped off the plates. After centrifugation, the levels of cAMP in the supernatant were determined as stated in Materials and Methods. A representative experiment is shown (mean of duplicate samples).

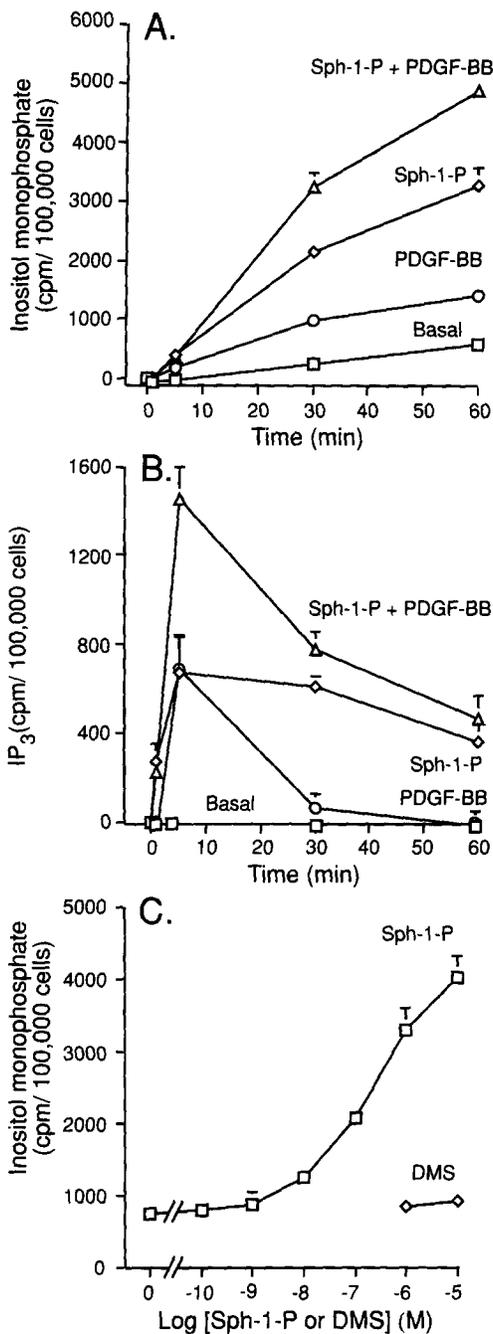


Figure 6. Sph-1-P stimulates PI turnover in human SMC. Levels of IP₁ and IP₃ were measured in cells incubated in 2 μ Ci/ml myo-[³H]inositol in Medium 199 containing 1% human PDS in 6-well trays for 24 h. Phosphatidylinositol turnover was blocked with 20 mM LiCl for 30 min, and the cells subsequently stimulated with PDGF-BB and/or Sph-1-P or DMS for the indicated periods of time at 37°C. Cells were extracted with TCA and free inositol was eluted from a Bio Rad 1-X8 AG column. IP₁ was eluted with 0.2 M ammonium formate and 0.1 M formic acid, and IP₃ was eluted with 0.8 M ammonium formate with 0.1 M formic acid. The results are shown as cpm/100,000 cells (mean \pm SD of triplicate samples). Time-course of the effects of 1 μ M Sph-1-P, 1 nM PDGF-BB or the combination of Sph-1-P and PDGF-BB on levels of IP₁ (A) and IP₃ (B) are shown. C shows a Sph-1-P dose-response curve for IP₁ levels following a 60-min stimulation with Sph-1-P or DMS. The experiment was repeated several times with similar results.

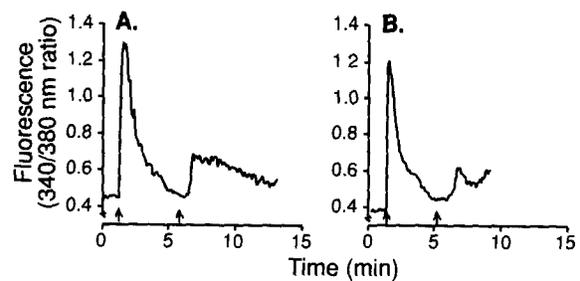


Figure 7. Sph-1-P increases calcium mobilization in human SMC. Cells grown on chambered cover glasses were loaded with 2 μ M fura-2 acetoxymethylester for 30 min at room temperature, washed and stimulated with (A) 100 nM Sph-1-P (left arrow) and 1 nM PDGF-BB (right arrow) or (B) 1 μ M Sph-1-P and 1 nM PDGF-BB. The intracellular changes in calcium in single cells were monitored using a Nikon fluorescence microscope and the software "Image 1" (Universal Image Corp.). Basal levels of intracellular calcium were 30–40 nM. The experiments were repeated three times with similar results.

the DMS-treated (or vehicle-treated) cells are spread, and \sim 50% of the cells have obvious actin filaments that terminate in typical focal adhesion sites containing α 2 integrin subunits (Fig. 11, A and B). In the presence of 1 nM PDGF-BB the fraction of cells showing clear actin filaments and focal adhesion sites increase to close to 90% (data not shown). The α 2 integrin immunostaining is colocalized with staining for β 1 integrin subunits and vinculin (data not shown).

Approximately 50% of the Sph-1-P-treated cells are still around 30 min after plating, and show no obvious formation of actin filaments (Fig. 11 D). Instead, most of the phalloidin stained actin is cortical, which is more readily observed when Sph-1-P-treated cells are studied with confocal microscopy (not shown). Furthermore, α 2 integrin subunits (as well as β 1 integrin subunits and vinculin) are not localized in typical focal adhesion sites, but are rather found in less well-defined patches (Fig. 11 C). There is no obvious difference when the Sph-1-P-treated cells are plated in the presence of 1 nM PDGF-BB, compared to the absence of PDGF-BB. At 2 h after plating, peripheral actin filaments are more frequently seen in the Sph-1-P-treated cells, and they display peripheral focal adhesion sites (data not shown).

Despite the slow formation of typical focal adhesion plaques in the presence of Sph-1-P, no inhibition of tyrosine phosphorylation of focal adhesion kinase (p125^{FAK}) is found. Immunoprecipitation of p125^{FAK} using 10 μ g of a mouse monoclonal p125^{FAK} IgG1 antibody (UBI, Lake Placid, NY) following a 10-min adherence of SMC to collagen type 1 in the presence or absence of Sph-1-P, DMS and/or PDGF-BB reveals no difference in tyrosine phosphorylation of p125^{FAK} (data not shown). At this time, Sph-1-P-treated cells are still round, whereas PDGF-treated cells are actively spreading.

Part of the Sph-1-P-induced Inhibition of Actin Filament Assembly, Spreading, and Chemotaxis Can Be Reversed by Inhibition of PKA

Activation of PKA inhibits actin filament assembly in human SMC. For example, activation of PKA with 1 μ M for-

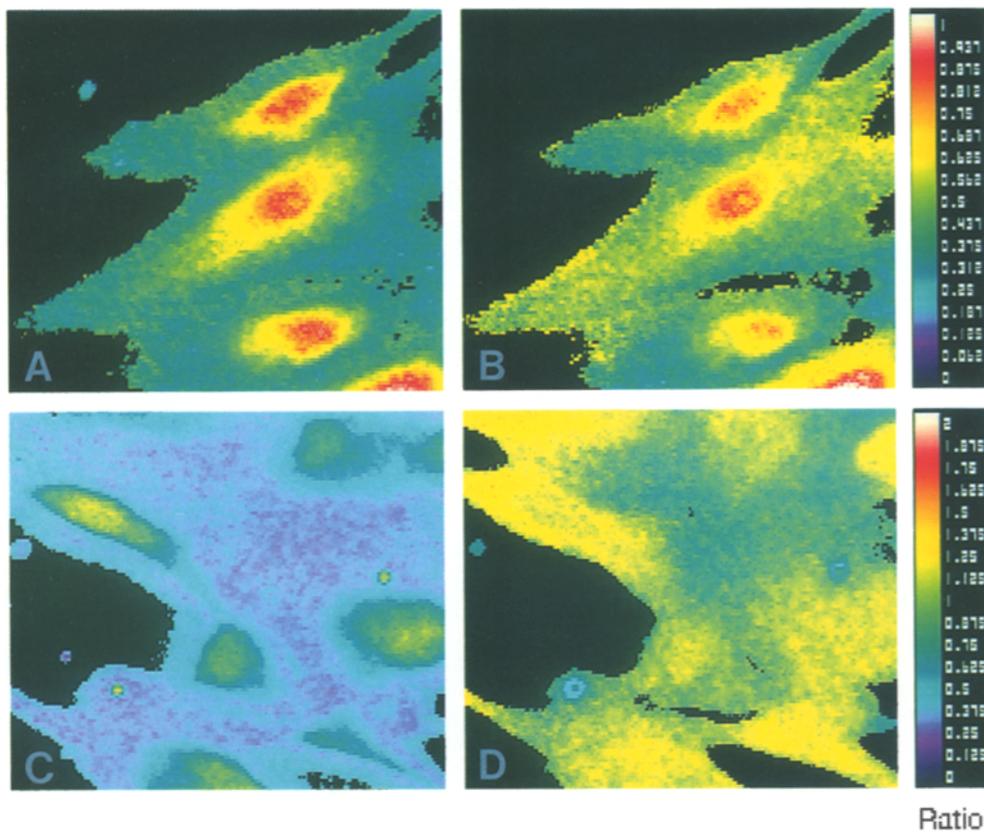


Figure 8. Spatial differences in calcium mobilization following stimulation of human SMC with PDGF-BB and Sph-1-P. The SMC were loaded with 2 μ M fura-2 acetoxyethyl ester as in Fig. 7. Changes in intracellular calcium were monitored in single cells following stimulation with PDGF-BB or Sph-1-P using a Nikon fluorescence microscope as in Fig. 7. The spatial patterns of calcium levels were monitored before (A) and after maximal stimulation with 1 nM PDGF-BB (B) and before (C) and after maximal stimulation with 100 nM Sph-1-P (D). Note that the scales are ranging from 0–1 in A and B and from 0–2 in C and D. The experiments were repeated three times with similar results.

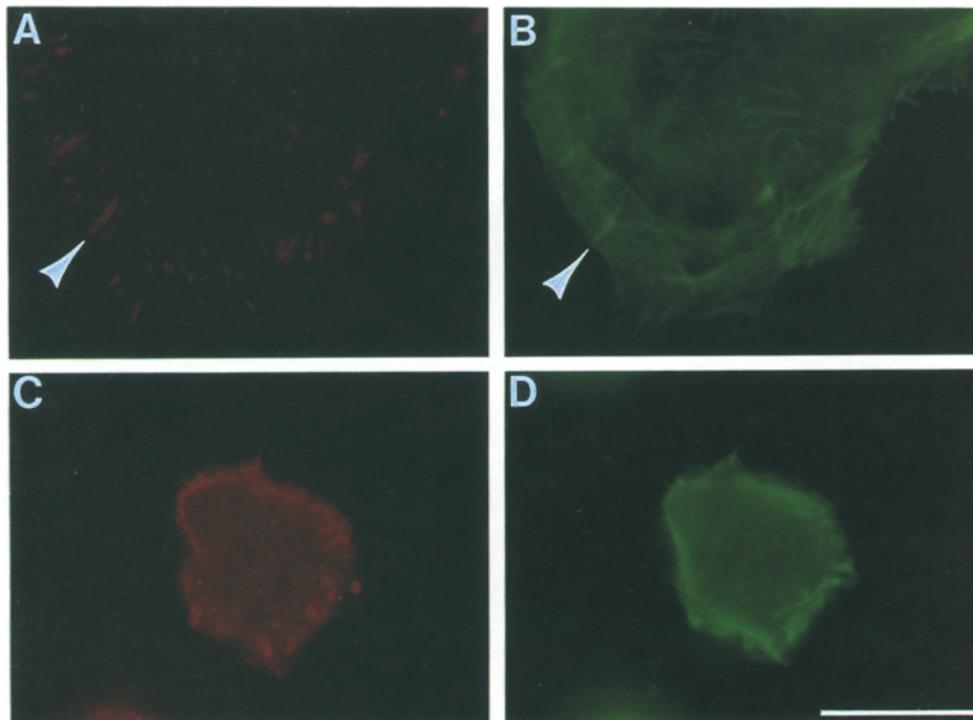


Figure 11. Sph-1-P inhibits actin filament assembly and formation of typical focal adhesion plaques. SMC were plated onto collagen type I-coated glass cover slips, and the cells were allowed to attach and spread for 30 min. The cells were then fixed in 2% formalin for 30 min at room temperature, permeabilized in 0.2% Triton X-100, and incubated for 1 h at room temperature with mouse monoclonal antibodies directed against α 2 integrin subunits (left panel) and with FITC-labeled phalloidin (right panel). Omitting the primary antibody resulted in no detectable immunostaining. The ventral plasma membrane is shown. The photos show a representative SMC plated in vehicle (A and B) or 1 μ M Sph-1-P (C and D) and subsequently stained with an α 2 integrin antibody (A and C) and double-stained with FITC-labeled phalloidin (B and D). Typical focal adhesion sites are marked by arrows. Bar, 20 μ M.

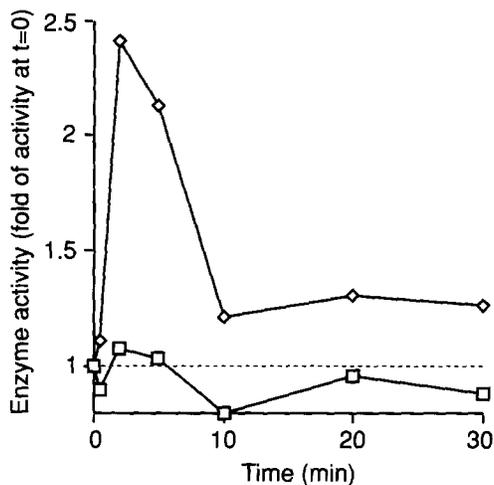


Figure 9. Sph-1-P activates PKA without activating MAP kinase. Activities of MAP kinase (\square) and PKA (\diamond) were measured in the same cell extracts following stimulation with $1 \mu\text{M}$ Sph-1-P for the indicated periods of time. MAP kinase activity was measured as in Fig. 2. PKA activity was assayed by measuring phosphorylation of Kemptide in the presence or absence of PKI peptide, and was calculated as the amount of Kemptide phosphorylated in the absence of PKI peptide minus that phosphorylated in the presence of PKI peptide. The results are expressed as fold increase in enzyme activity over basal levels. Similar results were obtained in three independent experiments.

skolin results in an almost complete loss of actin filament assembly in response to PDGF-BB 30 min after plating on collagen type I (data not shown). Based on this observation, it seemed possible that Sph-1-P activation of PKA might play a role in Sph-1-P inhibition of actin filament assembly and migration of SMC. To study this, PKA catalytic activity was down-regulated by an overnight treatment of cells with $25 \mu\text{M}$ forskolin/ $500 \mu\text{M}$ IBMX (Richardson et al., 1990), or PKA was inhibited with the PKA inhibitor H-89 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA).

Down-regulation of the PKA catalytic activity reverses part of the Sph-1-P inhibition of PDGF-induced spreading and actin filament assembly. 10 min after plating on collagen type I, cells lacking PKA catalytic activity are clearly spreading in response to PDGF-BB in the presence of $1 \mu\text{M}$ Sph-1-P (Fig. 5D) whereas cells not subjected to PKA down-regulation are still round in the presence of Sph-1-P and PDGF-BB and show characteristic blebbing (Fig. 5C). At later time-points (30 min), cells lacking PKA catalytic activity show increased actin filament assembly in response to PDGF-BB even in the presence of Sph-1-P (compare Fig. 5, E with F), whereas cells with normal PKA activity are lacking obvious actin filaments in the presence of Sph-1-P (Fig. 11D).

Down-regulation of the PKA catalytic activity also reverses part of the Sph-1-P inhibition of PDGF-induced migration and chemotaxis as shown in Table III. Although migration of SMC toward PDGF-BB in the presence of Sph-1-P was not fully recovered in cells lacking PKA catalytic activity, migration toward PDGF-BB in the presence of Sph-1-P was 49% of that seen in the absence of Sph-1-P

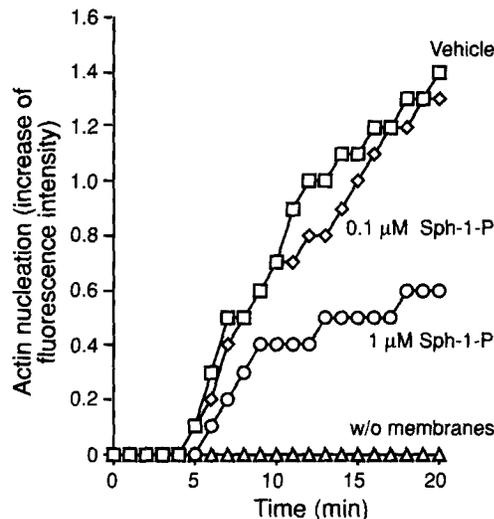


Figure 10. Sph-1-P inhibits actin nucleation in vitro. Plasma membranes from SMC were isolated by homogenization and ultra centrifugation. The membranes were incubated in the presence of pyrene-labeled actin and unlabeled actin in the presence or absence of the indicated concentrations of Sph-1-P. Actin nucleation was measured as increase in fluorescence at 407 nm (excitation 365 nm). The results are presented as mean of three independent experiments.

(Table III). In contrast, migration toward PDGF-BB in the presence of Sph-1-P was only 9% of that seen in the absence of Sph-1-P in control cells. Activation of PKA by $1 \mu\text{M}$ forskolin also inhibited PDGF-induced migration and chemotaxis in normal cells, and this effect was lost in cells lacking PKA catalytic activity (Table III), confirming that PKA was efficiently down-regulated by the procedure used. There is no obvious difference in ability of $1 \mu\text{M}$ Sph-1-P to stimulate PIP_2 hydrolysis and calcium mobilization in cells lacking PKA catalytic activity as compared to normal cells (data not shown).

Consistent with the results obtained in cells where the PKA catalytic activity has been down-regulated, inhibition of PKA by $20 \mu\text{M}$ H-89 (a concentration that completely inhibits PKA catalytic activity in human SMC following a 20 min preincubation), reverses Sph-1-P-induced actin filament disassembly and partly reverses the Sph-1-P inhibition of migration of SMCs toward PDGF-BB (data not shown).

Discussion

Sph-1-P Does Not Mimic or Affect PDGF Receptor Signaling Associated with Smooth Muscle Cell Proliferation

Two recent studies have implicated sphingolipids as second messengers in PDGF receptor mitogenic signal transduction. These studies show that formation of Sph-1-P and sphingosine are rapidly stimulated by PDGF in Swiss 3T3 cells (Olivera and Spiegel, 1993), and that levels of sphingosine are increased following PDGF stimulation of the rat arterial SMC-derived cell line A7r5 (Jacobs and Kester, 1993). The fact that Sph-1-P stimulates DNA synthesis in

Table III. Sph-1-P Inhibition of PDGF-stimulated Migration is Partly Mediated Through PKA

	Total migration	Chemotaxis
Control cells		
Basal	55 ± 4	0 ± 8
PDGF-BB	106 ± 9	42 ± 12
PDGF-BB + Sph-1-P	10 ± 4	2 ± 4
PDGF-BB + forskolin	43 ± 4	26 ± 4
Cells lacking PKA catalytic activity		
Basal	72 ± 7	5 ± 15
PDGF-BB	177 ± 12	66 ± 19
PDGF-BB + Sph-1-P	87 ± 5	37 ± 3
PDGF-BB + forskolin	176 ± 6	82 ± 15

The PKA catalytic activity was down-regulated by an overnight treatment with 25 μ M forskolin/500 μ M IBMX. The cells were then allowed to migrate toward 1 nM PDGF-BB in presence or absence of 1 μ M Sph-1-P or 1 μ M forskolin in the Boyden chamber for 4 h at 37°C. The chemotactic response was calculated as the number of migrated cells in presence of PDGF-BB only in the lower chamber (total migration) minus the number of cells in absence of a PDGF-gradient (equal concentrations of PDGF-BB on both sides of the filter; chemokinesis). The results are expressed as number of cells/400X HPF (mean \pm SD of triplicate samples). The experiment was repeated four times with similar results.

Swiss 3T3 cells approximately eightfold (Zhang et al., 1991), and that PDGF-induced DNA synthesis is inhibited by DL-threo-dihydrosphingosine (a sphingosine kinase inhibitor; Olivera and Spiegel, 1993) support the possible involvement of Sph-1-P in PDGF receptor mitogenic signaling. Further, binding of the transcription factor AP-1 (activator-protein 1) to c-Jun and c-Fos has recently been found to be increased by Sph-1-P in Swiss 3T3 cells (Su et al., 1994).

Endogenous basal levels of Sph-1-P in human arterial SMC are 2.5–5-fold those reported in quiescent Swiss 3T3 cells (Olivera and Spiegel, 1993). Thus, high levels of endogenous Sph-1-P in human SMC are consistent with a possible role for Sph-1-P in signal transduction in this non-transformed, diploid cell. However, in human SMC, Sph-1-P does not activate the MAP kinase cascade associated with stimulation of proliferation at concentrations that markedly stimulate PI-turnover and calcium mobilization. Only at concentrations of 20 μ M or more is a weak stimulation of the MAP kinase cascade observed. Sph-1-P (1 μ M) is able to induce an \sim 1.7-fold increase in DNA synthesis, compared to the fourfold increase by 1 nM PDGF-BB. The small increase in DNA synthesis induced by Sph-1-P does not lead to an increased cell number, even when 1 μ M Sph-1-P is added daily during a 6-d period, whereas PDGF-BB gives an \sim 70% increase of cell number within this period. Thus, although endogenous formation of Sph-1-P may play a role in PDGF receptor signaling in human SMC, the effect of PDGF on the MAP kinase cascade and the subsequent mitogenic effect of PDGF does not seem to be mediated by, or significantly modified by, Sph-1-P. In addition, DMS at a concentration that inhibits sphingosine kinase (20 μ M) better than DL-threo-dihydrosphingosine, does not significantly inhibit PDGF-activation of MAP kinase (our unpublished results). Taken together, these results do not agree with the notion of Sph-1-P as a ubiquitous mediator in PDGF receptor mitogenic signaling.

PKA can antagonize PDGF-induced activation of the MAP kinase cascade (Graves et al., 1993). Activation of PKA by Sph-1-P is \sim 30% of the PKA activity required to

maximally inhibit MAP kinase kinase and MAP kinase in these cells. The shift in the dose-response of MAP kinase kinase activation by PDGF-BB following preincubation with Sph-1-P suggests that the MAP kinase kinase is slightly inhibited by the PKA activation caused by Sph-1-P. This inhibition is potentially not marked enough to be transferred to measurable downstream changes in MAP kinase activity, or alteration of DNA synthesis or proliferation.

Finally, these studies show that extensive PIP₂ hydrolysis and calcium mobilization induced by Sph-1-P are not sufficient to induce proliferation in human SMC, and that activation of this pathway is distinct from signal transduction associated with stimulation of proliferation (as shown by Fig. 12). In contrast, in certain circumstances, and in certain cell types, activation of PLC γ is sufficient to induce a mitogenic response (Valius et al., 1993). In this context, it is important to bear in mind that similar signal transduction pathways can generate different biological responses depending on the strength and the duration of the signal (Marshall, 1995). Further, activation of a growth factor receptor may stimulate different signals depending on the balance of signaling molecules and on the state of differentiation of a certain cell type (Campbell et al., 1995). Thus, to further understand the biological effects of PDGF, it will be important to compare and contrast differences in PDGF receptor signal transduction in diploid cell types, such as human SMC, and in cell lines, such as 3T3 cells.

Sph-1-P Inhibits SMC Spreading and Migration by Excessively Mimicking PDGF Receptor Signaling Leading to Actin Filament Disassembly

In contrast to the lack of effect of Sph-1-P on proliferation, Sph-1-P markedly inhibits migration and chemotaxis of human SMC. The inhibitory effect of Sph-1-P on migration appears to be due to reduced ability of the SMC to extend leading lamellae and spread in the presence of Sph-1-P. These effects by Sph-1-P seem to be explained by an accelerated and prolonged actin filament disassembly, since actin nucleation and formation of actin filaments and focal adhesion sites are delayed in presence of Sph-1-P.

Actin filament disassembly and assembly play an important role in the leading edge of a cell migrating in a gradient of PDGF-BB, where these processes enable cytoplasmic flow and protrusion of new leading lamellae (for review see Stossel, 1993). During chemotaxis of a cell in a gradient of a chemoattractant, intracellular signal transduction must be localized and gradients of signaling molecules are crucial (Gilbert et al., 1994; for review see Beridge, 1994; Clapham, 1995). Thus, the local intracellular balance and cycling between actin filament disassembly and assembly must be finely regulated. What are the intracellular signal transduction events leading to the accelerated actin filament disassembly during chemotaxis toward PDGF? Results obtained by several different approaches imply a role for PLC γ in PDGF-induced chemotaxis (Matsui et al., 1989; Bornfeldt et al., 1994; Kundra et al., 1994). Hydrolysis of PIP₂ and the subsequent increase in intracellular calcium promotes actin filament disassembly by inducing capping of actin filament barbed ends and actin monomer sequestration (for review see Stossel, 1993).

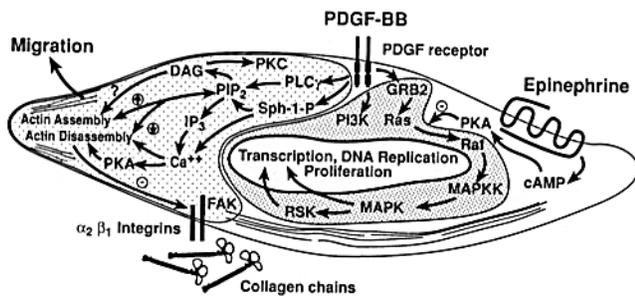


Figure 12. Signal transduction pathways associated with proliferation versus directed migration of human arterial smooth muscle cells. The PDGF-induced signal transduction pathways associated with proliferation (■) versus those associated with directed migration (□) are shown. Sph-1-P selectively mimics PDGF receptor signal transduction associated with migration but not with proliferation. Localized and temporal changes in actin filament disassembly and assembly by modulation of levels of PIP₂, calcium, and activation of PKA are crucial for directed cell migration in a gradient of PDGF. Sph-1-P mimics the signals favoring actin filament disassembly and when added exogenously, Sph-1-P disturbs the spatial and temporal balance between actin disassembly and assembly and inhibits migration. Local changes in concentration of endogenous Sph-1-P may potentially play an important role in regulating these events in a migrating human SMC. Localized and temporal interactions between the cell and the extracellular matrix is also a requisite for directed migration and can be mediated through modulation of $\alpha 2\beta 1$ integrin interaction with collagen type I chains. Activation of the MAP kinase cascade and PI 3-kinase are associated with the mitogenic effects of PDGF, and Sph-1-P does not significantly mimic or affect these signal transduction pathways or proliferation in human SMC. Although Sph-1-P-induced activation of PKA is important in regulating spreading and migration of SMC, the PKA activity is not sufficient to induce measurable inhibition of MAP kinase activity or proliferation.

Point mutation of Y1021 in the human PDGF receptor β -subunit disables activation of PLC γ and reduces PDGF-induced migration (Kundra et al., 1994).

The intracellular effects of Sph-1-P in SMC seem partly attributable to a marked hydrolysis of PIP₂, and a strong mobilization of calcium. Dose-response curves show that doses of Sph-1-P that increase PI-turnover and calcium mobilization also inhibit PDGF-induced cell migration. In other cell types, Sph-1-P has previously been described to increase PI-turnover (Peng Chao et al., 1994) and calcium release from intracellular stores (Ghosh et al., 1990; Mattie et al., 1994; Peng Chao et al., 1994). The effects of Sph-1-P on intracellular calcium stores are not due to a non selective increase in permeability of the membranes (Ghosh et al., 1990). Instead, Sph-1-P seems to release calcium mainly from the IP₃-sensitive calcium pool, but not directly through IP₃ receptors or ryanodine receptors (Ghosh et al., 1994). An effect mediated through IP₃ in the present study cannot be excluded, since Sph-1-P markedly increased levels of IP₃. The maximal effect of Sph-1-P on PIP₂ hydrolysis is stronger and more prolonged than that of PDGF, shifting the actin cycle to favor actin filament disassembly. The effect of Sph-1-P on calcium mobilization is also stronger, and calcium levels are increased in the entire cytosol of cells stimulated with concentrations

of Sph-1-P that inhibit chemotaxis, whereas the effect of PDGF is more localized. By disturbing the local balance of PIP₂ and calcium, exogenously added Sph-1-P may inhibit PDGF-induced events leading to localized actin filament disassembly and subsequent reassembly (see Fig. 12). An increase in endogenous levels of Sph-1-P following PDGF stimulation may also be involved in regulation of cytoskeletal reorganization required for cell movement.

The increased levels of intracellular calcium following stimulation with Sph-1-P lead to increased formation of cAMP. The fact that depletion of intracellular calcium stores by pretreatment with thapsigargin blocks the mobilization of calcium and generation of cAMP by Sph-1-P indicates that Sph-1-P may act through activation of calcium-dependent adenylate cyclase(s) with a subsequent activation of PKA. Activation of PKA leads to actin filament disassembly in many cell types. Numerous cytoskeletal proteins are phosphorylated by PKA, and elevation of cAMP has been found to result in disruption of actin filaments, diffusion of integrins from adhesion sites (Lampugnani et al., 1990; Glass et al., 1993) and inhibition of cell migration (Lampugnani et al., 1990). This is consistent with results from the SMC, where activation of PKA by forskolin inhibits PDGF-induced migration, and results in loss of actin filaments (data not shown). Down-regulation of the PKA catalytic activity, or inhibition of PKA with the selective inhibitor H-89, partly reverses the effects of Sph-1-P on migration, spreading and actin filament disassembly. Thus, part of the effects of Sph-1-P on migration and actin filament disassembly may be explained by activation of PKA.

Compartmentalized Changes in the Extent and Kinetics of Particular Signal Transduction Pathways May Be Critical to Cytoskeletal Connections to the Plasma Membrane

In human SMC, $\alpha 2\beta 1$ integrins seem to mediate migration toward PDGF-BB on collagen type I (Skinner et al., 1994). In the presence of Sph-1-P, localization of $\alpha 2\beta 1$ integrin (and vinculin) into focal adhesion sites is markedly slower than in the absence of Sph-1-P. In general, focal adhesion sites are also sites for actin filament attachment to the plasma membrane and actin nucleation sites (for review see Mitchison, 1992). The slower time-course of formation of focal adhesion sites when SMC are allowed to spread in the presence of Sph-1-P is likely to be related to formation of actin nucleation sites in the plasma membrane. Interestingly, it has recently been shown that localization of integrins in focal adhesion plaques seems to be required for their ability to mediate cell migration (Zhang et al., 1993). The impaired localization of $\alpha 2\beta 1$ integrins and other components of focal adhesion plaques in presence of Sph-1-P may lead to inhibition of integrin-transmembrane signaling.

Cell adhesion to the matrix is a requisite for cellular movement involving the generation of tractional forces. These cell-matrix interactions must be made and broken in a temporally coordinated fashion during the process of cell translocation. It is known that the chemotactic response to growth factors is bell-shaped (Grotendorst et al., 1982). This is most likely due to a loss of the chemotactic

gradient at higher concentrations of growth factors, which affects gradients of both receptor occupancy and intracellular signaling events. It is also known that PDGF stimulation of 3T3 cells induces tyrosine phosphorylation of paxillin and p125^{FAK}, both proteins associated with focal adhesions, with a bell-shaped dose response curve. In contrast, phosphorylation of PLC γ exhibits a sigmoidal dose-response curve (Rankin and Rozengurt, 1994). It has been proposed that in these cells, higher concentrations of PDGF disrupt the actin cytoskeleton, possibly through activation of PLC γ , stimulation of PIP₂ hydrolysis and calcium mobilization. PDGF is known to induce rapid and time-dependent alterations in the distribution of vinculin and actin (Herman and Pledger, 1985; Mellström et al., 1988; Latham et al., 1994). Consequently, higher concentrations of PDGF (greater than those giving a maximal chemotactic response) may be ineffective due to the dependence of particular signal transduction pathways on cytoskeletal integrity.

In our studies, Sph-1-P mimicked PDGF's early and rapid induction of actin disassembly, and induction of PI-turnover and calcium mobilization. However, PDGF-induced PI turnover is rapid and transient, while Sph-1-P changes in PI turnover are greater, and more sustained, than those induced by maximal concentrations of PDGF. Sph-1-P-stimulated calcium mobilization is also greater than that induced by PDGF and, in contrast to PDGF, the increase in calcium is propagated throughout the cytoplasm. Therefore, it is possible that the kinetics of the PDGF signaling response and/or the spatial localization of a particular response may be critical to allow the dynamic interaction of the cell with its matrix required for cell locomotion. Understanding which changes in intracellular signaling molecules are sufficient to inhibit migration, and whether endogenously produced Sph-1-P is involved in regulation of the signal transduction pathways, should allow further definition of the necessary cross-talk between growth factor-induced signaling and the cytoskeleton.

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