Intracellular signal transduction for migration and actin remodeling in vascular smooth muscle cells after sphingosylphosphorylcholine stimulation

Sheng Li,1,* Hideyuki Tanaka,2,# Hong Hui Wang,1 Shinji Yoshiyama,1 Hiroyuki Kumagai,1 Akio Nakamura,1 Dawn L. Brown,3 Sean E. Thatcher,3 Gary L. Wright,3 and Kazuhiro Kohama1

1Department of Molecular and Cellular Pharmacology, Gunma University Graduate School of Medicine, 2Department of Research Science, Gunma University School of Health Sciences, Gunma, Japan; and 3Department of Physiology, The Joan Edwards School of Medicine, Marshall University, Huntington, West Virginia

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Li, Sheng, Hideyuki Tanaka, Hong Hui Wang, Shinji Yoshiyama, Hiroyuki Kumagai, Akio Nakamura, Dawn L. Brown, Sean E. Thatcher, Gary L. Wright, and Kazuhiro Kohama. Intracellular signal transduction for migration and actin remodeling in vascular smooth muscle cells after sphingosylphosphorylcholine stimulation. Am J Physiol Heart Circ Physiol 291: H1262–H1272, 2006; doi:10.1152/ajpheart.00901.2005.—Molecular mechanisms underlying migration of vascular smooth muscle cells (VSMCs) toward sphingosylphosphorylcholine (SPC) were analyzed in light of the hypothesis that remodeling of the actin cytoskeleton should be involved. After SPC stimulation, mitogen-activated protein kinases (MAPKs), including p38 MAPK (p38) and p42/44 MAPK (p42/44), were found to be phosphorylated. Migration of cells toward SPC was reduced in the presence of SB-203580, an inhibitor of p38, but not PD-98059, an inhibitor of p42/44. Pertussis toxin (PTX), a G protein inhibitor, induced an inhibitory effect on p38 phosphorylation and VSMC migration. Myosin light chain (MLC) phosphorylation occurred after SPC stimulation with or without pretreatment with SB-203580 or PTX. The MLC kinase inhibitor ML-7 and the Rho kinase inhibitor Y-27632 inhibited MLC phosphorylation but only partially inhibited SPC-directed migration. Complete inhibition was achieved with the addition of SB-203580. After SPC stimulation, the actin cytoskeleton formed thick bundles of actin filaments around the periphery of cells, and the cells were surrounded by elongated filopodia, i.e., magunapodia. The peripheral actin bundles consisted of α- and β-actin, but magunapodia consisted exclusively of β-actin. Such a remodeling of actin was reversed by addition of SB-203580 and PTX, but not ML-7 or Y-27632. Taken together, our biochemical and morphological data confirmed the regulation of actin remodeling and suggest that VSMCs migrate toward SPC, not only by an MLC phosphorylation-dependent pathway, but also by an MLC phosphorylation-independent pathway.

arteriosclerosis; cytoskeletal remodeling

MIGRATION OF VASCULAR smooth muscle cells (VSMCs) from the media to the intima, resulting in intimal thickening, is a critical process in the development of arteriosclerosis and in restenosis after angioplasty (39). Elucidation of the mediators and knowledge of their mode of action may provide useful information for development of therapeutic treatments for these diseases (39, 43). Among the mediators (2, 6, 22, 26, 37), the report that sphingosylphosphorylcholine (SPC), generated by N-deacetylation of sphingomyelin, stimulates the migration of endothelial cells (7) and VSMCs (6) attracted our interest in its signaling transduction mechanism.

SPC can contract not only intact vascular smooth muscle tissue (35, 42), but also membrane-permeabilized cells (42), suggesting that it might act as a secondary messenger to activate protein(s) related to the intracellular signal transduction mechanism (42). However, many cellular events that occur in intact cells after SPC treatment, such as cell migration, are inhibited by pertussis toxin (PTX) (7). This suggests that SPC functions by interacting with its specific receptor, which has recently been reported to be coupled to G protein of ovarian cancer G protein-coupled receptor (GPR) 1, GPR4, and GPR12 (20, 32, 45). Mitogen-activated protein kinases (MAPKs), including p38 MAPK (p38) (9) and p42/44 MAPK (p42/44) (45), are located downstream from GPR. Both p38 and p42/44 are known to be phosphorylated in response to various chemotactic agents (15, 21, 22, 30). It is known that SPC can phosphorylate MAPKs in VSMC (9), whereas the effect of MAPKs on SPC-directed migration remains unclear.

Several isoforms of actin, classified as α-, β-, and γ-actin, have been identified in a variety of cells (see Ref. 23 for comparison). Although the primary structure of actin is highly conserved among the isoforms, chemical stimulation results in compartmentalization of α- and β-actin. For example, phorbol ester stimulation caused remodeling of the α-actin cytoskeleton of VSMCs but had no effect on the β-actin cytoskeleton (12, 29). Remodeling of β-actin is reported in fibroblasts and other cells (47, 50, 52). The main form of myosin in quiescent cells is the dephosphorylated form. The active form, which interacts with actin, is generated by phosphorylation of the 20-kDa regulatory myosin light chain (MLC20), a process that is catalyzed by MLC kinase (1, 2, 25). However, migration in VSMCs can be initiated without elevation of the phosphorylation level of MLC20. The finding that migration of VSMCs toward platelet-derived growth factor (PDGF)-BB was not associated with an elevation in MLC20 (25) suggests an alternative pathway of signal transduction. For example, VSMCs are the dephosphorylated form (13).

In the present study, we show that SPC can induce VSMC migration. We then investigate whether phosphorylation of

* S. Li and H. Tanaka contributed equally to this work.

Address for reprint requests and other correspondence: K. Kohama, Dept. of Molecular and Cellular Pharmacology, Faculty of Medicine, Gunma Univ. Graduate School of Medicine, 3-39-22 Showa-Machi, Maebashi, Gunma 371-8511, Japan (e-mail: kohamak@med.gunma-u.ac.jp).
MAPKs and MLC20 is involved in the SPC-directed migration of VSMCs. Remodeling of the actin cytoskeleton after SPC treatment is also described.

MATERIALS AND METHODS

Materials. SPC was purchased from Merck/EMD Bioscience (Darmstadt, Germany) and dissolved in chloroform-methanol (2:1, vol/vol). SB-203580, PD-98059, and PTX were purchased from Calbiochem (Darmstadt, Germany) and indomethacin and ML-7 from Sigma (St. Louis, MO). Y-27632 was generously donated by Yoshitomi Pharmaceutical Industries (Osaka, Japan). Fluorescein isothiocyanate (FITC)-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR), fura 2-AM from Dojin Laboratories (Kumamoto, Japan), monocular antibody (clone MY21) against MLC20 from Sigma, phosphorylated p38 MAPK (Thr180/Tyr182) and phosphorylated p42/44 MAPK (Thr202/Tyr204) antibodies from Cell Signaling Technology (Beverly, MA), and α-actin (clone 1A4) and β-actin (clone AC-15) antibodies from Sigma.

Cell culture. GbaSM-4 cells, a VSMC line derived from the brain basilar artery of guinea pigs (5), were maintained in Dulbecco’s air atmosphere). The cells were grown to confluence, and quiescence was achieved by overnight serum starvation.

Cell migration. GbaSM-4 cells, a VSMC line derived from the brain basilar artery of guinea pigs (5), were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a CO2 incubator (humidified 5% CO2-95% air atmosphere). The cells were grown to ~90% confluence, and quiescence was achieved by overnight serum starvation.

β-Actin-enhanced green fluorescent protein plasmid transfection. GbaSM-4 cells were transfected with α,β-actin-enhanced green fluorescent protein (EGFP) expression plasmid (Clontech, Palo Alto, CA), as described by Fultz et al. (12).

Migration assay. Cell migration was monitored using a Boyden chamber (Neuroprobe, Gaithersburg, MD) as previously described (25). In all experiments, collagen-coated 8-μm pore polycarbonate filters were used. GbaSM-4 cells (10^4 cells/well) suspended in serum-free DMEM containing 0.5% bovine serum albumin (DMEM-BSA) were loaded into the upper wells of the chamber. The lower wells were filled with DMEM-BSA containing 0.001–100 μmol/l SPC or vehicle. After 8 h of incubation at 37°C, the cells that had migrated to the lower surface of the filter were fixed in methanol, stained with Giemsa solution, and observed using a light microscope. Each condition was tested in quadruplicate, and the number of cells from a randomly chosen high-power field (×400 magnification) was counted in each well. In some experiments, the cells were pretreated with various inhibitors for 1 h. During the assay, the inhibitors at the same concentration were added to the upper and lower wells. The migration assay was performed in at least three independent experiments.

p38 and p42/44 activation assay. Treatment of GbaSM-4 cells with various agents was terminated by addition of trichloroacetic acid, and the total cellular proteins were extracted with SDS-sample buffer as previously described (2). An equal amount of protein (20 μg/lane) was separated by SDS-PAGE and transferred to a polyvinylindene difluoride membrane (Millipore, Billerica, MA). For determination of the phosphorylation activity of p38 and p42/44, the membrane was incubated with each phosphospecific antibody (36). Immunoreactivity was visualized using an enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ). The blots were scanned and quantified by densitometry using the Scion Image program (Beta 4.02 version for Windows, Scion, Frederick, MD).

MLC20 phosphorylation assay. MLC20 phosphorylation levels were determined by glycerol-PAGE followed by Western blot as previously described (25). Briefly, after treatment with various agents, the cells were fixed by the addition of trichloroacetic acid, and the total proteins were extracted into urea-sample buffer. Phosphorylated and unphosphorylated MLC20 was detected by Western blot using a monoclonal antibody (MY-21). The density of each band was quantified by densitometry as described above. MLC20 phosphorylation (monophosphorylated + diphosphorylated) is expressed as a percentage of total MLC20.

Confocal microscopy. Cells on collagen-coated glass coverslips were treated with various agents, fixed with 4% paraformaldehyde, and permeabilized with Triton X-100. The cells were blocked with 5% BSA in PBS and incubated with FITC-phalloidin (17) for examination of the total actin cytoskeleton or with α- and β-actin antibodies for examination of α- and β-actin cytoskeletal compartmentation (12). Coverslips were mounted with 20% (wt/vol) Mowiol 4-88 (Calbiochem) in PBS and viewed using a confocal laser scanning microscope (model MRC-1024, Bio-Rad Laboratories, Hercules, CA). Microscopy was carried out with at least three independent cultures. Live cell preparations expressing β-actin-EGFP fusion protein were continuously observed without fixation and permeabilization.

Measurement of intracellular Ca2+ concentration. Ca2+ concentration in GbaSM-4 cells on SPC stimulation was monitored using an Aquacosmos system equipped with an Orca II cold charge-coupled device (CCD) camera (Hamamatsu) as described by Kanzaki et al. (24). Fura 2-AM incorporated into the cells in modified Hanks’ solution was excited alternately at 340 and 380 nm, with emission at 510 nm. The ratio of the excitation signals was used to indicate intracellular Ca2+ concentration.

Statistical analysis. Values are means ± SE. Statistical significance was determined by one-way ANOVA and Newman-Keuls post hoc multiple comparison test. P < 0.05 was considered to be statistically significant.

RESULTS

VSMC migration toward SPC. GbaSM-4 cells that were added to the upper wells of the Boyden chamber and allowed to move to the lower wells, which contained 0.01–100 μmol/l SPC, migrated toward SPC in a dose-dependent manner (Fig. 1A). The greatest number of migrating cells occurred at 1 μmol/l SPC. The number of migrated cells decreased at higher SPC concentrations, yielding a typical bell-shaped dose-response curve.

Phosphorylation of p38 and p42/44 in SPC-treated cells. To test whether SPC can activate p38 and p42/44, we treated the cells with 1 μmol/l SPC for 2 min to 8 h and used Western blot with specific antibodies to detect the phosphorylated forms of p38 and p42/44 (36). SPC induced a transient increase in the phosphorylation of p38 and p42/44 (Fig. 1, A and C). Phosphorylation levels of p38 and p42/44 were maximal 15 min after SPC stimulation, with 3.8 ± 0.4-fold (n = 3) and 4.2 ± 0.6-fold (n = 3) increases, respectively, above the basal levels, and then declined, with a return to the basal levels after 1 and 2 h, respectively.

Involvement of p38, but not p42/44, in VSMC migration toward SPC. To investigate the possible involvement of p38 phosphorylation in SPC-directed VSMC migration, we pretreated GbaSM-4 cells with SB-203580, a compound that specifically inhibits p38 phosphorylation (22), and then stimulated the cells with 1 μmol/l SPC. SB-203580 caused dose-dependent inhibition of p38 phosphorylation to the basal level (Fig. 2, A and B). Migration assay showed a partial but significant reduction of migrated cells from 68 ± 9 (n = 3) in the absence of SB-203580 to 33 ± 6 (n = 3) in the presence of SB-203580.

To investigate whether p42/44 is involved in SPC-directed migration, we examined the effect of PD-98059, a specific inhibitor of MAPK kinase, which is the upstream activator of p42/44 (22). PD-98059 (50 μmol/l) reduced the maximal phosphorylation level of p42/44 from that stimulated by 1 μmol/l SPC to the basal level (Fig. 3, A and B). However, in...
sharp contrast to SB-203580, 5–50 μmol/l PD-98059 failed to block VSMC migration toward SPC (cf. Fig. 3C with Fig. 2C). These data suggest that phosphorylation of p38, but not p42/44, is functionally associated with SPC-directed migration. We also examined whether SB-203580 and PD-98059 inhibited the phosphorylation of p42/44 and p38, respectively. We failed to observe an inhibitory effect on these MAPK kinases (Figs. 2A and Fig. 3A), confirming the specificity of the inhibitors (see DISCUSSION).

Effect of SB-203580 and PTX on phosphorylation of MLC20. The time course of MLC20 phosphorylation after stimulation with 1 μmol/l SPC is shown in Fig. 4A. In the absence of SPC, the basal level of MLC20 phosphorylation was 29.1 ± 5.3% (n = 3). At 2 min after SPC stimulation, the level significantly increased to 93.78 ± 3.7% (n = 3). After 10 min of stimulation, the MLC20 phosphorylation level de-
there was no further decrease during the 8 h of experimentation. We then investigated whether SB-203580 at 50 μmol/l, the concentration that effectively inhibited p38 phosphorylation, could affect MLC20 phosphorylation. The time course of MLC20 phosphorylation in the presence of SB-203580 for 1 h and stimulated with 1 μmol/l SPC or vehicle for 15 min. A: p38 (top) and p42/44 (bottom) phosphorylation detected by Western blot. B: densitometric quantification of phosphorylation of p38 and p42/44. **P < 0.01 vs. control (SPC + 0 PD-98059). C: migration toward 1 μmol/l SPC in cells pretreated with PD-98059 for 1 h. PD-98059 failed to inhibit migration.

Because the effects of SB-203580 mimic those of PTX, as described above, we examined MLC20 phosphorylation in the presence and absence of 100 ng/ml PTX. After 2 min of SPC stimulation, MLC20 phosphorylation proceeded to 90–100%, irrespective of the presence of PTX (Fig. 4B). We confirmed that the time course of MLC20 phosphorylation in the presence of PTX was similar to that in the absence of PTX during 8 h of culture (data not shown). These data are consistent with the idea that Gi protein is located upstream from p38 in the SPC-directed migration.

Relation between migratory activity and MLC20 phosphorylation. Do changes in MLC phosphorylation modify migration to SPC? To answer this question, we investigated the effects of ML-7, a known inhibitor of MLC kinase (1), and Y-27632, an inhibitor of Rho kinase (2). After culture of GbaSM-4 cells in 10 μmol/l ML-7 or 10 μmol/l Y-27632, 1 μmol/l SPC was added. As shown in Fig. 5B, ML-7 or Y-27632 totally abolished the elevation of MLC20 phosphorylation. Such a low level was maintained during 8 h of experimentation (data not shown). In vehicle control migration assay (Fig. 5C), 73 ± 9 (n = 3) cells migrated toward 1 μmol/l SPC, whereas ML-7 and Y-27632 significantly reduced the number of migrating cells to 39 ± 5 (n = 3) and 36 ± 6 (n = 3), respectively. The inhibition was only partial, because in...
unstimulated samples only 14 ± 3 (n = 3) cells migrated. When SB-203580 was combined with ML-7 or Y-27632, however, migration was inhibited to the unstimulated control level. The implication of the additive features of these inhibitors is discussed in relation to MLC20 phosphorylation-dependent and -independent pathways of intracellular signal transduction for migration of VSMCs toward SPC.

**Fig. 5. Independence of the p38-related pathway from the MLC kinase-related pathway. A: effect of SB-203580 on MLC20 phosphorylation in response to SPC. GbaSM-4 cells were pretreated with 50 μmol/l SB-203580 or vehicle for 1 h, and SPC was added at a final concentration of 1 μmol/l for 2 min to 8 h. Total protein was extracted and subjected to Western blot using MLC20 antibody. Top: typical immunoblot. Bottom: quantification of MLC20 phosphorylation from 3 independent experiments. *P < 0.05; **P < 0.01 vs. unstimulated cells without SB-203580. †P < 0.05 vs. unstimulated cells with SB-203580. ††P < 0.01 vs. unstimulated cells with SB-203580. SB-203580 did not affect MLC20 phosphorylation. B: effects of ML-7 and Y-27632 on MLC20 phosphorylation in response to SPC. GbaSM-4 cells were pretreated with 10 μmol/l ML-7 or 10 μmol/l Y-27632 for 1 h, and MLC20 phosphorylation was examined 2 min after treatment with 1 μmol/l SPC. Top: representative immunoblot. Bottom: densitometric quantification of MLC20 phosphorylation. ***P < 0.01 vs. SPC alone. C: additive nature of effects of inhibitors on migration toward SPC in cells pretreated with 10 μmol/l ML-7, 10 μmol/l Y-27632, and/or 50 μmol/l SB-203580 and allowed to migrate toward 1 μmol/l SPC. *P < 0.05 vs. unstimulated cells without SB-203580. †P < 0.05 vs. unstimulated cells with SB-203580.**

To observe magunapodia formation after SPC stimulation, we expressed actin-EGFP in the living cells and observed the time course of their development. As shown in Fig. 6II, A, control cells had no peripheral cytoskeleton, few lamellipodia, and no filopodia. After SPC stimulation, intense staining at the cell periphery and lamellipodia developed with time (Fig. 6II, B–D).

The expressed actin-EGFP in Fig. 6II was β-actin. To determine whether α-actin is involved in magunapodia formation, we stained the SPC-treated cells with antibodies against α- and β-actin immediately after 15 min of SPC stimulation (Fig. 7). When observed just above the glass surface (<0.5 μm), β-actin antibody stained the entire actin cytoskeleton, including thick actin bundles, wide lamellipodia, and magunapodia (Fig. 7A). With the α-actin antibody, staining was limited to the cell periphery (Fig. 7B). On close observation, α-actin was present in the thick actin bundle and wide lamellipodia, but only at the foot of the magunapodia (cf. Fig. 7B). The yellow merge was detected in the thick actin bundles, but not in the magunapodia (Fig. 7C). Thus the dominant isoform of actin in the magunapodia characteristic of SPC treatment was β-actin. At 1.0 μm above the surface, staining of α-actin (Fig. 7E) predominated over staining of β-actin (Fig. 7D), and the actin cytoskeletal features of the magunapodia were obscured.
Effects of SB-203580 and PTX on remodeling of the actin cytoskeleton. To examine how the actin cytoskeleton of SPC-treated cells was modified by inhibitors of VSMC migration, we pretreated GbaSM-4 cells with 100 ng/ml PTX or 50 μmol/l SB-203580 for 1 h, stimulated the cells with 1 μmol/l SPC for 15 min, used FITC-phalloidin to stain for actin. These cells (Fig. 8II, A and B) maintained the features of quiescent cells before SPC treatment (Fig. 8II, A, see also Fig. 6A); i.e., thin stress fibers were parallel to each other, and development of filopodia and lamellipodia was hardly observed.

Although quantitative analysis of immunofluorescence data of the stress fibers and thick actin bundles in the cells was difficult, we were able to count the cells with and without lamellipodia and filopodia, the most characteristic change due to SPC stimulation. The incidence of filopodia was only 7.4% among cells in the control culture (Fig. 8II), increased to 92.3% with SPC stimulation, and decreased to 10.2% and 12.5% with SB-205380 and PTX treatment, respectively.

Figure 9 examines the effect of SPC and/or SB-205380 treatment on the cytoskeleton for as long as 8 h, which is required for the migration assay (Fig. 1A). Most non-SPC-stimulated cells contained thin stress fibers in association with a few lamellipodia and filopodia (Fig. 6f, A, and Fig. 8II, A). The thick actin bundles and augmented lamellipodia/filopodia (Fig. 9, A and B, see also Fig. 6f, D) were comparable to the staining patterns of the actin cytoskeleton (Fig. 6f, B–D). Such cytoskeletal features were detectable 8 h after SPC stimulation (Fig. 9C). In the presence of SB-203581, the cytoskeletal changes due to SPC were not obvious throughout the experimental period (Fig. 9, D–F).

Fig. 6. Remodeling of the actin cytoskeleton induced by SPC stimulation. I: confocal microscopic images of FITC-conjugated phalloidin staining of the actin cytoskeleton in GbaSM-4 cells before (A) and 15 min (B), 30 min (C), and 120 min (D) after addition of 1 μmol/l SPC. Scale bars, 10 μm. II: confocal microscopic images of changes in β-actin cytoskeleton of living GbaSM-4 cells transfected by the β-actin-enhanced green fluorescence protein (GFP) plasmid before (A) and 25 min (B), 45 min (C), and 75 min (D) after stimulation by 1 μmol/l SPC. Scale bar, 10 μm.

Fig. 7. Remodeling of α- and β-actin cytoskeleton in GbaSM-4 cells treated with 1 μmol/l SPC for 15 min, stained with antibodies against β-actin (A and D) and α-actin (B and E), and observed with a confocal microscope at 1.0 μm (A–C) and <0.5 μm (D–F) above the substrate. C and F: merged images.
Effect of ML-7 and Y-27632 on remodeling of the actin cytoskeleton. GbaSM-4 cells pretreated with 10 μmol/l ML-7 and stimulated with 1 μmol/l SPC did not retain the features of the actin cytoskeleton characteristic of quiescent cells: formation of filopodia and lamellipodia was reduced to 15.6%, and stress fibers were arranged in random directions (Fig. 8 II, E).

Among cells pretreated with 10 μmol/l Y-27632 and stimulated with SPC, only 5.1% formed filopodia and lamellipodia, and stress fibers appeared as dots (Fig. 8 II, F).

As controls of the above inhibitors, we observed the actin cytoskeleton of the cells cultured in 100 ng/l PTX, 50 μmol/l SB-203580, 10 μmol/l ML-7, or 10 μmol/l Y-27632 without SPC stimulation. PTX (Fig. 8 I, A) and SB-203580 (Fig. 8 I, B) did not alter the actin cytoskeleton. However, ML-7 and Y-27632 modified the cytoskeleton: ML-7 tended to increase the number of stress fibers (Fig. 8 I, C), and Y-27632 tended to disrupt the stress fibers into small fragments (Fig. 8 I, D).

Taken together with the cytoskeletal studies of the inhibitors of migration, we concluded that MLC kinase, which is inhibited by ML-7, and Rho kinase, which is inhibited by Y-27632, were not located downstream from the SPC receptor-Gi protein-p38 signal transduction cascade, an interpretation that is consistent with previous findings.

**Fig. 8.** Effect of inhibitors of signal transduction pathways on remodeling of the actin cytoskeleton. I: confocal microscopic images of FITC-phalloidin-stained GbaSM-4 cells pretreated for 1 h with 50 μmol/l SB-203580 (A), 100 ng/ml PTX (B), 10 μmol/l ML-7 (C), and 10 μmol/l Y-27632 (D). II: confocal microscopic images 15 min after stimulation with 1 μmol/l SPC in FITC-phalloidin-stained GbaSM-4 cells pretreated with SB-203580 (C), PTX (D), ML-7 (E), and Y-27632 (F). Circles show percentage of cells with filopodia among 100–200 cells. A: cells cultured in the presence of vehicle. B: cells pretreated with vehicle and stimulated with SPC for 15 min. Scale bars, 10 μm.
consistent with the biochemical data (Figs. 4 and 5). We also concluded that development of magunapodia by remodeling of the β-actin cytoskeleton, rather than the thick actin bundle composed of α- and β-actin, has an important role in migration to SPC.

**DISCUSSION**

The present study shows that SPC acts on VSMCs to induce MAPK p38 activation, MLC20 phosphorylation, actin cytoskeletal remodeling, and cell migration. PTX and SB-203580 blocked activation of MAPK p38 and remodeling of the cytoskeleton without changing the level of MLC20 phosphorylation but partially (~50%) reduced cell migration. An explanation for this finding is that SPC acts partially through a G protein-coupled receptor to activate p38, which initiates the cytoskeletal remodeling involved in cell migration (Fig. 10). Use of ML-7 to inhibit MLC kinase and Y-27632 to inhibit Rho kinase reduced the SPC-induced increase in MLC20 phosphorylation to the basal level. ML-7 and Y-27632 partially (~50%) blocked cell migration and partially affected cytoskeletal remodeling. These partial effects suggest a second pathway that involves MLC kinase and myosin ATPase (Fig. 10). As schematized in Fig. 10, there is an interplay between pathways that regulate MLC20 phosphorylation and those that regulate MAPK p38.

Activation of p38 by phosphorylation reached a maximum 15 min after SPC stimulation (Fig. 1), which was associated with remodeling of the actin cytoskeleton as characterized by development of magunapodia (Fig. 6). Although phosphorylation of p38 returned to the unstimulated control level within ~1 h (Fig. 1), magunapodia persisted for as long as 8 h (Fig. 9), which allowed GbaSM-4 cells to migrate in the Boyden chamber. Because development of magunapodia indicates high migration activity (19), the persistence can explain the time difference between the assays for phosphorylation of p38 and migration.

The actin cytoskeleton is obvious, even in non-SPC-stimulated GbaSM-4 cells (Fig. 8, A), and ML-7 and Y-27632 have profound effects on the cytoskeletal structure (Fig. 8, C and D). In the unstimulated cells, MLC20 was phosphorylated at ~30% (Figs. 4B and 5B), and ML-7 and Y27632 reduced the level of MLC20 phosphorylation (unpublished observation). We could relate the cytoskeletal change to MLC20 phosphorylation. However, it was difficult to relate the change to cell movement, because the cells hardly move. The effect of ML-7 and Y-27632 on SPC-induced actin rearrangement was associated with inhibition of cell movement and MLC20 phosphorylation, but only partial (~50%) inhibition of cell movement was observed (see above). Therefore, it is possible that the MAPK p38 pathway regulates cell movement. We are confi-

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**Fig. 9.** Actin cytoskeleton in GbaSM-4 cells during prolonged treatment of cells with SPC and/or SB-203580. A–C: cells cultured for 1, 2, and 8 h in the presence of 1 μmol/l SPC, fixed, stained by FITC-phalloidin, and viewed under a confocal microscope. D–F: cells cultured for 1, 2, and 8 h in the presence of 1 μmol/l SPC and 50 μmol/l SB-203580, fixed, stained by FITC-phalloidin, and viewed under a confocal microscope. Scale bars, 10 μm.

**Fig. 10.** Proposed cascades of signal transduction regulating VSMC migration toward SPC. One cascade is characterized by an SPC-G protein-coupled receptor (Receptor) interaction, which is mediated by G protein. MAPK p38 is proposed to mediate actin remodeling and induction of VSMC migration. MLC kinase is not located downstream from this cascade. The other cascade is related to MLC kinase, which phosphorylates MLC20 to activate myosin ATPase. Both cascades are required to remodel the actin cytoskeleton, causing migration to SPC.
consistent with previous findings (41), our data indicate that SPC can induce remodeling of the actin cytoskeleton. A growing body of evidence suggests that p38 is involved in cell migration by remodeling the actin cytoskeleton (4, 18, 31). The p38 pathway is a known downstream target of Cdc42 and Rac (11, 28, 46). These small G proteins are particularly important in the formation of polarized actin-containing structures such as filopodia and lamellipodia at the cell periphery (19). Activated p38 might phosphorylate actin-binding heat shock protein 27 to enhance actin polymerization, which is necessary for migration (17, 18, 31).

Numerous reports indicate that MLC20 phosphorylation is required for migration of VSMCs toward chemoattractants (1, 2, 25). Indeed, pretreatment of the cells with ML-7 or Y-27632 abolished the increase in MLC20 phosphorylation in response to SPC (Fig. 5B); however, these compounds only partially blocked the SPC-directed migration of VSMCs (Fig. 5C). This observation indicates that part of SPC-directed migration can occur without elevation of MLC20 phosphorylation. This finding is consistent with our previous observation that VSMCs migrate toward PDGF-BB without changing the MLC20 phosphorylation level (25).

Chin and Chueh (9) reported that SPC mediates Grcoupled increases in intracellular Ca2+ in a smooth muscle cell line in a dose-dependent manner (9). We found little inhibition of MLC phosphorylation by PTX, a Gi protein inhibitor (Fig. 4C). If we assume that SPC acts through a Ca2+-dependent pathway involving calmodulin and MLC kinase for MLC phosphorylation, we question whether SPC elevates Ca2+ in GbaSM-4 cells. To answer the question, we measured the intracellular Ca2+ concentration after stimulation by 100 μmol/l SPC. Ca2+ concentration was not increased by 1 and 10 μmol/l SPC, but a transient increase was observed at 100 μmol/l SPC (Fig. 11B). We interpret this result to indicate that Ca2+-dependent phosphorylation by MLC kinase is not the only cause of SPC-induced MLC phosphorylation. Other kinases that are able to phosphorylate MLC without Ca2+, such as Rho kinase (3), MAPKAP kinase (27), ZIP kinase (34), and integrin-linked kinase (44), may have contributed to our results. Furthermore, it is possible that Rho kinase causes Ca-independent inhibition of MLC phosphatase (3). However, GbaSM-4 migrated, even though SPC was increased to 100 μmol/l (Fig. 1A). In such a case, there may be cross talk between the pathways proposed in this study and the Ca2+-dependent pathway.

In conclusion, we have demonstrated that SPC can stimulate VSMC migration and that MLC20 phosphorylation-dependent and -independent pathways are involved in cell migration. An SPC receptor-Gc-p38 signal cascade is important for actin reorganization and cell migration in response to SPC. Inhibition of these pathways might have interesting therapeutic applications for controlling VSMC migration associated with arteriosclerosis and restenosis.

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Present address of S. Li: Department of Neurology, First Affiliated Hospital, Dalian Medical University, Dalian 116011, People’s Republic of China.

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SPC-DIRECTED VSMC MIGRATION WITH ACTIN REMODELING

Fig. 11. Effect of indomethacin on phosphorylation of p38 and changes in intracellular Ca2+ concentration. A: Western blot of p38 phosphorylation in GbaSM-4 cells pretreated with vehicle (0) or 10–50 μmol/l indomethacin and then with vehicle (0) or 1 μmol/l SPC. B: changes in intracellular Ca2+ concentration expressed as ratio of fluorescence at 340 nm to fluorescence at 380 nm (F340/F380) in fura 2-loaded cells treated with 1, 10, and 100 μmol/l SPC. As a positive control, cells were stimulated with 100 mmol/l KCl (in place of 100 mmol/l NaCl). Vehicle was used for negative control (traces not shown).

Consistent with previous findings (41), our data indicate that SPC can induce remodeling of the actin cytoskeleton. A growing body of evidence suggests that p38 is involved in cell migration by remodeling the actin cytoskeleton (4, 18, 31). The p38 pathway is a known downstream target of Cdc42 and Rac (11, 28, 46). These small G proteins are particularly important in the formation of polarized actin-containing structures such as filopodia and lamellipodia at the cell periphery (19). Activated p38 might phosphorylate actin-binding heat shock protein 27 to enhance actin polymerization, which is necessary for migration (17, 18, 31).

Numerous reports indicate that MLC20 phosphorylation is required for migration of VSMCs toward chemoattractants (1, 2, 25). Indeed, pretreatment of the cells with ML-7 or Y-27632 abolished the increase in MLC20 phosphorylation in response to SPC (Fig. 5B); however, these compounds only partially blocked the SPC-directed migration of VSMCs (Fig. 5C). This observation indicates that part of SPC-directed migration can occur without elevation of MLC20 phosphorylation. This finding is consistent with our previous observation that VSMCs migrate toward PDGF-BB without changing the MLC20 phosphorylation level (25).

Chin and Chueh (9) reported that SPC mediates Gc-coupled increases in intracellular Ca2+ in a smooth muscle cell line in a dose-dependent manner (9). We found little inhibition of MLC phosphorylation by PTX, a Gi protein inhibitor (Fig. 4C). If we assume that SPC acts through a Ca2+-dependent pathway involving calmodulin and MLC kinase for MLC phosphorylation, we question whether SPC elevates Ca2+ in GbaSM-4 cells. To answer the question, we measured the intracellular Ca2+ concentration after stimulation by 100 μmol/l SPC. Ca2+ concentration was not increased by 1 and 10 μmol/l SPC, but a transient increase was observed at 100 μmol/l SPC (Fig. 11B). We interpret this result to indicate that Ca2+-dependent phosphorylation by MLC kinase is not the only cause of SPC-induced MLC phosphorylation. Other kinases that are able to phosphorylate MLC without Ca2+, such as Rho kinase (3), MAPKAP kinase (27), ZIP kinase (34), and integrin-linked kinase (44), may have contributed to our results. Furthermore, it is possible that Rho kinase causes Ca-independent inhibition of MLC phosphatase (3). However, GbaSM-4 migrated, even though SPC was increased to 100 μmol/l (Fig. 1A). In such a case, there may be cross talk between the pathways proposed in this study and the Ca2+-dependent pathway.

In conclusion, we have demonstrated that SPC can stimulate VSMC migration and that MLC20 phosphorylation-dependent and -independent pathways are involved in cell migration. An SPC receptor-Gc-p38 signal cascade is important for actin reorganization and cell migration in response to SPC. Inhibition of these pathways might have interesting therapeutic applications for controlling VSMC migration associated with arteriosclerosis and restenosis.

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Present address of S. Li: Department of Neurology, First Affiliated Hospital, Dalian Medical University, Dalian 116011, People’s Republic of China.

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