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# $G_{12/13}$ and $G_q$ mediate $S1P_2$-induced inhibition of Rac and migration in vascular smooth muscle in a manner dependent on Rho but not Rho kinase

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**Aims** The lysophospholipid mediator sphingosine-1-phosphate (S1P) activates G protein-coupled receptors (GPCRs) to induce potent inhibition of platelet-derived growth factor (PDGF)-induced Rac activation and, thereby, chemotaxis in rat vascular smooth muscle cells (VSMCs). We explored the heterotrimeric G protein and the downstream mechanism that mediated S1P inhibition of Rac and cell migration in VSMCs.

**Methods and results** S1P inhibition of PDGF-induced cell migration and Rac activation in VSMCs was abolished by the selective $S1P_2$ receptor antagonist JTE-013. The C-terminal peptides of Gα subunits (Gα-CTs) act as specific inhibitors of respective G protein-GPCR coupling. Adenovirus-mediated expression of $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and $G\alpha_q$-CT, but not that of $G\alpha_s$-CT or LacZ or pertussis toxin treatment, abrogated S1P inhibition of PDGF-induced Rac activation and migration, indicating that both $G_{12/13}$ and $G_q$ classes are necessary for the S1P inhibition. The expression of $G\alpha_q$-CT as well as $G\alpha_{12}$-CT and $G\alpha_{13}$-CT also abolished S1P-induced Rho stimulation. C3 toxin, but not a Rho kinase inhibitor or a dominant negative form of Rho kinase, abolished S1P inhibition of PDGF-induced Rac activation and cell migration. The angiotensin II receptor $AT_1$, which robustly couples to $G_q$, did not mediate either Rho activation or inhibition of PDGF-induced Rac activation or migration, suggesting that activation of $G_q$ alone was not sufficient for Rho activation and resultant Rac inhibition. However, the $AT_1$ receptor fused to $G\alpha_{12}$ was able to induce not only Rho stimulation but also inhibition of PDGF-induced Rac activation and migration. Phospholipase C inhibition did not affect S1P-induced Rho activation, and protein kinase C activation by a phorbol ester did not mimic S1P action, suggesting that S1P inhibition of migration or Rac was not dependent on the phospholipase C pathway.

**Conclusion** These observations together suggest that $S1P_2$ mediates inhibition of Rac and migration through the coordinated action of $G_{12/13}$ and $G_q$ for Rho activation in VSMCs.

## 1. Introduction

Migration of medial vascular smooth muscle cells (VSMCs) into the intima, together with the targeting mobilization and differentiation into VSMCs of bone-marrow-derived vascular progenitor cells, is a crucial step that gives rise to the formation of vascular proliferative lesions including atheroma and post-angioplasty restenosis.[1,2] The control of vascular cell motility may confer a useful means to treat vascular proliferative lesions. Sphingosine-1-phosphate (S1P) is a recently established lipid mediator that exerts pleiotropic effects on diverse cell types including VSMCs and vascular endothelial cells.[3–6] These include regulation of cell migration, cell shape and cell-to-cell adhesion, and stimulation of mitogenesis, most of which are mediated via S1P-specific G protein-coupled receptor (GPCR) family, including $S1P_1$ receptor ($S1P_1R$), $S1P_2R$, and $S1P_3R$.[3–6] S1P exhibits unique bimodal activities as an extracellular regulator on cell motility. It stimulates or inhibits cell migration, in a manner apparently dependent on cell types;[7] S1P stimulates migration of vascular endothelial cells,[6] whereas S1P inhibits migration of VSMCs.[8] We previously demonstrated that this bimodal regulation by S1P is based upon a diversity of S1P receptor subtypes, which mediate receptor subtype-specific stimulatory or inhibitory regulation for cell migration.[9] Thus, $S1P_1R$ and

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$S1P_3R$ act as attractant receptors to mediate migration directed towards S1P, whereas $S1P_2R$ acts as a repellant receptor to mediate inhibition of chemotaxis towards a chemoattractant.

Rho family small GTPases, primarily Rac, Cdc42, and Rho, are well-known regulators of actin reorganization and myosin motor function and thereby of cell motility.[10] We observed that platelet-derived growth factor (PDGF) stimulates Rac, but not Cdc42 or Rho in VSMCs, and that Rac plays an essential role in PDGF-directed chemotaxis.[8] We found in heterologous expression of each S1P receptor subtype that the attractant receptors $S1P_1R$ and $S1P_3R$ mediate stimulation of Rac, whereas the repellant receptor $S1P_2R$ inhibits chemoattractant-induced Rac activation.[9] Thus, S1PRs exert bimodal regulation on cell migration primarily through positively or negatively regulating cellular Rac activity. In VSMCs, S1P induced inhibition of PDGF-triggered Rac activation as well as migration.[8]

Pleiotropic actions of GPCRs are mediated primarily by four classes of heterotrimeric G proteins, $G_s$, $G_i$, $G_q$, and $G_{12/13}$. Several GPCRs including prostaglandin $E_2$ ($PGE_2$) receptors, dopamine receptors, and adrenomedullin receptors mediate inhibition of motility of VSMCs. These receptors are all canonical $G_s$-coupled type of receptors, and we recently showed that upon $PGE_2$ receptor stimulation, cyclic AMP mediated Rac inhibition as a mechanism for inhibition of VSMC migration.[11] Different from these receptors, $S1P_2R$ is not a $G_s$-coupled type of receptor.[12,13] It is unknown which class of heterotrimeric G protein mediates S1P-induced inhibition of VSMC migration. Also, signalling mechanisms downstream of a heterotrimeric G protein for the inhibition of Rac and migration in VSMCs are not fully defined. In the present study, we show in VSMCs that $S1P_2R$ induces Rho activation via both the $G_q$ and $G_{12/13}$ classes, which in turn mediates the inhibition of Rac and chemotaxis in a manner dependent on Rho but independent of Rho kinase.

## 2. Methods

### 2.1 Materials

S1P and human PDGF-B chain were purchased from BIOMOL (Plymouth Meeting, PA, USA) and Peprotech (London, UK), respectively. A mouse monoclonal anti-Rac1 antibody (23A8) and rabbit polyclonal anti-phospho ($Thr^{850}$)-MYPT1 (36-003) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Mouse monoclonal anti-RhoA antibody (26C4) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-MYPT1 antibody (PRB-457C) was bought from Covance (Berkley, CA, USA). Mouse monoclonal anti-myc antibody (9E10) was obtained from ATCC (Rockville, MA, USA). JTE-013 and HA-1077 were kind gifts from Japan Tabacco (Takatsuki, Japan) and Asahi Chemical Industry (Fuji, Japan), respectively. U73122 and BAPTA-AM were purchased from Merck-Calbiochem Biosciences (Darmstadt, Germany), and phorbol 12,13-dibutyrate (PDBu) and G418 were obtained from Sigma (St Louis, MI, USA). Pertussis toxin was bought from List Biological Laboratories (Campbell, CA, USA). Botulinum C3 toxin, human PAK1 [amino acids 75–131 (CRIB-domain)], and mouse Rhotekin [amino acids 7–89 (Rho-binding domain)] that were fused to glutathione-*s*-Sepharose (GST) (GST-C3, GST-PAK1, and GST-Rhotekin, respectively) were prepared as described previously.[28]

### 2.2 Plasmids and adenoviruses

The mammalian expression plasmid for $S1P_2$, pME18S-$S1P_2$, was described previously.[9] Mouse $AT_1a$ receptor DNA encoding the entire coding region cloned from mouse genomic DNA by PCR was ligated onto pME18S at B*stXI* site. The fusion receptor $AT_1$-$G\alpha_{12}$, in which the full length $G\alpha_{12}$ is fused to the C-terminus of $AT_1a$, was generated by the PCR-based method as described previously.[13] Replication-deficient adenoviruses carrying myc-tagged $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, $G\alpha_q$-CT, and $G\alpha_s$–CT were described previously.[13] The adenovirus encoding β-galactosidase (LacZ) were kindly donated by I. Saito (Institute of Medical Sciences, University of Tokyo). The cells were infected with adenoviruses at a multiplicity of 200 by incubating VSMCs with an adenovirus-containing medium for 1 h, which conferred successful gene transduction in nearly 100% of cells without significant cell damage.[8,14] After recovery in a fetal calf serum (FCS)-containing growth medium for 24 h, the cells were subjected to migration assay or serum-deprived before each experiment.

### 2.3 Cells

Rat aortic VSMCs were isolated by the explant method from an 8-week old Wistar male rat, as described previously.[8] The present investigation using experimental animals conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). VSMCs were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS (Equitech-Bio, Ingram, TX, U.S.A.), 100U/ml of penicillin, and 100 μg/ml of streptomycin (Wako, Osaka, Japan). When indicated, VSMCs were pretreated with C3 toxin (10 μg/ml) in serum-free DMEM for 72 h.[13] The VSMCs stably overexpressing either $AT_1$ or $AT_1$-$G\alpha_{12}$ were established by co-transfecting VSMCs with one of the above expression plasmids and pKM3 vector that confers G418 resistance, and selecting in the presence of 0.2 mg/ml of G418 as described previously.[14] The stable VSMC transfectants were maintained in the presence of the same concentration of G418. VSMCs were treated with 10 ng/ml pertussis toxin for 24 h when indicated. Total RNA was isolated from VSMCs and analyzed for mRNA expression by Northern blotting using $^{32}P$-labelld cDNA probe as described peviously.[8,14]

### 2.4 Transwell migration assay

Transwell migration of VSMCs was determined in a modified Boyden chamber (Neuroprobe, Gaithersburg, MD) using polycarbonate filters with 8-μm pores as described in detail previously.[8,9] VSMCs ($1 \times 10^5$) in 200 μL of serum-free DMEM containing 0.1% fatty acid-free bovine serum albumin were loaded into the upper wells, whereas the lower wells were filled with the same medium containing various concentrations of S1P, angiotensin II, and/or PDGF. The cells were allowed to migrate across the porous filter for 5 h at 37°C in a tissue culture incubator. After staining with Diff-Quick (Sysmex, Kobe, Japan) and scraping the upper surface of the filter, the number of cells that migrated to the lower side of the filter was determined by measuring optical densities at 595 nm using a 96-well microplate reader Model 3550 (Bio-Rad, Hercules, CA). There was a linear positive relationship between an optical density (OD) value and a counted number of migrated cells through pores in a range of $<0.2$ of the OD value.[9] In the experiment to examine the effect of the expression of a dominant negative form of Rho kinase (DN-ROCK)[15] on transwell migration, VSMCs were co-transfected with the β-galactosidase expression vector (pCAGGS-LacZ) and either the DN-ROCK expression vector pME18S-DN-ROCK or the empty vector as described.[9] The filter was subjected to staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate and the migrated cells attached onto the lower side of the filters were counted under a microscope.[9]

### 2.5 Determination of the activities of Rho and Rac

Pull-down assays to determine GTP-bound active forms of RhoA and Rac1 were performed as described in detail previously.[8,9,13] Briefly, cells that had been serum-deprived for 72 h were stimulated and cell lysates were prepared as described. Cell extracts were

incubated with the GST-rhotekin (for determination of Rho activity) or the GST-PAK1 (for determination of Rac activity) that were immobilized onto glutathione-*s*-Sepharose 4B beads (GE Healthcare, Buckinghamshire, UK) at 4°C for 45 min, followed by three washes. Bound Rho and Rac proteins were detected by western blotting using specific monoclonal antibodies against RhoA and Rac1 and quantitated by densitometry, as described. One-hundredth of total RhoA and Rac1 present in the cell lysate of each sample were also shown in each figure. The amounts of GTP-bound active RhoA and Rac1 were normalized for the total amounts of RhoA and Rac1, respectively, and expressed as multiples of the value of non-treated control cells, which was expressed as 1.0.

### 2.6 Statistics

All data are shown as mean ± SEM and are representative of at least three independent experiments with similar results. ANOVA (analysis of variance) was followed by Bonferoni's test to determine the statistical significance of differences between mean values. For all statistical comparisons, $P < 0.05$ was considered significant.

## 3. Results

### 3.1 $S1P_2R$ mediates inhibition of PDGF-induced cell migration and Rac activation via $G_{12/13}$ and $G_q$

S1P inhibited migration directed towards PDGF-B chain of naive VSMCs and vector control VSMCs (data not shown) in a transwell migration assay in a dose-dependent manner with complete inhibition at 0.1 μM S1P (*Figure 1A*). S1P alone slightly inhibited random migration of VSMC. The selective $S1P_2$ receptor antagonist JTE-013[16] abolished S1P inhibition of both random migration and PDGF-directed migration at up to 0.1 μM S1P. On the other hand, overexpression of $S1P_2R$ in VSMCs shifted leftward the dose–response curve for S1P inhibition nearly by one order, compared with naive VSMCs. These observations indicate that S1P inhibition of cell migration is mediated via $S1P_2R$. PDGF stimulated Rac (*Figure 1B*), which is essential for PDGF-directed VSMC migration.[8] S1P inhibited PDGF-induced Rac activation in a dose-dependent manner with complete inhibition at 0.1 μM S1P. JTE-013 totally abolished S1P inhibition of PDGF-induced Rac stimulation (*Figure 1C*), indicating that Rac inhibition is also mediated via $S1P_2R$.

In order to identify which member of the heterotrimeric G proteins is responsible for $S1P_2R$-mediated migration inhibition, we determined the effects of specific inhibition of receptor-G protein coupling by adenovirus-mediated expression of the C-terminal peptides of heterotrimeric G protein α subunits (Gα-CTs) and pertussis toxin (PTX) pre-treatment. The effectiveness and specificity of each Gα-CT peptide was previously demonstrated by us and other groups.[13,17] The expression of each myc-tagged Gα-CT peptide in adenovirus-infected VSMCs was confirmed by western blot analysis (*Figure 2A*). The expression of either of $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and $G\alpha_q$-CT, but not that of $G\alpha_s$-CT or LacZ (*Figure 2B*) or PTX pre-treatment (data not shown), abolished S1P inhibition of PDGF-directed migration. The expression of any of these Gα-CT peptides or PTX pre-treatment in VSMCs did not affect PDGF-directed migration itself. Similarly, the expression of either of $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and $G\alpha_q$-CT, but not that of $G\alpha_s$-CT or LacZ (*Figure 2C*) or PTX pre-treatment (data not shown), abolished S1P inhibition of PDGF-induced Rac activation. These

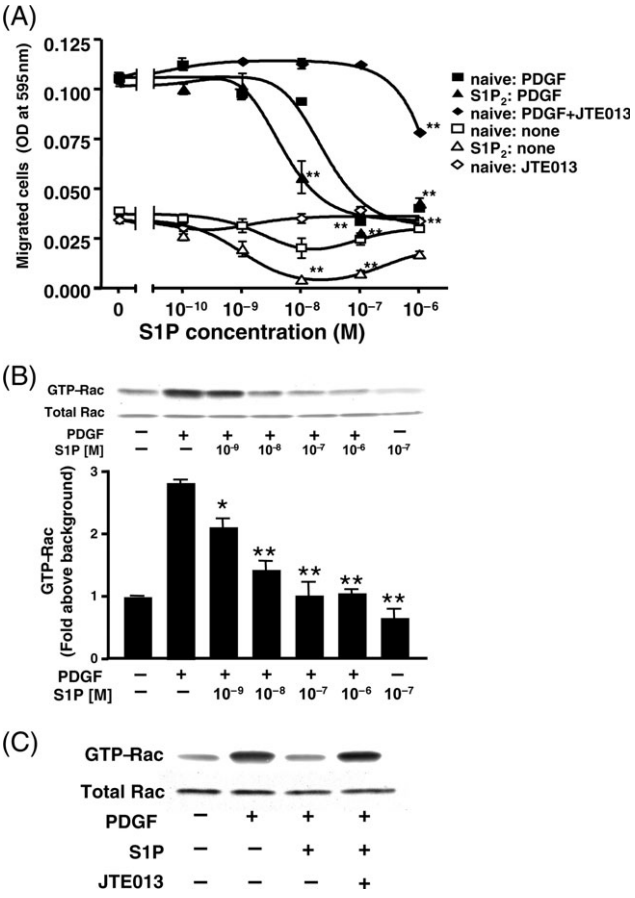


**Figure 1** Sphingosine-1-phosphate inhibits platelet-derived growth factor (PDGF)-triggered chemotaxis and Rac activation via $S1P_2R$ in vascular smooth muscle cells. (*A*) Dose-dependent inhibition of platelet-derived growth factor-directed migration. Transwell migration across the porous filter of non-transfected vascular smooth muscle cells (naive) and vascular smooth muscle cells stably overexpressing $S1P_2R$ ($S1P_2$) towards platelet-derived growth factor (0.41 nM) was determined in the presence and absence of various concentrations of sphingosine-1-phosphate and JTE-013 (1 μM) in the lower well of the Boyden chamber. OD, optical density. * and ** denote $P < 0.05$ and $P < 0.01$, respectively, compared with the values in the absence of sphingosine-1-phosphate. (*B*) Dose-dependent inhibition of platelet-derived growth factor-induced Rac activation by sphingosine-1-phosphate. Vascular smooth muscle cells were pre-treated with various concentrations of sphingosine-1-phosphate for 5 min and then stimulated with platelet-derived growth factor (0.41 nM) for 2 min. The cells were then subjected to a pulldown assay for GTP-Rac as described in Methods. One-hundredth of total Rac present in each sample is also shown to confirm loading of equal amounts of proteins (bottom). * and ** denote $P < 0.05$ and $P < 0.01$, respectively, compared with platelet-derived growth factor alone. (*C*) Blockade of sphingosine-1-phosphate inhibition of platelet-derived growth factor-induced Rac activation by the $S1P_2R$-selective inhibitor JTE-013. Vascular smooth muscle cells were pre-treated or not with JTE-013 (1 μM) for 10 min and treated with 0.1 μM sphingosine-1-phosphate for 5 min, followed by stimulation with platelet-derived growth factor (0.41 nM) for 2 min.

observations indicate that the $G_{12/13}$ and $G_q$ classes are both necessary for S1P-induced inhibition of cell migration and Rac.

### 3.2 $G_{12/13}$- and $G_q$-coupled Rho activation mediates S1P inhibition of cell migration and Rac in a Rho kinase-independent manner

S1P stimulated Rho activity in a dose-dependent manner with maximum activation at 0.1 μM S1P (*Figure 3A*). JTE-013 abolished S1P-induced Rho activation (*Figure 3B*),

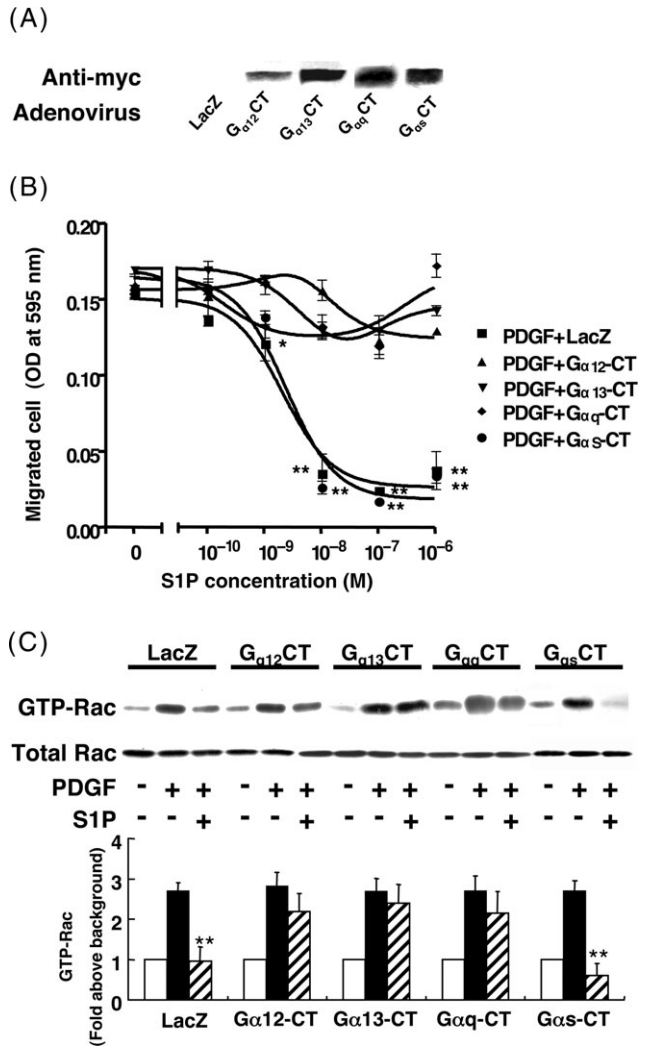


**Figure 2** Effects of selective blockade of $G_{12}$, $G_{13}$, $G_q$, and $G_s$ on sphingosine-1-phosphate inhibition of platelet-derived growth factor-induced chemotaxis and Rac activation in vascular smooth muscle cells. (A) Expression of myc-tagged $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, $G\alpha_q$-CT, and $G\alpha_s$-CT. Vascular smooth muscle cells were infected with indicated adenoviruses and analysed for expression of each Gα-CT peptide by western blotting using anti-myc antibody. (*B* and *C*) Effects of Gα-CT peptides on sphingosine-1-phosphate inhibition of platelet-derived growth factor-directed chemotaxis (*B*) and platelet-derived growth factor-induced Rac activation (*C*). Vascular smooth muscle cells were infected with adenovirus encoding $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, $G\alpha_q$-CT, $G\alpha_s$-CT, or LacZ as described in Methods, and then subjected to transwell migration assay (*B*). Vascular smooth muscle cells were stimulated with platelet-derived growth factor (0.41 nM). In (*C*), vascular smooth muscle cells were pre-treated with 0.1 μM sphingosine-1-phosphate for 5 min and then stimulated with platelet-derived growth factor (0.41 nM) for 2 min, followed by pulldown assay for GTP-Rac. ** denotes $P < 0.01$ compared with platelet-derived growth factor alone.

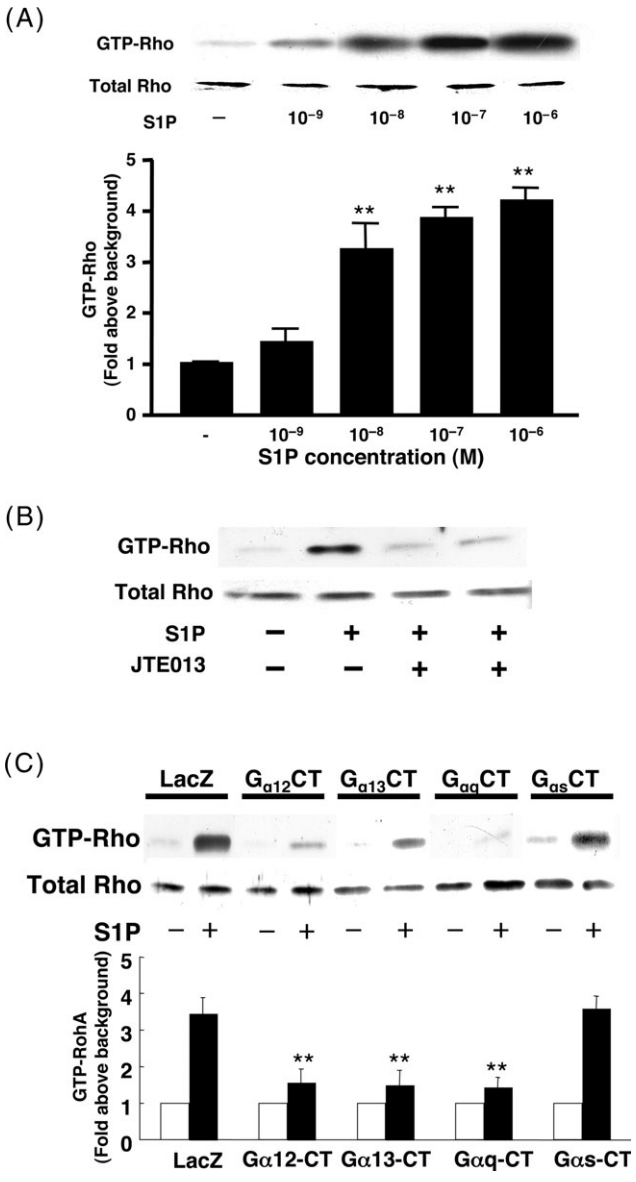


**Figure 3** Effects of selective blockade of $G_{12}$, $G_{13}$, $G_q$, and $G_s$ on sphingosine-1-phosphate-induced Rho activation in vascular smooth muscle cells. (*A*) Dose-dependent stimulation of Rho by sphingosine-1-phosphate. Vascular smooth muscle cells were stimulated with various concentrations of sphingosine-1-phosphate for 3 min and were subjected to a pulldown assay for GTP-Rho as described in Methods. One-hundredth of total Rho present in each sample is also shown to confirm loading of equal amounts of proteins (bottom). ** denotes $P < 0.01$ compared with no stimulation. (*B*) Blockade of sphingosine-1-phosphate-induced Rho activation by the $S1P_2R$-selective inhibitor JTE-013. Vascular smooth muscle cells were pre-treated or not with JTE-013 (1 μM) for 10 min and stimulated with 0.1 μM sphingosine-1-phosphate for 3 min. (*C*) Effects of Gα-CT peptides on sphingosine-1-phosphate-induced Rho activation. Vascular smooth muscle cells were infected with adenovirus encoding $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, $G\alpha_q$-CT, $G\alpha_s$-CT, or LacZ as described in Methods. Vascular smooth muscle cells were stimulated with 0.1 μM sphingosine-1-phosphate for 3 min. In (*C*), ** denotes $P < 0.01$ compared with sphingosine-1-phosphate stimulation in LacZ virus-infected cells.

indicating that $S1P_2R$ mediated Rho activation. The expression of either of $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and $G\alpha_q$-CT, but not that of $G\alpha_s$-CT or LacZ (*Figure 3C*) or PTX pre-treatment (data not shown), abolished S1P-induced Rho activation, indicating that Rho stimulation is mediated via both $G_{12/13}$ and $G_q$. Thus, Rac inhibition, migration inhibition, and Rho stimulation induced by $S1P_2R$ activation are all mediated via both $G_{12/13}$ and $G_q$.

In order to investigate the role of Rho in the inhibition of Rac and migration in VSMCs, we examined the effects of C3 toxin treatment on S1P inhibition of Rac and migration. Treatment of VSMCs with C3 toxin induced the mobility shift of RhoA which reflected ADP ribosylation, and abolished RhoA binding to the Rho-binding domain of the Rho effector Rhotekin, confirming effective inactivation of cellular Rho (*Figure 4A*). C3 toxin strongly suppressed S1P inhibition of PDGF-directed migration but did not affect PDGF-directed

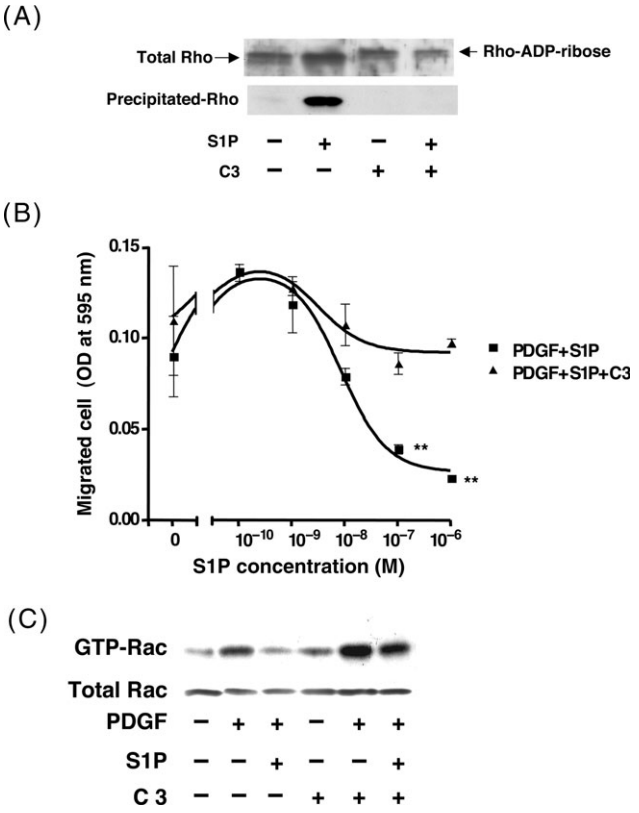


**Figure 4** Sphingosine-1-phosphate inhibition of platelet-derived growth factor-induced chemotaxis and Rac activation is dependent on Rho. (*A*) ADP-ribosylation of RhoA and inhibition of binding to rhotekin by C3 treatment. Note the mobility shift of RhoA from C3-pre-treated cells and the inability to bind to glutathione-*s*-Sepharose-rhotekin beads. In (*B* and *C*), vascular smooth muscle cells that had been pre-treated or not with C3 toxin were subjected to transwell migration (*B*) and pulldown assay for Rac (*C*). Vascular smooth muscle cells were treated with sphingosine-1-phosphate and stimulated with platelet-derived growth factor as described in *Figure 1A* and *2B*. In (*B*), ** denotes $P < 0.01$ compared with platelet-derived growth factor stimulation in the absence of sphingosine-1-phosphate.

migration itself (*Figure 4B*). Similarly, C3 toxin treatment nearly abolished S1P suppression of PDGF-induced Rac activation (*Figure 4C*). Thus, $S1P_2R$-mediated inhibition of PDGF-stimulated Rac activation and cell migration is dependent on Rho.

We examined the possible involvement of a Rho kinase, a downstream effector of Rho, in S1P inhibition of migration and Rac, because a Rho kinase inhibitor was previously reported to block S1P inhibition of cell migration in other cell types.[18] The Rho kinase inhibitor HA-1077 (fasudil) did not inhibit PDGF-directed cell migration itself or random migration (*Figure 5A*). HA-1077 slightly (at most by 20%) suppressed S1P-induced inhibition of PDGF-directed migration at the maximal concentration but did not block the most part of the S1P inhibition. Consistent with these observations, HA-1077 failed to reverse S1P inhibition of PDGF-induced membrane ruffling (*Figure 5B*) and Rac activation (*Figure 5C*). The effectiveness of HA-1077 in inhibiting Rho kinase in VSMCs was confirmed by the observations that this compound abolished S1P-induced stress fibre formation (*Figure 5B*) and S1P-induced stimulation of phosphorylation of the myosin-targeting subunit of myosin light chain phosphatase, MYPT1 (*Figure 5D*). Similar to the effect of HA-1077, the expression of a dominant negative form of Rho kinase, DN-ROCK, failed to suppress S1P-induced inhibition of PDGF-directed migration (*Figure 5E*). The observations suggest that $S1P_2R$-mediated inhibition of PDGF-stimulated Rac activation and cell migration is independent of Rho kinase.

### 3.3 $G_q$ is necessary but not sufficient for activation of Rho or inhibition of Rac and migration

The GPCR agonist angiotensin II (Ang II) effectively couples to $G_q$ to induce robust phospholipase C stimulation and $Ca^{2+}$ mobilization via $AT_1R$ in VSMCs.[19] Ang II did not stimulate Rho or inhibit PDGF-induced Rac activation or migration in vector-transfected (control) VSMCs (*Figure 6B–D*), suggesting that activation of $G_q$ alone is not sufficient for Rho stimulation or resultant inhibition of Rac and migration. In order to explore the role of $G_{12/13}$ in $AT_1R$ signalling, we established VSMCs stably overexpressing either wildtype $AT_1R$ or the $AT_1$-$G\alpha_{12}$ fusion receptor ($AT_1$-$G\alpha_{12}R$) in which $G\alpha_{12}$ is fused to $AT_1$ at its C-terminus and, thereby, the coupling to $G_{12}$ is facilitated (*Figure 6A*), and compared their abilities to stimulate Rho and to inhibit Rac and migration. In $AT_1R$-overexpressing VSMCs, Ang II tended to only slightly stimulate Rho, whereas Ang II induced a 1.8-fold increase in GTP-Rho in $AT_1$-$G\alpha_{12}R$-overexpressing cells (*Figure 6B*). Ang II did not at all affect PDGF-induced Rac activation in either vector-transfected or $AT_1R$-overexpressing VSMCs, but inhibited PDGF-induced Rac activation in AT1-$G\alpha_{12}$-R-overexpressing VSMCs approximately by 50% (*Figure 6C*). Consistent with this observation, Ang II inhibited PDGF-directed migration in AT1-$G\alpha_{12}R$-overexpressing VSMCs, but not in vector-transfected or $AT_1R$-overexpressing VSMCs (*Figure 6D*). Ang II alone slightly stimulated cell migration in vector control VSMCs. Thus, the $G_q$ signalling pathway by itself seems to be insufficient for stimulating Rho, but additional activation of the $G_{12/13}$ pathway results in the inhibition of Rac and cell migration with Rho stimulation.

### 3.4 $G\alpha_q$ mediates Rho activation through a $Ca^{2+}$- and protein kinase C-independent mechanism

$G_q$ mediates stimulation of phospholipase C (PLC), resulting in $Ca^{2+}$ mobilization and protein kinase C activation. Previous studies[20,21] suggested the involvement of $Ca^{2+}$ and protein kinase C in Rho-induced cellular responses. We examined the involvement of PLC, $Ca^{2+}$, and protein kinase C in S1P-induced Rho activation. The PLC inhibitor U73122, which totally inhibited S1P-induced increase in the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in VSMCs, did not affect S1P-induced Rho activation (see Supplementary material online, *Figure* S1A). The cell permeable $Ca^{2+}$-chelator BAPTA-AM did not inhibit S1P stimulation of Rho. The protein kinase C activator phorbol-12,13-dibutyrate (PDBu) did not stimulate Rho (see Supplementary material online, *Figure* S1B and S1C). Thus, the mechanism of the $G_q$ involvement in $S1P_2$-R-mediated Rho stimulation does not appear to involve PLC, $Ca^{2+}$, or protein kinase C.

## 4. Discussion

Migration of VSMCs into the intima is a critical step that gives rise to the formation of atheroma and vascular stenotic lesions.[1] Migration of VSMCs is positively and negatively regulated by chemoattractants and inhibitory mediators, respectively.[22] Upon ligand binding, chemoattractant

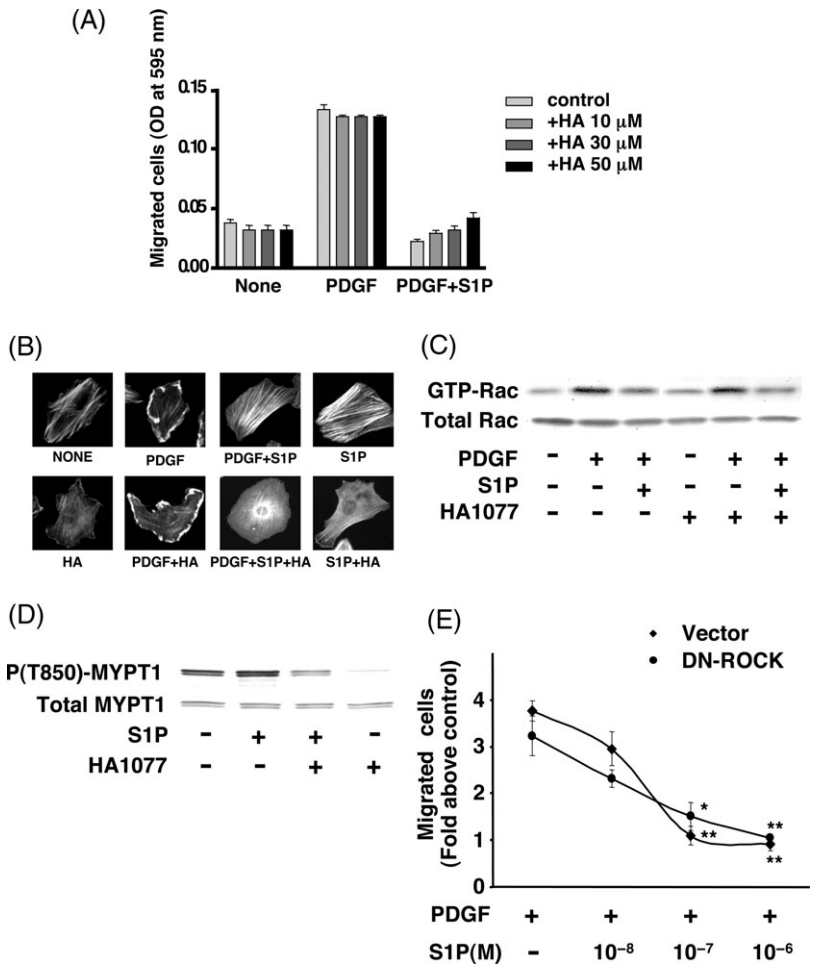


**Figure 5** Neither a Rho kinase inhibitor nor a dominant negative form of Rho kinase suppresses sphingosine-1-phosphate inhibition of platelet-derived growth factor-induced chemotaxis, lamellipodium formation, Rac activation, or MYPT1 phosphorylation. (*A*) Vascular smooth muscle cells were pre-treated or not with HA-1077 (HA) (10, 30, or 50 μM) for 15 min before transwell migration assay. Transwell migration was determined in the presence or absence of platelet-derived growth factor (0.41 nM) and sphingosine-1-phosphate (0.1 μM) in the lower chamber. HA-1077 was present in both the upper and lower chambers, where indicated. (*B*) Vascular smooth muscle cells were pre-treated or not with HA-1077 (20 μM) for 15 min and then treated with sphingosine-1-phosphate (0.1 μM) for 10 min when indicated. The cells were then stimulated with platelet-derived growth factor (0.41 nM) for 30 min. F-actin was visualized by staining with TRITC-labelled phalloidin as described in Methods. (*C*) Vascular smooth muscle cells were pre-treated or not with sphingosine-1-phosphate (0.1 μM) for 5 min and stimulated with platelet-derived growth factor (0.41 nM) for 2 min, followed by pulldown assay for GTP-Rac. HA-1077 (20 μM) was added 15 min before the addition of sphingosine-1-phosphate when indicated. (*D*) Vascular smooth muscle cells were pre-treated with HA-1077 or left untreated as in (*C*) and stimulated with sphingosine-1-phosphate (0.1 μM) for 3 min. The cell lysate was analysed for phosphorylation at $Thr^{850}$ of MYPT1 and total amount of MYPT1. (*E*) Vascular smooth muscle cells were co-transfected with LacZ-expression vector and either expression vector of a DN-ROCK or empty vector, and subjected to transwell migration assay followed by quantification of migrated cells as described in Methods. The numbers of migrated cells in the presence of platelet-derived growth factor alone were $72 \pm 8$ and $152 \pm 7$ cells/five low-power fields in vector- and DN-ROCK-transfection, respectively. * and ** denote $P < 0.05$ and $P < 0.01$, respectively, compared with the values in the presence of platelet-derived growth factor alone.

receptors, which include receptor tyrosine kinases, GPCRs, and cytokine type receptors, activate complex signalling cascades involving protein tyrosine kinases, PI (phosphoinositide) 3-kinase, and the small G proteins, particularly Rho family GTPases.[22] Among Rho family GTPases, Rac is activated by numerous chemoattractants and serves essential roles for cell migration, as well as Cdc42.[10] Compared with chemoattractant receptors, much less is known about the signalling mechanisms of inhibitory or repellant receptors. Among these, we identified $S1P_2R$ as the first example of a GPCR that mediates negative regulation of Rac and, thereby, cell migration in Chinese hamster ovary (CHO) cells overexpressing $S1P_2R$ and other cell types.[7,9]

We previously demonstrated in the same type of VSMCs as employed in the present study that the repellant receptor $S1P_2R$ is a predominantly expressed isoform, whereas the expression levels of the attractant receptors $S1P_1R$ and $S1P_3R$ are moderate or very low.[14] Consistent with these observations, S1P inhibited both random migration and PDGF-directed migration of VSMCs in a manner sensitive to the selective $S1P_2R$ antagonist JTE013 (*Figure 1A*). We previously showed in the CHO cells that $S1P_2R$-induced inhibition of Rac, a molecular switch for actin reorganization and cell migration, is a major mechanism for $S1P_2R$-mediated inhibition of cell migration.[9] S1P inhibited PDGF-induced Rac activation via $S1P_2R$ in VSMCs (*Figures 1B* and *2C*) with a similar dose–response relationship as that for inhibition of cell migration (*Figure 1A*), suggesting that $S1P_2R$-mediated Rac inhibition is at least in part responsible for S1P inhibition of migration in VSMCs.

$S1P_2R$ couples to multiple heterotrimeric G proteins.[9] We took advantage of adenovirus-mediated expression of specific inhibitor peptides and pertussis toxin to identify a

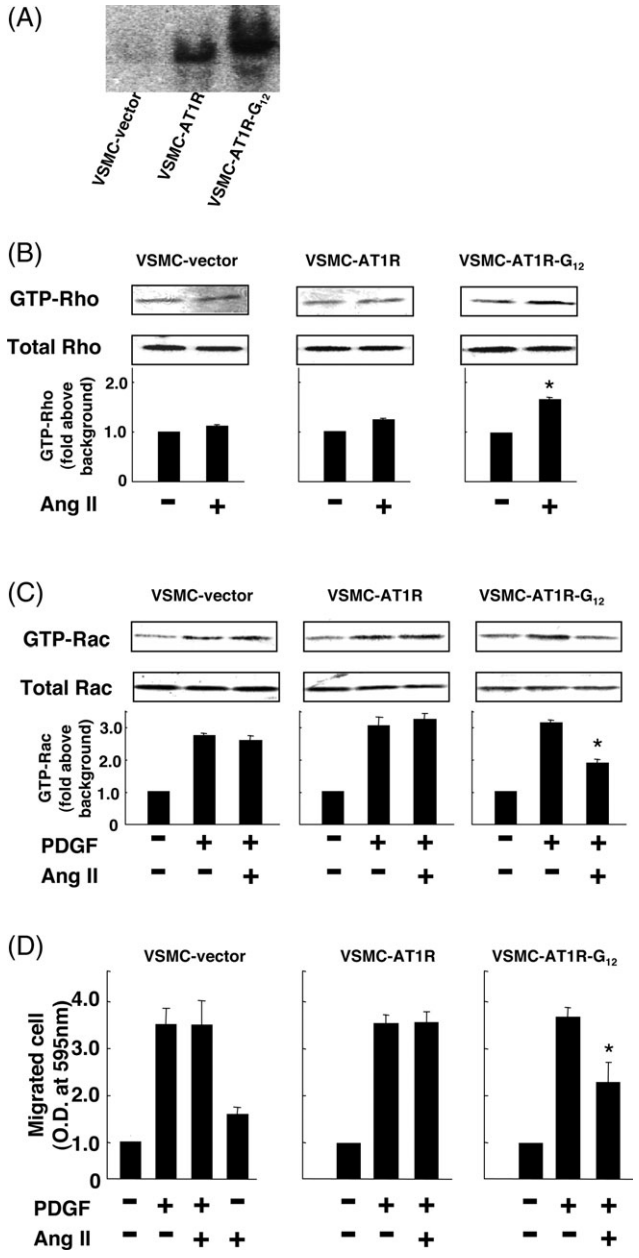


**Figure 6** Facilitation of $AT_1$ receptor coupling to $G_{12}$ enables stimulation of Rho and inhibition of Rac and migration. (*A*) Expression of $AT_1R$ and $AT_1$-$G\alpha_{12}$ fusion receptor ($AT_1$-$G\alpha_{12}R$) in vascular smooth muscle cells. Vascular smooth muscle cells were transfected with either of receptor expression vectors and empty vector and selected in the presence of G418 as described in Methods. The mRNA levels of $AT_1R$ and $AT_1$-$G\alpha_{12}R$ were analysed by northern analysis using $AT_1R$ cDNA. (*B–D*) The effects of angiotensin II (Ang II) on the amounts of GTP-Rho (*B*), platelet-derived growth factor-induced increases in GTP-Rac (*C*), and platelet-derived growth factor-directed cell migration (*D*) were determined. Vascular smooth muscle cells were pre-treated or not with 0.1 μM Ang II for 5 min, and stimulated with platelet-derived growth factor (0.41 nM) for 2 min (GTP-Rac assay) or for 3 min (GTP-Rho assay). The cells were then subjected to pulldown assays for GTP-Rac and GTP-Rho. Transwell migration of vascular smooth muscle cells towards platelet-derived growth factor (0.41 nM) was determined in the presence and absence of various concentrations of Ang II (0.1 μM) in the lower well of the Boyden chamber. In (*B*), * denotes $P < 0.05$ compared with no stimulation. In (*C* and *D*), * denotes $P < 0.05$ compared with platelet-derived growth factor alone.

heterotrimeric G protein that couples $S1P_2R$ to the inhibition of Rac and cell migration. The results showed that $S1P_2$-R-mediated inhibition of Rac and cell migration required both $G_q$ and $G_{12/13}$ in VSMCs (*Figure 2B* and *C*). $G_{12/13}$ is well known to mediate coupling of GPCR activation to Rho stimulation.[20] $S1P_2R$-mediated Rho activation in VSMCs was dependent on not only $G_{12/13}$ but also on $G_q$, similar to the inhibition of Rac and migration (*Figure 3*). We previously observed in CHO cells overexpressing $S1P_2R$ that $S1P_2$-R-mediated inhibition of Rac and cell migration was dependent on Rho.[13] This was also the case in VSMCs (*Figure 4*). Our previous study[9] showed that $G\alpha_{13}$-CT is a selective inhibitor for $G_{13}$, whereas $G\alpha_{12}$-CT inhibits both $G_{12}$ and $G_{13}$. In addition, either $G\alpha_{12}$-CT or $G\alpha_{13}$-CT does not affect the $G_q$ coupling, and vice versa. Therefore, our observations suggest that all of $G_{12}$, $G_{13}$, and $G_q$ are required, or both $G_{13}$ and $G_q$ are required for S1P-induced Rac inhibition in VSMCs. Previous studies[20,23] on non-muscle cells demonstrated that stimulation of GPCRs with various receptor agonists including lysophosphatidic acid, endothelin-1, and thrombin induced Rho activation through receptor coupling to the $G_{12/13}$ family of the heterotrimeric G proteins. Direct physical and functional interaction of $G\alpha_{12/13}$ with a group of Rho-GEFs with the conserved structural motif known as the regulator of G protein signalling domain (the RGS box), which include p115RhoGEF, PDZ-RhoGEF, and LARG, was demonstrated.[23] More recent studies[20] showed that $G_q$ also had the ability to mediate Rho stimulation through the similar mechanisms in certain non-muscle cell types.

In the present study, $G_q$ activation by Ang II did not induce either Rho stimulation or inhibition of Rac or cell migration (*Figure 6*) although $G\alpha_q$ was reported to directly associate with RhoGEFs in non-muscle cells.[20] The observations suggest that $G_q$ activation by itself is not sufficient for effective Rho activation and the following inhibition of Rac and migration in VSMCs. Forced coupling of $AT_1R$ to $G_{12}$ by fusing $G\alpha_{12}$ at the C-terminus of $AT_1R$ enabled Ang II-induced Rho activation and the inhibition of Rac and migration. These observations, together with the finding that $S1P_2$-R-mediated Rho activation is dependent on both $G_{12/13}$ and $G_q$, suggest that $G_q$ may exert a permissive effect on Rho activation in cooperation with $G_{12/13}$. A previous study[24] showed that the expression of activated forms of $G\alpha_{12}$ and $G\alpha_{13}$, but not $G\alpha_q$, in VSMCs induced contraction that was inhibited by $C_3$ toxin, which is also consistent with the notion that $G_q$ plays a permissive role in Rho activation in VSMCs. Previous investigations[20,21] in non-muscle cells showed the involvement of $Ca^{2+}$ and protein kinase C in Rho-dependent responses induced by the expression of a constitutively active mutant of $G\alpha_q$. However, it was unclear in these studies whether $Ca^{2+}$ and protein kinase C affected the process of Rho activation itself or at a site(s) downstream of Rho activation. Our results suggest that PLC or its downstream second messengers, $Ca^{2+}$ or protein kinase C, are likely not involved in $S1P_2R$-mediated Rho activation (see Supplementary material online, *Figure S1*).

A number of downstream effectors of Rho have been identified, including Rho kinase, Dia, protein kinase N (PKN), rhotekin, rhophilin, citron, and citron kinase.[25] Among them, Rho kinase has been well investigated and found to be essential for Rho-induced formation of stress fibres and focal adhesions and Rho-induced negative regulation of myosin phosphatase.[25] In the present study, either the Rho kinase inhibitor HA-1077 or the expression of a dominant negative form of Rho kinase did not suppress $S1P_2R$-mediated inhibition of Rac, cell migration, or

membrane ruffling although HA-1077 effectively inhibited $S1P_2R$-mediated Rho-dependent stress fibre formation (*Figure 5*). These results indicate that a Rho effector other than Rho kinase participates in $S1P_2R$-mediated Rac inhibition in VSMCs. The responsible Rho effector molecule remains to be identified.

Previous investigations suggested that S1P exerts both atherogenic and anti-atherogenic effects. $S1P_1R$ but not $S1P_3R$ was shown to suppress adhesion of leukocytes onto endothelial cells in the isolated aorta,[26] whereas in cultured endothelial cells both $S1P_1R$ and $S1P_3R$ were shown to stimulate the expression of adhesion molecules and monocytic cell adhesion.[27] In the latter investigations, $S1P_1R$ also exerted inhibitory effects on adhesion molecule expression through PI 3-kinase and eNOS in cultured endothelial cells. Thus, there is still some controversy concerning the roles of S1P receptors in the regulation of adhesion molecule expression in endothelial cells. In addition, $S1P_1R$ suppresses vascular permeability,[28] whereas $S1P_2R$ induces vascular hyperpermeability.[29] *In vivo*, the synthetic S1P analogue FTY720 inhibited the development of atherosclerosis in murine models of hypercholesterolaemia probably through modulating functions of lymphocytes and macrophages via $S1P_1R$ and $S1P_4R$, both of which are major targets of FTY720.[30] $S1P_1R$, which is upregulated in neointimal VSMCs, stimulates migration of neointimal VSMCs, suggesting its stimulatory role in neointima formation.[31] In contrast to $S1P_1R$, $S1P_2R$ inhibits migration of VSMCs. A very recent report[32] showed that neointima formation *in vivo* in the carotid artery ligation model is increased in $S1P_2R$-null mice compared with wildtype mice, indicating that $S1P_2R$ mediates an inhibitory effect on neointima formation. Thus, in atherosclerotic lesions and other vaso-occlusive lesions, S1P appears to exert complicated effects on lesion formation through multiple mechanisms in receptor subtype- and cell-type-specific manners. Selective activation of $S1P_2R$ in VSMCs by local drug delivery methods including a drug-eluting stent at stenotic sites could inhibit accumulation of VSMCs into the luminal surface by uniquely activating a chemorepellant activity.

## Supplementary material

Supplementary Material is available at *Cardiovascular Research* Online.

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