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Characterization of $S1P_1$ and $S1P_2$ receptor function in smooth muscle by receptor silencing and receptor protection

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Hu, Wenhui, Sunila Mahavadi, Jiean Huang, Fang Li, and Karnam S. Murthy. Characterization of $S1P_1$ and $S1P_2$ receptor function in smooth muscle by receptor silencing and receptor protection. *Am J Physiol Gastrointest Liver Physiol* 291: G605–G610, 2006. First published May 4, 2006; doi:10.1152/ajpgi.00147.2006.—Sphingosine-1-phosphate (S1P) induces an initial Ca^{2+}-dependent contraction followed by a sustained Ca^{2+}-independent, RhoA-mediated contraction in rabbit gastric smooth muscle cells. The cells coexpress $S1P_1$ and $S1P_2$ receptors, but the signaling pathways initiated by each receptor type and the involvement of one or both receptors in contraction are not known. Lentiviral vectors encoding small interfering RNAs were transiently transfected into cultured smooth muscle cells to silence $S1P_1$ or $S1P_2$ receptors. Phospholipase C (PLC)-β activity and Rho kinase activity were used as markers of pathways mediating initial and sustained contraction, respectively. Silencing of $S1P_1$ receptors abolished S1P-stimulated activation of $G\alpha_{i3}$ and partially inhibited activation of $G\alpha_{i1}$, whereas silencing of $S1P_2$ receptors abolished activation of $G\alpha_q$, $G\alpha_{13}$, and $G\alpha_{i2}$ and partially inhibited activation of $G\alpha_{i1}$. Silencing of $S1P_2$ but not $S1P_1$ receptors suppressed S1P-stimulated PLC-β and Rho kinase activities, implying that both signaling pathways were mediated by $S1P_2$ receptors. The results obtained by receptor silencing were corroborated by receptor inactivation. The selective $S1P_1$ receptor agonist SEW2871 did not stimulate PLC-β or Rho kinase activity or induce initial and sustained contraction; when this agonist was used to protect $S1P_1$ receptors so as to enable chemical inactivation of $S1P_2$ receptors, S1P did not elicit contraction, confirming that initial and sustained contraction was mediated by $S1P_2$ receptors. Thus $S1P_1$ and $S1P_2$ receptors are coupled to distinct complements of G proteins. Only $S1P_2$ receptors activate PLC-β and Rho kinase and mediate initial and sustained contraction.

small interfering RNAs; lentiviral vector; sphingosine-1-phosphate; phospholipase C-β; Rho kinase

THE LYSOPHOSPHOLIPIDS sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) possess a wide spectrum of biological activities including stimulation of cell growth, inhibition of apoptosis, induction of actin cytoskeletal reorganization, and stimulation of cell migration (1–3, 7, 29). S1P interacts with five of eight "endothelial differentiation gene" (EDG) receptors renamed in accordance with International Union of Pharmacology Societies nomenclature as $S1P_1$ (EDG_1), $S1P_2$ (EDG_5), $S1P_3$ (EDG_3), $S1P_4$ (EDG_6), and $S1P_5$ (EDG_8). LPA interacts with LPA_1 (EDG_2), LPA_2 (EDG_4), and LPA_3 (EDG_7) receptors. $S1P_1$, $S1P_2$, and $S1P_3$ receptors are widely distributed (1–3), whereas $S1P_4$ receptors are confined to lymphoid and hematopoietic tissues (9), and $S1P_5$ receptors are confined to oligodendrocytes (14).

Current understanding of G protein coupling of S1P receptors is based on receptor expression studies in cell lines. In cells expressing $S1P_1$ receptors, S1P stimulates phosphoinositide (PI) hydrolysis, Ca^{2+} mobilization, and ERK1/2 in a pertussis toxin (PTx)-sensitive fashion, implying coupling to G_i/G_o (27, 30); immunoprecipitation of $S1P_1$ receptors in these cells leads to coprecipitation of $G\alpha_{i1}$ and $G\alpha_{i3}$ (16). In cells expressing $S1P_2$ or $S1P_3$ receptors, S1P stimulates PI hydrolysis and Ca^{2+} mobilization in both a PTx-sensitive and -insensitive fashion and activates ERK1/2 in a PTx-sensitive, Ras-dependent fashion (6, 30); in addition, S1P activates RhoA and induces stress fiber formation and cell migration (6). Analysis of G protein coupling suggests that $S1P_2$, and probably $S1P_3$ receptors, are coupled to G_i, G_q, and G_{12}/G_{13} (6, 28). Rac1 is activated via $S1P_3$ and inhibited via $S1P_2$; Rac1 activation reflects predominant coupling of $S1P_3$ to G_i, and Rac1 inhibition reflects predominant coupling of $S1P_2$ to G_{12}/G_{13} (28). Recent studies (9) suggest that $S1P_4$ receptors regulate cell shape and motility via G_i and G_{12}/G_{13}. $S1P_5$ is coupled to $G_{i/o}$ and G_{12} but not to G_s or G_q (18).

Although cell lines transfected with individual S1P receptors can be a useful guide, G protein coupling in native cells may differ depending on the number of S1P receptors and/or the complement of G proteins expressed in these cells. Coronary arterial smooth muscle cells contract in response to S1P and express $S1P_2$ receptors and, to a lesser extent, $S1P_3$ and $S1P_1$ receptors (26). Gastric smooth muscle cells also contract in response to S1P and express both $S1P_2$ and $S1P_1$ receptors (31). All the G proteins (G_q, G_{i1}, G_{i2}, G_{i3}, and G_{13}) except for G_s are activated by S1P in gastric smooth muscle cells (31). Both G_q and G_i contribute to PI hydrolysis, Ca^{2+} release, and initial muscle contraction by activating $G\alpha_q$-dependent phospholipase (PLC)-β1 and $G\beta\gamma_i$-dependent PLC-β3, resulting in inositol 1,4,5-trisphosphate (IP_3)-stimulated Ca^{2+} release and activation of Ca^{2+}/calmodulin-dependent myosin light chain (MLC) kinase. Both G_q and G_{13} contribute to sustained muscle contraction by activating RhoA/Rho kinase/PKC-dependent pathways that mediate inhibition of MLC phosphatase and activation of Ca^{2+}-independent MLC kinase (20).

In the present study, we used molecular and pharmacological approaches to determine the functional contribution of each receptor type. Lentiviral vectors encoding small interfering RNAs (siRNAs) were transiently transfected into cultured smooth muscle cells to silence $S1P_1$ or $S1P_2$ receptors. In addition, $S1P_2$ receptors were chemically inactivated after protection of $S1P_1$ receptors with a selective $S1P_1$ receptor

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agonist. The results indicate that only S1P$_2$ receptors activate PLC-β- and RhoA-dependent pathways that mediate initial and sustained contraction, respectively.

MATERIALS AND METHODS

siRNA for S1P$_1$ and S1P$_2$ receptors. Lentiviral vector constructs encoding enhanced green fluorescent protein (EGFP) as an internal marker together with siRNA for S1P$_1$ or S1P$_2$ receptors were generated using a modified two-step PCR-based strategy (8, 11). Briefly, three siRNA expression cassettes for each receptor were generated through consequential two rounds of PCR and individually cloned into pLL3.7 lentiviral vector via *Xba*I/*Xho*I cloning sites. The sequence of each siRNA cassette was confirmed by restriction enzyme digestion with *Bam*HI/*Eco*RI and DNA sequencing. Silencing efficiency and specificity of these siRNA constructs were determined by Western blot and immunocytochemical studies in human embryonic kidney (HEK)-293T cells and RT-PCR analysis in gastric smooth muscle cells (11). The most effective siRNA constructs, S1P$_1$b, and S1P$_2$a, were used in the present study. The sequences for S1P$_1$b and S1P$_2$a were 5′-GAAGACCTGTGACATCCTGTA-3′ and 5′-ACCAAGGAGACGCTGGACATG-3′.

Lentiviral vector transfection into cultured smooth muscle cells. Dispersed gastric smooth muscle cells were prepared from the circular muscle layer of the rabbit distal stomach by sequential enzymatic digestion, filtration, and centrifugation as previously described (19, 21, 31). The cells were cultured to confluence in DMEM with 10% FBS plus antibiotics. Cells in first passage grown on six-well plates were transiently transfected with lentiviral vector encoding siRNA for S1P$_1$ or S1P$_2$ receptors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Lentiviral vector (2 μg) with or without siRNA in 125 μl Opti-MEM medium was mixed with 5 μl Lipofectamine 2000 in 125 μl Opti-MEM. The mixture was incubated at room temperature for 20 min and added to wells containing 1.5 ml DMEM-10% FBS for 1 day. The medium was then replaced with DMEM-10% FBS plus antibiotics for 2 days. The cells were maintained for a final 24 h in DMEM without FBS before experiments were started. Fluorescence analysis of EGFP showed a transfection efficiency of 60–70%.

G protein activation assay. Activation of specific G proteins was determined by measuring the increase in Gα binding to guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) on addition of agonist (S1P) as previously described (22, 31). Cells obtained 3 days after transfection with lentiviral siRNA vectors were homogenized in a medium consisting of 20 mM HEPES (pH 7.4), 2 mM $MgCl_2$, 1 mM EDTA, and 2 mM 1,4-dithiothreitol (DTT). The homogenate was centrifuged at 30,000 *g* for 30 min at 4°C, and the membranes were solubilized at 4°C in 20 mM HEPES (pH 7.4) buffer containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. The solubilized membranes were incubated at 37°C for 20 min in a medium containing 100 nM [^{35}S]GTPγS and 10 mM HEPES (pH 7.4) in the presence or absence of S1P. Ten volumes of 100 mM Tris·HCl (pH 8.0) containing 10 mM $MgCl_2$, 100 mM NaCl, and 20 μM GTP were used to stop the reaction. The membranes were incubated for 2 h on ice in wells separately coated with antibodies to Gα$_{i1}$, Gα$_{i2}$, Gα$_{i3}$, Gα$_q$, and Gα$_{13}$. After each well was washed with PBS, radioactivity was counted by liquid scintillation.

Assay for PLC-β activity. PLC-β activity was determined in smooth muscle from the formation of total inositol phosphates using ion-exchange chromatography as described previously (22). Cultured (after transfection) or freshly dispersed smooth muscle cells labeled with *myo*-[^{3}H]inositol (0.5 μCi/ml) in inositol-free DMEM without FBS were washed with PBS and treated with S1P or S1P$_1$ agonist (SEW2871; 1 μM) for 60 s in 1 ml of 25 mM HEPES medium (pH 7.4) containing (in mM) 115 NaCl, 5.8 KCl, 2.1 KH_2PO_4, 2 $CaCl_2$, and 14 glucose. The reaction was terminated by the addition of 940 μl of chloroform-methanol-HCl (50:100:1). The samples were extracted with chloroform and water, and the phases were separated by centrifugation for 15 min at 1,000 *g*. The aqueous phase was applied to a DOWEX AG-1 column, and [^{3}H]inositol phosphates were eluted with 0.8 M ammonium formamate-0.1 M formic acid. Radioactivity was determined by liquid scintillation and was expressed as counts per minute (cpm).

Assay for Rho kinase activity. Rho kinase activity was determined in cell extracts by immunokinase assay as described previously (24). Cultured (after transfection) or freshly dispersed smooth muscle cells were solubilized with lysis buffer containing 50 mM Tris·HCl (pH 7.5), 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. An equal amount of protein extracts was incubated with Rho kinase-2 antibody plus protein A/G agarose overnight at 4°C. The immunoprecipitates were washed twice in a medium containing 10 mM $MgCl_2$ and 40 mM HEPES (pH 7.4) and then incubated for 5 min on ice with 5 μg of myelin basic protein. The assay was initiated by the addition of 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 20 μM ATP, followed by incubation for 10 min at 37°C. The ^{32}P-labeled myelin basic protein was absorbed onto phosphocellulose disks, and free radioactivity was removed by repeated washings with 75 mM phosphoric acid. The amount of radioactivity on the disks was measured by liquid scintillation.

Selective protection of S1P$_1$ receptors and inactivation of S1P$_2$ receptors. A technique of selective preservation of one receptor type was used to determine the function of S1P$_1$ and S1P$_2$ receptors. This technique was extensively validated and used to determine the function of G protein-coupled receptors coexpressed in the same cell (e.g., 5-hydroxytryptamine receptors) (15, 19, 21, 23). The technique involves protection of one receptor type with a selective agonist or antagonist followed by inactivation of all unprotected receptors by brief treatment with a low concentration of *N*-ethylmaleimide. In the present study, the selective S1P$_1$ receptor agonist SEW2871 was used to protect S1P$_1$ and inactivate all other receptors including S1P$_2$ receptors. S1P was used as a control to preserve both S1P$_1$ and S1P$_2$ receptors and inactivate all other receptors. Freshly dispersed muscle cells were incubated with SEW2871 or S1P at 31°C for 2 min followed by addition of 5 μM *N*-ethylmaleimide for 20 min. The cells were centrifuged twice at 150 *g* for 10 min to eliminate the protective agonist and *N*-ethylmaleimide and resuspended in fresh HEPES buffer. The initial and sustained contractile response of cells treated in this fashion was compared with the response of untreated (naive) cells. Previous studies (15, 19, 21, 23) have shown that the coupling of protected receptors to signaling pathways remains intact. Smooth muscle cells incubated with *N*-ethylmaleimide without protective ligand did not respond to agonists but retained their ability to respond to agents that bypass receptors (e.g., ionomycin, KCl, and forskolin), implying that postreceptor mechanisms were intact (5, 10).

Measurement of contraction in dispersed smooth muscle cells. Muscle cell contraction was measured in freshly dispersed muscle cells by scanning microscopy as described previously (19, 20). Cell aliquots containing ~10^4 muscle cells/ml were treated with S1P or the selective S1P$_1$ receptor agonist SEW2871 in the presence or absence of the S1P$_1$ antagonist VPC23019; the reaction was terminated with 1% acrolein. The lengths of muscle cells treated with S1P or SEW2871 were compared with the lengths of untreated cells, and contraction was expressed as the percent decrease in cell length from control.

Statistical analysis. The results are expressed as means ± SE of *n* experiments and were analyzed for statistical significance using Student's *t*-test for paired or unpaired values.

Materials. S1P was obtained from BioMol Research Labs (Plymouth Meeting, PA), SEW2871 from Calbiochem (San Diego, CA), (*R*)-phosphoric acid mono-[2-amino-2-(3-octyl-phenylcarbamoyl)-ethyl] ester (VPC23019) from Avantis Polar Lipids (Alabaster, AL), [γ-^{32}P]ATP from Amersham Pharmacia Biotech (Piscataway, NJ), and *myo*-[^{3}H]inositol from DuPont New England Nuclear (Boston, MA). Gα and Rho kinase-2 antibodies were obtained from Santa Cruz

Biotechnology (Santa Cruz, CA). All the other reagents were from Sigma (St. Louis, MO).

RESULTS

Distinctive patterns of G protein activation by S1P$_1$ and S1P$_2$ receptors in smooth muscle. Previous studies (31) have demonstrated that S1P activates $G\alpha_q$, $G\alpha_{13}$, and all three isoforms of $G\alpha_i$ in gastric smooth muscle cells. Because these cells express both $S1P_1$ and $S1P_2$ receptors, it was not possible to determine the pattern of G protein activation by each receptor. In the present study, we measured G protein activation in response to S1P after selective silencing of $S1P_1$ or $S1P_2$ receptors in cultured gastric smooth muscle cells transiently transfected with lentiviral vectors encoding siRNAs for each receptor. Silencing of $S1P_1$ receptors abolished S1P-stimulated activation of $G\alpha_{i3}$ and inhibited activation of $G\alpha_{i1}$ by ~60% but had no effect on activation of $G\alpha_{i2}$, $G\alpha_q$, and $G\alpha_{13}$ (Fig. 1). In contrast, silencing of $S1P_2$ receptors abolished S1P-stimulated activation of $G\alpha_q$, $G\alpha_{13}$, and $G\alpha_{i2}$ and inhibited activation of $G\alpha_{i1}$ by ~40% but had no effect on activation of $G\alpha_{i3}$ (Fig. 1). Thus $S1P_1$ receptors are coupled to G_{i3} and G_{i1}, whereas $S1P_2$ receptors are coupled to G_q, G_{13}, G_{i2}, and G_{i1}. Only G_{i1} activation is shared by both receptors.

Silencing of S1P$_2$ receptors suppresses S1P-induced activation of PLC-β and Rho kinase in smooth muscle. We have previously shown that in smooth muscle cells expressing both receptors, S1P stimulated PLC-β activity in both a PTx-sensitive and -insensitive fashion, implying participation of both G_q and G_i (31). The involvement of both G proteins was confirmed by expression of $G\alpha_q$ or $G\alpha_i$ minigenes to inactivate G_q or all isoforms of G_i. Silencing of $S1P_2$ receptors abolished S1P-stimulated PLC-β activity (measured at 60 s), whereas silencing of $S1P_1$ receptors had no effect (Fig. 2). In light of our previous study (31), this implied that activation of PLC-β was mediated by G_q (PTx-insensitive component) and by G_{i2} and/or G_{i1} (PTx-sensitive component) but not by G_{i3}, which is exclusively coupled to $S1P_1$ receptors (Fig. 1).

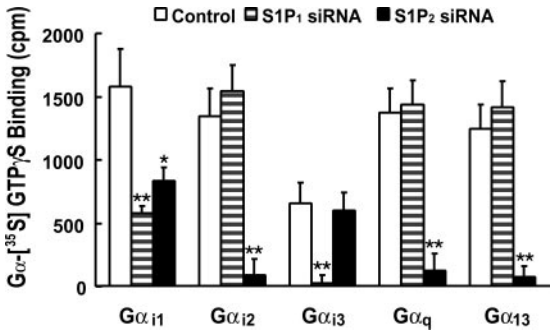

Fig. 1. Distinct patterns of G protein activation by sphingosine-1-phosphate $(S1P)_1$ and $S1P_2$ receptors. Cultured gastric smooth muscle cells (SMCs) in first passage were transfected with lentiviral vectors encoding small interfering RNA (siRNA) for $S1P_1$ or $S1P_2$ receptors or empty vector (control). After 3 days, G protein activity was determined from the difference in the binding of specific Gα subunits to 0.1 μM [^{35}S]GTPγS alone or in the presence of 1 μM S1P; the difference for each Gα subunit is shown by the open bars and expressed as counts per minute (cpm). Binding of GTPγS alone to Gα subunits ranged from 852 ± 47 to 1,034 ± 65 cpm. Silencing of $S1P_2$ receptors abolished S1P-stimulated $G\alpha_q$, $G\alpha_{13}$, $G\alpha_{i2}$ activity and partially inhibited $G\alpha_{i1}$ activity (~40%). Silencing of $S1P_1$ receptors abolished $G\alpha_{i3}$ activity and partially inhibited $G\alpha_{i1}$ activity (~60%). Values are means ± SE of 4 experiments. $^*P < 0.05$ and $^{**}P < 0.01$, significant differences from control Gα binding activity in response to S1P (shown by open bars for each Gα subunit).

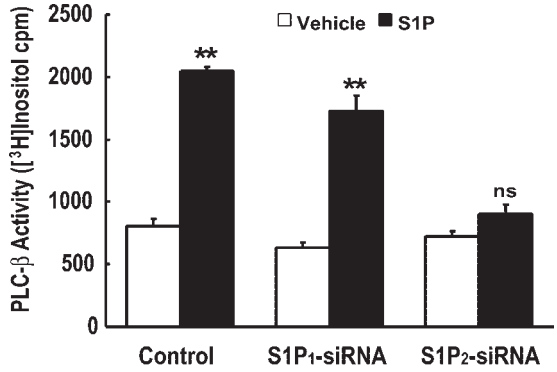

Fig. 2. Silencing of $S1P_2$ receptors abolishes S1P-induced activation of phospholipase C (PLC)-β. Cultured gastric SMCs in the first passage were transfected with lentiviral vectors encoding siRNA for $S1P_1$ or $S1P_2$ receptors or empty vector (control). After 2 days, cells were labeled with *myo*-[^{3}H]inositol and then treated with S1P (1 μM) or vehicle for 60 s. [^{3}H]inositol phosphate was determined as described in MATERIALS AND METHODS and expressed as cpm. Values are means ± SE of 3 experiments. $^{**}P < 0.01$, significant increase in inositol phosphate production above basal level (vehicle) in control cells and cells expressing $S1P_1$-siRNA. No significant (ns) increase in inositol phosphate production in cells expressing $S1P_2$-siRNA was found.

We have also previously shown that in smooth muscle cells expressing both receptors, S1P-stimulated Rho kinase activity was partially inhibited by expression of $G\alpha_q$ or $G\alpha_{13}$ minigenes and virtually abolished by coexpression of both minigenes, implying that RhoA was activated by both G_q and G_{13} (31). In the present study, silencing of $S1P_2$ receptors abolished S1P-stimulated Rho kinase activity (measured at 5 min), whereas silencing of $S1P_1$ receptors had no effect (Fig. 3). The involvement of $S1P_2$ receptors reflected their ability to activate G_q and G_{13} (Fig. 1).

Chemical inactivation of S1P$_2$ receptor suppresses S1P-induced smooth muscle contraction. To corroborate the results obtained by selective receptor silencing, we compared the contractile responses to S1P and a selective $S1P_1$ receptor agonist, SEW2871, and used the latter to protect $S1P_1$ recep-

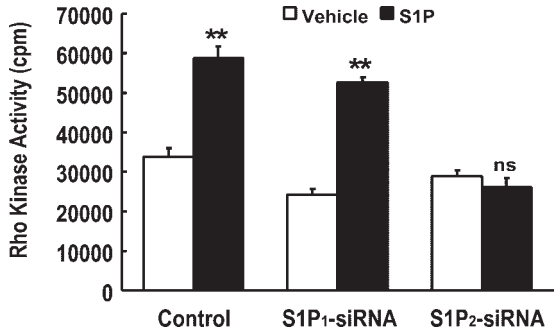

Fig. 3. Silencing of $S1P_2$ receptors abolishes S1P-induced activation of Rho kinase. Cultured gastric SMCs in first passage were transfected with lentiviral vectors encoding siRNA for $S1P_1$ or $S1P_2$ receptors or empty vector (control). After 3 days, cells were treated with S1P (1 μM) or vehicle for 5 min, and Rho kinase activity was determined by immunokinase assay as described in MATERIALS AND METHODS and expressed as cpm. Values are means ± SE of 3 experiments. $^{**}P < 0.01$, significant increase in Rho kinase activity above basal level (vehicle) in control cells and cells expressing $S1P_1$-siRNA. No significant increase in Rho kinase activity in cells expressing $S1P_2$-siRNA was found.

tors and chemically inactivate $S1P_2$ receptors. Treatment of freshly dispersed smooth muscle cells with SEW2871 did not elicit contraction, whereas treatment with S1P caused an initial contraction (measured at 30 s) and a sustained contraction (measured at 5 min; Fig. 4*A*).

Treatment of the cells with SEW2871 for 2 min to protect $S1P_1$ receptors followed by the addition of *N*-ethylmaleimide to inactivate all unprotected receptors including $S1P_2$ receptors abolished the contractile response to S1P (Fig. 4*B*). In contrast, treatment of the cells with S1P protected both $S1P_1$ and $S1P_2$ receptors and preserved the response to S1P (Fig. 4B). Studies to protect $S1P_2$ receptors and inactivate $S1P_1$ receptors could not be done because the $S1P_2$ receptor antagonist JTE013 reported in other studies (13, 26) is not commercially available.

Selective S1P$_1$ receptor agonists do not activate PLC-β or Rho kinase. Treatment of freshly dispersed muscle cells with S1P stimulated both PLC-β and Rho kinase activities, measured at 60 s and 5 min, respectively, whereas treatment of the cells with SEW2871 had no effect (Fig. 5). S1P-stimulated PLC-β and Rho kinase activities were not affected by pretreatment of the cells with VPC23019, a mixed $S1P_1$/$S1P_3$ antagonist (Fig. 5) (4). The results confirmed that $S1P_1$ receptors do not activate pathways that mediate initial and sustained muscle contraction.

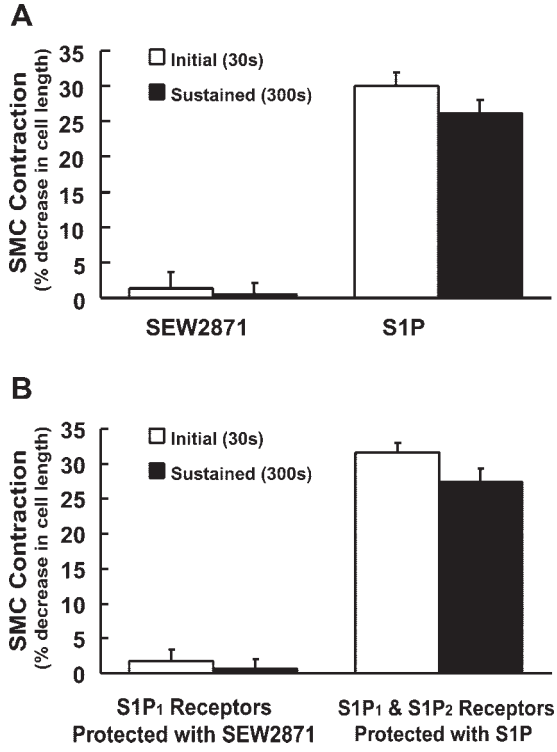

Fig. 4. Chemical inactivation of $S1P_2$ receptors abolishes S1P-induced smooth muscle contraction. *A*: freshly dispersed SMCs were treated with the selective $S1P_1$ receptor agonist SEW2871 (1 μM) or S1P (1 μM) for 30 s to determine initial contraction and for 5 min to determine sustained contraction. Cell length was measured by scanning microscopy and contraction was expressed as the %decrease in cell length from control. Values are means ± SE of 3–4 experiments. *B*: dispersed SMCs were incubated with SEW2871 (1 μM) or S1P (1 μM) for 2 min, after which 5 μM *N*-ethylmaleimide was added and incubation was maintained for 20 min to inactivate unprotected receptors. After the SMCs were washed, the initial and sustained contractile responses to S1P (1 μM) were measured as described above. Selective inactivation of $S1P_2$ receptors after protection of $S1P_1$ receptors with SEW2871 abolished the contractile response to S1P, whereas protection of $S1P_1$ and $S1P_2$ receptors after protection with S1P preserved the contractile response to S1P. Values are means ± SE of 3–4 experiments.

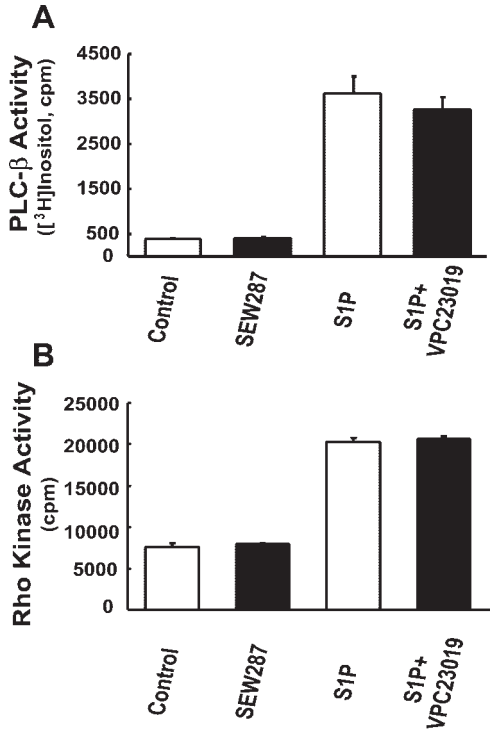

Fig. 5. A selective $S1P_1$ receptor agonist does not activate PLC-β or Rho kinase in freshly dispersed gastric SMCs. Cells were treated with a selective $S1P_1$ receptor agonist SEW2871 (1 μM) or S1P (1 μM) for 60 s for measurement of PLC-β activity (*A*) or for 5 min for measurement of Rho kinase activity (*B*). Activities were determined as described in MATERIALS AND METHODS and expressed as cpm. Pretreatment with the mixed $S1P_1$/$S1P_3$ receptor antagonist VPC23019 had no effect on the response to S1P. Values are means ± SE of 3 experiments.

DISCUSSION

The presence of two or more S1P receptor types on target cells complicates the analysis of signaling pathways, in particular the assigning of function to specific receptors. Recent studies (31) have demonstrated coexpression of $S1P_1$ and $S1P_2$ receptors in smooth muscle cells of the gut. In these cells, S1P activates a full complement of PTx-sensitive and -insensitive G proteins and initiates signaling cascades that mediate Ca^{2+}-dependent initial contraction and Ca^{2+}-independent sustained contraction (24, 31). The initial contraction reflected activation of PLC-β1 and PLC-β3 by PTx-insensitive (G_q) and -sensitive (G_i) G proteins, respectively, whereas sustained contraction reflected activation of RhoA via PTx-insensitive G proteins (G_q and G_{13}). In the present study, PLC-β (PI hydrolysis) and Rho kinase activities were used as specific markers of the pathways that mediate initial and sustained contraction, respectively.

Molecular (selective receptor silencing by siRNA) and pharmacological (selective receptor inactivation) approaches were used to determine whether $S1P_1$ and/or $S1P_2$ receptors mediated these signaling pathways. The siRNA constructs for $S1P_2$ and $S1P_1$ receptors used in the present study efficiently and stably silence $S1P_2$ and $S1P_1$ receptors endogenously ex-

pressed in gastric smooth muscle cells (11). In these studies, the lentiviral vectors encoding each siRNA were delivered into smooth muscle cells by infection with lentivirus (11). In the present study, the same vectors were transiently transfected into smooth muscle cells using Lipofectamine 2000. Transient silencing of S1P$_2$ but not S1P$_1$ receptors abolished S1P-induced PLC-β and Rho kinase activities (Figs. 2 and 3), confirming the results obtained on stable silencing of each receptor type by lentiviral infection (11).

Receptor silencing yielded a clear pattern of G protein activation by each receptor. S1P$_2$ receptors activated G_q, G_{13}, G_{i2}, and G_{i1} (~40%), whereas S1P$_1$ receptors activated G_{i3} and G_{i1} (~60%). Because S1P stimulated both PTx-sensitive and -insensitive PLC-β activities as previously shown (31) and silencing of S1P$_2$ receptors abolished PLC-β activity, it follows that PLC-β activity reflected activation of G_q (PTx-insensitive component) and G_{i2} and/or G_{i1} (PTx-sensitive component). It also follows that coupling of S1P$_1$ receptors to G_{i3} or G_{i1} did not lead to activation of PLC-β. The inability of S1P$_1$ receptors to activate PLC-β in smooth muscle cells contrasts with observations made in Chinese hamster ovary or human erythroleukemia cells, where expression of S1P$_1$ receptors resulted in S1P-induced, PTx-sensitive stimulation of PI hydrolysis and Ca^{2+} mobilization (27). Expression of S1P$_1$ receptors in insect *Spodoptera frugiperda* Sf9 or monkey kidney fibroblast (COS7) cells, however, did not result in Ca^{2+} mobilization (32). The contrasting results between various cell lines and between cell lines and native cells (e.g., smooth muscle cells) underline the importance of determining G protein coupling and signaling pathways in each cell type.

Similarly, because silencing of S1P$_2$ receptors (Fig. 3) or coexpression of G_q and G_{13} minigenes (31) abolished Rho kinase activity, it follows that Rho kinase activity reflected activation of G_q and G_{13} by S1P$_2$ receptors. The pattern is consistent with previous studies (25) showing that receptors coupled exclusively to G_i do not activate RhoA or Rho kinase.

The effect of receptor silencing on G protein activation and on specific markers of signaling pathways (PLC-β and Rho kinase) implied that initial and sustained muscle contraction was mediated exclusively by S1P$_2$ receptors. This conclusion was supported further by direct measurement of initial and sustained contraction in freshly dispersed smooth muscle cells. A selective S1P$_1$ receptor agonist did not elicit contraction or stimulate PLC-β and Rho kinase activities. When the agonist was used to protect S1P$_1$ receptor so as to allow chemical inactivation of all other receptors including S1P$_2$ receptors, the cells did not contract in response to S1P.

We have previously shown that some G_i-coupled receptors (e.g., opioid μ, δ, and κ, somatostatin $sstr_3$, and adenosine A_1 receptors) are capable of mediating sustained contraction via sequential activation of PI 3-kinase and integrin-linked kinase (ILK) by $G\beta\gamma_i$ (12). ILK activates CPI-17, a potent endogenous inhibitor of MLC phosphatase, and acts as a Ca^{2+}-independent MLC kinase, leading to sustained MLC_{20} phosphorylation and muscle contraction. This G_i-dependent mechanism did not contribute to sustained contraction mediated by S1P$_2$ receptors, because sustained contraction induced by S1P (unlike initial contraction) was insensitive to PTx and was virtually abolished by a combination of $G\alpha_q$ and $G\alpha_{13}$ antibodies (31).

The inability of G_{i1}/G_{i3}-coupled S1P$_1$ receptors to elicit initial or sustained muscle contraction deserves further comment. Muscarinic M_2 receptors, which activate PLC-β3 but do not stimulate initial or sustained contraction, are preferentially coupled via $G\beta\gamma_{i3}$ to sequential activation of PI 3-kinase, p21-activated protein kinase (PAK1), and p38-MAPK: PAK1 phosphorylates and inactivates MLC kinase, whereas p38-MAPK phosphorylates and inactivates ILK, thus precluding phosphorylation of MLC_{20} and smooth muscle contraction (12). It is possible that a mechanism involving inactivation of ILK by p38 MAPK underlies the inability of S1P$_1$ (or S1P$_2$) receptors to elicit $G\beta\gamma_i$-dependent sustained contraction. A more plausible mechanism, however, is akin to that identified recently for cannabinoid CB_1 receptors in gastric smooth muscle cells: these receptors are coupled to an atypical G protein in which the Gγ-like domain of RGS6 (regulator of G protein signaling) binds to $G\beta_5$ and $G\alpha_{i2}$ (17). On dissociation, the RGS6-Gβ5 complex, unlike a typical $G\beta\gamma_i$ dimer, does not activate downstream effector enzymes such as PLC-β3 or PI 3-kinase and thus does not initiate signaling cascades capable of stimulating initial or sustained contraction. This aspect will be explored in future studies.

We have previously shown that smooth muscle cells express two isoforms of sphingosine kinase (SPK1 and SPK2); these enzymes phosphorylate sphingosine, a metabolic product of ceramide, to yield S1P (31). The kinases are activated by various growth factors, cytokines, and G protein-coupled receptor agonists (e.g., acetylcholine). S1P formed within smooth muscle cells may be transported to the cell surface to activate S1P$_1$ and S1P$_2$ receptors and modulate the response to contractile agonists, growth factors, or cytokines. The functional significance of S1P in smooth muscle under physiological or pathological conditions remains to be explored.

In summary, the specific functions of S1P$_1$ and S1P$_2$ receptors coexpressed in smooth muscle of the gut were characterized by selective receptor silencing with siRNA and by selective chemical inactivation. Each receptor was shown to couple to a distinct complement of G proteins, and only S1P$_2$ receptors were shown to activate signaling pathways that mediate initial and sustained muscle contraction.

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