Characterization of S1P₁ and S1P₂ 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 S1P₁ and S1P₂ 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 Ca²⁺-dependent contraction followed by a sustained Ca²⁺-independent, RhoA-mediated contraction in rabbit gastric smooth muscle cells. The cells coexpress S1P₁ and S1P₂ 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 S1P₁ or S1P₂ receptors. Phospholipase C (PLC)-β activity and Rho kinase activity were used as markers of pathways mediating initial and sustained contraction, respectively. Silencing of S1P₁ receptors abolished S1P-stimulated activation of Gα₁₃ and partially inhibited activation of Gα₁₁, whereas silencing of S1P₂ receptors abolished activation of Gα₁₁, Gα₁₃, and Gα₁₂ and partially inhibited activation of Gα₁₁. Silencing of S1P₂, but not S1P₁ receptors, suppressed S1P-stimulated PLC-β and Rho kinase activities, implying that both signaling pathways were mediated by S1P₂ receptors. The results obtained by receptor silencing were corroborated by receptor inactivation. The selective S1P₁ 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 S1P₁ receptors so as to enable chemical inactivation of S1P₁ receptors, S1P did not elicit contraction, confirming that initial and sustained contraction was mediated by S1P₁ receptors. Thus S1P₁ and S1P₂ receptors are coupled to distinct complements of G proteins. Only S1P₂ 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 (SIP) 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 growth factor receptor-bound protein 2 (Grb2) and activation of 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 S1P₂ and probably S1P₃ receptors, are coupled to G₁₁, G₁₄, and G₁₂/G₁₃ (6, 28). Rac1 is activated via S1P₃ and inhibited via S1P₂; Rac1 activation reflects predominant coupling of S1P₃ to G₁₁ and Rac1 inhibition reflects predominant coupling of S1P₂ to G₁₂/G₁₃ (28). Recent studies (9) suggest that S1P₄ receptors regulate cell shape and motility via G₁₁ and G₁₂/G₁₃. S1P₅ is coupled to G₁₂ and G₁₃ but not to G₁₁ or G₁₄ (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 S1P₁ receptors and, to a lesser extent, S1P₁ and S1P₂ receptors (26). Gastric smooth muscle cells also contract in response to S1P and express both S1P₁ and S1P₂ receptors (31). All the G proteins (G₁₁, G₁₄, G₁₂, G₁₃, and G₁₅) except for G₁₅ are activated by S1P in gastric smooth muscle cells (31). Both G₁₁ and G₁₄ contribute to PI hydrolysis, Ca²⁺ release, and initial muscle contraction by activating G₁₁-dependent phospholipase (PLC)-β₁ and G₁₂/G₁₃-dependent PLC-β₃, resulting in inositol 1,4,5-trisphosphate (IP₃)-stimulated Ca²⁺ release and activation of Ca²⁺/calmodulin-dependent myosin light chain (MLC) kinase. Both G₁₁ and G₁₃ contribute to sustained muscle contraction by activating RhoA/Rho kinase/ PKC-dependent pathways that mediate inhibition of MLC phosphatase and activation of Ca²⁺-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 S1P₁ or S1P₂ receptors. In addition, S1P₂ receptors were chemically inactivated after protection of S1P₁ receptors with a selective S1P₁ receptor

Current understanding of G protein coupling of S1P receptors is based on receptor expression studies in cell lines. In cells expressing S1P₁ receptors, S1P stimulates phosphoinositide (PI) hydrolysis, Ca²⁺ mobilization, and ERK1/2 in a pertussis toxin (PTx)-sensitive fashion, implying coupling to G₁₁ and G₁₃ (16). In cells expressing S1P₂ or S1P₃ receptors, S1P stimulates PI hydrolysis and Ca²⁺ 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 S1P₂ and probably S1P₃ receptors, are coupled to G₁₁, G₁₄, and G₁₂/G₁₃ (6, 28). Rac1 is activated via S1P₃ and inhibited via S1P₂; Rac1 activation reflects predominant coupling of S1P₃ to G₁₁ and Rac1 inhibition reflects predominant coupling of S1P₂ to G₁₂/G₁₃ (28). Recent studies (9) suggest that S1P₄ receptors regulate cell shape and motility via G₁₁ and G₁₂/G₁₃. S1P₅ is coupled to G₁₂ and G₁₃ but not to G₁₁ or G₁₄ (18).

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agonist. The results indicate that only S1P2 receptors activate PLC-β- and RhoA-dependent pathways that mediate initial and sustained contraction, respectively.

MATERIALS AND METHODS

siRNA for S1P1 and S1P2 receptors. Lentiviral vector constructs encoding enhanced green fluorescent protein (EGFP) as an internal marker together with siRNA for S1P1 or S1P2 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 XbaI/Xhol cloning sites. The sequence of each siRNA cassette was confirmed by restriction enzyme digestion with BamHI/EcoRI 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, S1P1b, and S1P2a, were used in the present study. The sequences for S1P1 and S1P2a were 5'-GAAGACCTGTGACATCCTGTA-3' and 5'-AC-CAAGGAGACGCTGACATG-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 S1P1 or S1P2 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%. Dispersed gastric smooth muscle cells were treated with S1P2 agonist SEW2871 (1 µM) to protect S1P1 and inactivate all other receptors. Freshly dispersed muscle cells were incubated with SEW2871 or S1P at 31°C for 2 min followed by inactivation of all unprotected receptors by 10 µCi of [γ-32P]ATP (3,000 Ci/mmol) and 20 µM ATP, followed by incubation for 10 min at 37°C. The [γ-32P]ATP-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 S1P1 receptors and inactivation of S1P2 receptors. A technique of selective preservation of one receptor type was used to determine the function of S1P1 and S1P2 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 S1P1 receptor agonist SEW2871 was used to protect S1P1, and inactivate all other receptors including S1P2 receptors. S1P was used as a control to preserve both S1P1, and S1P2 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 (naïve) 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 ~10^6 muscle cells/ml were treated with S1P or the selective S1P1 receptor agonist SEW2871 in the presence or absence of the S1P1 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-32P]ATP from Amersham Pharmacia Biotech (Piscataway, NJ), and myo-[3H]inositol from DuPont New England Nuclear (Boston, MA). Gx 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 S1P1 and S1P2 receptors in smooth muscle. Previous studies (31) have demonstrated that S1P activates Goq, Go13, and all three isoforms of Go1 in gastric smooth muscle cells. Because these cells express both S1P1 and S1P2 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 S1P1 or S1P2 receptors in cultured gastric smooth muscle cells transiently transfected with lentiviral vectors encoding siRNAs for each receptor. Silencing of S1P1 receptors abolished S1P-stimulated activation of Go3 and inhibited activation of Go1 by ~60% but had no effect on activation of Go2, Goq, and Go13 (Fig. 1). In contrast, silencing of S1P2 receptors abolished S1P-stimulated activation of Goq, Go13, and Go12 and inhibited activation of Go1 by ~40% but had no effect on activation of Go13 (Fig. 1). Thus S1P1 receptors are coupled to Go3 and Go1, whereas S1P2 receptors are coupled to Go2, Go13, Go12, and Go11. Only Gi1 activation is shared by both receptors.

Silencing of S1P2 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 Gq and Gi (31). The involvement of both G proteins was confirmed by expression of Goq or Goi minigens to inactivate Gi or all isoforms of Go. Silencing of S1P2 receptors abolished S1P-stimulated PLC-β activity (measured at 60 s), whereas silencing of S1P1 receptors had no effect (Fig. 2). In light of our previous study (31), this implied that activation of PLC-β was mediated by Gi (PTx-insensitive component) and by Gq (PTx-sensitive component) and that Rho kinase activity was partially inhibited by expression of Goq or Go13 mini-gens and virtually abolished by coexpression of both mini-gens, implying that RhoA was activated by both Gq and Go13 (31). In the present study, silencing of S1P2 receptors abolished S1P-stimulated Rho kinase activity (measured at 5 min), whereas silencing of S1P1 receptors had no effect (Fig. 3). The involvement of S1P2 receptors reflected their ability to activate Gi and Go13 (Fig. 1).

Chemical inactivation of S1P2 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 S1P1 receptor agonist, SEW2871, and used the latter to protect S1P1 recep-

![Fig. 1. Distinct patterns of G protein activation by sphingosine-1-phosphate (S1P1) and S1P2 receptors. Cultured gastric smooth muscle cells (SMCs) in first passage were transfected with lentiviral vectors encoding small interfering RNA (siRNA) for S1P1 or S1P2 receptors or empty vector (control). After 3 days, G protein activity was determined from the difference in the binding of specific Gs subunits to 0.1 μM [3H]GTPγS alone or in the presence of 1 μM S1P; the difference for each Go subunit is shown by open bars and expressed as counts per minute (cpm). Binding of GTPγS alone to Go subunits ranged from 852 ± 47 to 1,034 ± 65 cpm. Silencing of S1P2 receptors abolished S1P-stimulated Goq, Go13, Go12 activity and partially inhibited Go11 activity (~40%). Silencing of S1P1 receptors abolished Go13 activity and partially inhibited Go11 activity (~60%). Values are means ± SE of 4 experiments. *P < 0.05 and **P < 0.01, significant differences from control Go binding activity in response to S1P (shown by open bars for each Go subunit).](http://ajpgi.physiology.org/)

![Fig. 2. Silencing of S1P2 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 S1P1 or S1P2 receptors or empty vector (control). After 2 days, cells were labeled with myo-[3H]inositol and then treated with S1P (1 μM) or vehicle for 60 s. [3H]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 S1P1-siRNA. No significant (ns) increase in inositol phosphate production in cells expressing S1P2-siRNA was found.](http://ajpgi.physiology.org/)

![Fig. 3. Silencing of S1P2 receptors abolishes S1P-induced activation of Rho kinase. Cultured gastric SMCs in first passage were transfected with lentiviral vectors encoding siRNA for S1P1 or S1P2 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 S1P1-siRNA. No significant increase in Rho kinase activity in cells expressing S1P2-siRNA was found.](http://ajpgi.physiology.org/)
tors and chemically inactivate S1P2 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. 4A).

Treatment of the cells with SEW2871 for 2 min to protect S1P1 receptors followed by the addition of N-ethylmaleimide to inactivate all unprotected receptors including S1P2 receptors abolished the contractile response to S1P (Fig. 4B). In contrast, treatment of the cells with S1P protected both S1P1 and S1P2 receptors and preserved the response to S1P (Fig. 4B). Studies to protect S1P2 receptors and inactivate S1P1 receptors could not be done because the S1P2 receptor antagonist JTE013 reported in other studies (13, 26) is not commercially available.

Selective S1P1 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 S1P1/S1P3 antagonist (Fig. 5) (4). The results confirmed that S1P1 receptors do not activate pathways that mediate initial and sustained muscle contraction.

**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 S1P1 and S1P2 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 Ca2+-dependent initial contraction and Ca2+-independent sustained contraction (24, 31). The initial contraction reflected activation of PLC-β1 and PLC-β3 by PTx-insensitive (Gq) and -sensitive (Gi) G proteins, respectively, whereas sustained contraction reflected activation of RhoA via PTx-insensitive G proteins (Gq and G13). 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 S1P1 and/or S1P2 receptors mediated these signaling pathways. The siRNA constructs for S1P2 and S1P1 receptors used in the present study efficiently and stably silence S1P2 and S1P1 receptors endogenously ex-

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**Fig. 4.** Chemical inactivation of S1P2 receptors abolishes S1P-induced smooth muscle contraction. A: freshly dispersed SMCs were treated with the selective S1P1 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 S1P2 receptors after protection of S1P1 receptors with SEW2871 abolished the contractile response to S1P, whereas protection of S1P1 and S1P2 receptors after protection with S1P preserved the contractile response to S1P. Values are means ± SE of 3–4 experiments.

**Fig. 5.** A selective S1P1 receptor agonist does not activate PLC-β or Rho kinase in freshly dispersed gastric SMCs. Cells were treated with a selective S1P1 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 S1P1/S1P3 receptor antagonist VPC23019 had no effect on the response to S1P. Values are means ± SE of 3 experiments.
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 S1P2 but not S1P1 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. S1P2 receptors activated Gq, G13, G12, and Gi1 (~40%), whereas S1P1 receptors activated Gi3 and Gi1 (~60%). Because S1P stimulated both PTx-sensitive and -insensitive PLC-β activities as previously shown (31) and silencing of S1P2 receptors abolished PLC-β activity, it follows that PLC-β activity reflected activation of Gq (PTx-insensitive component) and G12 and/or Gi1 (PTx-sensitive component). It also follows that coupling of S1P1 receptors to Gi3 or Gi1 did not lead to activation of PLC-β. The inability of S1P1 receptors to activate PLC-β in smooth muscle cells contrasts with observations made in Chinese hamster ovary or human erythroleukemia cells, where expression of S1P1 receptors resulted in S1P-induced, PTx-sensitive stimulation of PI hydrolysis and Ca2+ mobilization (27). Expression of S1P1 receptors in insect Spodoptera frugiperda Sf9 or monkey kidney fibroblast (COS7) cells, however, did not result in Ca2+ 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 S1P2 receptors (Fig. 3) or coexpression of Gq and G13 minigenes (31) abolished Rho kinase activity, it follows that Rho kinase activity reflected activation of Gq and G13 by S1P2 receptors. The pattern is consistent with previous studies (25) showing that receptors coupled exclusively to Gi 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 S1P2 receptors. This conclusion was supported further by direct measurement of initial and sustained contraction in freshly dispersed smooth muscle cells. A selective S1P1 receptor agonist did not elicit contraction or stimulate PLC-β and Rho kinase activities. When the agonist was used to protect S1P1 receptor so as to allow chemical inactivation of all other receptors including S1P2 receptors, the cells did not contract in response to S1P.

We have previously shown that some G13-coupled receptors (e.g., opioid μ, δ, and κ, somatostatin sstr3, and adenosine A1 receptors) are capable of mediating sustained contraction via sequential activation of PI 3-kinase and integrin-linked kinase (ILK) by Gβγ1 (12). ILK activates CPI-17, a potent endogenous inhibitor of MLC phosphatase, and acts as a Ca2+-independent MLC kinase, leading to sustained MLC20 phosphorylation and muscle contraction. This G13-dependent mechanism did not contribute to sustained contraction mediated by S1P2 receptors, because sustained contraction induced by S1P (unlike initial contraction) was insensitive to PTx and was virtually abolished by a combination of Goq and Gα13 antibodies (31).

The inability of Gi1/G13-coupled S1P1 receptors to elicit initial or sustained muscle contraction deserves further comment. Muscarinic M2 receptors, which activate PLC-β3 but do not stimulate initial or sustained contraction, are preferentially coupled via Gβγ13 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 MLC20 and smooth muscle contraction (12). It is possible that a mechanism involving inactivation of ILK by p38 MAPK underlies the inability of S1P1 (or S1P3) receptors to elicit Gβγ-dependent sustained contraction. A more plausible mechanism, however, is akin to that identified recently for cannabinoid CB1 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 Gβ and Go12 (17). On dissociation, RGS6-Gβ5 complex, unlike a typical Gβγ 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 S1P1 and S1P2 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 S1P1 and S1P2 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 S1P2 receptors were shown to activate signaling pathways that mediate initial and sustained muscle contraction.

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