Characterization of S1P$_1$ and S1P$_2$ receptor function in smooth muscle by receptor silencing and receptor protection

Wenhui Hu, Sunila Mahavadi, Jiean Huang, Fang Li, and Karnam S. Murthy

Departments of Physiology and Medicine, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia

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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)-$\beta$ 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$_{i3}$ and partially inhibited activation of G$_{i1}$, whereas silencing of S1P$_2$ receptors abolished activation of G$_{i4}$, G$_{i13}$, and G$_{i12}$ and partially inhibited activation of G$_{i11}$. Silencing of S1P$_2$ receptors but not S1P$_1$ receptors suppressed S1P-stimulated PLC-$\beta$ 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-$\beta$ 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-$\beta$ and Rho kinase and mediate initial and sustained contraction.

Small interfering RNAs; lentiviral vector; sphingosine-1-phosphate; phospholipase C-$\beta$; Rho kinase

THE LYSOPHOSPHOLIPIDS sphingosine-1-phosphate (SIP) and lysophosphatic 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 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 named 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$_7$). LPA interacts with LPA$_1$ (EDG$_2$), LPA$_2$ (EDG$_6$), 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$_3$ 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$_{i1}$, G$_{i3}$, and G$_{i13}$ (16). In cells expressing S1P$_2$ or S1P$_3$ receptors, S1P stimulates PI hydrolysis and Ca$^{2+}$ mobilization in both a PTx-sensitive and -independent 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$_{i4}$, G$_{i4}$, and G$_{i12}$/G$_{i13}$ (6, 28). Rac1 is activated via S1P$_3$ and inhibited via S1P$_2$; Rac1 activation reflects predominant coupling of S1P$_3$ to G$_{i6}$ and Rac1 inhibition reflects predominant coupling of S1P$_2$ to G$_{i12}$/G$_{i13}$ (28). Recent studies (9) suggest that S1P$_4$ receptors regulate cell shape and motility via G$_{i4}$ and G$_{i12}$/G$_{i13}$. S1P$_5$ is coupled to G$_{i6}$ and G$_{i12}$ but not to G$_{i4}$ or G$_{i13}$ (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$_4$ receptors (26). Gastric smooth muscle cells also contract in response to S1P and express both S1P$_2$ and S1P$_3$ receptors (31). All the G proteins (G$_{i1}$, G$_{i1}$, G$_{i2}$, G$_{i3}$, and G$_{i13}$) except for G$_{i4}$ are activated by S1P in gastric smooth muscle cells (31). Both G$_{i4}$ and G$_{i1}$ contribute to PI hydrolysis, Ca$^{2+}$ release, and initial muscle contraction by activating G$_{i4}$-dependent phospholipase (PLC)-B1 and G$_{i4}$-dependent PLC-$\beta$-3, 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$_{i4}$ and G$_{i13}$ 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

Address for reprint requests and other correspondence: W. Hu, Dept. of Physiology, P.O. Box 980551, Medical College of Virginia Campus, Virginia Commonwealth Univ., Richmond, VA 23298 (e-mail: whu@vcu.edu).

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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 carcinoma and specificity of these siRNA constructs were determined by measuring the increase in G

**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), and [3H]inositol from DuPont New England Nuclear (Boston, MA). Gx and Rho kinase-2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Rho kinase-2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**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.
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 Gqq₁, Gq₁₃, and all three isoforms of Gq₁₁ 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 Gq₁₃ and inhibited activation of Gq₁₁ by ~60% but had no effect on activation of Gq₁₂, Gq₁₉, and Gq₁₃ (Fig. 1). In contrast, silencing of S1P₂ receptors abolished S1P-stimulated activation of Gq₁₆, Gq₁₃, and Gq₁₂ and inhibited activation of Gq₁₁ by ~40% but had no effect on activation of Gq₁₃ (Fig. 1). Thus S1P₁ receptors are coupled to Gq₁₃ and Gq₁₁, whereas S1P₂ receptors are coupled to Gq₁₂, Gq₁₉, Gq₁₇, and Gq₁₁. Only Gq₁₁ 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 G₁₁ (31). The involvement of both G proteins was confirmed by expression of Gq₁₂ or Gq₁₆ minigenes to inactivate Gq or all isoforms of G₁₁. Silencing of S1P₁ receptors abolished S1P-stimulated PLC-β activity (measured at 60 s), whereas silencing of S1P₂ receptors had no effect (Fig. 2). In light of our previous study (31), this implied that activation of PLC-β was mediated by Gq (PTx-insensitive component) and by Gq₁₂.

![Fig. 1. Distinct patterns of G protein activation by sphingosine-1-phosphate (S1P₁ and S1P₂ receptors.](image1)

![Fig. 2. Silencing of S1P₂ receptors abolishes S1P-induced activation of phospholipase C (PLC)-β.](image2)

![Fig. 3. Silencing of S1P₂ receptors abolishes S1P-induced activation of Rho kinase.](image3)

We have also previously shown that in smooth muscle cells expressing both receptors, S1P-stimulated Rho kinase activity was partially inhibited by expression of Gq₁₂ or Gq₁₆ minigenes and virtually abolished by coexpression of both minigenes, implying that RhoA was activated by both Gq₁₂ and Gq₁₆ (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 Gq₁₂ and Gq₁₆ (Fig. 1).

Chemical inactivation of S1P₂ receptor suppresses S1P₁-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-
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+ /H11001-dependent initial contraction and Ca2+ /H11001-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-
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 Gαi1, G13, G2α, and G1α (~40%), whereas S1P1 receptors activated G1α3 and G1α (~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 Gαi (PTX-insensitive component) and G1α2 and/or G1α (PTX-sensitive component). It also follows that coupling of S1P1 receptors to G1α3 or G1α1 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 G1α and G1α3 minigenes (31) abolished Rho kinase activity, it follows that Rho kinase activity reflected activation of G1α and G1α3 by S1P2 receptors. The pattern is consistent with previous studies (25) showing that receptors coupled exclusively to G1α 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 G1α-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 PI-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 G1α-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 Gαq and Gαq13 antibodies (31).

The inability of Gαi1/G1α3-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 Gβγi3 to sequential activation of PI 3-kinase, p21-activated protein kinase (PAK1), and p38-MAKP: PAK1 phosphorylates and inactivates MLC kinase, whereas p38-MAKP 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 Gβγ1-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 Gβ3 and Goi12 (17). On dissociation, the RGS6-Gβγ5 complex, unlike a typical Gβγ complex, 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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SIP2 mediates smooth muscle contraction


