Sphingosine 1-Phosphate Causes Airway Hyper-Reactivity by Rho-Mediated Myosin Phosphatase Inactivation

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ABSTRACT

In the present study, we investigated whether extracellular sphingosine 1-phosphate (S1P) is involved in airway hyper-reactivity in bronchial asthma. The effects of S1P on the response to methacholine was examined in the fura-2-loaded strips of guinea pig tracheal smooth muscle using simultaneous recording of the isometric tension and the ratio of fluorescence intensities at 340 and 380 nm (F340/F380). A 15-min pretreatment with S1P (>100 nM) markedly enhanced methacholine-induced contraction without elevating F340/F380. This effect of S1P was suppressed in the presence of Y-27632 (R)-(+)-trans-4-(1-aminoethyl)-cyclohexane-carboxamide, a selective inhibitor of Rho-kinase, in a concentration-dependent manner. Moreover, pretreatment with pertussis toxin caused an inhibition in S1P-induced hyper-reactivity to methacholine in a time- and concentration-dependent manner. In contrast, although S1P-induced Ca2+ mobilization was attenuated by SKF96365 and verapamil, the subsequent response to methacholine was unaffected. A 15-min pretreatment with lower concentrations of S1P (<100 nM), which is clinically attainable, did not increase methacholine-induced contraction. However, when the incubation was lengthened to 6 h, S1P (<100 nM) enhanced the subsequent response to methacholine. Next, application of S1P to cultured human bronchial smooth muscle cells increased the proportion of active RhoA (GTP-RhoA) and phosphorylation of myosin phosphatase target subunit 1 (MYPT1). This phosphorylation of MYPT1 was significantly inhibited by application of Y-27632 and by pretreatment with pertussis toxin. Our findings demonstrate that exposure of airway smooth muscle to S1P results in airway hyper-reactivity mediated by Ca2+ sensitization via inactivation of myosin phosphatase, which links G- and RhoA/Rho-kinase processes.

Sphingosine 1-phosphate (S1P), a bioactive lysophospholipid, is generated in the plasma membrane of various cells by sphingolipid metabolism. It is generally considered that S1P is capable of a wide spectrum of biological effects, including cell growth, cell differentiation, cell survival, and cell migration (Spiegel and Milstien, 2000). Recent clinical trials have demonstrated that the level of S1P is dramatically elevated in bronchoalveolar lavage (BAL) fluid from patients with asthma following allergen challenges (Ammit et al., 2001; Jolly et al., 2001). In vitro, FceRI cross-linking, which elicits the IgE-mediated allergic reaction, activates sphingosine kinase, resulting in the conversion of sphingosine to S1P (Jolly et al., 2004). S1P is released from mast cells and plays an important role not only in regulating mast cell functions but also eosinophil chemotaxis (Rosenfeldt et al., 2003; Roviezzo et al., 2004). Moreover, extracellular S1P modulates the production of proinflammatory cytokines and cell proliferation. Therefore, S1P may act as a lipid mediator in the pathophysiology of bronchial asthma.

External application of S1P to smooth muscle in a various tissues causes contraction with an increase in the concentrated intracellular Ca2+ ([Ca2+]i) (Watterson et al., 2005). The Ca2+ mobilization induced by S1P is mediated by Ca2+ influx pass through voltage-dependent L-type Ca2+ channels and store-operated Ca2+ entry (Ghosh et al., 1990; Bischoff et al., 2000; Rosenfeldt et al., 2003). On the other hand, Rho, a small GTPase, and Rho-kinase, a specific effector of Rho, are involved in the postreceptor signal transduction pathways of S1P (Coussiun et al., 2002; Rosenfeldt et al., 2003; Salomone et al., 2003; Zhou and Murthy, 2004). However, in smooth muscle contraction induced by S1P, the role of Ca2+ sensitization that relates to Rho/Rho-kinase pathways remains un-
clear. Extracellular S1P acts as a ligand for a family of specific G-protein coupled receptors (S1P<sub>1-5</sub>), which are coupled to a variety of G proteins that are linked, in turn, to downstream signaling pathways (Payne et al., 2002; Siehler et al., 2003). Previous studies in cultured human ASM cells using mechanically loaded collagen matrix have shown that S1P causes contraction by activating voltage-dependent L-type Ca<sup>2+</sup> channels (Rosenfeldt et al., 2003). Moreover, the S1P-induced contraction in ASM cells is mediated by pertussis toxin (PTX)-insensitive processes (Rosenfeldt et al., 2003). However, it has been proposed that G<sub>i</sub> may participate in S1P-induced contraction of smooth muscle in other tissues (Bischoff et al., 2000; Salomone et al., 2003; Zhou and Murphy, 2004). Little is currently known about S1P action in ASM, although it could act as a mediator of the interaction between inflammatory cells and ASM in bronchial asthma. This study was designed to determine whether extracellular S1P is involved in airway hyper-reactivity, which is implicated in the pathophysiology of bronchial asthma. To identify the molecular mechanisms of regulating ASM contractility following continuous exposure to S1P, we focused on the role of Ca<sup>2+</sup> mobilization and Ca<sup>2+</sup> sensitization in the postreceptor signal transduction pathways activated by S1P.

**Materials and Methods**

**Tissue Preparation and Solution.** Male Hartley guinea pigs (300–350 g) were killed by injection of overdose of anesthetics (150 mg/kg pentobarbital i.p.), and tracheas were excised. The tracheal rings were opened by cutting longitudinally at the cartilaginous region, and the epithelium was dissected out. The normal bathing solution was composed of 137 mM NaCl, 5.9 mM KHCO<sub>3</sub>, 2.4 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, and 11.8 mM glucose, bubbled with a mixture of 99% O<sub>2</sub> and 1% CO<sub>2</sub> (pH 7.4). The organ bath was filled with the bathing solution at a constant flow of 3 ml/min, and the temperature of the organ bath was maintained at 37°C. All animal procedures were approved by The Animal Care and Use Committee, Nagoya University Graduate School of Medicine.

**Isometric Tension Recording and Measurement of Fura-2 Fluorescence.** Isometric tension and fura-2 fluorescence were measured essentially as described previously (Kume and Takagi, 1987; Kume et al., 2001). Muscle strips containing four cartilaginous rings, one for isometric tension recording and three for measurement of [Ca<sup>2+</sup>]<sup>i</sup>, were prepared. Muscle strips were treated with 10 μM acetylcholine ester of fura-2 for 4 h at room temperature (22–24°C). The nontoxic detergent, Pluronic F-127 (0.01% v/v), was added to increase the solubility of fura-2. After the loading, the chamber was filled with the normal bathing solution at 37°C for 50 min to wash out the extracellular fura-2 prior to the measurements. Isometric tension and the fura-2 fluorescence of the muscle strips were measured simultaneously, using a displacement transducer and a spectrofluorometer (CAB-110; Japan Spectroscopic, Tokyo, Japan). The intensities of fluorescence due to excitation at 340 nm (F<sub>340</sub>) and 380 nm (F<sub>380</sub>) were measured after background subtraction. The absolute amount of [Ca<sup>2+</sup>]<sup>i</sup> was not calculated because the dissociation constant of fura-2 for Ca<sup>2+</sup> in smooth muscle cytoplasm is different from that obtained in vitro (Konishi et al., 1988). Therefore, the ratio F<sub>340/F<sub>380</sub></sub> was used as a relative indicator of [Ca<sup>2+</sup>]<sup>i</sup>. Muscle tension and F<sub>340/F<sub>380</sub></sub> in the resting state were taken as 0%, and the values of percent contraction and percent F<sub>340/F<sub>380</sub></sub> were expressed by taking response to 1 μM methacholine (MCh) or 10 μM histamine at each experimental condition as 100%. Time-matched control tissues were treated similarly to the test tissues but exposed continuously to the normal bathing solution instead of S1P and PTX. The resting tone was abolished by addition to 2 μM indomethacin throughout the experiments.

**Cell Culture.** Primary cultures of normal human bronchial smooth muscle (BSM) cells from multiple donors were obtained from Cambrex (Walkersville, MD). The cells were maintained in culture medium containing 5% fetal bovine serum, human recombinant epidermal growth factor (1 ng/ml), insulin (10 μg/ml), human recombinant fibroblast growth factor (2 ng/ml), gentamicin (50 μg/ml), and amphotericin B (0.05 mg/ml) (SmGM-2 BulletKit; Cambrex) in an atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. Cells at the 5th to 7th passages were used for the experiments.

**RhoA Activation Assay.** The amount of activated Rho (GTP-Rho) was determined using a Rho activation assay kit (Upstate Biotechnology, Inc., Lake Placid, NY). The human BSM cells were grown to confluence and then placed in serum-free medium (Dulbecco’s modified Eagle’s medium/F-12; Invitrogen, Carlsbad, CA) containing antibiotics-antimycotic (100 units/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B; Invitrogen) for 24 h. After stimulation by S1P, the cells were washed with ice-cold phosphate-buffered saline (PBS). Cellular lysates were prepared according to the manufacturer’s instructions.

**Phosphorylation of Myosin-Binding Subunit.** Phosphorylation of myosin-binding subunit (MBS) was evaluated with specific antibodies for myosin phosphatase target subunit 1 (MYPT1). The human BSM cells were grown to confluence and then placed in serum-free medium for 24 h. After exposure to agents, the BSM cells were washed with ice-cold PBS. Whole-cell lysates were prepared by treating the cells with lysis buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, and 5% 2-mercaptoethanol).

**Western Blot.** Protein contents of cellular lysates were measured using a Bio-Rad protein assay reagent kit (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Equal amounts of lysates, adjusted to protein content, were resolved by SDS-polyacrylamide gel electrophoresis using a 4 to 20% linear gradient running gel. Proteins were transferred to a nitrocellulose membrane, and the membrane was incubated at room temperature in PBS containing 0.2% Tween 20 for 1 h. Immunoblotting was performed using antibodies against RhoA, MYPT1 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-phospho-MYPT1 (Thr<sup>560</sup>) (Upstate Biotechnology, Inc.). Immunodetection was accomplished using a sheep anti-mouse secondary antibody or donkey anti-rabbit secondary antibody and the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ). The intensity was quantified using Scion image software (Scion Image, Frederick, MD).

**Experimental Protocols.** To examine the effects of S1P on ASM, the fura-2-loaded tissues of guinea pig tracheal smooth muscle were treated with S1P for 15 min. Before and after application of S1P, the strips were washed out for 10 min with the normal bathing solution. To examine the effects of S1P on airway hyper-reactivity, the contraction and F<sub>340/F<sub>380</sub></sub> in response to 1 μM MCh and 10 μM histamine were measured before and after exposure to S1P. To determine the involvement of Ca<sup>2+</sup> mobilization by S1P in the pathogenesis of airway hyper-reactivity, the tension and F<sub>340/F<sub>380</sub></sub> in response to MCh were examined before and after exposure to S1P in the absence and presence of Ca<sup>2+</sup> channel blockers such as SKF96365, a nonselective inhibitor of Ca<sup>2+</sup> channels, and verapamil, a selective inhibitor of voltage-dependent Ca<sup>2+</sup> channels. To determine the involvement of Ca<sup>2+</sup> sensitization by S1P in these mechanisms, response to MCh was similarly examined in the absence and presence of Y-27632, a selective inhibitor of Rho-kinase, PD98059, an inhibitor of mitogen-activated protein kinase kinase, and bisindolylmaleimide, an inhibitor of protein kinase C. When the period for incubation with S1P (<100 nM) was lengthened to 6 h, the strips were not loaded with fura-2. To determine the involvement of G<sub>i</sub>, the inhibitory G protein of adenyl cyclase, in the augmentation of MCh-induced contraction by S1P, the strips were pretreated with...
−1 μg/ml PTX for 6 h, and then the response to MCh was examined after exposure to S1P. To determine relationships between S1P and RhoA, after treating the human BSM cells with −3 μM S1P for 15 min, GTP-RhoA was measured using the RhoA activation assay. Moreover, to confirm the effects of S1P on RhoA, phosphorylation of MYPT1, a direct target substrate of Rho-kinase, was measured in the human BSM cells using Western blots. To determine the involvement of Rho-kinase and extracellular signal-regulated kinase in the stimulation of MYPT1 phosphorylation by S1P, the cells were treated with Y-27632 and PD98059, respectively, 30 min before stimulation by S1P. To determine the involvement of G1 in this phosphorylation, the cells were treated with 100 ng/ml PTX for 4 h before stimulation with S1P.

**Agents.** MCh, indomethacin, PTX, verapamil, bisindolylmaleimide, histamine, and Pluronic F-127 were obtained from Sigma (St. Louis, MO). S1P was obtained from BIOMOL (Plymouth Meeting, PA). Y-27632 was obtained from Wako (Osaka, Japan). SKF96365 and PD98059 were obtained from Calbiochem (La Jolla, CA). Fura-2/AM was obtained from Dojin Laboratories (Kumamoto, Japan).

**Statistics.** All data were expressed as means ± S.D., and response to an agent under each experimental condition was described as the percentage of the control condition. Values of concentration of agents that produces a 50% response (EC50) of contraction by 1 μM MCh were determined using regression analysis applied to the linear portion of each concentration-response curve. Statistical significance was assessed by Student’s t test or repeated-measures analysis of variance, followed by Bonferroni post hoc test. *p* < 0.05 was considered to be a significant difference.

**Results**

**Role of Ca2+ Sensitization in the Induction of Airway Hyper-Reactivity by S1P.** In fura-2-loaded tissues, S1P (0.03–10 μM) caused a concentration-dependent generation of force with an increase in $F_{340}/F_{380}$. The concentration-response curve for $F_{340}/F_{380}$ induced by S1P was upper area than that curve for tension induced by S1P (Fig. 1B). The value of percent contraction in response to 3 μM S1P was 52.4 ± 10.5%, whereas the value of percent $F_{340}/F_{380}$ in response to an equimolar of S1P was 95.1 ± 3.1% (*n* = 10, *p* < 0.01, Fig. 1B). A 15-min exposure to 3 μM S1P markedly

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**Fig. 1.** Augmentation of MCh-induced contraction by S1P in ASM. A, typical example of a simultaneous record of tension (top trace) and $F_{340}/F_{380}$ (bottom trace) in response to 1 μM MCh before and after 3 μM S1P for 15 min. B, concentration-response curves for S1P on tension (open circles) and $F_{340}/F_{380}$ (closed circles). The values of percent response to S1P were expressed taking response to 1 μM MCh as 100%. C, values of percent contraction (open columns) and $F_{340}/F_{380}$ (closed columns) for MCh after exposure to the normal bathing solution (control) and S1P between 0.03 and 3 μM for 15 min. D, values of percent contraction (open columns) and $F_{340}/F_{380}$ (closed columns) for histamine after exposure to S1P in the same way in C. E, concentration-response curves for MCh after exposure to the normal bathing solution (open circles), 0.003 μM (closed circles), and 0.03 μM S1P (open squares) for 6 h. The abscissa in B and E express molar concentrations on a log scale. *p* < 0.05; **, *p* < 0.01.
enhanced MCh-induced contraction without affecting $F_{340}/F_{380}$ (Fig. 1A). As shown in Fig. 1C, pretreatment with S1P (0.03–3 μM) caused an increase in the contraction induced by MCh in a concentration-dependent manner. The values of percent contraction for MCh after treatment without and with 3 μM S1P were 99.6 ± 9.8% (n = 10) and 138.5 ± 9.2% (n = 10, p < 0.01), respectively. In contrast, the values of percent $F_{340}/F_{380}$ for MCh were 96.8 ± 9.8% (n = 10) and 100.8 ± 9.4% (n = 10, not significant), respectively. Contractions induced by 10 μM histamine were also augmented without elevating $F_{340}/F_{380}$ after exposure to equimolar S1P for an equivalent time (Fig. 1D). The values of percent contraction and percent $F_{340}/F_{380}$ after exposure to 3 μM S1P were 129.8 ± 8.4 (p < 0.01) and 98.6 ± 7.8% (not significant), respectively (n = 8). Next, MCh was applied cumulatively to the fura-2-unloaded tissues between 0.001 and 10 μM before and after incubation with S1P (0.003–0.03 μM) for 6 h. Under this experimental condition, the concentration-response curves for MCh were markedly shifted to the left in a concentration-dependent manner (Fig. 1E). The values of $EC_{50}$ for the curves after treatment without and with 0.03 μM S1P were 0.59 ± 0.18 (n = 12) and 0.05 ± 0.02 μM (n = 12, p < 0.01), respectively.

### Involvement of Ca$^{2+}$ Sensitization by Rho-Kinase in the Induction of Airway Hyper-Reactivity by S1P.

In the presence of 1 μM Y-27632, 3 μM S1P-induced contraction was markedly attenuated without reducing $F_{340}/F_{380}$ (Fig. 2A). After exposure to 3 μM S1P with 1 μM Y-27632, both contraction and $F_{340}/F_{380}$ induced by 1 μM MCh were not augmented (Fig. 2A). Application of Y-27632 (0.01–1.0 μM) caused an inhibition in the enhancement of MCh-induced contraction by S1P in a concentration-dependent manner. The value of percent contraction for MCh after exposure to 3 μM S1P with 1 μM Y-27632 was decreased to 100.4 ± 9.4% (n = 8, p < 0.01) (Fig. 2B). In contrast, the values of percent for $F_{340}/F_{380}$ for MCh were unaffected (Fig. 2B). After exposure of the tissues to Y-27632 without S1P, MCh-induced contraction was not reduced (data not shown). MCh (0.001–10 μM) was cumulatively applied to the fura-2-unloaded tissues before and after exposure to 0.03 μM S1P with Y-27632 (0.1–1.0 μM) for 6 h. The concentration-response curve for MCh after exposure to S1P was shifted to the right by addition of Y-27632 in a concentration-dependent manner. The values for $EC_{50}$ for the curves for MCh after exposure to 0.03 μM S1P in the absence and presence of 1.0 μM Y-27632...
were 0.06 ± 0.04 (n = 10) and 0.41 ± 0.16 μM (n = 10, p < 0.01), respectively (Fig. 2C).

Next, in the presence of 30 μM PD98059, response to MCh was examined before and after exposure to 3 μM SIP for 15 min. However, under this experimental condition, the contraction induced by 1 μM MCh following exposure to SIP was increased without an elevation in F$_{340}$, similar to the result shown in Fig. 1A. The values of percent contraction and percent F$_{340}$ for MCh were 134.9 ± 12.4 (not significant) and 105.2 ± 9.2% (not significant), respectively (n = 6, Fig. 2D).

Ca$^{2+}$ Mobilization Is Not Involved in the Induction of Airway Hyper-Reactivity by SIP. SKF96365 (100 μM) resulted in a marked suppression of both tension and F$_{340}$ for MCh (Fig. 3A). However, after exposure to SIP with SKF96365, 1 μM MCh-induced contraction was markedly increased without elevating F$_{340}$, similar to the result shown in Fig. 1A. The values of percent contraction and percent F$_{340}$ for MCh after exposure to SIP with SKF96365 were 129.7 ± 12.9% (n = 6, not significant) and 96.3 ± 9.8% (n = 6, not significant), respectively (Fig. 3B). Verapamil (10 μM) caused a modest inhibition in the augmented tension and F$_{340}$ by equimolar SIP (data not shown). Moreover, after exposure to SIP in the presence of verapamil, 1 μM MCh-induced contraction was markedly increased independent of F$_{340}$ similar to the results shown in Fig. 1A. The values of percent contraction and percent F$_{340}$ for MCh after exposure to SIP in the presence of verapamil were 134.1 ± 12.1 (n = 6, not significant) and 102.4 ± 13.1% (n = 6, not significant), respectively (Fig. 3B).

Involvement of G$_i$ in the Induction of Airway Hyper-Reactivity Induced by SIP. Pretreatment of the fura-2-unloaded tissues with PTX 1 μg/ml for ~6 h caused a marked attenuation of the augmented response to 1 μM MCh after exposure to 3 μM SIP in a time-dependent manner (Figs. 4, A and B). The values of percent contraction for MCh after exposure to SIP, followed by treatment without and with PTX for 6 h, were 134.6 ± 12.8 (n = 8) and 102.5 ± 8.6% (n = 8, p < 0.01), respectively (Fig. 4B). MCh (0.001–10 μM) was cumulatively applied to the tissues before and after incubation with 0.1 and 1.0 μg/ml PTX in the presence of 0.05 μM SIP for 6 h. The concentration-response curves for MCh after exposure to SIP with PTX were shifted to the right in a concentration-dependent manner (Fig. 4C). The values of EC$_{50}$ for the curves for MCh after exposure to SIP without and with 1.0 μg/ml PTX for 6 h were 0.08 ± 0.05 (n = 8) and 0.48 ± 0.18 μM (n = 8, p < 0.01), respectively (Fig. 4C).

Effects of SIP on RhoA Activation in ASM Cells. After cultured human BSM cells were exposed for 15 min to serum-free medium in the absence (control) and presence of 3 μM SIP, relative proportion of RhoA-GTP to total RhoA (RhoA-GTP/total RhoA) was measured using the Rhotekin binding domain assay. The value of RhoA-GTP/total RhoA was increased to 2.3 ± 0.2-fold (n = 4) by application of 3 μM SIP, compared with the control (p < 0.01, Fig. 5).

Effects of SIP on the Phosphorylation of MYPT1 in ASM Cells. Application of SIP (0.3–3 μM) for 15 min caused a concentration-dependent increase in MYPT1 (Thr$^{850}$) phosphorylation in human BSM cells (Fig. 6A). An increase in phosphorylation of MYPT1 (Thr$^{850}$) by 3 μM SIP was significantly inhibited in the presence of Y-27632 (0.1–1.0 μM) in a concentration-dependent manner (Fig. 6B). In contrast, under the experimental condition of exposure to SIP in the presence of 30 μM PD98059, phosphorylation of MYPT1 (Thr$^{850}$) by SIP was not affected (Fig. 6C). However, when the human BSM cells were pretreatment with 0.1 μg/ml PTX for 4 h, the effect of SIP on phosphorylation of MYPT1 (Thr$^{850}$) was markedly suppressed (Fig. 6C).

**Discussion**

The principal observations in this study are as follows. SIP directly suppresses myosin phosphatase (MP) activity by the phosphorylation of MYPT1, which is a direct target substrate of Rho-kinase. Pretreatment with SIP leads to an increase in MCh-induced contraction mediated by Ca$^{2+}$ sensitization via the MP inactivation processes. PTX-sensitive processes are involved in the functional effects of SIP on the induction of airway hyper-reactivity.

The contractile effect of SIP on ASM has not been previously investigated in tissue samples, although studies in vascular smooth muscle have demonstrated that SIP induces contraction at concentrations above 100 nM (Bischoff et al.,
Effects of S1P on Airway Hyper-Reactivity by MYPT1

Fig. 4. Involvement of Gi in the induction of airway hyper-reactivity induced by S1P. A, typical record of tension by 1 μM MCh before and after exposure to 3 μM S1P for 15 min subsequent to incubation with 1.0 μg/ml PTX (top trace) and the normal bathing solution (sham, bottom trace) for 6 h. B, values of percent contraction for MCh after exposure to 3 μM S1P subsequent to incubation with the normal bathing solution (control), and an equivalent concentration of PTX for 2 to 6 h. C, concentration-response curves for MCh after exposure to 0.03 μM S1P in the absence (open circles) and presence of 0.1 μg/ml (closed circles), and 1.0 μg/ml (open squares) PTX for 6 h. The abscissa in C expresses molar concentrations on a log scale. *, p < 0.05; **, p < 0.01.

2000; Tosaka et al., 2001; Salomone et al., 2003), similar results were observed in ASM (Fig. 1B). Although the level of S1P in BAL fluid is elevated by an antigen challenge in patients with asthma, the concentration is less than 100 nM (Ammit et al., 2001; Jolly et al., 2001). Hence, extracellular S1P may not have a direct role in bronchoconstriction associated with bronchial asthma. Pretreatment with concentrations of S1P below 100 nM for 15 min also did not enhance subsequent responses to MCh or histamine (Fig. 1A, C, and D). However, when the period of incubation with S1P was extended to 6 h, lower concentrations of S1P (3–30 nM), which are similar to the level attained in BAL fluid after an antigen challenge, caused a marked enhancement of the response to MCh (Fig. 1E). These results in isolated guinea pig ASM tissues were also observed in isolated human ASM tissues effects (data not shown). Therefore, although whether S1P is related to airway hyper-reactivity in patients with asthma remains unknown, our results suggest that extracellular S1P, which is released from inflammatory cells, can lead to airway hyper-reactivity clinically.

As shown in Figs. 1B and 3A, S1P is less potent in enhancing the sensitivity to intracellular Ca2+ than MCh, and Ca2+ influx passing through Ca2+ channels nonselectively plays a functional important role in S1P-induced contraction. S1P has modest effects on verapamil-sensitive Ca2+ channels. In addition, similar to our observations, previous patch-clamp studies have shown that in endothelial cells, S1P activates nonselective cation channels (Muraki and Imaizumi, 2001). In contrast, our results indicate that Ca2+ sensitization mediated by Rho-kinase is involved in the functional effects of S1P on ASM (Fig. 2A). External application of S1P augments RhoA activity and the phosphorylation of MYPT1 (Figs. 5 and 6), demonstrating that a loss of MP activity via activation of the RhoA/Rho-kinase-mediated sensitization appears to be the inhibition in MP activity due to phosphorylation of MBS (Kimura et al., 1996; Matsui et al., 1996; Fukata et al., 2001). As shown in this study, Rho-kinase acts on MYPT1 in ASM, which is similar to findings in vascular smooth muscle (Velasco et al., 2002; Liu et al., 2004; Wilson et al., 2005). Therefore, phosphorylation of myosin light chain is an indirect response to S1P as a result of inhibition in MP activity by Rho-kinase.

We also examined the involvement of Ca2+ mobilization and Ca2+ sensitization in the induction of airway hyper-reactivity after exposure to S1P. As shown in Fig. 1, A and C, Ca2+ sensitization is involved in an increase in MCh-induced contraction after exposure to S1P. When S1P-induced contraction was suppressed in the presence of Y-27632 independent of [Ca2+]i, the augmented response to MCh after exposure to S1P was abolished in a concentration-dependent manner (Fig. 2, A and B). Moreover, Y-27632 also suppressed the ability of an extended treatment with the clinical levels of
SIP to enhance the effects of MCh (Fig. 2C). Therefore, Ca\(^{2+}\) sensitization via Rho-kinase-dependent inactivation of MP participates in the ability of SIP to induce airway hyper-reactivity. In contrast, even though both tension and [Ca\(^{2+}\)], by SIP was suppressed in the presence of SKF96365 and verapamil, the ability of SIP to enhance MCh-induced contraction was unaffected by these Ca\(^{2+}\) antagonists (Fig. 3, A and B). These results provide further support of the idea that Ca\(^{2+}\) sensitization but not Ca\(^{2+}\) mobilization is involved in the effect of SIP on the induction of airway hyper-reactivity in ASM. However, as shown in Fig. 2D, extracellular signal-regulated kinase and protein kinase C do not appear to be involved in the Ca\(^{2+}\) sensitization by SIP (Shirao et al., 2002).

Next, we sought to determine the involvement of the receptor and postreceptor signal transduction pathways in the induction of airway hyper-reactivity after treatment with SIP. As shown in Fig. 4, A to C, preincubation with PTX causes an inhibition in the enhancement of MCh-induced contraction by SIP in time- and concentration-dependent manners. Since exposure to PTX causes a functional uncoupling of G\(_i\), the inhibitory heterotrimeric G protein of adenylyl cyclase, from its associated receptor via the ADP ribosylation, G\(_i\) may mediate the ability of SIP to cause hyper-reactivity to MCh. A previous report has indicated that MCh-induced contraction in ASM is partly attenuated after exposure to PTX (Kume et al., 1995). Although the inhibitory effects of PTX on the SIP-induced hyper-reactivity to MCh may be due to muscarinic inhibition by PTX, incubation with PTX directly suppressed the phosphorylation of MYPT1 by SIP (Fig. 6C). Moreover, in ASM, the hyperresponsiveness to histamine was similarly observed by pretreatment with SIP (Fig. 1D). These results indicate that an inhibition in the muscarinic hyper-reactivity by SIP is due to a PTX-sensitive pathway that alters the action of SIP rather than a direct effect of the PTX-sensitive pathway on muscarinic action. In endothelial cells, activation of nonselective cation channels by SIP is also suppressed by pretreatment with PTX (Muraki and Imai, 2001). Although it remains unclear whether G\(_i\) is involved in the effect of SIP on ASM, our results demonstrate that G\(_i\) is related to SIP-induced hyper-reactivity to MCh via activation of MP. In smooth muscle other than ASM, external application of SIP augmented both force generation and [Ca\(^{2+}\)], via a PTX-sensitive process (Bischoff et al., 2000; Salomone et al., 2003; Zhou and Murthy, 2004). Moreover, in non smooth muscle, the effect of SIP is antagonized by PTX (Liu et al., 2001; Rouach et al., 2006). As shown in Fig. 6, the phosphorylation of MYPT1 was markedly attenuated after exposure to 0.1 μg/ml PTX for 4 h. In ASM cells, the inhibitory effects of MCh on Ca\(^{2+}\)-activated K\(^{+}\) channel activity is eliminated by a 4-h pretreatment with PTX (Kume and Kotlikoff, 1991; Kume et al., 1992). In ASM tissues, in contrast, more than 4 h are needed for the maximal effect of PTX.

It is generally considered that SIP\(_{3}\) receptors are coupled to G\(_i\) (Anfellin and Hla, 1999; Hla et al., 2001; Siehler and Manning, 2002) and that it enhances Rho activity (Paik et al., 2001) and Ca\(^{2+}\) influx (Ishii et al., 2002). Suramin is a nonselective antagonist of SIP\(_{3}\); however, this agent is also used as an inhibitor of SIP (Anfellin and Hla, 1999). In the presence of suramin, the enhancement of MCh-induced contraction by pretreatment with SIP was markedly attenuated in a concentration-dependent manner (data not shown), suggesting that SIP\(_{3}\) is involved in the SIP-induced hyper-reactiveness to MCh in ASM. Although the SIP receptor subtype and the postreceptor pathway have not been fully elucidated yet in this study, loss of MP activity by the relationship between SIP/G\(_i\) and Rho/Rho-kinase may play a fundamentally functional role in the induction of hyper-reactivity to MCh by SIP in ASM.

In conclusion, pretreatment of ASM with the clinical level of SIP leads to an enhancement of contractility of ASM due to Ca\(^{2+}\) sensitization via Rho-mediated MP inactivation. Although clinical relevance of these results remains unknown, our observation may provide the evidence that SIP acts as a lipid mediator and that extracellular SIP causes airway hyper-reactivity implicated in the pathophysiology of bronchial asthma.
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References


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