View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

*Allergology International. 2012;61:311-322*
*DOI: 10.2332/allergolint.11-OA-0350*

ORIGINAL ARTICLE

# Role of Sphingosine-1-Phosphate in β-adrenoceptor Desensitization via $Ca^{2+}$ Sensitization in Airway Smooth Muscle

Yasushi Makino[1], Hiroaki Kume[2], Tetsuya Oguma[1], Mihoko Sugishita[1], Akira Shiraki[1], Yoshinori Hasegawa[1], Haruo Honjo[3] and Kaichiro Kamiya[3]

## ABSTRACT

**Background:** The correlation between inflammatory cells and airway smooth muscle plays fundamental roles in the pathophysiology of asthma. This study was designed to determine whether pre-exposure of airway smooth muscle to sphingosine-1-phosphate (S1P), which is released from mast cells by allergic reactions, causes a deterioration of β-adrenoceptor function.

**Methods:** Isometric tension and the ratio of fluorescence intensities at 340 and 380 nm ($F_{340}/F_{380}$), an indicator of intracellular $Ca^{2+}$ levels, were simultaneously measured using fura-2 loaded guinea-pig tracheal tissues. Intracellular cAMP levels were also measured.

**Results:** Pre-exposure to S1P caused a reduction in the inhibitory effects of 0.3 μM isoprenaline, a β-adrenoceptor agonist, and 10 μM forskolin, a direct activator of adenylyl cyclase, against 1 μM methacholine-induced contraction in concentration- and time- dependent manners. In contrast, the values of $F_{340}/F_{380}$ were not augmented under this experimental condition. After incubation with S1P in the presence of 0.001-1 μM Y-27632, a Rho-kinase inhibitor, the reduced responsiveness to forskolin induced by S1P was reversed in a concentration-dependent manner. Moreover, pre-treatment with pertussis toxin (PTX), an inhibitor of $G_i$, suppressed the loss of forskolin-induced relaxation induced by S1P. Pre-exposure to S1P markedly inhibited the augmentation of cAMP accumulation induced by forskolin. However, addition of Y-27632 and pre-exposure to PTX returned forsokin-induced cAMP accumulation to the control level.

**Conclusions:** Pre-exposure to S1P causes heterologus desensitization of β-adrenoceptors by increasing the sensitivity of airway smooth muscle to intracellular $Ca^{2+}$. $Ca^{2+}$ sensitization regulated by $G_i$ and Rho-kinase is involved in this phenomenon.

## KEY WORDS

bronchial asthma, Rho-kinase, tracheal smooth muscle, Y-27632, β-adrenoceptor agonists

## INTRODUCTION

Sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite, is released from inflammatory cells such as mast cells that are implicated in the pathophysiology of asthma. In vitro, FcεRI cross-linking, which elicits the IgE-mediated allergic reaction, activates sphingosine kinase, resulting in the conversion of sphingosine to S1P. Extracellular S1P not only effects eosinophil infiltration of the airway wall[1] but also affects smooth muscle by augmenting the production of inflammatory cytokines, contraction, and the response to contractile agonists.[2-4] Moreover, S1P disrupts the epithelial cell barrier integrity (tight junctions) in the respiratory system.[5] Previous clinical trials have demonstrated that the level of S1P is markedly elevated in bronchoalveolar lavage (BAL) fluid from patients with asthma after allergen challenges.[3,6] Therefore, external S1P may be involved in the pathophysiology of asthma by acting as a lipid mediator.

The physiological response induced by S1P was

[1]Department of Respiratory Medicine, Nagoya University Graduate School of Medicine, [3]Department of Cardiovascular Research Institute of Environmental Medicine, Nagoya University, Aichi and [2]Department of Respiratory Medicine and Allergology, Kinki University Faculty of Medicine, Osaka, Japan.
Conflict of interest: No potential conflict of interest was disclosed.
Correspondence: Hiroaki Kume, MD, PhD, Department of Respiratory Medicine and Allergology, Kinki University Faculty of Medicine, 377-2 Ohnohigashi, Osakasayama, Osaka 589-8511, Japan.
Email: hkume@med.kindai.ac.jp
Received 1 July 2011. Accepted for publication 8 December 2011.
©2012 Japanese Society of Allergology

shown to be mediated by binding to a family of specific GTP-binding (G)-protein coupled receptors ($S1P_{1-5}$) that are linked to downstream signal transduction pathways.[7,8] It is generally considered that $S1P_2$, which is coupled to the inhibitory trimeric G-protein of adenylyl cyclase, $G_i$, may play an important physiological role in the respiratory system.[2] In smooth muscle including airways and other tissues, S1P causes contraction and increases the concentration of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$). These effects are mediated by pertussis toxin-sensitive processes.[9-11] This S1P-induced $Ca^{2+}$ dynamics is due to voltage-dependent $Ca^{2+}$ channels[2] or nonselective $Ca^{2+}$ influx[4] in airway smooth muscle.

RhoA, a monomeric G protein, and Rho-kinase, an effector molecule of RhoA, are involved in the downstream signal transduction pathways of $S1P_2/G_i$.[12] The RhoA/Rho-kinase processes cause the contraction, proliferation and migration of airway smooth muscle cells and inflammatory cells by increasing cellular sensitivity to intracellular $Ca^{2+}$.[13-16] These processes may be closely involved in most of the basic pathophysiological features of asthma, such as airflow limitation, eosinophil recruitment, airway hyperresponsiveness and airway remodeling.[15,16]

Pre-exposure to S1P enhances methacholine (MCh)-induced contraction of isolated airway smooth muscle by $Ca^{2+}$ sensitization via inactivation of RhoA-mediated myosin phosphatase (myosin phosphatase target subunit 1, MYPT1).[4] Moreover, in vivo augmented responsiveness to MCh following allergen challenges is suppressed by a sphingosine kinase inhibitor.[17,18] These results indicate that the stimulatory linkage between S1P and RhoA plays a key role in airway hyperresponsiveness. However, little is currently known about involvement of this linkage in the pathophysiology of asthma.

This study was designed to determine whether pre-exposure of airway smooth muscle to S1P leads to a deterioration of β-adrenoceptor function. Moreover, we examined the relationship between $Ca^{2+}$ signaling and post-receptor pathways that are known intracellular mechanisms underlying β-adrenergic desensitization by S1P, with a focus on $S1P/G_i/RhoA$ processes.

## METHODS

### TISSUE PREPARATION AND TENSION RECORDS

The methods were essentially similar to those described previously.[19] The tracheas were excised from male Hartley guinea-pigs (300 - 350 g) after injection of pentobarbital (150 mg/kg intraperitoneally [i.p.]). The tracheal rings were opened by cutting them longitudinally in the cartilaginous region, and the epithelium was dissected away. The muscle strips containing one cartilage ring were removed and were placed vertically in an organ bath to measure tension isometrically. The passive tension was adjusted to 0.5 g after equilibration of the preparation in the normal bathing solution for 60 min. After the control response to 1 μM methacholine (MCh) was established, the experiments were started. The normal bathing solution was composed of 137 mM NaCl, 5.9 mM $KHCO_3$, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 11.8 mM glucose, and was bubbled with a gas mixture of 99% $O_2$ - 1% $CO_2$ (pH 7.4). The bathing solution was perfused at a constant flow of 3 ml/min. Indomethacin (2 μM) was perfused throughout the isometric tension recording to abolish the spontaneous mechanical tone. The temperature of the organ bath was maintained at 37℃. All animal procedures were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine.

### THE SIMULTANEOUS MEASUREMENT OF ISOMETRIC TENSION AND FURA-2 FLUORESCENCE

The methods have been described in detail previously.[20] Briefly, muscle strips were treated with 10 μM of fura-2/acetoxymethyl (fura-2/AM) for 4 h at room temperature (22 - 24℃). The non-cytotoxic detergent, pluronic F-127 (0.01% wt/vol), was added to increase the solubility of fura-2/AM. After the loading, the chamber was filled with the normal solution. Isometric tension and the fura-2 fluorescence of muscle strips were measured simultaneously, using a displacement transducer and a spectrofluorometer (CAF-110, Japan Spectroscopic, Tokyo, Japan). The intensity of fura-2 fluorescence due to excitation at 340 ($F_{340}$) and 380 ($F_{380}$) nm was measured after background subtraction. The absolute amount of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was not calculated because the dissociation constant of fura-2 for $Ca^{2+}$ in smooth muscle cytoplasm is known to be different from that obtained in vitro.[21] The ratio of $F_{340}$ to $F_{380}$ ($F_{340}/F_{380}$) was used as a relative indicator of $[Ca^{2+}]_i$. After equilibration of the response to MCh, the experiments were started. Muscle tension and $F_{340}/F_{380}$ in response to the normal bathing were taken as 0%, and the values of percent contraction and $F_{340}/F_{380}$ at each experimental condition were expressed by taking the values induced by 1 μM of MCh under each experimental condition as 100%. Indomethacin was also perfused throughout the experiments to abolish the spontaneous tone.

### EXPERIMENTAL PROTOCOL OF TENSION RECORDS

To determine the effect of pre-treatment of S1P on β-adrenergic action, the inhibitory action of isopreneline (ISO), a β-adrenoceptor agonist, on MCh-induced contraction was examined before and after exposure to S1P. S1P (<1 μM) was applied because pre-exposure to S1P did not cause augmentation of MCh-induced contraction.[4] MCh (1 μM) was applied to the tissues in the presence of 0.3 μM ISO for 10

min, and the tissue was then washed by perfusion with normal bathing solution for 15 min. Next, the tissues were exposed to S1P (0.001 - 0.1 μM) for 15 - 60 min. After washing out S1P by perfusing the normal bathing solution for 15 min, MCh with ISO was also applied to the identical tissues in the same way. To determine the effect of pre-treatment with S1P on the inhibitory action of cAMP-related agents that bypass β-adrenoceptors, the inhibitory effects of forskolin, a direct activator of adenylyl cyclase, on MCh-induced contraction were examined before and after exposure to S1P in the same way. To examine the involvement of $Ca^{2+}$ signaling in the effects of S1P, MCh was applied in the presence of ISO before and after exposure to S1P in the same way using the fura-2 loaded tissues. In the fura-2 experiments, because the experiments must be carried out under the condition that the loaded fluorescence is steady, the periods for application and washing described earlier were shortened as described in a previous report.[22] To determine the involvement of $Ca^{2+}$ sensitization mediated by RhoA, the tissues were exposed to S1P in the presence of Y-27632, a selective inhibitor of Rho-kinase. To determine the involvement of $Ca^{2+}$ dynamics mediated by $Ca^{2+}$ influx, the tissues were exposed to S1P in the presence of SKF-96365, a nonselective inhibitor of $Ca^{2+}$ channels. To determine the involvement of $G_i$, the inhibitory G protein of adenylyl cyclase, the strips were pre treated with 1 μg/ml PTX for -6 h. Time-matched control tissues were treated similarly to the test tissues, but were exposed continuously to the normal bathing solution (sham incubation) instead of to S1P and PTX.

### cAMP MEASUREMENT

Measurement of cAMP was performed as previously described.[23-26] Muscle strips of guinea-pig trachea (-2 × 2 × 10 mm) were prepared by dissecting parallel to the longitudinal axis of the muscle. The strips were equilibrated in the normal bathing solution at 37℃ for 60 min, and the experiments were then started. All experiments were conducted in the presence of 2 μM indomethacin. At the end of each incubation period, the strips were rapidly removed, blotted dry, frozen with liquid nitrogen and stored at -80℃. Frozen strips were homogenized in cold 6% trichloroacetic acid at 2-8℃ and the homogenate was centrifuged at 2000 *g* for 15 min at 4℃. Precipitated protein was separated and used for the measurement of protein content using bovine serum albumin as a standard. The supernatant was washed four times with 5 volumes of water-saturated diethyl ether and the aqueous extract remaining was lyophilized for assay of cAMP content. The concentrations of cAMP were estimated using a commercially available enzyme-immunoassay kit (RPN 225; Amersham Life Sciences, Buckinghamshire, UK) without acetylation. cAMP content was expressed as picomoles of cAMP per milligram of protein.

### MATERIALS

MCh, ISO, PTX, forskolin, SKF-96365, pluoronic F127 and indomethacin were obtained from Sigma Chemical (St. Louis, MO, USA). Y-27632 was obtained from Wako (Osaka, Japan). S1P was obtained from BIOMOL (Plymouth Meeting, PA). Fura-2/AM was obtained from Dojin Laboratories (Kumamoto, Japan).

### ANALYSIS OF RESULTS

All data are expressed as means ± standard deviation (SD). The response to an agent is described as a percentage of the maximal response. Parameters were compared using Student's t-test and ANOVA, followed by the Bonferroni *post hoc* test. A probability below 0.05 ($P < 0.05$) was considered to be a significant difference.

## RESULTS

### EFFECT OF PRE-TREATMENT WITH S1P ON THE SUBSEQUENT RESPONSE TO β-ADRENOCEPTOR AGONISTS

Application of 0.3 μM ISO to airway smooth muscle caused a marked inhibition of 1 μM MCh-induced contraction, which was markedly attenuated by pre-exposure of the tissues to S1P (0.1 μM) for 60 min. SIP exposure alone did not augment MCh-induced contraction (Fig. 1A). The values of percent contraction for MCh with ISO inhibition after exposure to the normal bathing solution (control) and to 0.1 μM S1P for 60 min were 24.8 ± 4.4 ($n$ = 10) and 98.3 ± 5.3% ($n$ = 10), respectively ($p < 0.05$). After exposure to S1P (0.001 - 0.1 μM), the inhibitory effects of ISO were attenuated in a concentration-dependent manner (Fig. 1B). Moreover, following exposure to 0.1 μM S1P for 15 - 60 min, the subsequent response to ISO was markedly attenuated in a time-dependent manner. The values of percent contraction after exposure to 0.1 μM S1P for 15 and 30 min were 82.0 ± 5.7 ($n$ = 10, $P < 0.05$) and 94.6 ± 5.3% ($n$ = 10, $P < 0.05$), respectively (Fig. 1C).

### EFFECT OF PRE-TREATMENT WITH S1P ON THE SUBSEQUENT RESPONSE TO cAMP RELATED AGENTS THAT BYPASS β-ADRENOCEPTORS

After the tissues were exposed to 0.001 μM S1P for 60 min, the inhibitory effects of 10 μM forskolin on 1 μM MCh-induced contraction were not significantly attenuated (Fig. 2A). The values of percent contraction for MCh with forskolin after exposure to the normal bathing solution and to 0.001 μM S1P for 60 min were 19.6 ± 12.6 ($n$ = 8) and 23.3 ± 9.4% ($n$ = 8), respectively (not significant, Fig. 2B). However, after exposure to 0.1 μM S1P for 60 min, the inhibitory effects of forskolin were markedly attenuated (Fig. 2A,

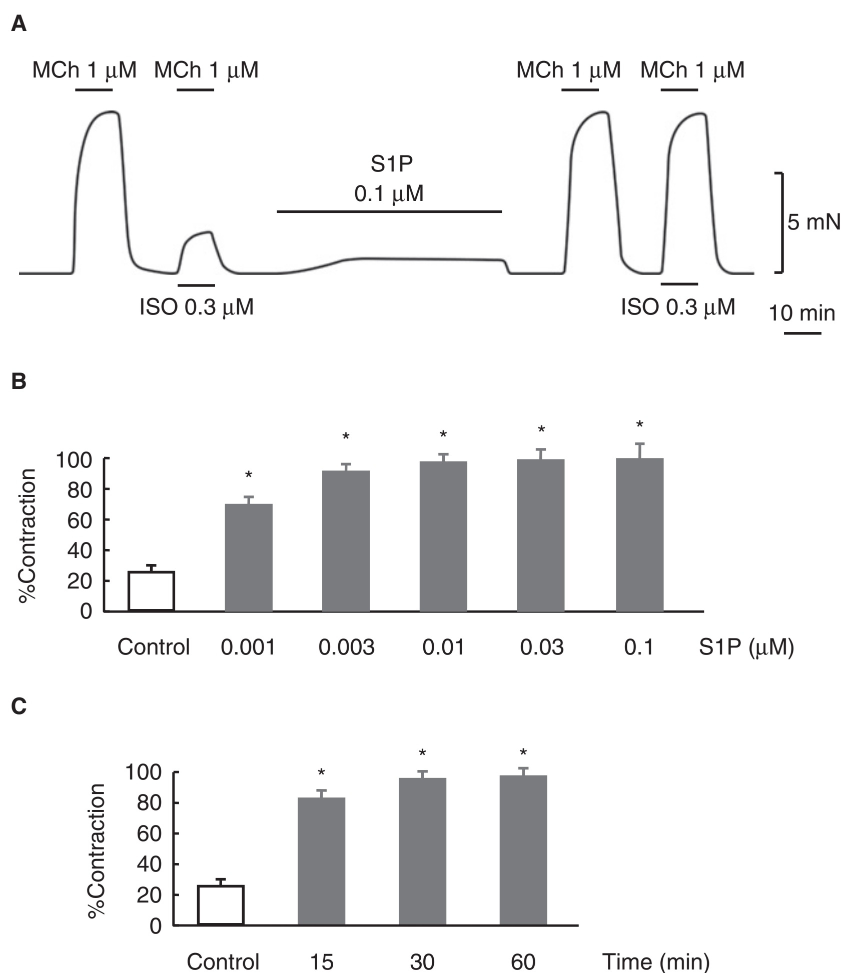


**Fig. 1** β-Adrenergic action after exposure to S1P. **A**: A typical example of the inhibitory effect of 0.3 μM isoprenaline (ISO) on 1 μM methacholine (MCh)-induced contraction before and after exposure to 0.1 μM S1P for 60 min. **B**: The values of percent contraction for MCh with ISO after exposure to the normal bathing solution (control, open columns) and to S1P (0.001-0.1 μM, closed columns) for an equivalent number of min. **C**: The values of percent contraction for MCh with ISO after exposure to the normal bathing solution (control, open columns) and to 0.1 μM S1P (closed columns) for 15, 30, and 60 min, respectively. $^*P < 0.05$.

B). Under this experimental condition, the values of percent contraction for MCh with forskolin were increased to 58.2 ± 11.1% ($n$ = 10, $P < 0.01$). After exposure to S1P (0.001 - 0.1 μM), the inhibitory effects of forskolin were attenuated in a concentration-dependent manner (Fig. 2B). Moreover, the subsequent response to forskolin was markedly attenuated in a time-dependent manner (Fig. 2C). The values of percent contraction after exposure to 0.1 μM S1P for 15, 30 and 60 min were 42.8 ± 7.8 ($n$ = 10, $P < 0.05$), 53.2 ± 8.8 ($n$ = 10, $P < 0.05$) and 58.2 ± 11.1% ($n$ = 10, $p < 0.05$), respectively (Fig. 2C).

## INVOLVEMENT OF RHO-KINASE IN THE REDUCED RESPONSIVENESS TO FORSKOLIN INDUCED BY S1P

After the tissues were exposed to 0.1 μM S1P in the presence of 0.001 μM Y-27632 for 60 min, the inhibitory effects of 10 μM forskolin on 1 μM MCh-induced contraction were significantly attenuated (Fig. 3A). However, after exposure to 0.1 μM S1P together with 1 μM Y-27632 for an equivalent time, the inhibitory effects of forskolin were not attenuated (Fig. 3A). Y-27632 (0.001 - 1 μM) inhibited the reduced responsiveness to forskolin induced by S1P in a concentration-dependent manner (Fig. 3B). The values of percent contraction for MCh with forskolin after exposure to 0.1 μM S1P in the presence of 0.01

**Fig. 2** Response to cAMP-related agents after exposure to S1P. **A**: A typical example of the inhibitory effect of 10 μM forskolin on 1 μM MCh-induced contraction before and after exposure to 0.001 (upper trace) and 0.1 μM S1P (lower trace) for 60 min. **B**: The values of percent contraction for MCh with forskolin after exposure to the normal bathing solution (control, open column) and to S1P (0.001-0.1 μM, closed columns) for an equivalent number of min. **C**: The values of percent contraction for MCh with forskolin after exposure to the normal bathing solution (control, open column) and to 0.1 μM S1P (closed columns) for 15, 30, and 60 min, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$.

and 1 μM Y-27632 were 46.4 ± 10.2 ($n$ = 10, $P < 0.05$) and 28.9 ± 13.4% ($n$ = 10, $P < 0.05$), respectively (Fig. 3B).

## INVOLVEMENT OF $Ca^{2+}$ SENSITIZATION IN THE REDUCED RESPONSIVENESS TO FORSKOLIN INDUCED BY S1P

When S1P was applied to fura-2 loaded strips, force

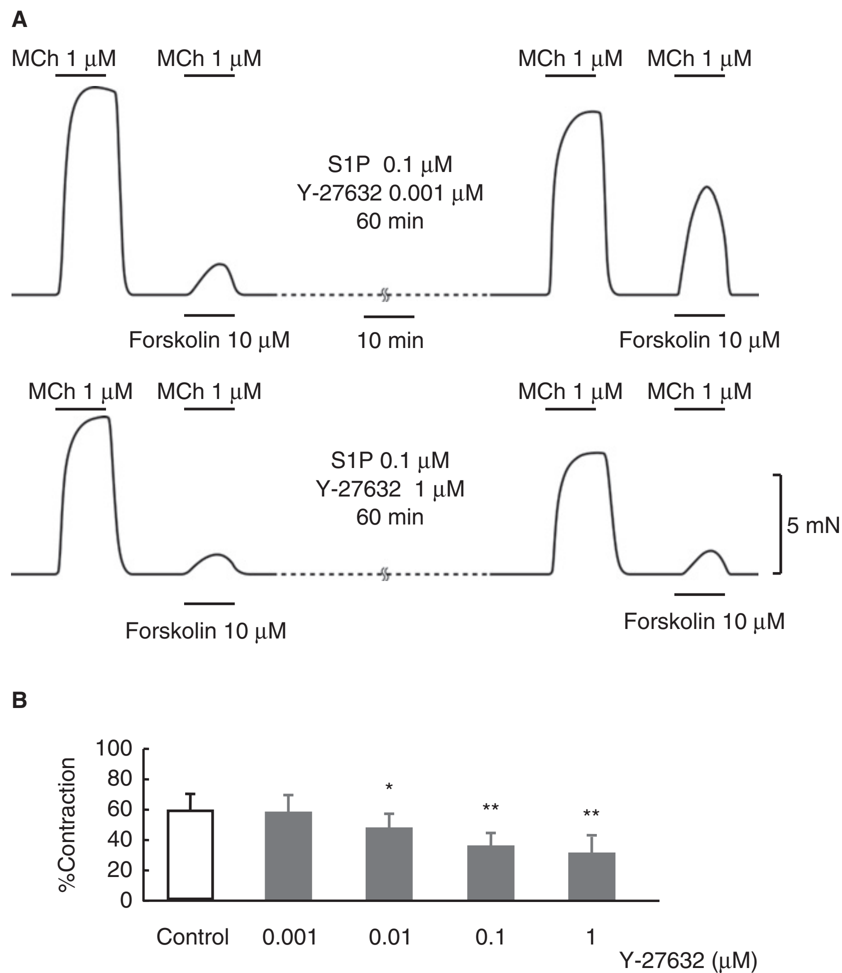


**Fig. 3** The effect of Rho-kinase on S1P-induced β-adrenergic desensitization. **A**: A typical example of the inhibitory effect of forskolin on MCh-induced contraction before and after exposure to 0.1 μM S1P in the presence of 0.001 (upper trace) and 1 μM Y-27632 (lower trace) for 60 min. **B**: The values of percent contraction for MCh with forskolin after exposure to an equi-molar amount of S1P in the absence (control, open column) and presence of Y-27632 (0.001-1 μM, closed columns) . $^*P < 0.05$, $^{**}P < 0.01$.

was generated with an increase in $[Ca^{2+}]_i$. The values of percent contraction and percent $F_{340}/F_{380}$ for 0.1 μM S1P were 8.6 ± 7.9 and 18.1 ± 13.6% ($n$ = 8), respectively (Fig. 4A). The effect of S1P on both tension and $[Ca^{2+}]_i$ had disappeared within 5 min after washing out (Fig. 4A). After exposure to 0.1 μM S1P for 15 min, the inhibitory effect of 10 μM forskolin on 1 μM MCh-induced contraction was markedly attenuated, whereas the inhibitory effect of forkolin on MCh-induced $[Ca^{2+}]_i$ was not affected (Fig. 4A). The values of percent contraction for MCh with forskolin after exposure to the normal bathing solution and to 0.1 μM S1P for 15 min were 12.1 ± 7.7 ($n$ = 8) and 63.1 ± 9.6% ($n$ = 8), respectively ($p < 0.01$, Fig. 4A). The values for percent $F_{340}/F_{380}$ were 36.4 ± 9.3 ($n$ = 8) and 41.6 ± 11.3% ($n$ = 8), respectively (not significant, Fig. 4B).

## INVOLVEMENT OF Ca2+ SIGNALING BY S1P IN THE SUBSEQUENT RESPONSE TO FORSKOLIN

When Y-27632 (0.0001-0.1 μM) was cumulatively applied, 0.1 μM S1P-induced contraction was inhibited in a concentration-dependent manner, whereas the SIP-induced $[Ca^{2+}]_i$ increase was not affected (data not shown). The values of percent contraction and percent $F_{340}/F_{380}$ for 0.1 μM S1P with 0.1 μM Y-27632 were 0.0 ± 0.0 ($p < 0.01$, $n$ = 8) and 16.1 ± 7.8% (not significant, $n$ = 8), respectively (Fig. 5A). After incubation with 0.1 μM S1P in the presence of 0.1 μM Y-27632 for 15 min, the reduced responsiveness to forskolin induced by pre-exposure to S1P returned to the control level without a reduction in $[Ca^{2+}]_i$. The

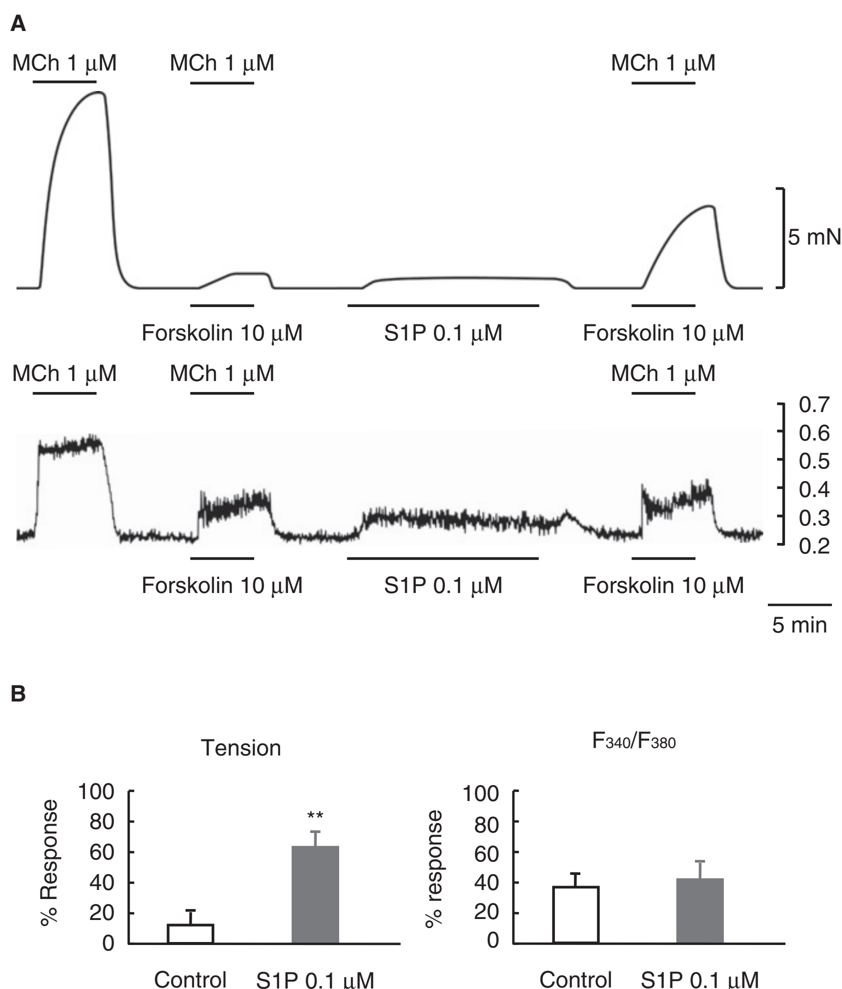


**Fig. 4** The effect of $Ca^{2+}$ sensitization on S1P-induced β-adrenergic desensitization. **A**: A typical example of a simultaneous record of tension (upper trace) and $F_{340}/F_{380}$ (lower trace) in the inhibitory effects of forskolin on MCh-induced contraction before and after exposure to 0.1 μM S1P for 15 min. **B**: The values of percent contraction and $F_{340}/F_{380}$ for MCh with forskolin before and after exposure to the normal bathing solution (control, open columns) and to 0.1 μM S1P (closed columns) for 15 min. $^{**}P < 0.01$.

value of percent contraction and percent $F_{340}/F_{380}$ were 26.1 ± 5.4 ($P < 0.01$, $n = 8$) and 38.6 ± 18.2% (not significant, $n = 8$), respectively (Fig. 5B). In contrast, SKF-96365 inhibited S1P-induced contraction with reducing $[Ca^{2+}]_i$ in a concentration-dependent manner (data not shown). SKF-96365 (0.1 μM) completely inhibit both the tension and the increased $[Ca^{2+}]_i$ induced by 0.1 μM S1P (Fig. 5A), however, after exposure to S1P in the presence of SKF-96365, the reduced responsiveness to forskolin induced by S1P was not reversed. The values of percent contraction and percent $F_{340}/F_{380}$ were 61.4 ± 22.7 (not significant) and 40.9 ± 20.9% (not significant, $n = 8$), respectively (Fig. 5B).

## INVOLVEMENT OF $G_i$ IN THE INDUCTION OF S1P-INDUCED β-ADRENERGIC DESENSITIZATION

Pre-treatment of the fura-2-unloaded tissues with 1 μg/ml PTX for 6 h caused inhibition of the reduced responsiveness to forskolin induced by S1P. Values of percent contraction for MCh with forskolin after exposure to 0.1 μM S1P for 60 min followed by treatment without and with 1 μg/ml PTX for 6 h, were 66.9 ± 12.8 ($n = 8$) and 25.5 ± 8.0% (n = 8, $P < 0.01$), respectively (Fig. 6A, B). Pre-treatment of the tissues with PTX (0.001 - 1 μg/ml) inhibited the reduced responsiveness to forskolin induced by S1P in a concentration-dependent manner (Fig. 6B).

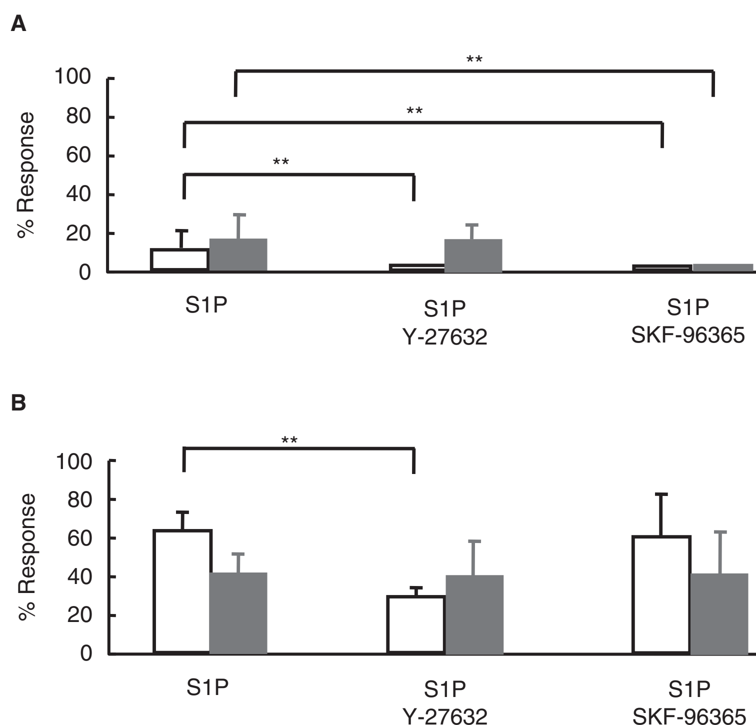


**Fig. 5** The effect of S1P-induced $Ca^{2+}$ sensitization on β-adrenergic desensitization. **A**: Values of percent contraction (open columns) and $F_{340}/F_{380}$ (closed columns) in response to S1P, S1P with Y-27632, and S1P with SKF-96365. **B**: Values of percent contraction (open columns) and $F_{340}/F_{380}$ (closed columns) for MCh with forskolin after exposure to S1P, S1P with Y-27632, and S1P with SKF-96365 for 15 min. $^{**}P < 0.01$.

### THE EFFECTS OF S1P, PTX AND Y-27632 ON THE INTRACELLULAR LEVEL OF cAMP INDUCED BY FORSKOLIN

Application of 10 μM forskolin caused an increase in the intracellular level of cAMP from 4.2 ± 3.1 to 358.2 ± 168.4 pmol/mg protein in tracheal smooth muscle (Fig. 7). However, when the tissues were exposed to 0.1 μM S1P for 60 min, the intracellular level of cAMP induced by 10 μM forskolin was markedly reduced to 116.2 ± 66.8 pmol/mg protein ($n = 8$, $P < 0.05$). When the tissues were treated with 0.1 μg/ml PTX for 6 h before exposure to S1P, a reduction in the intracellular level of cAMP by S1P returned to the control level. The value of the cAMP level induced by forskolin was significantly increased to 304.8 ± 116.4 pmol/mg protein ($n = 10$, $P < 0.05$). Moreover, when the tissues were exposed to 0.1 μM S1P with 1 μM Y-27632 for 60 min, a reduction in the intracellular level of cAMP by S1P was also reversed. The value of the cAMP level induced by forskolin was significantly increased to 392.4 ± 190.6 pmol/mg protein ($n = 10$, $P < 0.05$).

## DISCUSSION

The major findings of the present study are summarized as follows: 1) External application of S1P causes the heterologus desensitization of β-adrenoceptors in airway smooth muscle. 2) Y-27632-sensitive $Ca^{2+}$ sensitization is involved in this reduced responsiveness to β-agonists induced by pre-exposure to S1P. 3) $G_i$ processes are essential for the $Ca^{2+}$ sensitization induced by pre-exposure to S1P.

As shown in Figure 1, 2, pre-exposure to S1P impairs the function of β-adrenoceptors/AC processes. After exposure to S1P (<0.01 μM), response to ISO was markedly attenuated, on the other hand, response to forskolin was not attenuated, indicating that S1P impairs β-adrenoceptors, but AC is still active under this experimental condition. Therefore, at less than 0.01 μM of S1P, pre-exposure to S1P causes β-adrenergic desensitization independent of PKA activity. To determine whether cAMP/PKA processes are involved in this phenomenon, the tissues were exposed to S1P in the presence of 300 μM Rp-cAMP, a membrane-permeable PKA inhibitor. Reduced responsiveness to ISO was not significantly inhibited (data not shown), similar to a previous report shown that reduced responsiveness to ISO after excessive exposure to ISO is not influenced in the presence of Rp-cAMP.[27] There are two pathways in β-adrenergic

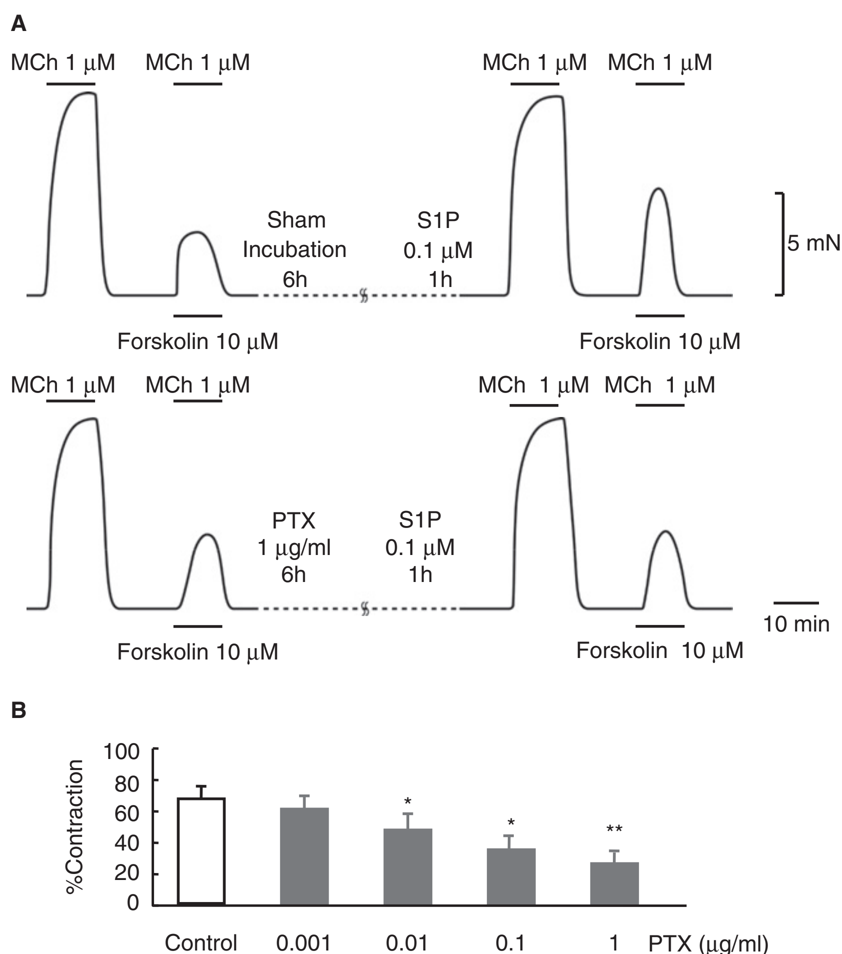


**Fig. 6** The effect of PTX on β-adrenergic desensitization by S1P. **A**: Relaxant effects of 10 μM forskolin on 1 μM MCh-induced contraction after exposure to 0.1 μM S1P for 1 h subsequent to treatment with the normal bathing solution (sham incubation, upper trace) and 1 μg/ml PTX (lower trace) for 6 h. **B**: The values of percent contraction for MCh with forskolin after exposure to an equi-molar amount of S1P for 1 h subsequent to treatment with the normal bathing solution (control, open column) and PTX (0.001-1 μg/ml, closed columns) for 6 h. $^{*}P < 0.05$, $^{**}P < 0.01$.

action on airway smooth muscle, i.e. 1) cAMP-dependent protein kinase processes (the classical pathway), 2) cAMP-independent processes (probably G protein direct action).[28-30] Under this experimental condition of pre-exposure to S1P (<0.01 μM), cAMP-independent, G protein direct processes described above may be impaired.

The ability of pre-exposure to S1P to reduce the response to β-adrenoceptor agonists is mediated by heterologous desensitization because not only a β-adrenoceptor agonist but also a cAMP-related agent was affected. This observation is consistent with the reduced responsiveness to β-agonists after exposure to TGF-β1 and PDGF, growth factors[23,31] and IL-1β, a proinflammatory cytokine.[32] On the other hand, pre-exposure to lysophosphatidylcholine (Lyso-PC), another lysophospholipid synthesized by phospholipase $A_2$ ($PLA_2$),[22] and tryptase, a trypsin-like neural serine-class protease that is released from mast cells,[33] cause β-adrenergic desensitization mediated by homologous processes, which differs from pre-exposure to S1P. Little is currently known about the clinical relevance for difference between homologous and heterologous desensitizastion of β-adrenoceptors.

Subsensitivity to forskolin mediated by pre-exposure to S1P was significantly inhibited in the presence of 0.01 μM Y-27632 (Fig. 3B), similar to the inhibition induced by pre-exposure to Lyso-PC[22] and tryptase.[33] The inhibition constant ($K_i$) value of Y-27632 for inhibition of Rho-kinase is generally considered to be 0.14 μM in vitro.[34] These results suggest that Rho-kinase is related to β-adrenergic desensitiza-

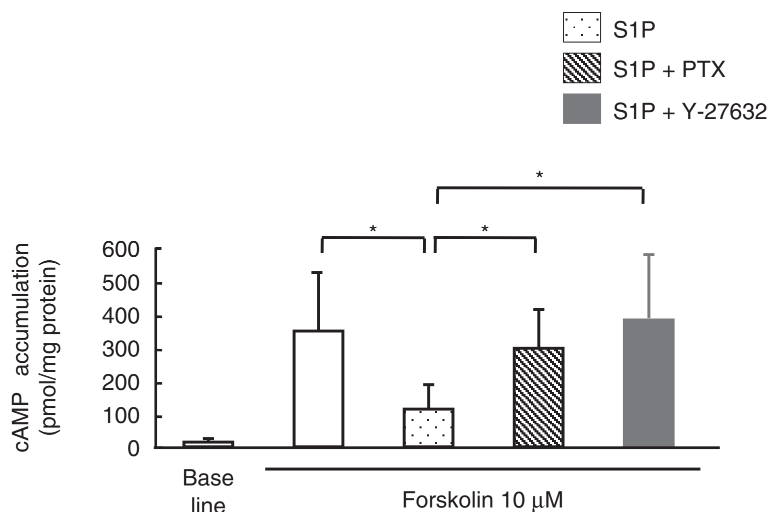


**Fig. 7** The effects of S1P, PTX and Y-27632 on cAMP accumulation induced by forskolin. Values of the intracellular concentrations of cAMP in strips exposed to 10 μM forskolin in the absence and presence of 0.1 μM S1P for 60 min, and the values in strips exposed to an equi-molar concentration of forskolin and S1P in the presence of 1 μg/ml PTX for 6 h and 1 μM Y-27632 for 60 min. $^{*}P < 0.05$.

tion by pre-treatment with S1P. On the other hand, S1P (0.3 - 3 μM) causes an increase in activity of MYPT1 in a concentration-dependent manner.[4] 0.1 μM S1P caused a significant, but modest activation of MYPT1 in human airway smooth muscle (data not shown), similar to 0.3 μM S1P. Although 0.1 μM S1P may activate Rho-kinase in guinea pig tracheal smooth muscle, its action may not be so meaningful for the physiological response. Rho-kinase mediated pathways independent of MYPT-1 may be involved in this phenomenon.

$Ca^{2+}$ sensitization is involved in β-adrenergic desensitization induced by pre-exposure to S1P (Fig. 4A, B), similar to that induced by pre-exposure to Lyso-PC[22] and tryptase.[33] Y-27632 inhibited S1P-induced contraction with no change in $[Ca^{2+}]_i$, and the reduced responsiveness to forskolin was reversed to the control level with no change in $[Ca^{2+}]_i$ after exposure to S1P in the presence of Y-27632 (Fig. 5A, B). This result indicates that S1P-induced $Ca^{2+}$ sensitization leads to the β-adrenergic desensitization mediated by Rho-kinase. β-Adrenoceptor agonists and cAMP-related agents inhibit MCh-induced contraction with a decrease in $[Ca^{2+}]_i$ ($Ca^{2+}$ dynamics).[35] However, inactivation of not only $Ca^{2+}$ dynamics but also $Ca^{2+}$ sensitization results in relaxation of airway smooth muscle when β-adrenoceptor agonists and other cAMP-related agents were applied.[36] On the other hand, to determine involvement of Rac, other Rho family, the tissues were incubated with S1P in the presence of NSC23766, an inhibitor of Rac, and PD98059, an inhibitor of mitogen-activated protein kinase kinase. The reduced responsiveness to forskolin induced by pre-exposure to S1P was not attenuated under these experimental conditions (data not shown). $Ca^{2+}$ sensitization is also mediated by protein kinase C/CPI-17 processes. To determine involvement of protein kinase C, the tissues were incubated with bisindolylmaleimide, an inhibitor of protein kinase C. The effects of pre-exposure to S1P were not inhibited (data not shown). Mechanisms other than Rho-kinase may not be responsible for this phenomenon

S1P also causes contraction in airway smooth muscle that is accompanied by an increase in $[Ca^{2+}]_i$ via $Ca^{2+}$ influx passing through $Ca^{2+}$ channels nonselectively.[4] Although SKF-96365, a nonselective inhibitor of $Ca^{2+}$ channels, suppressed S1P-induced contraction and reduced $[Ca^{2+}]_i$, pre-treatment with S1P in the presence of SKF-96365 did not reverse the reduced responsiveness to forskolin induced by S1P (Fig. 5B). This observation is consistent with β-adrenergic desensitization induced by pre-exposure to tryptase as shown previously.[33] S1P-induced $Ca^{2+}$ dynamics does not lead to the heterologous desensitization of β-aderenoceptors.

We sought to determine the involvement of post receptor signal transduction pathways in the S1P-induced β-adrenergic desensitization via $Ca^{2+}$ sensitization. As shown in Figure 6, the loss of effects of forskolin induced by S1P was significantly inhibited after exposure to 1 μg/ml PTX. Since exposure to PTX causes a functional uncoupling of $G_i$ from its associated receptor via its ADP ribosylation, $G_i$ may me-

diate the S1P-induced heterologous desensitization of β-adrenoceptors. Pre-exposure to S1P (>0.1 μM) enhances MCh-induced contraction mediated by an augmentation in sensitivity to intracellular $Ca^{2+}$ via $G_i$/RhoA/Rho-kinase processes.[4] Under this experimental condition of S1P (>0.1 μM), the inhibitory effects of ISO on MCh (β-adrenergic action) are not able to evaluate accurately. In this study, β-adrenergic action was examined after exposure to S1P (≤0.1 μM). Consequently, ISO- and forskolin-induced action was markedly attenuated mediated by the same processes. Hence, S1P/$G_i$ linkage causes $Ca^{2+}$ sensitization mediated by activation of Rho-kinase, leading to hypersensitivity to MCh (airway hyperresponsiveness), and subsensitivity to β-adrennoceptor agonists (β-adrenergic desensitization) in airway smooth muscle.

The role of concentrations of intracellular cAMP in this pre-exposure to S1P mediated β-adrenergic desensitization was examined as shown in Figure 7. S1P reduced the concentrations of intracellular cAMP that were increased by forskolin, indicating that β-adrenoceptor phosphorylation via PKA activation is unrelated to this phenomenon. Incubation with S1P in the presence of PTX and Y-27632 reversed the concentrations of intracellular cAMP that were reduced by S1P to the control level (Fig. 7), also indicating that phosphorylation induced by cAMP/PKA processes is unrelated to this phenomenon. In this study, the concentrations of intracellular cAMP are consequent to the activity of $G_i$, and are not related to the induction of β-adrenoceptor desensitization. Previous reports have demonstrated that cAMP/PKA inhibits the activation of RhoA,[37,38] and that $G_i$ activates RhoA.[4] This present study demonstrates that the $G_i$/RhoA/Rho-kinase process plays a fundamental role for this pre-exposure to S1P-induced $\beta_2$-adrenergic desensitization. However, this signal transduction pathway has never demonstrated in molecular biological technique.[39] Further studies for this pathway may contribute to developing a novel strategy of therapeutics in bronchial asthma.

Since cytosolic $PLA_2$ ($cPLA_2$) and cyclooxygenase-2 (COX-2) are activated by S1P,[40] prostaglandins are synthesized in the cytosolic side of airway smooth muscle cells. Pre-exposure to S1P may cause β-adrenergic desensitization mediated by increasing concentrations of endogenous prostaglandins. However, in this study, indomethacin, a nonselective inhibitor of COX, was applied throughout these experiments to abolish the spontaneous mechanical tone. Hence, an augmentation in COX-2 activity may not be related to the pre-exposure to S1P-induced β-adrenergic desensitization. On the other hand, pre-exposure to Lyso-PC, which is synthesized from $PLA_2$ in the cell membrane, causes β-adrenergic desensitization via $Ca^{2+}$ sensitization induced by Rho-kinase in airway smooth muscle[22] as same as the observations shown in this study using pre-exposure to S1P. Activation of $PLA_2$ may be involved in this β-adrenergic desensitization induced by S1P.

In conclusion, although the clinical relevance of our observation remains unknown, these results may provide evidence that S1P, which is released from mast cells, brings about not only the principal pathophysiology of bronchial asthma (airway obstruction, airway hyperresponsiveness, pro-inflammatory cytokine production, eosinophil recruitment and edema in the airway walls) but also dysfunction of β-adrenoceptors in patients with this disease. $Ca^{2+}$ sensitization regulated by the $G_i$/Rho-kinase pathway may be a therapeutic target for this disease.

## ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Kume H, 19590891, 22590846).

## REFERENCES

1. Jolly PS, Bektas M, Olivera A *et al*. Transactivation of sphingosine 1-phosphate receptors by FcεRI triggering is required for normal mast cell degranulation and chemotaxis. *J Exp Med* 2004;**199**:959-70.
2. Rosenfeldt HM, Amrani Y, Watterson KR, Murthy KS, Panettieri RA Jr, Spiegel S. Sphingosine 1-phosphate stimulates contraction of human airway smooth muscle cells. *FASEB J* 2003;**17**:1789-99.
3. Ammit AJ, Hastie AT, Edsall LC *et al*. Sphingosine 1-phosphate modulates human airway smooth muscle cell function that promote inflammation and airway remodeling in asthma. *FASEB J* 2001;**15**:1212-4.
4. Kume H, Takeda N, Oguma T *et al*. Sphingosine 1-phosphate causes airway hyper-reactivity by Rho-mediated myosin phosphatase inactivation. *J Pharmacol Exp Ther* 2007;**320**:766-73.
5. Gon Y, Wood MR, Kiosses WB *et al*. S1P3 receptor-induced reorganization of epithelial tight junctions compromises lung barrier integrity and is potentiated by TNF. *Proc Natl Acad Sci U S A* 2005;**102**:9270-5.
6. Jolly PS, Rosenfeldt HM, Milstien S, Spiegel S. The role of sphingosine 1-phosphate in asthma. *Mol Immunol* 2001;**38**:1239-45.
7. Siehler S, Manning DR. Pathways of transduction engaged by sphingosine 1-phosphate through G protein-coupled receptors. *Biochim Biophys Acta* 2002;**1582**:94-9.
8. Pyne S, Pyne N. Sphingosine 1-phosphate signalling via the endothelial differentiation gene family of G-protein-coupled receptors. *Pharmacol Ther* 2000;**88**:115-31.
9. Salomone S, Yoshimura S, Reuter U *et al*. S1P3 receptors mediate the potent constriction of cerebral arteries by sphingosine-1-phosphate. *Eur J Pharmacol* 2003;**469**:125-34.
10. Zhou H, Murthy KS. Distinctive G protein-dependent signaling in smooth muscle by sphingosine 1-phosphate receptors S1P1 and S1P2. *Am J Physiol* 2004;**286**:C1130-8.
11. Bischoff A, Czyborra P, Meyer Zu, Heringdorf D, Jacobs KH, Michel MC. Sphingosine-1-phosphate reduces rat renal and mesenteric blood flow in vivo in a pertussis toxin-sensitive manner. *Br J Pharmacol* 2000;**130**:1878-83.
12. Watterson KR, Ratz PH, Spiegel S. The role of sphingosine-1-phosphate in smooth muscle contraction.

*Cell Signal* 2005;**17**:289-98.
13. Adachi T, Vita R, Sannohe S *et al*. The functional role of rho and rho-associated coiled-coil forming protein kinase in eotaxin signaling of eosinophils. *J Immunol* 2001;**15**: 4609-15.
14. Takeda N, Kondo M, Ito S, Ito Y, Shimokata K, Kume H. Role of RhoA inactivation in reduced cell proliferation of human airway smooth muscle by simvastatin. *Am J Respir Cell Mol Biol* 2006;**35**:722-9.
15. Schaafsma D, Gosens R, Zaagsma J, Halayko AJ, Meurs H. Rho kinase inhibitors: a novel therapeutical intervention in asthma? *Eur J Pharmacol* 2008;**585**:398-406.
16. Kume H. RhoA/Rho-kinase as a therapheutic target in asthma. *Curr Med Chem* 2008;**15**:2876-85.
17. Roviezzo F, Di Lorenzo A, Bucci M *et al*. Sphingosine-1-phosphate/sphingosine kinase pathway is involved in mouse airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 2007;**36**:757-62.
18. Nishiuma T, Nishimura Y, Okada T *et al*. Inhalation of sphingosine kinase inhibitor attenuates airway inflammation in asthmatic mouse model. *Am J Physiol Lung Cell Mol Physiol* 2008;**294**:L1085-93.
19. Kume H, Mikawa K, Takagi K, Kotlikoff MI. Role of G proteins and $K_{Ca}$ channels in the muscarinic and β-adrenergic regulation of airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 1995;**268**:L221-9.
20. Ito S, Kume H, Honjo H *et al*. Possible involvement of Rho kinase in $Ca^{2+}$ sensitization and mobilization by MCh in tracheal smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2001;**280**:L1218-24.
21. Konishi M, Olson A, Hollongwort S, Baylor SM. Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements. *Biophys J* 1998;**54**: 1089-104.
22. Kume H, Ito S, Ito Y, Yamaki K. Role of lysophosphatidylcholine in the desensitization of β-adrenergic receptors by $Ca^{2+}$ sensitization in tracheal smooth muscle. *Am J Respir Cell Mol Biol* 2001;**25**:291-8.
23. Ishikawa T, Kume H, Kondo M, Ito Y, Yamaki K, Shimokata K. Inhibitory effects of interferon-gamma on the heterologous desensitization of beta-adrenoceptors by transforming growth factor-beta 1 in tracheal smooth muscle. *Clin Exp Allergy* 2003;**33**:808-15.
24. Devillier P, Corompt E, Breant D, Caron F, Bessard G. Relaxation and modulation of cyclic AMP production in response to atrial natriuretic peptides in guinea pig tracheal smooth muscle. *Eur J Pharmacol* 2001;**430**:325-33.
25. Cortijo J, Villagrasa V, Pons R *et al*. Bronchodilator and anti-inflammatory activities of glaucine: *in vitro* studies in human airway smooth muscle and polymorphonuclear leukocytes. *Br J Pharmacol* 1999;**127**:1641-51.
26. Fujii K, Kohrogi H, Iwagoe H *et al*. Novel phosphodiesterase 4 inhibitor T-440 reverses and prevents human bronchial contraction induced by allergen. *J Pharmacol Exp Ther* 1998;**284**:162-9.
27. Kume H, Takagi K. Inhibition of β-adrenergic desensitization by $K_{Ca}$ channels in human trachealis. *Am J Respir Crit Care Med* 1999;**159**:452-60.
28. Kume H, Takai A, Tokuno H, Tomita T. Regulation of $Ca^{2+}$-dependent $K^+$-channel activity in tracheal myocytes by phosphorylation. *Nature* 1989;**341**:152-4.
29. Kume H, Graziano MP, Kotlikoff MI. Stimulatory and inhibitory regulation of calcium-activated potassium channels by guanine nucleotide-binding proteins. *Proc Natl Acad Sci U S A* 1992;**89**:11051-5.
30. Kume H, Hall IP, Washabau RJ, Takagi K, Kotlikoff MI. β-Adrenergic agonists regulate $K_{Ca}$ channels in airway smooth muscle by cAMP-dependent and-independent mechanisms. *J Clin Invest* 1994;**93**:371-9.
31. Ikenouchi T, Kume H, Oguma T *et al*. Role of $Ca^{2+}$ mobilization in desensitization of β-adrenoceptors by platelet-derived growth factor in airway smooth muscle. *Eur J Pharmacol* 2008;**591**:259-65.
32. Koto H, Mak JC, Haddad EB *et al*. Mechanisms of impaired beta-adrenoceptor-induced airway relaxation by interleukin-1 beta in vivo in the rat. *J Clin Invest* 1996;**98**: 1780-7.
33. Kobayashi M, Kume H, Oguma T, Makino Y, Ito Y, Shimokata K. Mast cell tryptase causes homologous desensitization of β-adrenoceptors by $Ca^{2+}$ sensitization in tracheal smooth muscle. *Clin Exp Allergy* 2008;**38**:135-44.
34. Uehata M, Ishizaki T, Satoh H *et al*. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997;**389**:990-4.
35. Hiramatsu T, Kume H, Kotlikoff MI, Takagi K. Role of calcium-activated potassium channels in the relaxation of tracheal smooth muscles by forskolin. *Clin Exp Pharmacol Physiol* 1994;**21**:367-75.
36. Oguma T, Kume H, Ito S *et al*. Involvement of reduced sensitivity to $Ca^{2+}$ in β-adrenergic action on airway smooth muscle. *Clin Exp Allergy* 2006;**36**:183-91.
37. Lang P, Gesbert F, Delespine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *EMBO J* 1996;**15**:510-9.
38. Whitehead IP, Campbell S, Rossman KL, Der CJ. Dbl family proteins. *Biochim Biophys Acta* 1997;**1332**:F1-F23.
39. Takuwa Y, Okamoto Y, Yoshioka K, Takuwa N. Sphingosine-1-phosphate signaling and biological activities in the cardiovascular system. *Biochim Biophys Acta* 2008;**1781**:483-8.
40. Nixon GF. Sphingolipids in inflammation: pathological implications and potential therapeutic targets. *Br J Pharmacol* 2009;**158**:982-93.