J. Biochem. **135**, 673–681 (2004)
DOI: 10.1093/jb/mvh081

Sphingosine 1-Phosphate Inhibits Migration of RBL-2H3 Cells *via* $S1P_2$: Cross-Talk between Platelets and Mast Cells

Eiko Yokoo[1], Yutaka Yatomi[*,2], Toshiro Takafuta[3], Makoto Osada[3], Yoshitaka Okamoto[†,1] and Yukio Ozaki[3]

Departments of [1]Otolaryngology and [3]Laboratory Medicine, University of Yamanashi Faculty of Medicine, 1110 Tamaho, Nakakoma, Yamanashi 409-3898; and [2]Department of Laboratory Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655

Received January 14, 2004;; accepted April 2, 2004

To analyze the involvement in allergic reactions of platelets and sphingosine 1-phosphate (Sph-1-P), a lysophospholipid mediator released from activated platelets, the effects of Sph-1-P and a supernatant prepared from activated platelets on mast cell line RBL-2H3 were examined. Sph-1-P strongly inhibited the migration of both non-stimulated and fibronectin-stimulated RBL-2H3 cells, which was reversed by JTE-013, a specific antagonist of G protein-coupled Sph-1-P receptor $S1P_2$; $S1P_2$ was confirmed to be expressed in these cells. A similar anti-motility effect of Sph-1-P was observed in a phagokinetic assay. Consistent with these results, treatment of RBL-2H3 cells with Sph-1-P resulted in a rounded cell morphology, which was blocked by JTE-013. Under the present conditions, Sph-1-P failed to induce intracellular Ca^{2+} mobilization or histamine degranulation, responses postulated to be elicited by intracellular Sph-1-P. Importantly, the Sph-1-P effect, *i.e.*, the regulation of RBL-2H3 cell motility, was mimicked by the supernatant (both with and without boiling) prepared from activated platelets, and this effect of the supernatant was also blocked by JTE-013. Our results suggest that the motility of mast cells can be regulated by Sph-1-P and also platelets (which release Sph-1-P), *via* cell surface receptor $S1P_2$ (not through intracellular Sph-1-P actions, postulated previously in the same cells).

Key words: allergy, lysophospholipid, platelets, RBL-2H3 cells, S1P/Edg receptor, sphingosine 1-phosphate.

Abbreviations: FcεRI, high affinity IgE receptor; LPA, lysophosphatidic acid; Sph, sphingosine; Sph-1-P, sphingosine 1-phosphate.

Mast cells play a central role in triggering IgE-mediated allergic reactions. Cross-linking of allergen-specific IgE bound to the high affinity IgE receptor (FcεRI) expressed on the surface of mast cells, upon challenge with polyvalent allergens, results in the release of several chemical mediators (*1*, *2*). This leads to the manifestation of allergic symptoms in atopic diseases such as bronchial asthma, atopic dermatitis, and allergic rhinitis (*1*, *2*). However, like most other biological reactions, allergy should be considered as an integrated group of multicellular events; interactions between mast cells and various cell types existing at sites of allergic inflammation should be involved.

It is now established that blood platelets are involved in a variety of biological reactions other than thrombosis and hemostasis, and allergic reaction is no exception (*3*–*6*). For example, the involvement of platelets in bronchial asthma has been postulated. Platelets are reportedly released from megakaryocytes in the capillary bed of the lungs (*7*), and found in bronchoalveolar lavage from asthmatic patients and allergic rabbits with allergen-induced responses (*8*, *9*). Furthermore, platelet-specific proteins platelet factor 4 and β-thromboglobulin, and RANTES (which is abundant in platelets) have been reported to be released into the circulation and bronchoalveolar lavage fluid during provoked or spontaneous asthmatic attacks *in vivo* (*3*, *5*, *10*, *11*), while agonist-mediated activation of platelets *in vitro* has been shown to be augmented in asthmatics (*12*). Finally, functional expression of FcεRI, as well as the low-affinity IgE receptor (CD23), has been reported in platelets (*13*). Platelet involvement in allergic reactions has been ascribed to the release, upon stimulation, of a number of bioactive substances such as thromboxane A_2, serotonin, histamine, platelet-derived growth factor, IgE, and chemokines such as RANTES and platelet factor 4 (*3*–*6*, *10*–*12*, *14*, *15*). Importantly, platelets of atopic individuals differ in their granular contents and in the amounts of biologically active mediators released, compared with platelets of non-atopic subjects (*15*). Analysis of the chemical mediators released from platelets may lead to a new therapeutic strategy for controling allergic diseases. In fact, the role of thromboxane A_2 in the pathogenesis of allergy, especially asthma, is now well established, and the therapeutic usefulness of thromboxane A_2 synthase inhibitors and receptor antagonists is widely known (*16*, *17*).

Sphingosine 1-phosphate (Sph-1-P) was recently added to the list of bioactive lipids released from activated

[*]To whom correspondence should be addressed. Tel: +813-5800-8730, Fax: +81-3-5689-0495, E-mail: yatomiy-lab@h.u-tokyo.ac.jp
[†]Present address: Department of Otolaryngology, Graduate School of Medicine, Chiba University.

platelets (*18*, *19*). This phosphorylated sphingoid base induces a variety of biological responses in diverse cell types, mainly through interaction with the cell surface receptors S1Ps (*20–22*). Although its role in allergic diseases is not established completely, it was recently shown that Sph-1-P is increased in bronchoalveolar lavage fluid collected from asthmatic subjects (*23*), and the involvement of this bioactive lipid in asthma has been suggested (*24*, *25*). Since blood platelets store abundant Sph-1-P (*18*, *26*) and release it upon activation (*18*, *19*), it is important to examine the effects of this bioactive lipid on mast cell functions from the viewpoint of platelet-mast cell interactions; mast cells exist abundantly along blood vessels. In this study, we examined the effect of Sph-1-P on rat basophilic leukemia cell line RBL-2H3, a tumor mast cell line used frequently as an experimental model of mucosal mast cells (*27*). We also analyzed the relative involvement of this bioactive lipid in platelet-mast cell interactions with the use of a supernatant prepared from activated platelets and a specific S1P antagonist.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the indicated suppliers: Sph-1-P (Biomol, Plymouth Meeting, PA, USA); fibronectin (from bovine plasma, 0.1% solution), lysophosphatidic acid (LPA), and tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma, St. Louis, MO, USA); thrombin (Mochida Pharmaceuticals, Tokyo); fura2-AM (Dojin Chemicals, Kumamoto). Convulxin was a gift from Prof. Takashi Morita (Meiji Pharmaceutical University, Tokyo).

The pyrazolopyridine derivative JTE-013 was a gift from the Central Pharmaceutical Research Institute, Japan Tobacco Incorporation, Osaka. JTE-013 is a specific $S1P_2$ antagonist; see "Pyrazolopyridine compounds and use thereof as drugs. PCT (WO) Patent: Publication number, WO 01/98301; Publication date, December 27, 2001". It has been confirmed that JTE-013 inhibits the specific binding of radio-labeled Sph-1-P to the cell membranes of Chinese hamster ovary cells stably transfected not only with human $S1P_2$ but also rat $S1P_2$ (*28*, *29*).

Cell Culture—The rat basophilic leukemia RBL-2H3 (JCRB0023) cells (*27*) were obtained from HSRRB, Japan Health Science Foundation (Osaka), and grown in Eagle's minimal essential medium (Sigma) containing 10% fetal bovine serum (ICN Biomedicals, Aurora, OH, USA), penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) at 37°C under an atmosphere of 5% CO_2 and 95% room air. The RBL-2H3 cells were harvested by treatment with 0.25% trypsin plus 0.02% EDTA for 3 min at 37°C.

Washed platelets were prepared from healthy donors as described previously (*19*). Washed platelet suspensions (cell density, 5×10^8/ml) were stimulated with 50 ng/ml of convulxin, a potent platelet stimulant (*30*), for 15 min at 37°C. Then, supernatants were obtained by centrifugation. It was confirmed that convulxin, by itself, failed to affect the response of RBL-2H3 cells in the present study.

RT-PCR Analysis—Total RNA was prepared from RBL-2H3 cells with Trizol reagent (Gibco BRL, Life Technologies, Rockville, MD, USA), and the isolation of polyA$^+$ RNA was performed with a polyA$^+$ RNA purification kit (Takara Biomedicals, Shiga), according to the manufacturer's instructions. The isolated mRNA was reverse transcribed using a SuperScript™ Preamplification System (Gibco BRL, Life Technologies, Rockville, MD, USA). This reverse transcribed cDNA and eight normalized, first-strand cDNA preparations from rat tissues (Rat MTC™ Panel I; Clontech Laboratories, Palo Alto, CA) were amplified in a Perkin-Elmer 9600R thermal cycler (The Perkin-Elmer Corp., Norwalk, CT, USA) using Ex Taq™ (Takara Biomedicals).

The full-length sequences of rat $S1P_1$ (NM 017301), $S1P_2$ (NM 017192), and $S1P_5$ (AF233649) were obtained from the GenBank database. Since the full-length sequences of rat $S1P_3$ and $S1P_4$ were not available in the database, we searched for rat genomic DNA sequences encoding rat $S1P_3$ and $S1P_4$ using the Trace Blast program. The oligonucleotide primer pairs designed and used for PCR amplification were as follows: $S1P_1$-1 (sense), 5′-ATGGTGTCCTCCACCAGC-3′, and $S1P_1$-2 (antisense), 5′-AGTTCCAGCCCATGATGG-3′; $S1P_2$-3 (sense), 5′-AGCAAGTTCCACTCAGCC-3′, and $S1P_2$-2 (antisense), 5′-CATAGAGGGGCAGCACAG-3′; $S1P_3$-1 (sense), 5′-ATGGCATCCACGCATGCG-3′, and $S1P_3$-3 (antisense), 5′-CATTCACTTGCAGAGGAC-3′; $S1P_3$-4 (sense), 5′-AACTTGGCTCTCTGCGAC-3′, and $S1P_3$-2 (antisense), 5′-CAGTCGGGAAAGTTCTCC-3′; $S1P_4$-4 (sense), 5′-TGGGTGTACTACTGCCTC-3′, and $S1P_4$-2 (antisense), 5′-GCGCACACACAGTTCCAG-3′; $S1P_5$-1 (sense), 5′-ATGGAGTCCGGGCTACTG-3′, and $S1P_5$-2 (antisense), 5′-TAGGCCTTGGCGTAGAGC-3′; and $S1P_5$-3 (sense), 5′-TTACCTTGTCGGACCTGC-3′, and $S1P_5$-4 (antisense), 5′-TCCCAAGCAGTTCCAGTT-3′.

When primers $S1P_1$-1 and $S1P_1$-2 were used, a 553 bp S1P1 fragment was amplified. With primers $S1P_2$-3 and $S1P_2$-2, a 376 bp fragment of $S1P_2$ was amplified. To amplify the $S1P_3$ cDNA, we performed nested PCR; the first PCR was performed using primers $S1P_3$-1 and $S1P_3$-3, and a 317 bp fragment was amplified by the second PCR using primers $S1P_3$-4 and $S1P_3$-2. With primers $S1P_4$-4 and $S1P_4$-2, a 326 bp fragment of $S1P_4$ was amplified. To amplify the $S1P_5$ cDNA, we performed nested PCR; the first PCR was performed using primers $S1P_5$-1 and $S1P_5$-2, and a 298 bp fragment was amplified by the second PCR using primers $S1P_5$-3 and $S1P_5$-4.

Immunoprecipitation and Immunoblotting—These procedures were performed basically as described previously (*28*). Cell lysates were immunoprecipitated and then immunoblotted with 2 μg/ml of anti-human and rat EDG-5 ($S1P_2$) C-terminal monoclonal antibodies (Exalpha Biologicals, Boston, MA, USA). Antibody binding was detected using peroxidase-conjugated anti-mouse IgG (ICN Biomedicals, Aurora, OH, USA) and visualized with ECL chemiluminescence reaction reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Migration Assay—RBL-2H3 cell migration was assessed by means of a modified Boyden's chamber assay, *i.e.*, in Transwell cell culture chambers (Costar, Cambridge, MA, USA). Polycarbonate filters with 8 μm pores, used to separate the upper and lower chambers, were coated with Vitrogen 50 (purified collagen) (Cohesion, Palo Alto, CA, USA). The coated filters were washed with a serum-free medium and dried immediately. Then RBL-2H3 cells were added to the upper compartment of the

chamber at a density of 1×10^5/100 μl of medium containing 0.1% bovine serum albumin and incubated for 4 h at 37°C. RBL-2H3 cells were allowed to migrate toward an indicated reagent in the lower chamber. After the reaction, the filters were fixed and stained with trypan blue. After removal of non-migrating cells by wiping with cotton swabs, cells that had migrated through the filter to the lower surface were counted manually under a microscope in five predetermined fields at a magnification of ×200. When checkerboard analysis was performed, 0, 0.01, 0.1, 1, or 10 μM Sph-1-P was added to the upper and/or lower chamber.

Phagokinetic Assay on Gold Sol-coated Plates—Random cell motility and phagocytotic activity were jointly estimated as the area of phagokinetic tracks on gold sol particle-coated plates. Briefly, 3.5 cm dishes coated with 0.2% gelatin were incubated with colloidal gold for 45 min, and then washed twice with PBS. RBL-2H3 cells (2,000 cells) were added to each dish. After 24 h at 37°C, phagokinetic tracks were visualized using dark-field illumination under a confocal microscope. The area cleared of gold particles was measured after photography by cutting out and weighing the cleared area, and the mean value for 20 cells was calculated in each experiment.

Actin Staining—For actin staining, cells were fixed with 3% paraformaldehyde in PBS for 40 min and then permeabilized with 0.2% Triton X-100 for 8 min. Actin filaments were detected by staining with 0.1 μg/ml of tetramethylrhodamine isothiocyanate-conjugated phalloidin. Actin staining was observed and photographed under a confocal microscope.

Measurement of the Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$)—$[Ca^{2+}]_i$ measurement was performed with the use of Ca^{2+}-sensitive fluorophore fura2. Confluent RBL-2H3 cells were harvested by trypsinization, and then the cells were incubated with 3 μM fura2-AM. After 30 min at 37°C, the cells were washed twice, adjusted to 2×10^6/ml, and then supplemented with 1 mM $CaCl_2$. Fluorescence measurements were made with an FS100 (Kowa, Tokyo). The $[Ca^{2+}]_i$ values were determined from the ratio of fura2 fluorescence intensity with 340 and 380 nm excitation.

Histamine Release Assay—The levels of histamine in the medium were measured, after its acylation, using a Histamine ELISA (ICN Biomedicals, Aurora, OH, USA). The procedures were based on the manufacturer's instructions.

Data Presentation and Statistics—The data are presented as the means ± SD ($n = 3$) or representative of 3 or 4 separate experiments. When indicated, the statistical significance of the difference between the two groups was determined by means of Student's t test. $P < 0.05$ was considered significant.

RESULTS

S1P Expression in RBL-2H3 Cells—Many, if not all, of the biological responses induced by Sph-1-P are mediated by its cell surface receptors, *i.e.* S1Ps (*21*, *22*). Although $S1P_1$ (EDG-1), $S1P_2$ (EDG-5), and $S1P_3$ (EDG-3) seem widely expressed, $S1P_4$ (EDG-6) and $S1P_5$ (EDG-8) each exhibit a limited expression pattern (*21*, *22*, *31*). The $S1P_4$ expression profile is largely confined to the tissues and cells of the hematopoietic system (*32*), while $S1P_5$ is known to be expressed in the brain (*33*). As shown in Fig. 1A, the PCR products of $S1P_1$ through $S1P_4$ were amplified from RBL-2H3 cells. However, when the S1P expression was compared with that in other rat tissues, the most obvious finding was strong $S1P_2$ expression in RBL-2H3 cells. $S1P_1$, known to be widely expressed, and $S1P_4$, mainly expressed in the hematopoietic systems, were confirmed to be expressed in RBL-2H3 cells; $S1P_5$ expression was not detected.

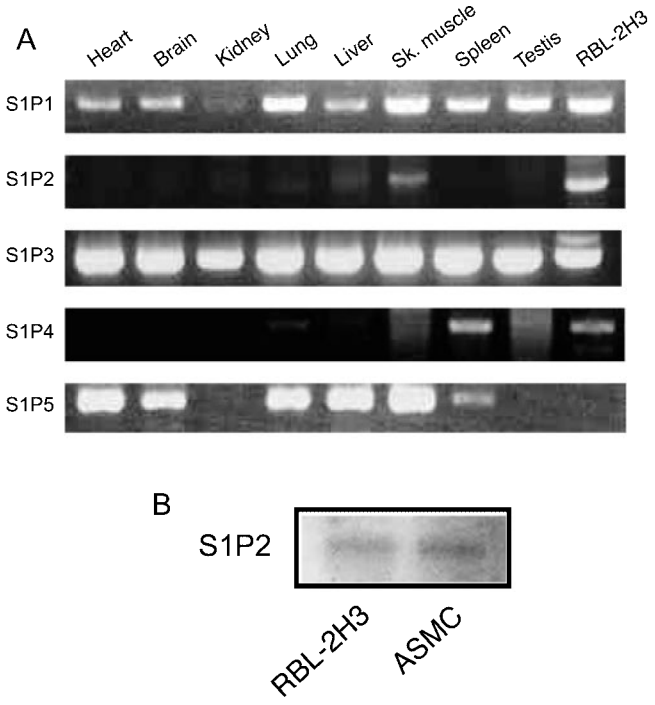

Fig. 1. **Expression of S1Ps in RBL-2H3 cells.** (A) Detection of expression of mRNA for S1Ps in RBL-2H3 cells, in comparison with in various rat tissues, was performed by RT-PCR. cDNA preparations from RBL-2H3 cells and various rat tissues were amplified for $S1P_{1-5}$. The products were resolved on 2% agarose gels. Sk. muscle, skeletal muscle. (B) Detection of $S1P_2$ protein in RBL-2H3 cells and vascular smooth muscle cells. Lysates obtained from RBL-2H3 cells and aortic smooth muscle cells (ASMC) were immunoprecipitated and then immunoblotted with anti-$S1P_2$ antibodies.

We next confirmed the protein expression of $S1P_2$ in these cells using vascular smooth muscle cells as a positive control (*21*, *22*). RBL-2H3 cells were found to express $S1P_2$ protein (Fig. 1B), although precise quantitation was difficult due to the employment of immunoprecipitation.

Inhibition of RBL-2H3 Cell Migration by Sph-1-P and Its Reversal by a Specific $S1P_2$ Antagonist—One of the most unique characteristics of S1P receptors is their receptor isotype-specific, bimodal regulatory activity on cell migration (*22*, *34*). While $S1P_1$ acts as a typical chemotactic receptor, $S1P_2$ acts as a chemorepellant one (*22*, *34*). Since RBL-2H3 cells expressed both S1P receptors, it was considered important to examine the effect of Sph-1-P on the migration of these cells. When examined by means of the modified Boyden's chamber assay, incubation of RBL-2H3 cells in the absence of any treatment was found to lead to significant basal migration across the membrane (Fig. 2A and Table 1). Sph-1-P strongly

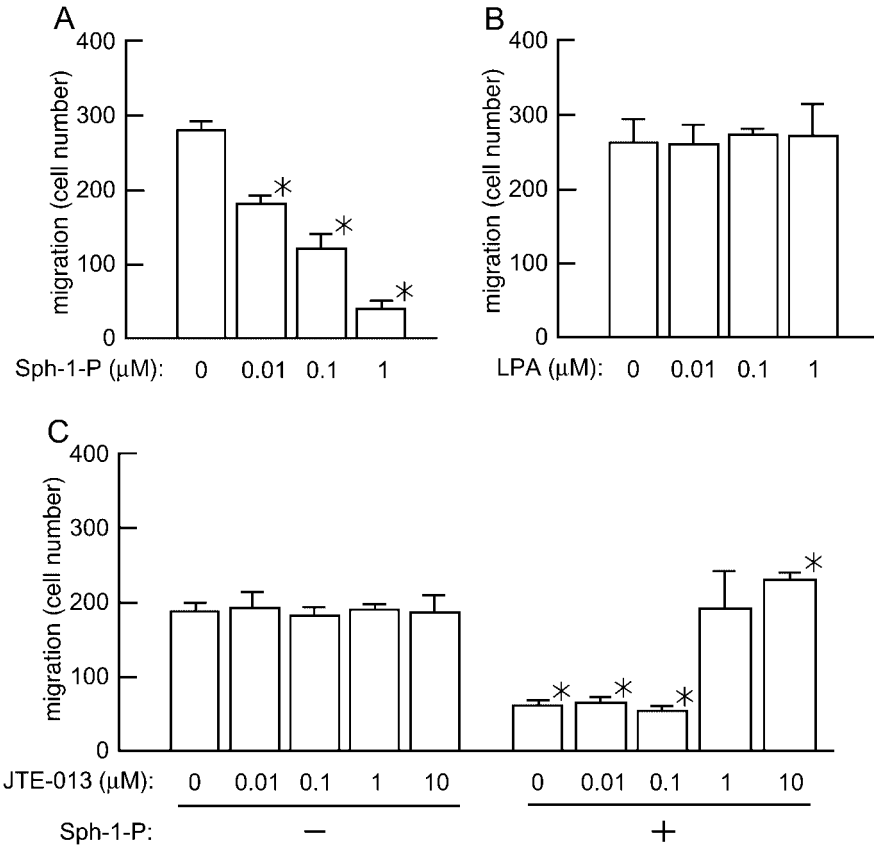

Fig. 2. **Inhibition of RBL-2H3 cell migration by Sph-1-P and its reversal by JTE-013, as assessed with a modified Boyden's chamber assay.** (A and B) RBL-2H3 cells were allowed to migrate for 4 h to the lower chamber, where various concentrations of Sph-1-P (A) or LPA (B) were placed. (C) RBL-2H3 cells preincubated with various concentrations of JTE-013 for 10 min were allowed to migrate for 4 h toward the lower chamber, where 100 nM Sph-1-P was present (+) or absent (–). *Statistically significant compared with the control cells (without Sph-1-P/JTE-013 treatment).

inhibited this basal RBL-2H3 cell migration in a concentration-dependent manner (Fig. 2A). To determine whether or not this migration inhibition depends on the presence of a concentration gradient of Sph-1-P between the lower and upper chambers, checkerboard experiments were conducted. Marked inhibition of migration was observed not only in the presence of a Sph-1-P concentration gradient, but also with equal concentrations of Sph-1-P below and above the membranes (Table 1). These results indicate that Sph-1-P inhibits chemokinesis (random motility) of RBL-2H3 cells, as well as chemotaxis. In contrast, LPA, which is structurally similar to Sph-1-P as a lysophospholipid and interacts with $LPA_{1\text{-}3}$ receptors (*21*, *31*), failed to affect the migration response (Fig. 2B). Furthermore, the strong migration inhibition by Sph-1-P (observed with the use of RBL-2H3 cells) was not observed for other hematopoietic cells such as human neutrophils, lymphocytes, eosinophils, monocytic leukemia U937 cells, and myeloma-derived ARH77 cells (data not shown), indicating the uniqueness of this tumor mast cell line.

Since chemorepellant receptor $S1P_2$, strongly expressed in RBL-2H3 cells (see Fig. 1), was the most probable candidate receptor involved in the Sph-1-P inhibition of cell motility, the effect of the $S1P_2$ antagonist JTE-013 (*28*, *29*) was examined. This compound, by itself, failed to affect the basal RBL-2H3 migration (Fig. 2C). When RBL-2H3 cells were pretreated with JTE-013, the inhibition induced by Sph-1-P was reversed (Fig. 2C), indicating Sph-1-P inhibition of RBL-2H3 cell migration through $S1P_2$. It should be noted that the cell migration after treatment with Sph-1-P plus JTE-013 was even enhanced compared with the basal migration without any treatment (Fig. 2C).

Fibronectin is known to enhance RBL-2H3 cell migration (*35*), which was confirmed under the present conditions (Fig. 3A). Similar to the basal RBL-2H3 cell migration, that enhanced by fibronectin was inhibited by Sph-1-P, which was reversed by JTE-013 (Fig. 3B). Further-

Table 1. **Checkerboard analysis of RBL-2H3 cells.** Different concentrations of Sph-1-P were added to the upper and/or lower chamber, and then RBL-2H3 cells were allowed to migrate for 4 h.

[Sph-1-P, Lower chamber] (μM)	[Sph-1-P, Upper chamber] (μM)				
	0	0.01	0.1	1	10
0	206 ± 30	184 ± 13	203 ± 26	187 ± 30	182 ± 21
0.01	131 ± 8	111 ± 10	118 ± 6	111 ± 7	122 ± 15
0.1	70 ± 18	68 ± 17	55 ± 11	62 ± 10	49 ± 8
1	53 ± 17	44 ± 14	50 ± 9	40 ± 4	40 ± 6
10	33 ± 4	23 ± 3	33 ± 11	30 ± 6	27 ± 7

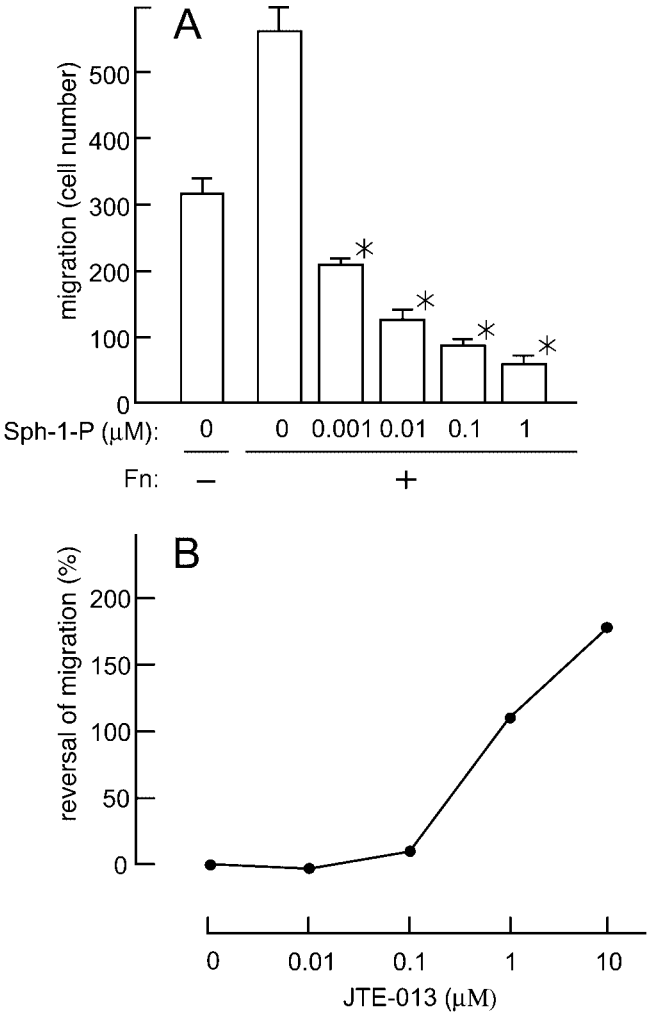

Fig. 3. **Inhibition of fibronectin-induced RBL-2H3 cell migration by Sph-1-P and its reversal by JTE-013.** (A) Various concentrations of Sph-1-P were placed in the lower chamber with (+) or without (–) 100 μg/ml of fibronectin (Fn). RBL-2H3 cells were allowed to migrate for 4 h. *Statistically significant compared with the control fibronectin-treated cells (without Sph-1-P treatment). (B) RBL-2H3 cells preincubated with various concentrations of JTE-013 for 10 min were allowed to migrate for 4 h toward the lower chamber, where 100 nM Sph-1-P was present or absent, together with 100 μg/ml of fibronectin. The reversal by JTE-013 of the Sph-1-P-inhibited migration (%) was calculated as ([migrating cells in the presence of JTE-013/Sph-1-P] − [migrating cells in the presence of Sph-1-P]) / ([migrating cells in the absence of JTE-013/Sph-1-P] – [migrating cells in the presence of Sph-1-P]) × 100.

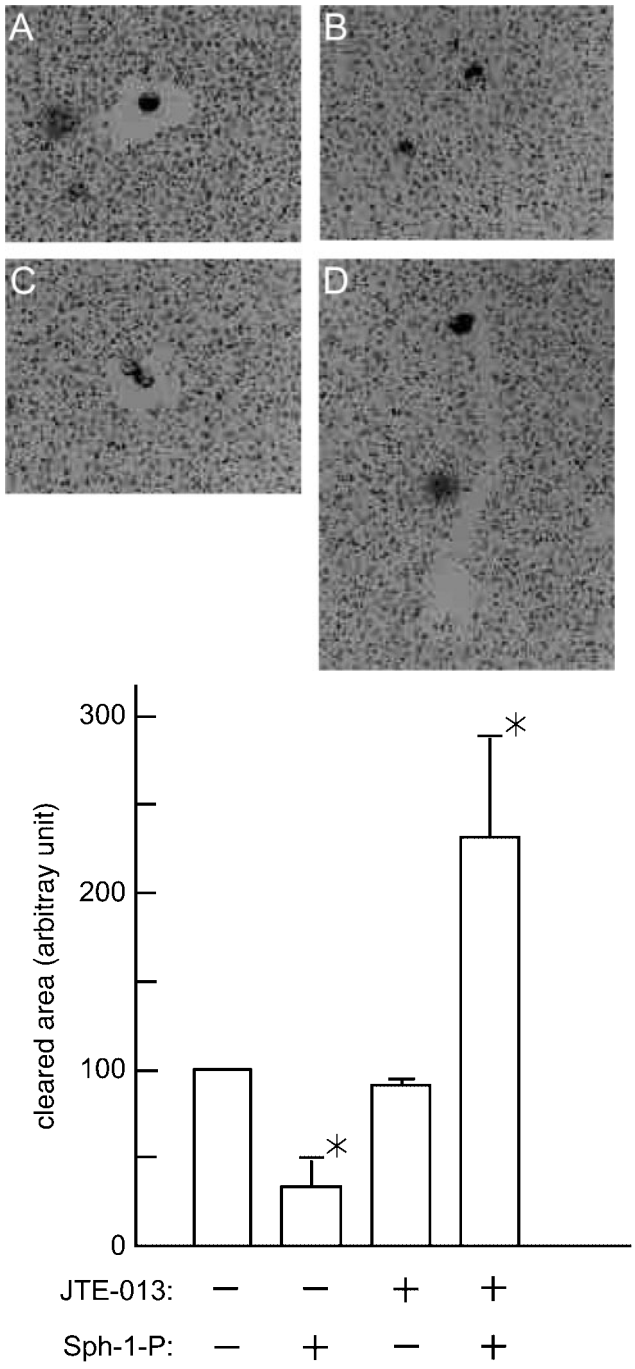

Fig. 4. **Inhibition of RBL-2H3 cell migration by Sph-1-P and its reversal by JTE-013, as assessed with a phagokinetic assay.** (Upper panel) RBL-2H3 cells were preincubated without (A and B) or with (C and D) 10 μM JTE-013 for 10 min, and then challenged without (A and C) or with (B and D) 1 μM Sph-1-P for 24 h. Chemokinesis was evaluated with a phagokinetic assay using gold sol-coated plates. (Lower panel) RBL-2H3 cells were treated as described for the upper panel, and the area cleared of gold particles for each cell was measured. *Statistically significant compared with the control cells (without Sph-1-P/JTE-013 treatment).

more, the fibronectin-stimulated cell migration in the presence of Sph-1-P plus JTE-013 was greater than that without Sph-1-P/JTE-013 (Fig. 3B).

Inhibition of RBL-2H3 Cell Phagokinesis by Sph-1-P and Its Reversal by a Specific $S1P_2$ Antagonist—To confirm the anti-motility effect of Sph-1-P, we also performed a phagokinetic track assay. The phagokinetic activity of RBL-2H3 cells on gold sol-coated plates was inhibited by Sph-1-P (Fig. 4). JTE-013, which by itself failed to affect the response, reversed the Sph-1-P-inhibited phagokinesis (Fig. 4). These results further strengthen the idea that Sph-1-P inhibits RBL-2H3 motility through $S1P_2$. Again, the response in the presence of Sph-1-P plus JTE-013 was greater than that without any treatment (Fig. 4).

Cytoskeletal Reorganization of RBL-2H3 Cells Treated with Sph-1-P—We further evaluated cytoskeletal reor-

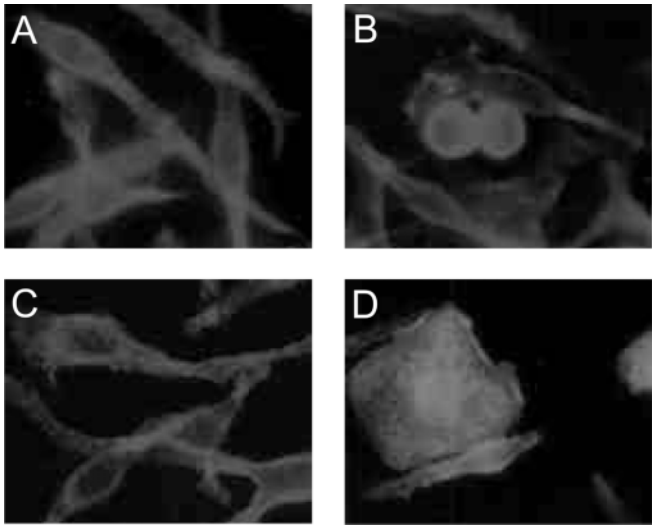

Fig. 5. **Cytoskeletal Reorganization of RBL-2H3 cells treated with JTE-013 and/or Sph-1-P.** RBL-2H3 cells were pretreated without (A and B) or with (C and D) 1 μM JTE-013 for 10 min, and then stimulated without (A and C) or with (B and D) 100 nM Sph-1-P for 30 min. Then, the cells were fixed, permeabilized, and stained with tetramethylrhodamine isothiocyanate-phalloidin for actin staining.

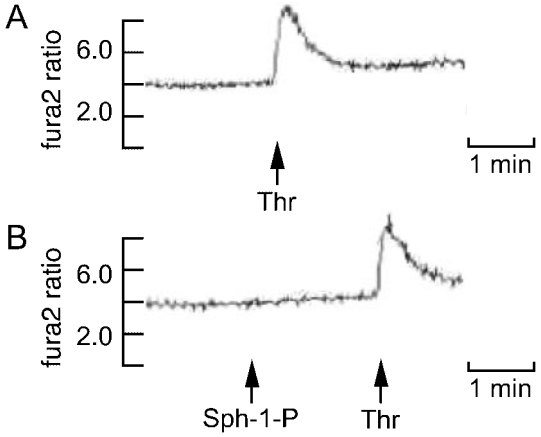

Fig. 6. **Measurement of RBL-2H3 cell $[Ca^{2+}]_i$ Fura2-loaded RBL-2H3 cells were stimulated with 1U/ml of thrombin (A) or 100 nM Sph-1-P and then 1U/ml of thrombin (B).** $[Ca^{2+}]_i$ was monitored as the ratio of fura2 fluorescence.

ganization under the conditions in which Sph-1-P exerted its anti-motility effect; RBL-2H3 cells stained to visualize F-actin are shown in Fig. 5. Low nanomolar concentrations of Sph-1-P induced stress fiber formation in RBL-2H3 cells (data not shown), possibly through Rho activation (*36*). When higher concentrations (100 nM – 1 μM) of Sph-1-P were employed, marked changes in cell morphology and increased numbers of rounded cells were observed (Fig. 5, A and B). The $S1P_2$ antagonist JTE-013, which, by itself, failed to affect the morphology of RBL-2H3 cells, blocked the Sph-1-P-induced cell shape change (Fig. 5), indicating Sph-1-P-induced cell rounding *via* $S1P_2$, as previously reported for transfected HEK293 cells (*37*). Instead, membrane ruffling-like structures were observed (Fig. 5D).

Effects of Sph-1-P on Intracellular Ca^{2+} Mobilization and Histamine Release in RBL-2H3 Cells—We next examined intracellular Ca^{2+} mobilization using Ca^{2+}-sensitive fluorophore fura2. As previously reported (*38*), thrombin caused a rapid and transient increase in $[Ca^{2+}]i$ (Fig. 5A). However, Sph-1-P, at concentrations capable of exerting the anti-motility effect, neither elicited an increase in $[Ca^{2+}]i$ nor affected the response induced by thrombin (Fig. 6B). When very high concentrations (above 10 μM) of Sph-1-P were employed, a small but significant increase was observed (data not shown), which should be independent of the effect of this bioactive lipid on RBL-2H3 cell migration shown above.

Consistent with the inability of Sph-1-P to induce intracellular Ca^{2+} mobilization (at least at nanomolar concentrations), this bioactive lysophospholipid failed to elicit histamine degranulation (data not shown), which is dependent on the Ca^{2+} signal (*39*, *40*).

Imitation by a Supernatant Prepared from Activated Platelets of the Effect Triggered by Sph-1-P—We finally analyzed platelet-mast cell interactions and the relative involvement of Sph-1-P in this cell-cell communication. For this purpose, we prepared a supernatant from an activated platelet suspension. This supernatant strongly inhibited RBL-2H3 cell migration (Table 2), as did Sph-1-P. Similar results were obtained when the boiled (instead of non-boiled) supernatant was used to eliminate the effect of peptide mediators (data not shown). Accordingly, not a protein but probably a lipid component seemed to be responsible for the migration inhibition by the platelet supernatant. Although a variety of bioactive substances are released from activated platelets, ones interacting with RBL-2H3 cells or mast cells have hardly been reported. Accordingly, we postulated that the observed effect of the supernatant may be due to the presence of Sph-1-P released from platelets, based on the resemblance of the effects of Sph-1-P and the platelet supernatant. We tested this possibility by examining the effect of the $S1P_2$ antagonist JTE-013 on the response induced by the supernatant; Sph-1-P inhibits RBL-2H3 migration through $S1P_2$, as shown above. As expected, the migration inhibition of RBL-2H3 cells by both the boiled (Fig. 7A) and non-boiled (Fig. 7B) activated platelet supernatants was completely reversed by JTE-013. Furthermore, pretreatment with high concentrations of this $S1P_2$ antagonist before the addition of the supernatant even enhanced the response compared with the control without any treatment (Fig. 7).

Table 2. **Inhibition of RBL-2H3 cell migration by a supernatant prepared from activated platelets.** RBL-2H3 cells were allowed to migrate for 4 h to the lower chamber, where various concentrations of a supernatant prepared from activated platelets were placed.

Experiment	Supernatant from activated platelets				
	0%	0.1%	1%	10%	100%
1	240	250	200	80	5
2	230	230	202	85	8
3	145			50	2
4	143			54	3

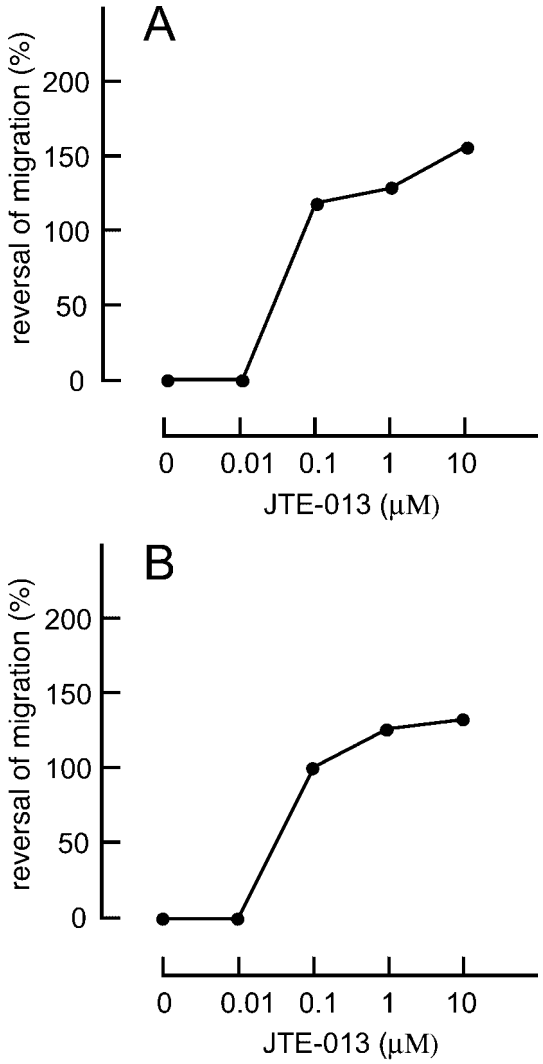

Fig. 7. **The reversal by JTE-013 of Sph-1-P-inhibited RBL-2H3 cell migration.** RBL-2H3 cells preincubated with various concentrations of JTE-013 for 10 min were allowed to migrate for 4 h toward the lower chamber, where a buffer containing 10% of the supernatant prepared from activated platelets was placed. The supernatant was boiled (B) or not boiled (A). The reversal by JTE-013 of the supernatant-inhibited migration (%) was calculated as ([migrating cells in the presence of JTE-013/the supernatant] – [migrating cells in the presence of the supernatant]) / ([migrating cells in the absence of JTE-013/the supernatant] – [migrating cells in the presence of the supernatant]) × 100.

DISCUSSION

Sph-1-P Inhibition of RBL-2H3 Cell Migration—In this study, we found that Sph-1-P strongly inhibits the migration of RBL-2H3 cells through the G protein-coupled receptor $S1P_2$. This is consistent with the facts that $S1P_2$ is strongly expressed in these cells (this study), and that $S1P_2$ negatively regulates membrane ruffling and the resultant cell migration (*22*, *34*). Although $S1P_2$ has been reported to be a chemorepellant receptor (*22*, *34*), our present results show that Sph-1-P interaction with $S1P_2$ leads to inhibition of both chemokinesis (random motility) and chemotaxis, since the phagokinetic activity of RBL-2H3 cells on gold sol-coated plates was inhibited by Sph-1-P, which was reversed by the $S1P_2$ antagonist JTE-013. Whether this difference is due to the choice of cell type remains to be solved.

It should be noted that, in cells pretreated with JTE-013 and then challenged with Sph-1-P, the migration and phagokinesis responses inhibited by Sph-1-P were not only blocked but also enhanced compared with those without Sph-1-P/JTE-013. RBL-2H3 cells express both $S1P_1$ and $S1P_2$. $S1P_1$ and $S1P_2$ exert contrasting effects on cell motility; the former stimulates membrane ruffling and migration in a Rac-dependent manner, while the latter inhibits these responses (*22*, *34*). Accordingly, when the $S1P_2$-mediated effect was blocked by its specific antagonist, the $S1P_1$-mediated effect, *i.e.*, Rac-dependent stimulation of migration, should be observed in Sph-1-P-challenged RBL-2H3 cells; Rac is involved in the migration of these cells (*41*). This is consistent with the morphological study in which a rounded cell shape (without cytosolic extension) was observed for RBL-2H3 cells incubated with Sph-1-P, while this rounded shape was abolished and membrane ruffling-like structures were observed when JTE-013 was pretreated before Sph-1-P addition.

Sph-1-P in RBL-2H3 Cells or Mast Cells: an Extracellular First Messenger or an Intracellular Second Messenger?—Sph-1-P is now considered to be a unique cell signaling molecule, functioning as both an extracellular first messenger and an intracellular second messenger (*20*). We believe that the regulation of RBL-2H3 cell motility by Sph-1-P reported in this study can be best explained by its extracellular action via the $S1P_2$ receptor since (i) $S1P_2$ is actually expressed in these cells, (ii) Sph-1-P is capable of eliciting the response at low nanomolar concentrations, corresponding to the Kd values of S1P receptors (*21*, *22*), and (iii) most importantly, the Sph-1-P-induced response was specifically blocked by an $S1P_2$ antagonist.

Our present paper is not the first to report the effect of exogenous Sph-1-P on mast cells or RBL-2H3 cells. Choi *et al.* reported that Sph-1-P increases intracellular Ca^{2+} in RBL-2H3 cells, although an extremely high concentration of Sph-1-P (such as 25 μM) was employed (*40*). It is hard to speculate that that high concentration of Sph-1-P can be attained *in vivo*; even the Sph-1-P concentration in serum, where the highest concentration of Sph-1-P could be attained given that the most important source of extracellular Sph-1-P is blood platelets, is less than 1 μM (*26*). In fact, it was postulated by Choi *et al.* that Sph-1-P, transiently formed intracellularly by sphingosine (Sph) kinase, acts as an intracellular messenger in signaling by the FcεRI antigen receptor (*40*). This Sph kinase-mediated Ca^{2+} signal, which is responsible for FcεRI-triggered mast cell degranulation, was later shown in human bone marrow-derived mast cells (*39*). Furthermore, high intracellular levels of Sph-1-P were shown to activate the mitogen-activated protein kinase pathway; again, as high as 10 μM Sph-1-P was employed in that study (*42*). Judging from the above together with our present study, it may be possible to speculate that both types of Sph-1-P action, *i.e.*, those as an intracellular messenger and an extracellular mediator, can be seen in mast cells; neither Ca^{2+} mobilization nor histamine release, possibly due to the intracellular Sph-1-P action, was observed with the

present conditions under which the anti-motility effect of extracellular Sph-1-P through $S1P_2$ was observed. This is not so surprising since a similar situation can be found in several systems (*18*, *20*). For example, in human umbilical vein endothelial cells, Sph-1-P induces migration, proliferation, angiogenesis, and nitric oxide formation through cell surface receptors $S1P_1$ and $S1P_3$ (*43*, *44*), while the Sph kinase pathway, through the generation of intracellular Sph-1-P, is critically involved in mediation of TNFα-induced endothelial activation (including adhesion molecule expression) (*45*).

Cross-talk between Mast Cells and Platelets—Another important finding in this study is that platelets seem to regulate mast (RBL-2H3) cell motility by releasing Sph-1-P. This is based on the facts that the Sph-1-P effect, *i.e.*, regulation of RBL-2H3 cell motility, is mimicked by a supernatant (both with and without boiling) prepared from activated platelets and that this effect of the supernatant was blocked by an $S1P_2$ antagonist. Given that 140 pmol Sph-1-P is stored in 10^8 platelets (*26*) and that 30% of it is released upon activation (*19*), the concentration of Sph-1-P in the supernatant prepared from activated platelets (5×10^8/ ml) is calculated to be 210 nM. Based on our results regarding the concentration-dependent effect of Sph-1-P (see Figs. 2A and 3A), this concentration of Sph-1-P is expected to exert an anti-motility effect on RBL-2H3 cells. At a site of inflammation, injury, or hemorrhage, platelets extravasate from the blood and adhere to the subendothelial tissue, which leads to their activation, while mast cells are residential cells adjacent to the endothelium in the connective and mucosal tissues. Therefore, activated platelets recruited to the inflamed or damaged site may modulate mast cell functions *in vivo*. The limitation of this study is the use of tumor mast cell line RBL-2H3, although this has been used frequently as an experimental model of mucosal mast cells (*27*). In this context, it should be noted that $S1P_1$ and $S1P_2$ are reportedly expressed not only in RBL-2H3 cells but also in primary bone marrow-derived mast cells (*25*). Furthermore, to strengthen our conclusion, other approaches than the usage of $S1P_2$ antagonists, *e.g.*, knockdown strategies involving siRNA, may be needed in the future.

We recently examined the effect of Sph-1-P on eosinophils. Sph-1-P acts as a chemoattractant for these cells (Yokoo, E., Yatomi, Y., Takafuta, T., and Ozaki, Y., unpublished observation), in contrast to mast cells. Accordingly, it is possible to speculate that Sph-1-P may inhibit and stimulate the locomotion of mast cells and eosinophils, respectively, thereby promoting interaction between mucosal mast cells (residing around the blood vessels) and infiltrating eosinophils. Recently, it was shown that nerve growth factor collaboratively worked with membrane lysophosphatidylserine of activated platelets to induce mast cell activation, and it was proposed that NGF released in response to inflammatory stimuli may contribute to mast cell activation in collaboration with locally activated platelets in the process of inflammation and tissue repair (*46*). We feel more attention should be paid to cross-talk between mast cells and platelets, although the significance of this cell-cell communication *in vivo* remains to be clarified in further studies.

The authors wish to thank the Japan Tobacco Incorporation and Prof. Takashi Morita for donating the JTE-013 and convulxin, respectively. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by the Mitsubishi Pharma Research Foundation.

REFERENCES

1. Turner, H. and Kinet, J.P. (1999) Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature* **402**, B24–30
2. Nadler, M.J., Matthews, S.A., Turner, H., and Kinet, J.P. (2000) Signal transduction by the high-affinity immunoglobulin E receptor Fc epsilon RI: coupling form to function. *Adv. Immunol.* **76**, 325–355
3. Gresele, P., Ribaldi, E., Grasselli, S., Todisco, T., and Nenci, G.G. (1987) Evidence for platelet activation in allergic asthma. *Agents Actions* **21**, 119–128
4. Page, C.P. (1989) Platelets as inflammatory cells. *Immunopharmacology* **17**, 51–59
5. Averill, F.J., Hubbard, W.C., Proud, D., Gleich, G.J., and Liu, M.C. (1992) Platelet activation in the lung after antigen challenge in a model of allergic asthma. *Am. Rev. Respir. Dis.* **145**, 571–576
6. Mannaioni, P.F., Di Bello, M.G., and Masini, E. (1997) Platelets and inflammation: role of platelet-derived growth factor, adhesion molecules and histamine. *Inflamm. Res.* **46**, 4–18
7. Zucker-Franklin, D. and Philipp, C.S. (2000) Platelet production in the pulmonary capillary bed: new ultrastructural evidence for an old concept. *Amer. J. Pathol.* **157**, 69–74
8. Metzger, W.J., Sjoerdsma, K., Richerson, H.B., Moseley, P., Zavala, D., Monick, M., and Hunninghake, G.W. (1987) Platelets in bronchoalveolar lavage from asthmatic patients and allergic rabbits with allergen-induced late phase responses. *Agents Actions* **21**, 151–159
9. Beasley, R., Roche, W.R., Roberts, J.A., and Holgate, S.T. (1989) Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am. Rev. Respir. Dis.* **139**, 806–817
10. Yamamoto, H., Nagata, M., Tabe, K., Kimura, I., Kiuchi, H., Sakamoto, Y., Yamamoto, K., and Dohi, Y. (1993) The evidence of platelet activation in bronchial asthma. *J. Allergy Clin. Immunol.* **91**, 79–87
11. Chihara, J., Yasuba, H., Tsuda, A., Urayama, O., Saito, N., Honda, K., Kayaba, H., Yamashita, T., Kurimoto, F., and Yamada, H. (1997) Elevation of the plasma level of RANTES during asthma attacks. *J. Allergy Clin. Immunol.* **100**, S52–55
12. Moritani, C., Ishioka, S., Haruta, Y., Kambe, M., and Yamakido, M. (1998) Activation of platelets in bronchial asthma. *Chest* **113**, 452–458
13. Hasegawa, S., Pawankar, R., Suzuki, K., Nakahata, T., Furukawa, S., Okumura, K., and Ra, C. (1999) Functional expression of the high affinity receptor for IgE (FcepsilonRI) in human platelets and its intracellular expression in human megakaryocytes. *Blood* **93**, 2543–2551
14. Kameyoshi, Y., Dorschner, A., Mallet, A.I., Christophers, E., and Schroder, J.M. (1992) Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J. Exp. Med.* **176**, 587–592
15. Klouche, M., Klinger, M.H., Kuhnel, W., and Wilhelm, D. (1997) Endocytosis, storage, and release of IgE by human platelets: differences in patients with type I allergy and nonatopic subjects. *J. Allergy Clin. Immunol.* **100**, 235–241
16. Yoshimi, Y., Fujimura, M., Myou, S., Tachibana, H., and Hirose, T. (2001) Effect of thromboxane A2 (TXA2) synthase inhibitor and TXA2 receptor antagonist alone and in combination on antigen-induced bronchoconstriction in guinea pigs. *Prostaglandins Other Lipid Mediat.* **65**, 1–9
17. Dogne, J.M., de Leval, X., Benoit, P., Rolin, S., Pirotte, B., and Masereel, B. (2002) Therapeutic potential of thromboxane inhibitors in asthma. *Expert Opin. Investig. Drugs* **11**, 275–281

18. Yatomi, Y., Ozaki, Y., Ohmori, T., and Igarashi, Y. (2001) Sphingosine 1-phosphate: synthesis and release. *Prostaglandins Other Lipid Mediat.* **64**, 107–122
19. Yatomi, Y., Yamamura, S., Ruan, F., and Igarashi, Y. (1997) Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J. Biol. Chem.* **272**, 5291–5297
20. Spiegel, S. and Milstien, S. (2002) Sphingosine 1-phosphate, a key cell signaling molecule. *J. Biol. Chem.* **277**, 25851–25854
21. Kluk, M.J. and Hla, T. (2002) Signaling of sphingosine-1-phosphate *via* the S1P/EDG-family of G-protein-coupled receptors. *Biochim. Biophys. Acta* **1582**, 72–80
22. Takuwa, Y. (2002) Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta* **1582**, 112–120
23. Ammit, A.J., Hastie, A.T., Edsall, L.C., Hoffman, R.K., Amrani, Y., Krymskaya, V.P., Kane, S.A., Peters, S.P., Penn, R.B., Spiegel, S., and Panettieri, R.A., Jr. (2001) Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J.* **15**, 1212–1214
24. Racke, K., Hammermann, R., and Juergens, U.R. (2000) Potential role of EDG receptors and lysophospholipids as their endogenous ligands in the respiratory tract. *Pulm. Pharmacol. Ther.* **13**, 99–114
25. Jolly, P., Rosenfeldt, H., Milstien, S., and Spiegel, S. (2002) The roles of sphingosine-1-phosphate in asthma. *Mol. Immunol.* **38**, 1239–1245
26. Yatomi, Y., Igarashi, Y., Yang, L., Hisano, N., Qi, R., Asazuma, N., Satoh, K., Ozaki, Y., and Kume, S. (1997) Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J. Biochem.* **121**, 969–973
27. Barsumian, E.L., Isersky, C., Petrino, M.G., and Siraganian, R.P. (1981) IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. *Eur. J. Immunol.* **11**, 317–323
28. Osada, M., Yatomi, Y., Ohmori, T., Ikeda, H., and Ozaki, Y. (2002) Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist. *Biochem. Biophys. Res. Commun.* **299**, 483–487
29. Ikeda, H., Satoh, H., Yanase, M., Inoue, Y., Tomiya, T., Arai, M., Tejima, K., Nagashima, K., Maekawa, H., Yahagi, N., Yatomi, Y., Sakurada, S., Takuwa, Y., Ogata, I., Kimura, S., and Fujiwara, K. (2003) Antiproliferative property of sphingosine 1-phosphate in rat hepatocytes involves activation of Rho via Edg-5. *Gastroenterology* **124**, 459–469
30. Cicmil, M., Thomas, J.M., Sage, T., Barry, F.A., Leduc, M., Bon, C., and Gibbins, J.M. (2000) Collagen, convulxin, and thrombin stimulate aggregation-independent tyrosine phosphorylation of CD31 in platelets. Evidence for the involvement of Src family kinases. *J. Biol. Chem.* **275**, 27339–27347
31. Lynch, K.R. (2002) Lysophospholipid receptor nomenclature. *Biochim. Biophys. Acta* **1582**, 70–71
32. Graler, M.H., Bernhardt, G., and Lipp, M. (1998) EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics* **53**, 164–169
33. Im, D.S., Heise, C.E., Ancellin, N., O'Dowd, B.F., Shei, G.J., Heavens, R.P., Rigby, M.R., Hla, T., Mandala, S., McAllister, G., George, S.R., and Lynch, K.R. (2000) Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J. Biol. Chem.* **275**, 14281–14286
34. Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H., and Takuwa, Y. (2000) Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol. Cell. Biol.* **20**, 9247–9261
35. Suzuki, T., Shoji, S., Yamamoto, K., Nada, S., Okada, M., Yamamoto, T., and Honda, Z. (1998) Essential roles of Lyn in fibronectin-mediated filamentous actin assembly and cell motility in mast cells. *J. Immunol.* **161**, 3694–3701
36. Yamamura, S., Hakomori, S., Wada, A., and Igarashi, Y. (2000) Sphingosine-1-phosphate inhibits haptotactic motility by overproduction of focal adhesion sites in B16 melanoma cells through EDG-induced activation of Rho. *Ann. N. Y. Acad. Sci.* **905**, 301–307
37. Van Brocklyn, J.R., Tu, Z., Edsall, L.C., Schmidt, R.R., and Spiegel, S. (1999) Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. *J. Biol. Chem.* **274**, 4626–4632
38. Ali, H., Tomhave, E.D., Richardson, R.M., Haribabu, B., and Snyderman, R. (1996) Thrombin primes responsiveness of selective chemoattractant receptors at a site distal to G protein activation. *J. Biol. Chem.* **271**, 3200–3206
39. Melendez, A.J. and Khaw, A.K. (2002) Dichotomy of Ca^{2+} signals triggered by different phospholipid pathways in antigen stimulation of human mast cells. *J. Biol. Chem.* **277**, 17255–17262
40. Choi, O.H., Kim, J.-H., and Kinet, J.-P. (1996) Calcium mobilization via sphingosine kinase in signalling by the FceRI antigen receptor. *Nature* **380**, 634–636
41. Woo, C.H., Jeong, D.T., Yoon, S.B., Kim, K.S., Chung, I.Y., Saeki, T., and Kim, J.H. (2002) Eotaxin induces migration of RBL-2H3 mast cells *via* a Rac-ERK-dependent pathway. *Biochem. Biophys. Res. Commun.* **298**, 392–397
42. Prieschl, E.E., Csonga, R., Novotny, V., Kikuchi, G.E., and Baumruker, T. (1999) The balance between sphingosine and sphingosine-1-phosphate is decisive for mast cell activation after Fc epsilon receptor I triggering. *J. Exp. Med.* **190**, 1–8
43. Lee, M.J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I., and Hla, T. (1999) Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* **99**, 301–312
44. Lee, M.J., Thangada, S., Paik, J.H., Sapkota, G.P., Ancellin, N., Chae, S.S., Wu, M., Morales-Ruiz, M., Sessa, W.C., Alessi, D.R., and Hla, T. (2001) Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. *Mol. Cell* **8**, 693–704
45. Xia, P., Gamble, J.R., Rye, K.A., Wang, L., Hii, C.S., Cockerill, P., Khew-Goodall, Y., Bert, A.G., Barter, P.J., and Vadas, M.A. (1998) Tumor necrosis factor-a induces adhesion molecule expression through the sphingosine kinase pathway. *Proc. Natl Acad. Sci. USA* **95**, 14196–14201
46. Kawamoto, K., Aoki, J., Tanaka, A., Itakura, A., Hosono, H., Arai, H., Kiso, Y., and Matsuda, H. (2002) Nerve growth factor activates mast cells through the collaborative interaction with lysophosphatidylserine expressed on the membrane surface of activated platelets. *J. Immunol.* **168**, 6412–6419