Full Paper

Cell-Culture–Dependent Change of Ca^{2+} Response of Rat Aortic Myocytes to Sphingosine-1-PhosphateRyoichi Tanaka¹, Katsuhiko Muraki², Susumu Ohya¹, Yuka Itoh², Noriyuki Hatano², and Yuji Imaizumi^{1,*}¹Department of Molecular and Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuhoku, Nagoya 467-8603, Japan²Department of Cellular Pharmacology, School of Pharmacy, Aichi-Gakuin University, 1-100 Kusumotocho, Chikusaku, Nagoya 464-8650, Japan

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Abstract. We characterized the effects of sphingosine-1-phosphate (S1P) on rat aortic myocytes with or without culture. Application of S1P induced a small Ca^{2+} response in 40% freshly dispersed aortic myocytes, whereas S1P caused a larger Ca^{2+} response in 90% myocytes cultured for 72 h. Concentration–response relationships of S1P in cultured myocytes were significantly different from that in non-cultured myocytes. Analysis of the expression of S1P-receptor mRNA transcripts revealed that S1P-receptor type 3 (S1P_3) was significantly increased when myocytes were cultured for 24 h. Neither the removal of serum from culture medium nor pretreatment with pharmacological agents, such as ERK, Rho, and PI3 kinase inhibitors, affected the progression of the S1P-induced Ca^{2+} response during culture. The sustained component of the Ca^{2+} response to S1P was sensitive to the removal of external Ca^{2+} and was effectively inhibited by inorganic Ca^{2+} -channel blockers such as Gd^{3+} , Cd^{2+} , and Ni^{2+} . However, application of S1P did not induce any contraction in organ-cultured as well as the intact aorta muscle strip. Aortic myocytes freshly dispersed from the organ-cultured muscle were also ineffective against S1P. Taken together, cell-culture changes the S1P_3 -mediated Ca^{2+} response to S1P in rat aortic myocytes.

Keywords: sphingosine-1-phosphate (S1P), S1P_3 receptor, rat aorta, cell culture

Introduction

Sphingosine-1-phosphate (S1P) is a bioactive lipid eliciting dramatic effects in a variety of cell types, including cardiovascular organs. Signaling by S1P is transduced by a family of five G protein-coupled receptors named S1P_{1-5} (1–3). These receptors are widely expressed in a variety of tissues and cell types, and their cellular effects may contribute to important biological and pathological functions of S1P in many processes, including vascular development, lymphocyte trafficking, angiogenesis, and cancer (4–7). A recent pre-clinical development in this field is the finding that fingolimod (FTY-720) stimulates S1P receptors after its phosphorylation by sphingosine kinase in the serum and has anti-immuno activity (8, 9). Moreover, a monoclonal

antibody that binds S1P slows tumor progression and the associated angiogenesis in several animal models of human cancer, suggesting that S1P permits or is required for the actions of angiogenic factors (10).

In cardiovascular organs, S1P acts as a lipid mediator to regulate various cellular functions (11–14). For example, S1P consistently activates acetylcholine (ACh)-sensitive K^+ channels in the atrium of guinea pigs, mice, rabbits, and rats via the activation of pertussis toxin (PTX)-sensitive G-protein (15–17). S1P-induced lowering of the heart rate was abolished in S1P_3 -knockout mice, strongly suggesting that activation of S1P_3 is responsible for chronotropic effects of S1P (17); however, this effect may not be of sufficient magnitude to be detectable in all cases, particularly in vivo. Previous studies have also revealed the importance of S1P and its receptor in vascular development (4, 18). S1P can be effectively administered to endothelial cells as well as vascular smooth myocytes in vivo since it is released in high concentration after platelet activation.

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In primary cultured vascular smooth muscle cells, including aortic myocytes, S1P effectively elevates intracellular Ca^{2+} concentration and facilitates cell-proliferation (19); however, in freshly isolated vascular smooth muscle, the effects of S1P vary from vessel to vessel. Large arteries, such as the aorta, renal, femoral, and mesenteric arteries, are apparently insensitive to S1P, and application of even a high concentration of S1P ($\sim 10 \mu\text{M}$) induced only a slight elevation of $[\text{Ca}^{2+}]_i$ and contraction (20, 21). It is not clear whether these responses mediate the activation of S1P receptors because a high concentration of S1P causes nonspecific actions (12, 22). In contrast, the basilar artery is substantially sensitive to S1P, suggesting that S1P receptors are expressed in the plasma membrane and S1P plays an obligatory role in the regulation of basilar arterial tone (20). Nevertheless, it is not concluded that the S1P-induced response and the expression of S1P receptors in vascular myocytes have plasticity in accordance with cell conditions.

In the present study, we compared the response to S1P of rat aorta before and after cell culture to measure Ca^{2+} fluorescence signal. Furthermore, mRNA expression of S1P receptors was determined in cultured and non-cultured aortic myocytes.

Materials and Methods

Cell dispersion and culture

All experiments were carried out in accordance with guiding principles for the care and use of laboratory animals (Science and International Affairs Bureau of the Japanese Ministry of Education, Culture, Sports, Science, and Technology) and also with the approval of the ethics committee of Nagoya City University. Wistar ST rats (Japan SLC Inc., Shizuoka) weighing 180–250 g were anesthetized with ether and then killed by exsanguination (23). After opening the chest, an approximately 3-cm-long segment of thoracic aorta was excised. After removing connective tissues and rubbing the inner wall of the vessel with a cotton pad to remove endothelium, the whole aorta was incubated in nominally Ca^{2+} - and Mg^{2+} -free Hanks solution for 5 min and then in solution containing 2 mg/ml collagenase (Amano, Nagoya) and 1 mg/ml papain (Sigma, St. Louis, MO, USA) for 45 min. Thereafter, the enzyme-treated strip was mechanically agitated in fresh Ca^{2+} - and Mg^{2+} -free Hanks solution that did not contain digestion enzymes. Freshly dissociated cells were used within 6 h after cell dispersion. Ca^{2+} - and Mg^{2+} -free Hanks solution for cell dispersion contained 137 mM NaCl, 5.4 mM KCl, 0.168 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 5.55 mM glucose, and 4.17 mM NaHCO_3 (pH 7.45). The purity of

smooth muscle cells with no response to $10 \mu\text{M}$ ACh was confirmed by staining with anti-smooth muscle-specific α -actin (SM α -actin) antibody (24). In some experiments, the dispersed aortic myocytes were placed on several cover glasses in DMEM with or without 10% FCS and cultured for 1–3 days. When the organ culture method was applied, the aorta strip was organ-cultured for 1–3 days in DMEM with 10% FCS.

Measurement of tension development

After connective tissue and adventia were removed carefully, 3-mm lengths of vessel ring were cut from the aorta. Endothelial cells were removed by rubbing the inner wall of the vessel with a cotton pad. The vessel ring was set in a 4-ml organ bath to measure isometric tension with a force transducer and perfused with Krebs' solution maintained at $37 \pm 1^\circ\text{C}$ and gassed with 95% O_2 and 5% CO_2 . The composition of Krebs' solution was 117 mM NaCl, 1.2 mM MgCl_2 , 2.2 mM CaCl_2 , 4.7 mM KCl, 1.2 mM KH_2PO_4 , 14 mM glucose, and 25 mM NaHCO_3 .

RNA extraction and quantitative PCR

Total RNAs were extracted from freshly dissociated and cultured aortic myocytes by the acid guanidium thiocyanate–phenol method following digestion with RNase-free DNase, and RT was performed according to the manufacturer's instructions (25). Real-time quantitative PCR was performed with the use of SYBR Green Chemistry on a Thermal Cycler Dice Real Time System (Takara Bio, Inc., Tokyo). Standard curves were generated for the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from regression analysis of the mean values of RT-PCRs for the $\log_{10}$ diluted cDNA. Unknown quantities relative to the standard curve for a particular set of primers were calculated, yielding transcriptional quantification of gene products relative to the endogenous standard (GAPDH). Each cDNA sample was tested in triplicate. Oligonucleotide sequences of primers specific for S1P₁ (EDG1), S1P₂ (EDG5), and S1P₃ (EDG3) of the rat (sense and antisense, 5' to 3') were TGGCCCCTCCTC AAAGACTAA and TTTGTCATTACAGTGAGAGAG CACATC for S1P₁, TCACCATCGTACTGGGTGTT TTC and CCGGACGGGACAGGTAGAGT for S1P₂, and GCGGCACCTGACCATGAT and GAAGGAACA CGCGGTGCTT for S1P₃ (19). As a control signal, GAPDH (sense and antisense, 5' to 3') expression was analyzed (CATGGCCTTCCGTGTTCT, CTGCT TCACCACCTTCTTGA).

Measurement of Ca^{2+} fluorescence ratio

Aortic myocytes were loaded with $2.5 \mu\text{M}$ Fura-2

acetoxymethyl ester (Fura-2) in standard HEPES solution for 45 min at room temperature (24). Fura-2 fluorescence signals were measured using an Argus/HisCa imaging system (Hamamatsu Photonics, Hamamatsu) and Imagework Bench 6.0 system (INDEC BioSystems, Santa Clara, CA, USA). The frequency of image acquisition was 0.1 Hz, unless otherwise specified. In the present experiments, we considered that cells having a Ca^{2+} response of less than 0.02 Ca^{2+} fluorescence ratio (ΔCa^{2+}_i) had no response.

Drugs

The following drugs were used: Fura-2 AM (Calbiochem, CA, La Jolla, USA), lysophosphatidic acid (LPA, Sigma), sphingosine-1-phosphate (S1P, Sigma), wortmannin (Sigma), endothelin-1 (ET; Peptide, Inc., Osaka), U0126 (Sigma), Y27632 (Sigma), suramin (Sigma), and SEW-2871 (Funakoshi, Tokyo). Solvents used to dissolve each drug (distilled water, methanol, and DMSO) had no effect on Ca^{2+}_i . All drug concentrations are expressed as the final concentration in solutions and the pH of the solution was readjusted after the addition of drugs. A change of solution could be achieved within 30 s.

Data analysis

Data are expressed as a mean $\pm$ S.E.M. Statistical

significance between two and among multiple groups was examined by using Student's *t*-test and one-way ANOVA, respectively. Statistical significance at *P* values of 0.05 and 0.01 are indicated in the figures and text by * (or #) and **, respectively.

Results

To reexamine whether rat aortic myocytes without culture were sensitive to S1P- and LPA-receptor agonists, S1P, and LPA, we applied 300 nM S1P and 300 nM LPA to acutely dispersed aortic myocytes while measuring $[\text{Ca}^{2+}]_i$ (Fig. 1A). In about 40 of the studied cells (25 and 21 of 56 cells, respectively, for S1P and LPA), these agonists induced a moderate elevation of $[\text{Ca}^{2+}]_i$ (peak ΔCa^{2+}_i by S1P and LPA: 0.15 ± 0.03 , $n = 25$ and 0.15 ± 0.02 , $n = 21$, respectively). On the other hand, 73% aortic myocytes were sensitive to 10 nM ET ($n = 37$, peak ΔCa^{2+}_i , 0.19 ± 0.01). In contrast, aortic myocytes cultured in MEM medium containing 10% FCS for 48 h had an augmented response to both S1P and LPA (Fig. 1Ab); in particular, 80% myocytes cultured for 48 h were sensitive to 300 nM S1P and the Ca^{2+} response was significantly larger (Fig. 1: Ba, Bb). As shown in Fig. 1B, the increase in Ca^{2+} response to S1P appeared in myocytes cultured for longer than 24 h and was sustained within 72 h after the start of culture.

Fig. 1. Effects of sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) on Ca^{2+} response in rat aortic myocytes with or without culture. A: Serial application of 300 nM LPA and S1P to non-cultured (0 h, Aa) and cultured (48 h, Ab) aortic myocytes loaded with Fura-2. The fluorescence ratio ($F_{340/380}$) was calculated and plotted against time. B: Summary of effects of S1P and LPA on Ca^{2+} response. Open and closed circles in Ba indicate percent cells responsive to 300 nM LPA and S1P, respectively, against the culture period. Number in parentheses denotes number of cells used. The change of fluorescence ratio (ΔCa^{2+}_i) induced by 300 nM LPA (open bars, # $P < 0.05$ vs 0 h) and S1P (closed bars, ** $P < 0.01$ vs 0 h) was plotted against culture period (Bb). Number in each column indicates the number of cells responding to LPA or S1P. Bar = S.E.M.

Fig. 2. Change of fluorescence ratio by S1P applied to non-cultured (0 h) and cultured (24, 48, and 72 h) aortic myocytes was summarized as concentration–response relationships. Number in parentheses indicates number of cells employed. Two-way ANOVA was applied to test differences among groups; * $P < 0.05$ vs 0 h. Bar = S.E.M.

While the Ca^{2+} response to LPA was increased in myocytes cultured for 24 h, the change of the response was small and appeared to be reversed. Figure 2 shows concentration–response relationships of S1P in cultured and non-cultured aortic myocytes. To remove the possible desensitization of S1P receptors, each concentration of S1P was applied once to each aortic myocyte. While freshly isolated aortic myocytes (0 h) were almost insensitive to S1P less than 30 nM, myocytes cultured for 24 h and for longer than 48 h had a substantial response to 30 nM and 3 nM S1P, respectively. Concentration–response relationships of S1P in myocytes cultured for 24, 48, and 72 h were significantly changed from that without culture ($P < 0.05$, closed circles).

In Fig. 3A, we evaluated the expressions of S1P₁ (EDG₁), S1P₂ (EDG₅), and S1P₃ (EDG₃) mRNAs in non-cultured and cultured rat aortic myocytes. Total RNA isolated from acutely dispersed (0 h) and cultured (24, 48, and 72 h) aortic myocytes was subjected to RT; and the mRNA expression levels of S1P₁, S1P₂, and S1P₃ were determined based on real-time PCR. As shown in Fig. 3A, S1P₁ mRNA was markedly reduced during culture, while S1P₂ mRNA was comparable. The analysis also demonstrated that the increase in S1P₃ mRNA was the most extensive in myocytes cultured for 24 h, suggesting that cell culture facilitates the expression of S1P₃. In order to confirm that S1P₃ protein was functionally expressed in cultured myocytes, effects of suramin, a S1P₃ antagonist, on S1P-induced Ca^{2+} response were examined. As shown in Fig. 3B, the presence of 10 μM suramin effectively inhibited the

Fig. 3. The mRNA expression of S1P-receptor subtypes in non-cultured (0 h) and cultured aortic myocytes (24–72 h) and effect of a S1P₁ agonist and a S1P₃ antagonist on S1P-induced Ca^{2+} response. A: Quantitative analyses for S1P-receptor subtypes were performed by real-time PCR. Relative expression of S1P₁, S1P₂, and S1P₃ were compared among culture periods ($n = 3$). B: Effect of SEW-2871 and suramin, a S1P₁ agonist and a S1P₃ antagonist, respectively, on Ca^{2+} response in myocytes cultured for 48 h were examined. The change of fluorescence ratio under various experimental conditions was summarized. Number in parentheses denotes number of cells used. ** $P < 0.01$. Bar = S.E.M.

S1P-induced Ca^{2+} response. In contrast, a potent and selective S1P₁ agonist, SEW-2871, had no effect on Ca^{2+} response in cultured myocytes.

To examine the mechanisms involved in the progression of S1P₃ expression, we tested the effects of serum (FCS) deficiency from standard culture medium (MEM + 10% FCS) on the Ca^{2+} response to S1P. As shown in Fig. 4A, the removal of FCS [FCS(–)] reduced the Ca^{2+} response to 300 nM S1P in myocytes cultured for 24 h, possibly due to low viability in FCS(–) (compare this with the results in Fig. 1B). Nevertheless, culture of myocytes in FCS(–) for 48 and 72 h augmented the Ca^{2+} response to S1P to 2.5- and 3-fold of

Fig. 4. Increase in Ca^{2+} response to S1P of aortic myocytes cultured in FCS(-) and in the presence of pharmacological agents. A: Closed circles in Aa indicate percent cells responsive to 300 nM S1P against culture period in FCS(-). Number in parentheses denotes number of cells used. In Ab, the change of fluorescence ratio induced by 300 nM S1P was plotted against culture period. ** $P < 0.01$ vs 24 h. Number in parentheses indicates number of cells responding to S1P. B: After aortic myocytes were cultured for 72 h in standard culture medium with or without 10 μ M U0126, 3 μ M Y-27632, and 100 nM wortmannin, each myocyte was exposed to 300 nM S1P to obtain the Ca^{2+} response. The change of fluorescence ratio induced by 300 nM S1P was plotted in B. The dotted line indicates the amplitude of S1P-induced Ca^{2+} fluorescence ratio without culture. Bar = S.E.M.

that for 24 h, respectively (Fig. 4Ab). The number of cells responding to 300 nM S1P also gradually increased in accordance with culture, even without FCS (Fig. 4Ab), demonstrating that FCS has a minor role in the progression of the response to S1P and expression of $S1P_3$ during culture. In Fig. 4B, the involvement of signaling pathways in the progression of the S1P response during culture was examined using pharmacological agents such as U0126, Y27632, and wortmannin. Myocytes were cultured with or without these agents for 72 h. Although the Ca^{2+} response to 300 nM S1P varied from cell to cell even under control conditions, these agents did not affect the response.

In Fig. 5, the mechanisms involved in S1P-induced

Fig. 5. Effects of removal of extracellular Ca^{2+} and inorganic Ca^{2+} -channel blockers on Ca^{2+} response of cultured aortic myocytes to S1P. A: In the presence and absence of external Ca^{2+} , 300 nM S1P was applied to aortic myocytes cultured for 72 h. Peak and sustained components of the Ca^{2+} response were summarized as the change of fluorescence ratio (ΔCa^{2+}_i). ** $P < 0.01$. B: Inhibition by Ni^{2+} , Cd^{2+} , and Gd^{3+} of the sustained component of the S1P-induced Ca^{2+} response. Aortic myocytes were cultured for 72 h in standard culture medium. Number in parentheses denotes number of cells used. Bar = S.E.M.

elevation of $[Ca^{2+}]_i$ were examined using cultured aortic myocytes for 72 h. Removal of Ca^{2+} from the bathing solution significantly reduced the transient Ca^{2+} response to 300 nM S1P and almost abolished the sustained component, suggesting that at least two different pathways, release of Ca^{2+} from the Ca^{2+} store site and influx of Ca^{2+} from the extracellular space, are responsible for the S1P-induced Ca^{2+} response in cultured aortic myocytes. To further confirm activation of the Ca^{2+} -entry pathway during stimulation with S1P, di- and tri-valent cations, which have Ca^{2+} -channel blocking action, were applied in Fig. 5B. Exposure to 100 nM Gd^{3+} markedly inhibited the Ca^{2+} response to 300 nM S1P. Moreover, Cd^{2+} and Ni^{2+} effectively inhibited the Ca^{2+} response, strongly demonstrating that Ca^{2+} entry has an obligatory role in the sustained component of Ca^{2+} response to S1P.

Although 45% rat aortic myocytes without culture

Fig. 6. Response to high K⁺ and S1P of aorta muscle strip with or without organ culture. **A:** Organ culture was performed in the presence of 10% FCS. Effects of 60 mM K⁺ and 3 μM S1P on tension development in the aorta muscle strip with or without organ culture for 72 h were examined. Endothelium in aorta was denuded by rubbing the inner wall of the muscle strip with a cotton pad. **B:** Ca²⁺ response of aortic myocytes dispersed from aorta with organ culture for 48 and 72 h. Percent cells (**Ba**) and the change of Ca²⁺ fluorescence ratio (ΔCa^{2+}_i , **Bb**) responsive to 300 nM S1P was plotted against organ culture period. The dotted line in **Bb** indicates the amplitude of S1P-induced Ca²⁺ fluorescence ratio without culture. Number in parentheses in **Ba** and **Bb** denotes number of cells used and those responding to S1P, respectively. Bar = S.E.M.

were slightly sensitive to 300 nM S1P (peak ΔCa^{2+}_i : 0.15 ± 0.03 , Fig. 1B), it is not clear that S1P induces vaso-contraction in the rat aorta muscle strip. In Fig. 6A, therefore, the effects of 3 μM S1P on tension development in the rat aorta muscle strip were examined. Contraction was not produced by 3 μM S1P in endothelium-denuded aorta, while it was induced by 60 mM high K⁺. In addition, endothelium-denuded aorta with organ culture for 72 h with 10% FCS had no contractile response to 3 μM S1P. Relative amplitude of the contractile response to 3 μM S1P with that to 60 mM high K⁺ was $5.6 \pm 1.5\%$ ($n = 4$) and $2.1 \pm 1.1\%$ ($n = 6$)

in the aorta muscle strip with (72 h) and without organ culture, respectively. Dispersed aortic myocytes prepared from aorta organ culture for 48 and 72 h with 10% FCS also had a weak response to 300 nM S1P, compared with those cultured for 24–72 h (Fig. 6B, see also Fig. 1B).

Discussion

Similar to previous reports, S1P exerts a weak stimulus to induce the Ca²⁺ response under physiological conditions, but did not elicit vasoconstriction in the rat aorta; however, in the present study, we have shown that the response of rat aortic myocytes to S1P progressively increases during culture. In addition, our results suggest that the mechanism by which this facilitatory response occurs is associated with change of S1P₃ expression during culture.

S1P caused slight vasoconstriction in rat renal and mesenteric micro-vessels (26). In addition, S1P did not induce any contractile response in coronary, femoral, and carotid arteries isolated from rats (21). Consistently, in our study, the stimulation of freshly dispersed aortic myocytes by S1P at high concentrations (1–3 μM) produced only a small Ca²⁺ response. In contrast, cultured rat aortic myocytes had significant Ca²⁺ response to S1P at submicromolar concentrations (19, 27), suggesting that under certain conditions, aortic myocytes are highly sensitive to S1P. Results from the present study indicate that cell dispersion and subsequent incubation in culture medium with/without FCS induced a cellular response to S1P, possibly via the progressive expression of S1P₃. Despite the non-responsiveness to S1P of the rat coronary, carotid and femoral arterial smooth muscles, all S1P receptor mRNA transcripts were detected in these microvessels as well as the aorta (21). In our study, S1P₃ mRNA transcript was increased in rat aortic myocytes cultured for 24 h and the S1P-induced Ca²⁺ response was effectively inhibited by a S1P₃ antagonist, strongly suggesting that S1P₃ was progressively expressed during culture. Compared with cerebral smooth muscle, the relative expression of S1P-receptor subtypes in the intact aorta is substantially low (20), correlating with a lack of vasoconstriction and a global increase in $[\text{Ca}^{2+}]_i$ by S1P. Although analysis of the protein expression of S1P receptors is required, cell dispersion could induce the expression of S1P₃. However, we can not rule out the possibility that other S1P subtypes also mediate the S1P-induced Ca²⁺-response in cultured aortic myocytes because suramin is a multi-effective drug and a part of the Ca²⁺ response was resistant to suramin (Fig. 3B).

Receptor-binding of S1P stimulates different signaling pathways via heterotrimeric G proteins. In the

heterologous expression system, S1P₃ can couple to G_i, G_q, and G_{12/13} (28). Moreover, the downstream signaling activated by S1P binding to S1P₃ has been extensively studied in different types of cells. S1P₃ mediates the activation of extracellular signaling-regulated kinase (ERK) via G_i, tightly coupled to phospholipase C (PLC) via G_q and can also mediate Rho kinase activation through G_{12/13} (29). Despite these findings, physiological coupling of S1P₃ to G proteins and the subsequent downstream signaling varies from cell to cell due to the different expression of G proteins and signaling molecules in each cell. In vascular smooth muscles, S1P elevates [Ca²⁺]_i via the activation of G_i and G_q because pharmacological agents such as pertussis toxin (PTX) and U73122 effectively inhibited the elevation of [Ca²⁺]_i by S1P (20, 27, 30). Activation of G_q leads to the release of Ca²⁺ from the Ca²⁺ store, supported by evidence that the Ca²⁺ response to S1P was abolished by thapsigargin, but not by removal of external Ca²⁺ (20). In the present study, removal of external Ca²⁺ abolished a sustained component of the Ca²⁺ response to S1P, but only reduced part of the transient component. These results imply that both Ca²⁺ influx from outside and Ca²⁺ release from the Ca²⁺ store are responsible for these Ca²⁺ responses in cultured aortic myocytes. Furthermore, the effective inhibition of sustained Ca²⁺ response by inorganic Ca²⁺-channel blockers provides a chain of evidence for the involvement of Ca²⁺ influx in the S1P-induced Ca²⁺ response through Ca²⁺-permeable channels. Signaling molecules produced by S1P₃ stimulation could activate such a channel (12), but it is possible that S1P directly activates cation channels (31).

Removal of FCS from culture medium did not affect progression of the Ca²⁺ response to S1P in cultured aortic myocytes. Moreover, sensitivity to S1P of myocytes dispersed from aorta organ culture in standard culture medium containing FCS had low sensitivity to S1P. These findings suggest that FCS does not play any critical role in progression of the S1P response and expression of S1P₃. Since the plasma concentration of S1P is 200–350 nM, that the effect of S1P on Ca²⁺ response occurs, *in vitro*, in the submicromolar range is of particular relevance. Although we can not identify the mechanisms involved in the change of the S1P response, possibly followed by enhancement of S1P₃ expression during culture, this is the first evidence that S1P₃ is up-regulated during culture. S1P₁ expression was decreased in proliferating vascular endothelial cells and hepatocytes (32, 33). Vascular endothelial growth factor (VEGF) induces the expression of S1P₁ in endothelial cells via tyrosine kinase- and protein kinase C-mediated pathways (34). On the other hand, enhanced expression of S1P₂ was observed in primary cultured rat hepato-

cytes depending on cell-cycle transition from G0 to G1, and thus S1P₂ expression was up-regulated during proliferating status (33). F9 embryonic carcinoma cells showed down-regulation for S1P₂ expression when differentiated (35). In addition, myogenic differentiation in C2C12 myoblasts was accompanied with down-regulation of S1P₂ (36). Cell proliferation is not responsible for the enhancement of the Ca²⁺ response to S1P and S1P₃ expressions in cultured rat aortic myocytes because serum deprivation, which arrests the cell cycle in the non-proliferating status, did not affect the enhancement of the Ca²⁺ response during culture. Moreover, the involvement of MEKs, PI3 kinase, and Rho kinase could be excluded because effective inhibitors of these kinases were ineffective for enhancement; however, we can not rule out the possibility that unknown factors released from aortic myocytes might affect the enhancement of the Ca²⁺ response to S1P and S1P₃ expressions. Alternatively, it is possible that cell-to-cell contact in aortic myocytes has an obligatory role in enhancement. In rat hepatocytes, harvested cell density was critical in the up-regulation of S1P₂ expression during progression of the proliferating status (33). Moreover, S1P has an attachment-dependent regulatory effect on invasion of epithelial ovarian cancer cells, suggesting that phenotype-dependent change of extracellular matrix such as N-cadherin, gamma- and beta-catenins, and/or integrins affects S1P-mediated Ca²⁺ responses in cultured smooth muscle cells (37).

Physiological roles of the induction of S1P₃ in aortic myocytes remain unclear. S1P had little effect on mesenteric, coronary, renal, and carotid arteries isolated from rats (21), consistent with our preliminary results of the non-responsiveness of rat renal, femoral, and mesenteric arteries to S1P (K. Muraki, unpublished observation). Moreover, S1P₃ null mouse is phenotypically normal (38), strongly suggesting that S1P₃ has minor roles in the regulation of vascular tone under physiological conditions. In contrast, both S1P and FTY720, an effective analogue of S1P, induced endothelial nitric oxide (NO)-dependent arterial vasodilation via the activation of S1P₃ in the endothelium (39). In a similar manner, high-density lipoprotein (HDL), which is a carrier of bioactive lysophospholipids including S1P, causes NO-dependent vasodilation (40). The present results suggest that the migration of vascular smooth muscle cells could induce S1P₃ expression and the subsequent stimulation by S1P initiated variable cellular signalings in the vasculature. S1P stimulates the proliferation and migration of vascular smooth muscle and endothelial cells through S1P₃ as well as S1P₁. Further extensive studies are required, but the induction of S1P₃ in vascular smooth muscle cells might be

involved in cardiovascular functions and/or diseases.

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