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Sphingosine 1-Phosphate (S1P) Regulates Vascular Contraction via $S1P_3$ Receptor: Investigation Based on a New $S1P_3$ Receptor Antagonist[S]

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ABSTRACT

Sphingosine 1-phosphate (S1P) induces diverse biological responses in various tissues by activating specific G protein-coupled receptors ($S1P_1$–$S1P_5$ receptors). The biological signaling regulated by $S1P_3$ receptor has not been fully elucidated because of the lack of an $S1P_3$ receptor-specific antagonist or agonist. We developed a novel $S1P_3$ receptor antagonist, 1-(4-chlorophenylhydrazono)-1-(4-chlorophenylamino)-3,3-dimethyl-2-butanone (TY-52156), and show here that the S1P-induced decrease in coronary flow (CF) is mediated by the $S1P_3$ receptor. In functional studies, TY-52156 showed submicromolar potency and a high degree of selectivity for $S1P_3$ receptor. TY-52156, but not an $S1P_1$ receptor antagonist [(*R*)-phosphoric acid mono-[2-amino-2-(3-octyl-phenylcarbamoyl)-ethyl] ester; VPC23019] or $S1P_2$ receptor antagonist [1-[1,3-dimethyl-4-(2-methylethyl)-1*H*-pyrazolo[3,4-*b*]pyridin-6-yl]-4-(3,5-dichloro-4-pyridinyl)-semicarbazide; JTE013], inhibited the decrease in CF induced by S1P in isolated perfused rat hearts. We further investigated the effect of TY-52156 on both the S1P-induced increase in intracellular calcium ($[Ca^{2+}]_i$) and Rho activation that are responsible for the contraction of human coronary artery smooth muscle cells. TY-52156 inhibited both the S1P-induced increase in $[Ca^{2+}]_i$ and Rho activation. In contrast, VPC23019 and JTE013 inhibited only the increase in $[Ca^{2+}]_i$ and Rho activation, respectively. We further confirmed that TY-52156 inhibited FTY-720-induced $S1P_3$ receptor-mediated bradycardia in vivo. These results clearly show that TY-52156 is both sensitive and useful as an $S1P_3$ receptor-specific antagonist and reveal that S1P induces vasoconstriction by directly activating $S1P_3$ receptor and through a subsequent increase in $[Ca^{2+}]_i$ and Rho activation in vascular smooth muscle cells.

Sphingosine 1-phosphate (S1P) is a bioactive lysophospholipid mediator that is mainly released from activated platelets and induces many biological responses, including angiogenesis, vascular development, and cardiovascular function (Siess, 2002; Yatomi, 2006; Takuwa et al., 2008). A wide variety of biological cellular responses to S1P have been ascribed to the presence of five S1P receptors, $S1P_1$ to $S1P_5$ receptors, that belong to the family of G protein-coupled receptors (GPCRs). Furthermore, a variation of heterotrimeric G protein downstream of S1P receptors accounts for the diversity of cellular responses to S1P (Rosen et al., 2009). In addition to the coupling of S1P receptors and G proteins, the expression of the combi-

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ABBREVIATIONS: S1P, sphingosine 1-phosphate; GPCR, G protein-coupled receptor; TY-52156, 1-(4-chlorophenylhydrazono)-1-(4-chlorophenylamino)-3,3-dimethyl-2-butanone; MAPK, mitogen-activated protein kinase; CF, coronary flow; HUVEC, human umbilical vein endothelial cell; HCASMC, human coronary artery smooth muscle cell; SBP, systemic blood pressure; HR, heart rate; MBP, mean blood pressure; JTE013, 1-[1,3-dimethyl-4-(2-methylethyl)-1*H*-pyrazolo[3,4-*b*]pyridin-6-yl]-4-(3,5-dichloro-4-pyridinyl)-semicarbazide; VPC23019, (*R*)-phosphoric acid mono-[2-amino-2-(3-octyl-phenylcarbamoyl)-ethyl] ester; SD, Sprague-Dawley; Ik_{Ach}, cardiac G protein-gated potassium channel; Eu-GTP, europium-GTP; LC/MS/MS, liquid chromatography/tandem mass spectrometry; U46619, 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$; FTY-720, fingolimod; Y-27632, (*R*)-(+)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate; SEW2871, 5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole.

nation of S1P receptors determines multiple cellular responses. To identify the signaling that is specific for each receptor, S1P receptor antagonists have been developed and have contributed to our understanding of S1P-mediated signaling (Huwiler and Pfeilschifter, 2008).

$S1P_1$ to $S1P_5$ receptors couple to different G proteins upon binding to S1P. Whereas $S1P_1$, $S1P_4$, and $S1P_5$ receptors mainly couple to G_i, $S1P_2$ and $S1P_3$ receptors couple to G_i, G_q, and $G_{12/13}$ (Rosen et al., 2009). The signal that converges from G_i-coupled S1P receptors inhibits the activation of adenylate cyclase and induces the activation of p44/p42 mitogen-activated protein kinase (MAPK). Although the $S1P_1$ receptor slightly increases intracellular calcium ($[Ca^{2+}]_i$) through $G\beta\gamma$, $S1P_2$ and $S1P_3$ receptors mainly increase $[Ca^{2+}]_i$ through the activation of phospholipase Cβ from G_q (Watterson et al., 2005). The deletion of $S1P_3$ but not $S1P_2$ receptor in mouse embryonic fibroblasts led to the marked inhibition of S1P-induced phospholipase C activation, which suggests that $S1P_3$ receptor plays an important role in the S1P-induced increase in $[Ca^{2+}]_i$ (Ishii et al., 2002).

$S1P_2$ and $S1P_3$ receptors also couple to $G_{12/13}$ protein to activate a small GTPase, Rho, which is involved in the regulation of actin-cytoskeleton (Ryu et al., 2002; Sugimoto et al., 2003). Rho kinase is activated by Rho through the $G_{12/13}$-Rho guanine nucleotide exchange factor family. S1P-induced Rho activation has been shown to be significantly reduced in $S1P_2$ but not $S1P_3$ receptor-null mouse embryonic fibroblasts (Ishii et al., 2002). Meanwhile, an association between $S1P_3$ receptor and Rho activation has been reported in cells expressing $S1P_3$ receptor (Sugimoto et al., 2003). An S1P-induced contraction of vascular smooth muscle cells has been ascribed to an increase in $[Ca^{2+}]_i$ and Rho activation (Ohmori et al., 2003; Watterson et al., 2005). S1P-induced vasoconstriction is significantly inhibited in cerebral arteries isolated from $S1P_3$ receptor-null mice but not in those from $S1P_2$ receptor-null mice (Salomone et al., 2008). In addition, Y-27632, a selective Rho kinase inhibitor, inhibits S1P-induced vasoconstriction in canine cerebral arteries (Tosaka et al., 2001), indicating that $S1P_3$ receptor plays an indispensable role in S1P-induced vasoconstriction mediated by Rho-Rho kinase signaling. Although S1P decreases coronary flow (CF) in isolated perfused canine heart, the receptor subtype that is responsible for the S1P-induced reduction of CF has not yet been fully identified. To distinguish $S1P_3$ receptor-dependent signal from $S1P_2$ receptor-dependent signal, an $S1P_3$ receptor-specific antagonist has been needed.

We have developed an $S1P_3$ receptor antagonist, TY-52156. By confirming that TY-52156 has a selective antagonistic effect toward $S1P_3$ receptor, we can delineate the role of $S1P_3$ receptor-specific signaling in vascular contraction. Moreover, the effectiveness of TY-52156 in vivo was bolstered by evidence that $S1P_3$ receptor-dependent bradycardia was suppressed by the oral administration of TY-52156.

Materials and Methods

Materials

TY-52156 was synthesized in our laboratories. Materials were purchased from the following suppliers: S1P was from BIOMOL Research Laboratories (Plymouth Meeting, PA); SEW2871, FTY-720 and FTY-720-(*S*)-Phosphate were from Cayman Chemical (Ann Arbor, MI); VPC23019 was from Avanti Polar Lipids, Inc. (Alabaster, AL); JTE013 was from Tocris Bioscience (Southampton, UK); U46619 was from Calbiochem (Darmstadt, Germany); HuMedia-EG2 and HuMedia-SG2 were from Kurabo (Osaka, Japan); and membranes containing human $S1P_1$, $S1P_2$, $S1P_3$, or $S1P_5$ receptors were from Millipore (Billerica, MA).

Synthesis of TY-52156

5,5-Dimethyl-2,4-dihexanone (1). Diisopropyl ether (3 liters) was placed in a 5-liter three-neck round-bottomed flask and stirred mechanically. Potassium *tert*-butoxide (324 g, 2.25 mol, 1.5 eq) was suspended in the diisopropyl ether at 0°C. Pinacolone (150 g, 1.50 mol, 1.0 eq) in ethyl acetate (440 ml, 4.50 mol, 3.0 eq) was slowly added drop-wise so that the temperature would remain below 10°C under ice-bath cooling. The reaction mixture was then stirred for 20 h at ambient temperature. Water (1 liter) was added slowly so that the temperature would remain below 10°C under ice-bath cooling. The separated organic layer was extracted with 1 N sodium hydroxide (150 ml). The basic aqueous layer was carefully acidified with 6 N HCl (750 ml, 2.0 equivalents of base) at 0°C and then extracted twice with petroleum ether (750 ml). The combined organic layer was washed with water (475 ml) and saturated brine (475 ml), dried over anhydrous sodium sulfate, filtered, and evaporated at 200 mm Hg and ambient temperature. The remaining liquid was distilled under reduced pressure with heating up to 80°C. The compound (**1**) was obtained as a colorless oil (126 g, 0.89 mol, 59%); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.17 (s, 9 H), 2.08 (s, 3 H), 5.61 (s, 1 H); boiling point: 62 to 69°C (20 mm Hg).

3-Chloro-5,5-dimethyl-2,4-dihexanone (2). 5,5-Dimethyl-2,4-dihexanone (**1**) (35.4 g, 249 mmol, 1.0 eq) was dissolved in chloroform (700 ml) and stirred mechanically. Sulfuryl chloride (260 ml, 324 mmol, 1.3 eq) in chloroform (130 ml) was slowly added drop-wise so that the temperature would remain below 5°C under ice-bath cooling. The reaction mixture was then stirred for 2 h at 25°C and then quenched with water (500 ml) at 0°C. The separated organic layer was washed three times with water (500 ml), dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was purified by distillation under reduced pressure with heating to 70°C. The title compound (**2**) was obtained as a yellow oil (41.5 g, 235 mmol, 94%); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.23 (s, 9 H), 2.38 (s, 3 H), 5.09 (s, 1 H); boiling point: 67 to 69°C (1.5 mm Hg).

[1-Chloro-1-(4-chlorophenylhydrazono)]-3,3-dimethyl-2-butanone (3). 4-Chloroaniline (29 g, 226 mmol, 1.0 eq) was added to 6 N hydrochloric acid (158 ml, 951 mmol, 4.2 eq) and water (68 ml) and stirred for 15 min at 0°C. Sodium nitrite (17 g, 249 mmol, 1.1 eq) in water (90 ml) was slowly added drop-wise so that the temperature would remain below 5°C under ice-bath cooling. The reaction mixture was stirred for 1 h at 0°C to prepare a solution of diazonium salt. 3-Chloro-5,5-dimethyl-2,4-dihexanone (2) (40 g, 226 mmol, 1.0 eq) was dissolved in pyridine (158 ml) and water (158 ml) at 0°C. The previously prepared diazonium salt solution was slowly added drop-wise so that the temperature would remain below 10°C, and the resulting mixture was then vigorously stirred for 2 h with warming from 0 to 25°C. The reaction mixture was extracted with ethyl acetate (452 ml), washed twice with 2 N HCl (1 liter) and saturated brine (452 ml), dried over anhydrous sodium sulfate, filtered, and evaporated. The resulting crude product was diluted with methanol (226 ml, 1 M solution), refluxed for 1 h and then cooled to 0°C. The precipitated crude crystals were collected by filtration, washed with petroleum ether, and dried under reduced pressure to give the title compound (**3**) as a yellow solid (24 g, 89 mmol, 39%); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.43 (s, 9H), 7.12 (d, J = 8.7 Hz, 2H), 7.33 (d, J = 8.7 Hz, 2H), 8.34 (s, 1H); melting point: 129 to 131°C.

TY-52156. [1-Chloro-1-(4-chlorophenylhydrazono)]-3,3-dimethyl-2-butanone (**3**) (22.4 g, 82.1 mmol, 1.0 eq) and 4-chloroaniline (11.5 g, 90.4 mmol, 1.1 eq) were dissolved in ethanol (274 ml), and triethylamine (13.7 ml, 98.6 mmol, 1.2 eq) was then added at 0°C. The

resulting mixture was stirred for 3 h at ambient temperature. The reaction mixture was evaporated, quenched with water (82 ml), and diluted with ethyl acetate (165 ml). The organic layer was washed with water (165 ml) and saturated brine (165 ml), dried over anhydrous sodium sulfate, filtered, and evaporated. The resulting crude crystals were washed with a solvent mixture of hexane-ethyl acetate (20:1) and dried under reduced pressure to obtain TY-52156 as a yellow powder (24.8 g, 68.1 mmol, 83%); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.48 (s, 9H), 6.55 (d, J = 8.4 Hz, 2H), 6.76 (s, 1H), 6.98 (d, J = 8.8 Hz, 2H), 7.19–7.26 (m, 5H); electrospray ionization-mass spectrometry m/z = 362 $(M\text{-}H)^-$; melting point: 90 to 91°C.

Cell Culture

Chinese hamster ovary K1 cells that stably expressed human $S1P_1$ ($S1P_1$-CHO), $S1P_2$ ($S1P_2$-CHO), or $S1P_3$ ($S1P_3$-CHO) receptors were maintained as described previously (Koide et al., 2007). A human recombinant $S1P_4$ receptor-expressing cell line ($S1P_4$-Chem) was purchased from Millipore. Human umbilical vein endothelial cells (HUVECs) purchased from DS Pharma Biomedical (Osaka, Japan) were cultured on collagen-coated dishes in HuMedia-EG2. Human coronary artery smooth muscle cells (HCASMCs) purchased from Kurabo were cultured in HuMedia-SG2.

Measurement of the Intracellular Calcium Concentration

$[Ca^{2+}]_i$ in $S1P_1$-, $S1P_2$-, $S1P_3$-CHO, and $S1P_4$-Chem was measured using the calcium-sensitive dye Fura-2 acetoxymethyl ester, as described previously (Koide et al., 2007). The fluorescence (excitation at 340 and 380 nm, emission at 510 nm) was measured with a FLEXStation II (Molecular Devices, Sunnyvale, CA). The ratio of the fluorescence intensity at two wavelengths (FR340/380) was calculated. The K_i value for TY-52156 was estimated from Ca^{2+} responses as described previously (Ohta et al., 2003).

[^{3}H]S1P Binding Assay

A [^{3}H]S1P binding assay was performed as described by Lim et al. (2003) with minor modifications. The cell membrane (60 μg/ml) was incubated with binding buffer containing [^{3}H]S1P (1 nM, approximately 40,000 dpm/well) and vehicle or each concentration of TY-52156 (micromolar) for 30 min at 25°C. Radioactivity was measured by a liquid scintillation counter after the addition of scintillation cocktail solution. Nonspecific binding was defined as the amount of radioactivity bound to the cells in the presence of unlabeled S1P (3.0 μM). Specific binding was calculated by subtracting nonspecific binding from total binding.

GTP Binding Assay

Europium-GTP (Eu-GTP) binding was determined using a DELFIA GTP Binding Assay Kit (PerkinElmer Life and Analytical Sciences, Turku, Finland). Samples were incubated in AcroWell filter plates (PALL, Ann Arbor, MI) for 60 ($S1P_1$ and $S1P_5$) or 90 min ($S1P_2$ and $S1P_3$) at 30°C. The reaction was started by adding membranes (48 μg/ml) containing human $S1P_1$, $S1P_2$, $S1P_3$, or $S1P_5$ receptors to the assay buffer (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl, 1.2 mg/ml saponin, 10 μM GDP, and 10 nM Eu-GTP at 30°C) including S1P (0.1 μM) and vehicle or the desired concentration (micromolar) of the test drug (TY-52156 or VPC23019). The reaction was terminated by rapid filtration, and the filter was washed five times with 200 μl of ice-cold washing solution in a vacuum manifold. The plate was measured by time-resolved fluorescence (340 nm excitation/615 nm emission) using EnVision (PerkinElmer Life and Analytical Sciences).

Western Blot Analysis for p44/p42 MAPK

$S1P_1$-, $S1P_2$-, and $S1P_3$-CHO (2.0×10^5 cells) were plated on six-well plates and cultured with Nutrient Mixture F-12 Ham (Sigma-Aldrich, St. Louis, MO) containing 1% fetal bovine serum for 4 h before the experiments. The cells were treated with vehicle, TY-52156 (10 μM), VPC23019 (10 μM), or JTE013 (1.0 μM) for 10 min and then with vehicle or S1P (0.1 μM) for 5 min at 37°C. The cells were lysed in CelLytic M containing Protease Inhibitor Cocktails and Phosphatase Inhibitor Cocktails (Sigma-Aldrich) for 10 min at 4°C. The lysate was centrifuged at 13,000g for 15 min at 4°C, and supernatant was transferred to a fresh tube. The protein concentration was determined using the Bradford method. Equal amounts of proteins were resuspended in 4× sample buffer (Wako Pure Chemicals, Tokyo, Japan), boiled for 5 min, and separated by 10% SDS-polyacrylamide gel electrophoresis. After being transferred to a polyvinylidene difluoride membrane, the membranes were blocked in Block Ace (DS Pharma Biomedical, Osaka, Japan) and immunoblotted with antibodies of phospho-p44/p42 MAPK or p44/p42 MAPK (1:1000; Cell Signaling Technology, Danvers, MA). The signals were visualized by an Amplified Alkaline Phosphatase Goat Anti-Rabbit Immuno-Blot Assay Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Quantitative analyses of immunoblots were performed using Quantity One version 4.2.2 software (Bio-Rad Laboratories). The relative percentage compared with the vehicle was calculated and expressed as the mean ± S.E.M.

Measurement of Coronary Flow

All animal experiments were reviewed and approved by the Experimental Animal Committee in our laboratories. Male Sprague-Dawley (SD) rats (300–350 g; Nihon Bunko, Tokyo, Japan) were anesthetized by the injection of pentobarbital (50 mg/kg i.p.). After thoracotomy, their hearts were rapidly excised and perfused at 37°C in a Langendorff manner with Krebs-Henseleit bicarbonate buffer (constant perfusion pressure of 70 ± 5 mm Hg) of the following composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$; 2.5 mM $CaCl_2$, 24.9 mM $NaHCO_3$, 0.027 mM EDTA/2Na^+, 0.057 mM ascorbic acid, and 11.1 mM glucose, pH 7.4, at 37°C, bubbled with 95% O_2/5% CO_2 (pO_2 > 550 mm Hg). A modified water-filled latex balloon (Technical Service Corporation, Louisville, KY) was inserted into the left ventricle via the left atrium with a pressure transducer (Ohmeda PPD, Liberty Corner, NJ) connected to an amplifier (Nihon Kohden). Left ventricular end-diastolic pressure was adjusted to approximately 5 to 10 mm Hg. To measure CF, a Cannulating-Type Flow Probe (Nihon Kohden) connected to an electromagnetic blood flowmeter (Nihon Kohden) was inserted to the perfusion line that was connected to the heart. After a 15-min period for equilibration, vehicle, TY-52156 (0.1 μM), VPC23019 (0.1 μM), or JTE013 (0.1 μM) was infused for 10 min by an infusion pump (Harvard Apparatus Inc., Holliston, MA) through a drug-infusion line connected to the main perfusion line at a flow rate of 1/100 the CF rate. After drug treatment, vehicle or the indicated concentration of S1P (micromolar) or U46619 (0.1 μM) was added to the same line. CF was measured before and 10 min after the infusion of S1P or U46619. The relative percentage compared with the vehicle was calculated from the CF rate.

Contractile Response in Cerebral Arteries

Beagle dogs (Oriental Yeast, Tokyo, Japan) were anesthetized by the injection of pentobarbital (30 mg/kg i.p.). The cerebral arteries were rapidly excised and mounted in organ chambers containing Krebs buffer of the following composition: 118.0 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, and 11.0 mM glucose, pH 7.2, at 37°C, bubbled with 95% O_2/5% CO_2. After equilibration, cerebral arteries were exposed to 60 mM KCl until the contractile responses were stabilized. After washout and recovery, contractile responses to S1P were measured every 10 min after the addition of the indicated concentration of S1P (micromolar). In another experiment, the cerebral arteries were contracted by S1P (5.0 μM), and then an increasing amount of vehicle or TY-52156 (up to 10 μM) was applied to the organ chambers. Relaxation responses were measured every 10 min after the addition of the indicated concentration of vehicle or TY-52156 (micromolar). The

degree of contraction compared with vehicle was calculated with Prism (ver. 4; GraphPad Software Inc., San Diego, CA) and expressed as the percentage of contraction compared with that induced by S1P.

Immunoprecipitation and Western Blot

HCASMCs were seeded at 5×10^5 cells in a culture dish. After they reached confluence, cells were pretreated with vehicle or the indicated concentration (micromolar) of test drug for 10 min and then with vehicle or S1P (0.1 μM) for 3 min at 37°C. The amounts of activated Rho (GTP-Rho) were measured by a Rho Activation Assay Biochem Kit (Cytoskeleton, Denver, CO) according to the manufacturer's instructions. Quantitative analysis was performed as described above. The density ratio of GTP-Rho to total Rho was measured, and its vehicle value was set to 1.0.

Pharmacokinetic Analysis

Male SD rats were purchased from Nihon SLC. Blood samples were collected from the jugular vein at 1, 2, 4, 6, 8, and 24 h after the start of the administration of TY-52156-HCl. Samples were placed into sodium-heparinized tubes and subjected to centrifugation at 14,000*g* for 10 min at 4°C to separate the plasma. Plasma concentrations were quantified by an API 4000 LC/MS/MS System (Applied Biosystems/MDS Sciex, Foster City, CA). The mean peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were estimated from actual measurements. The half-life ($t_{1/2}$) was calculated with WinNonlin version 2.1 software (Pharsight, Mountain View, CA).

Measurement of Systemic Blood Pressure, Heart Rate, and Mean Blood Pressure

Male SD rats (290–340 g) were purchased from Nihon SLC. Systemic blood pressure (SBP) and heart rate (HR) were measured in the conscious state with a tail-cuff blood pressure analyzer (Muromachi-kikai, Tokyo, Japan). In another experiment, SD rats were anesthetized by the injection of pentobarbital (50 mg/kg i.p.), and cannulae were placed in a carotid artery and femoral vein. Mean blood pressure (MBP) was measured with a pressure transducer that was connected to the cannula placed in a carotid artery. HR was measured with a tachometer (NEC-San-ei Instruments, Tokyo, Japan). MBP and HR were measured before and 10 and 20 min after the injection of FTY-720 (1.0 mg/kg i.v.).

Statistical Analysis

Experimental values are expressed as mean ± S.E.M. The Student's *t* test or analysis of variance followed by Dunnett's multiple-comparison test was used to statistically analyze differences between groups. $P < 0.05$ was considered to be significant.

Results

TY-52156 Is a Potent $S1P_3$ Receptor Antagonist. $S1P_3$ receptor contributes to the S1P-induced increase in $[Ca^{2+}]_i$ (Ishii et al., 2002). To identify potent $S1P_3$ receptor antagonists, first, we screened a diverse compound collection (7500 compounds) by a Ca^{2+} fluorescent assay using $S1P_3$-CHO (see Supplementary Methods). The hit criterion was defined as more than 50% inhibition of the S1P (0.01 μM)-induced increase in $[Ca^{2+}]_i$ at 10 μM. Second, several possible compounds were pruned to confirm the dose-dependent and specific inhibition of $S1P_3$ receptor. Third, we synthesized derivatives of the active compounds to improve their potency and selectivity toward $S1P_3$ receptor. As a result, we identified TY-52156 as a potent $S1P_3$ receptor antagonist (Fig. 1A). TY-52156 preferentially inhibited the S1P-induced increase in $[Ca^{2+}]_i$ in $S1P_3$-CHO rather than $S1P_1$-, $S1P_2$-CHO, and $S1P_4$-Chem (Fig. 1B and Supplementary Fig. 1 as the direct ratio of fluorescence). The dose-dependent $[Ca^{2+}]_i$ increase elicited by S1P in $S1P_3$-CHO was inhibited by TY-52156 in a competitive manner (Fig. 1C), and the K_i value was estimated to be 110 nM for $S1P_3$ receptor.

In addition to S1P receptor-expressing cell lines, we used HUVECs to confirm the specificity of TY-52156 for endogenous $S1P_3$ receptor. $S1P_1$ and $S1P_3$ receptors but not other S1P receptors are expressed on HUVECs and induce $[Ca^{2+}]_i$ elevation (Sensken et al., 2008), which is consistent with the finding that TY-52156 and VPC23019 inhibited the S1P-induced increase in $[Ca^{2+}]_i$ in HUVECs (Supplementary Fig. S2). Furthermore, the combination of TY-52156 and VPC23019 showed greater inhibitory activity than either compound alone (Supplementary Fig. S2). Thus, S1P induces $[Ca^{2+}]_i$ elevation via $S1P_1$ and $S1P_3$ receptors in HUVECs. To assess the inhibitory effect of the TY-52156 on S1P-induced increase in $[Ca^{2+}]_i$ through $S1P_3$, but not $S1P_1$, receptor, the SEW2871 (an $S1P_1$ receptor-specific agonist)-induced increase in $[Ca^{2+}]_i$ in HUVECs was evaluated with or without TY-52156. Pre-

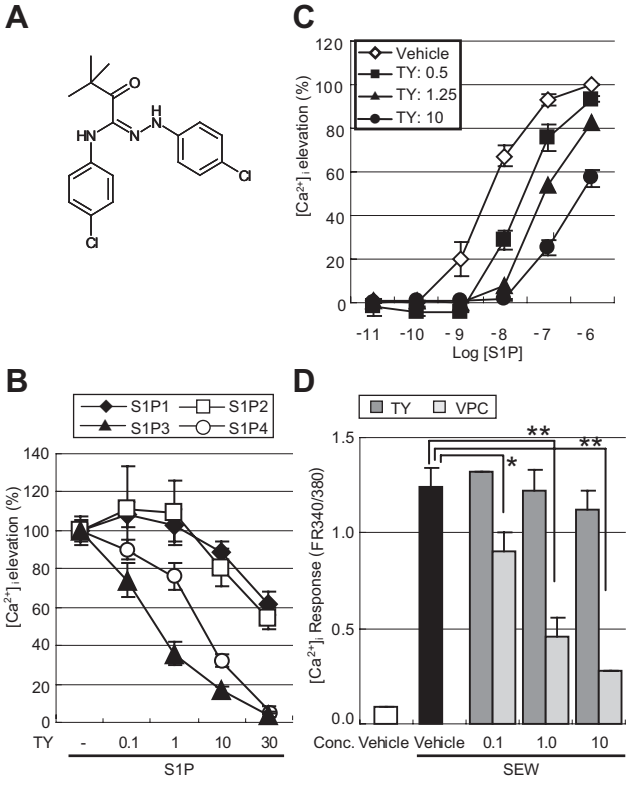

Fig. 1. Effects of S1P receptor antagonist on the S1P-induced increase in $[Ca^{2+}]_i$. A, chemical structure of TY-52156. B, $S1P_1$-, $S1P_2$-, $S1P_3$-CHO or $S1P_4$-Chem were pretreated with vehicle or the indicated concentration (micromolar) of test drug for 20 min and then treated with vehicle (−) or S1P (0.01 μM) to determine the increase in $[Ca^{2+}]_i$. The results are representative of three or four independent experiments. The relative percentage compared with the vehicle was calculated and expressed as mean ± S.E.M. C, $S1P_3$-CHO were pretreated with vehicle or the indicated concentration (micromolar) of TY-52156 for 20 min, and then treated with vehicle or the indicated concentration of S1P (logM) to determine the increase in $[Ca^{2+}]_i$. The relative percentage compared with the S1P (1 μM, vehicle treatment) was calculated and expressed as mean ± S.E.M. D, HUVECs were pretreated with vehicle or the indicated concentration (micromolar) of the test drug for 20 min, and then treated with vehicle or SEW2871 (SEW) (5.0 μM). The results are representative of three or four independent experiments. The ratio of the fluorescence intensity was calculated. The results are expressed as mean ± S.E.M. *, $P < 0.05$, **, $P < 0.01$ versus S1P alone (Dunnett's test).

treatment with VPC23019 (an $S1P_1$ receptor antagonist) significantly inhibited the SEW2871-induced increase in $[Ca^{2+}]_i$ through $S1P_1$ receptor (Fig. 1D). In contrast, this inhibition was blunted by pretreatment with TY-52156. These results suggest that TY-52156 inhibits the $S1P_3$ receptor-dependent increase in $[Ca^{2+}]_i$.

TY-52156 Is a Selective $S1P_3$ Receptor Antagonist. Using a [^{3}H]S1P binding assay, we found that TY-52156 inhibited the specific binding of [^{3}H]S1P to the membrane fraction of $S1P_3$-CHO in a dose-dependent manner (Fig. 2A). The total [^{3}H]S1P binding and nonspecific binding were 2156 ± 315 and 883 ± 109, respectively (mean dpm values ± S.E.M., $n = 5$). To further characterize the antagonist actions of TY-52156, we performed Eu-GTP binding to membranes prepared from cells expressing the human $S1P_1$, $S1P_2$, $S1P_3$, or $S1P_5$ receptors. The Eu-GTP binding assay has become a powerful alternative to the [^{35}S]GTPγS binding assay (Moreland et al., 2004). TY-52156 showed submicromolar potency and a high degree of selectivity for $S1P_3$ receptor (Fig. 2B).

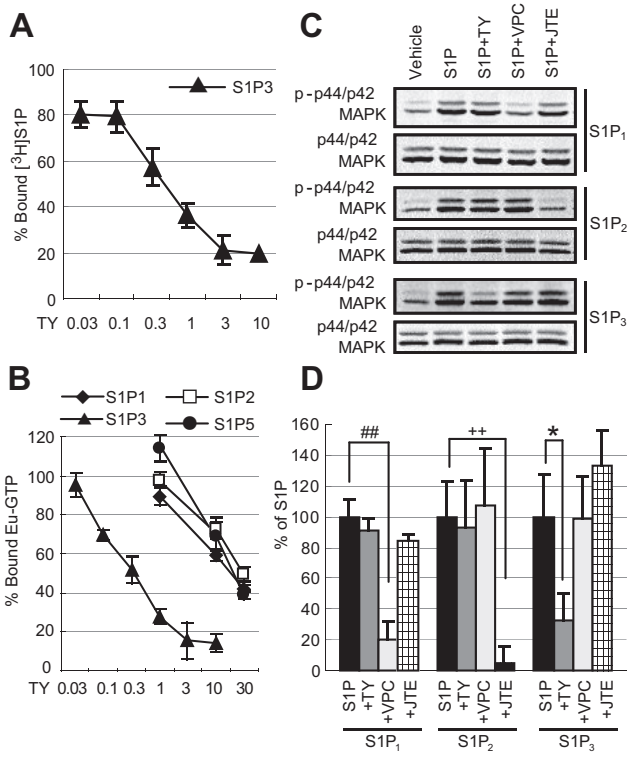

Fig. 2. Selectivity of TY-52156 toward $S1P_3$ receptor. A, the antagonistic effect of TY-52156 was determined by a radioligand binding study based on [^{3}H]S1P. Results are representative of three independent experiments. The relative percentage of specific binding compared with the vehicle was calculated and expressed as mean ± S.E.M. B, the antagonistic effect of TY-52156 was determined by a fluorescence-based Eu-GTP binding activity. The reaction was started by adding membranes containing human $S1P_1$, $S1P_2$, $S1P_3$, or $S1P_5$ receptors to the assay buffer including S1P (0.1 μM) and the desired concentration of TY-52156 (TY) (micromolar). The results are representative of three or four independent experiments. The relative percentage compared with the vehicle was calculated and expressed as mean ± S.E.M. C, $S1P_1$-, $S1P_2$-, or $S1P_3$-CHO were pretreated with vehicle, TY-52156 (TY) (10 μM), VPC23019 (VPC) (10 μM), or JTE013 (JTE) (1.0 μM) for 10 min and then treated with vehicle or S1P (0.1 μM) to determine p44/p42 MAPK phosphorylation. D, the data obtained from three independent experiments in C were quantitatively analyzed. ##, $P < 0.01$ versus S1P alone in $S1P_1$-CHO, ++, $P < 0.01$ versus S1P alone in $S1P_2$-CHO, *, $P < 0.05$ versus S1P alone in $S1P_3$-CHO (Dunnett's test).

We examined the selective inhibitory effect of TY-52156 on S1P-induced p44/p42 MAPK phosphorylation in $S1P_1$-, $S1P_2$- and $S1P_3$-CHO. TY-52156, VPC23019, and JTE013 inhibited S1P-induced p44/p42 MAPK phosphorylation only in $S1P_3$-, $S1P_1$-, and $S1P_2$-CHO, respectively (Fig. 2, C and D). Although VPC23019 is at least one-fifth less active toward $S1P_3$ receptor than $S1P_1$ receptor (Davis et al., 2005), it did not have an inhibitory effect on S1P-induced p44/p42 MAPK phosphorylation in $S1P_3$-CHO under our experimental conditions.

We further confirmed the selectivity of TY-52156 (10 μM) by examining its inhibitory effects on 24 GPCRs and three ion channels (all percentage inhibitions <30%, see Supplemental Table). These results indicate that TY-52156 is a potent $S1P_3$ receptor-selective antagonist.

S1P Reduces Coronary Flow via $S1P_3$ Receptor. S1P is released from activated platelets and thus is believed to be involved in thrombosis-related vascular diseases such as acute coronary syndrome (Siess, 2002). Recent studies have reported that intravenous injection of S1P decreases myocardial perfusion via $S1P_3$ receptor in vivo (Levkau et al., 2004). To investigate whether TY-52156 regulates CF, we examined its effect on S1P-dependent CF regulation. Consistent with previous reports (Sugiyama et al., 2000), we found that S1P dose-dependently decreased CF in perfused rat heart (Fig. 3A). Hearts were perfused with a solution containing each S1P receptor antagonist before S1P treatment. TY-52156, but not VPC23019 or JTE013, significantly restored the S1P-dependent reduction of CF (Fig. 3B). Meanwhile, TY-52156 did not affect the reduction of CF caused by a stable analog of thromboxane A_2, U46619. These results indicate that S1P reduces CF via $S1P_3$ receptor.

S1P Induces the Vasoconstriction of Canine Cerebral Arteries via $S1P_3$ Receptor. To investigate whether $S1P_3$ receptor expressed in the vasculature plays a role in S1P-induced vasoconstriction, we focused on the effect of TY-52156 on S1P-induced vasoconstriction in isolated canine cerebral arteries. S1P dose-dependently induced vasoconstriction (Fig. 4A), which is consistent with previous reports (Tosaka et al., 2001). TY-52156 cumulatively induced the relaxation of canine cerebral arteries that had been precontracted by S1P (Fig. 4B). These results suggest that S1P induces the vasoconstriction of isolated canine cerebral arteries via the $S1P_3$ receptor.

S1P Induces Both Rho Activation and the Increase in Ca^{2+} via $S1P_3$ Receptor in HCASMCs. Vascular tone balances relaxation and constriction in smooth muscle cells (Watterson et al., 2005). Vasorelaxation is mainly mediated by nitric oxide released from endothelial cells, where S1P activates endothelial nitric-oxide synthase. Vasoconstriction is presumably regulated by S1P-induced Rho activation and the increase in $[Ca^{2+}]_i$ in vascular smooth muscle cells. Because arteries were contracted by S1P stimulation, we assumed that S1P-mediated contraction dominated S1P-mediated relaxation in isolated perfused heart and arteries. Therefore, we hypothesized that the S1P-induced decrease in CF in isolated perfused rat heart might be ascribed to the contraction of coronary artery smooth muscle cells expressing $S1P_3$ receptor. We tested whether TY-52156 inhibited S1P-induced Rho activation and the increase in $[Ca^{2+}]_i$ in HCASMCs.

S1P-induced Rho activation in HCASMCs was inhibited by TY-52156 (Fig. 5A). JTE013 also inhibited S1P-induced Rho activation (Fig. 5B), which is consistent with previous studies (Arikawa et al., 2003). The combination of TY-52156 and JTE013 showed greater inhibitory activity than either compound alone (Fig. 5C). In contrast, VPC23019 did not inhibit S1P-induced Rho activation. SEW2871 did not induce Rho activation (Fig. 5D). Pretreatment with TY-52156 prevented the S1P-induced increase in $[Ca^{2+}]_i$ in HCASMCs (Fig. 6). Meanwhile, pretreatment with VPC23019, but not JTE013, attenuated the S1P-induced increase in $[Ca^{2+}]_i$ in HCASMCs. Collectively, these results suggest that $S1P_3$ receptor is responsible for both S1P-induced Rho activation and the increase in $[Ca^{2+}]_i$, whereas $S1P_1$ and $S1P_2$ receptors are involved in the increase in $[Ca^{2+}]_i$ and Rho activation, respectively.

TY-52156 Suppresses $S1P_3$ Receptor-Induced Bradycardia In Vivo. Finally, we sought to confirm the effects of TY-52156 in living animals. To test whether TY-52156 inhibits its $S1P_3$ receptor in vivo, we examined the antagonistic effects of TY-52156 on the FTY-720 (nonselective S1P receptor agonist)-induced transient reduction of HR.

The oral bioavailability of TY-52156 in SD rats was estimated to be 70.9% (Fig. 7A). To determine the pretreatment time before FTY-720 injection, the plasma concentrations of TY-52156 were measured (Fig. 7B). We also confirmed that oral administration of TY-52156 did not affect either HR or SBP in conscious rats (Fig. 7, C and D).

Although FTY-720 is a broad agonist of S1P receptors, it induces bradycardia, which has been shown to be mediated by the activation of $S1P_3$ receptor using $S1P_3$ receptor-null mice (Forrest et al., 2004; Sanna et al., 2004). FTY-720 (intravenous)-induced sinus bradycardia was observed from 10 to 20 min (Fig. 7E). Pretreatment with TY-52156 partially but significantly attenuated FTY-720-induced bradycardia but did not affect the FTY-720-induced elevation of MBP in unconscious rats (Fig. 7, E and F).

To complement the observation that TY-52156 inhibited FTY-720-induced bradycardia in vivo, dose-dependent inhibition of the FTY-720-induced cellular response was clarified. FTY-720 is phosphorylated to the active metabolite FTY-720 phosphate (FTY-720-P) in vivo (Zemann et al., 2006). We examined the inhibitory effect of TY-52156 on the FTY-720-P-induced increase in $[Ca^{2+}]_i$ in $S1P_3$-CHO. Pretreatment with TY-52156 prevented the FTY-720-P-induced increase in $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 8). Collectively, these results indicate that the oral administration of TY-52156 inhibits $S1P_3$ receptor-dependent bradycardia in vivo.

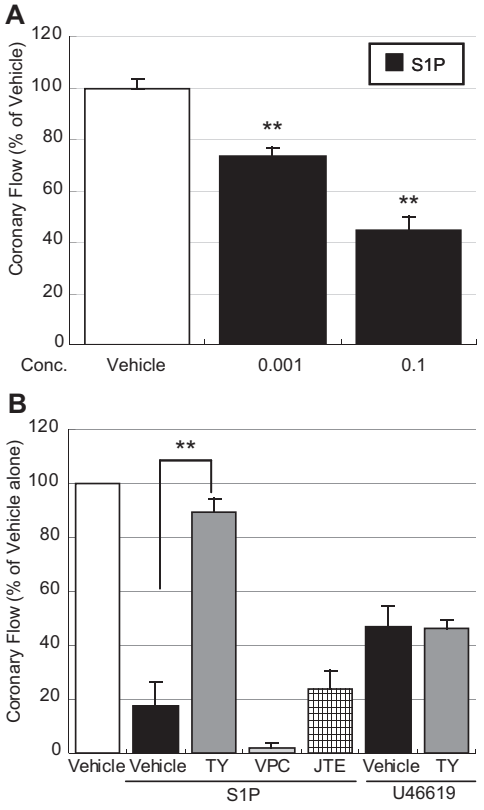

Fig. 3. Effects on the S1P- or U46619-induced change in cardiac coronary flow in isolated perfused rat hearts. A and B, isolated rat hearts were perfused at 37°C in a Langendorff manner with Krebs-Henseleit bicarbonate buffer at a constant perfusion pressure (70 ± 5 mm Hg). A, S1P dose-dependently (0.001 or 0.1 μM) decreased CF. Results are representative of five independent experiments. **, $P < 0.01$ versus vehicle (Dunnett's test). B, perfused rat hearts were pretreated with TY-52156 (TY) (0.1 μM), VPC23019 (VPC) (0.1 μM), or JTE013 (JTE) (0.1 μM) for 10 min and then treated with vehicle, S1P (0.1 μM), or U46619 (0.1 μM). Results are representative of five independent experiments. **, $P < 0.01$ versus S1P alone (Dunnett's test).

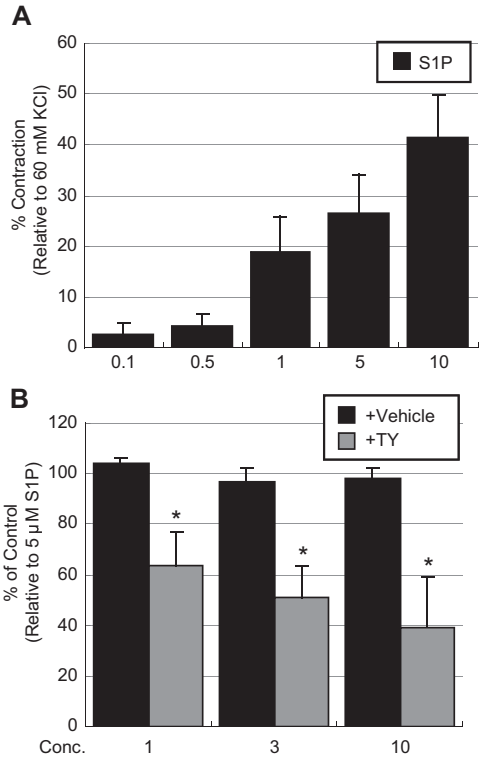

Fig. 4. Effects of TY-52156 on the S1P-induced contractile response in isolated canine cerebral arteries. A, S1P dose-dependently (0.1 to 10 μM) induced vasoconstriction in isolated canine cerebral arteries. Results are representative of three independent experiments. B, canine cerebral arteries were contracted by S1P (5.0 μM). After the maximum contractile response was observed, an increasing amount of TY-52156 (up to 10 μM) was applied to the organ chambers. Relaxation responses were measured every 10 min after the addition of the indicated concentration of vehicle or TY-52156 (micromolar). Results are representative of four independent experiments. *, $P < 0.05$, versus indicated dose of vehicle (Student's t test).

Discussion

TY-52156 was identified as a potent and selective antagonist of $S1P_3$ receptor. Based on its ability to inhibit Ca^{2+} responses and the results of an Eu-GTP binding assay, TY-52156 was approximately 10 to 30 times more potent for $S1P_3$ receptor than for $S1P_1$, $S1P_2$, $S1P_4$, or $S1P_5$ receptor, and the K_i value for $S1P_3$ receptor was estimated to be 110 nM. TY-52156 caused a parallel rightward shift of the dose-response curve for the S1P-induced increase in $[Ca^{2+}]_i$, which suggested competitive antagonism for $S1P_3$ receptor. Furthermore, TY-52156 did not have any significant effects on 24 GPCRs or three ion channels. Therefore, TY-52156 is a useful tool for studying $S1P_3$ receptor signaling.

VPC23019 has been described as an $S1P_1$ and $S1P_3$ receptor antagonist, with pK_b values (−logM) of 7.49 ± 0.15 and 5.98 ± 0.08 for the $S1P_1$ and $S1P_3$ receptors, respectively (Davis et al., 2005). Although VPC23019 (10 μM) did not inhibit p44/p42 MAPK phosphorylation in $S1P_3$-CHO, dose-dependent inhibition was observed at higher concentrations (30–100 μM) (Supplementary Fig. 3A). On the other hand, VPC23019 dose-dependently inhibited Eu-GTP binding to membranes ($S1P_3$) from 1 to 10 μM (Supplementary Fig. 3B), which is similar to previous findings (Davis et al., 2005). One possible explanation is that VPC23019 did not inhibit the cellular response on $S1P_3$-CHO because of the difference in sensitivity between membrane-based and whole cell-based assay conditions. In addition, because VPC23019 has been characterized in T24 cells (human bladder carcinoma) that stably expressed $S1P_3$ receptor (Davis et al., 2005), host-cell–specific differences between T24 cells and our Chinese hamster ovary K1 cells might play a role.

We demonstrated that $S1P_3$ receptor is central to S1P-regulated CF. CF is increased or decreased physiologically in response to the oxygen demand of the heart muscle. Thrombosis and atherosclerosis decrease CF by releasing S1P, thromboxane A_2, and platelet-derived growth factor from activated platelets and by narrowing the lumen of the coronary arteries, respectively (Pomposiello et al., 1997; Heldin and Westermark, 1999). Although S1P has been reported to decrease CF, it is not well known how S1P induces vasoconstriction. The deletion of $S1P_3$ receptor in mice led to inhibition of the S1P-induced decrease in myocardial perfusion (Levkau et al., 2004). We found that TY-52156, but not VPC23019 or JTE013, attenuated the S1P-dependent reduction of CF. Therefore, $S1P_3$ receptor is responsible for the S1P-induced decrease in cardiac coronary flow. Because $S1P_3$ receptor is highly expressed in the smooth muscle of small coronary vessels (Himmel et al., 2000; Mazurais et al., 2002), reduction of CF by S1P may primarily depend on the vasoconstriction of microvascular smooth muscle cells.

We focused on the mechanism by which $S1P_3$ receptor regulates the contraction of smooth muscle cells. There are two main signals that induce actomyosin-based contraction: an increase in $[Ca^{2+}]_i$, and Rho activation (Watterson et al., 2005). We observed that TY-52156 inhibited the S1P-induced increase in $[Ca^{2+}]_i$ and Rho activation in HCASMCs. In clear contrast, VPC23019 and JTE013 only inhibited the increase in $[Ca^{2+}]_i$ and Rho activation, respectively. Thus, $S1P_3$ receptor-mediated signal through both the increase in $[Ca^{2+}]_i$ and Rho activation, which lead to vasoconstriction, can account for our finding that TY-52156, but not VPC23019 or JTE013, preserved the S1P-dependent reduction of CF in perfused rat heart. Although Rho kinase inhibition has been believed to cause vasorelaxation (Tosaka et al., 2001), it is unclear why JTE013 did not alter the S1P-dependent reduction of CF. One possible explanation is the difference in the expression of S1P receptor subtypes in the vasculature (Coussin et al., 2002; Mazurais et al., 2002).

Sustained vascular spasm after subarachnoid hemorrhage and cerebral infarction has been shown to result in the extension of brain damage (Tosaka et al., 2001). The involvement of $S1P_3$ receptor in vasospasm has been reported using $S1P_3$ receptor-null mice (Salomone et al., 2008). The intracarotid injection of S1P decreases cerebral blood flow in vivo (Salomone et al., 2003). Thus, TY-52156 may potentially inhibit the S1P-induced vasospasm of cerebral arteries, because we found that TY-52156 attenuated S1P-induced vascular contraction in canine cerebral arteries.

S1P has opposite effects on vasculature: vasorelaxation and vasoconstriction. $S1P_1$ and $S1P_3$ receptors have been linked to the activation of NO synthesis in vascular endothelial cells (Igarashi and Michel, 2000; Dantas et al., 2003; Nofer et al., 2004). However, we confirmed that activation of $S1P_3$ receptor led to vasoconstriction in smooth muscle cells. Therefore, the net effect of $S1P_3$ receptor on vasorelaxation and vasoconstriction depends on the function of vascular endothelial cells or the expression profile of S1P receptor subtypes in the vasculature (Fig. 9). Endothelial-dependent vasorelaxation is supported by the fact that various vasoconstrictors, including acetylcholine and ergonovine, cause endothelium-dependent vasorelaxation via a NO-dependent mechanism in healthy subjects (Kugiyama et al., 1996; Davignon and Ganz, 2004; Kawano and Ogawa, 2004). This vasorelaxation is impaired in patients with endothelial dysfunction. Thus, S1P at least contributes to pathological processes that involve endothelial dysfunction, such as vasospasm and myocardial infarction.

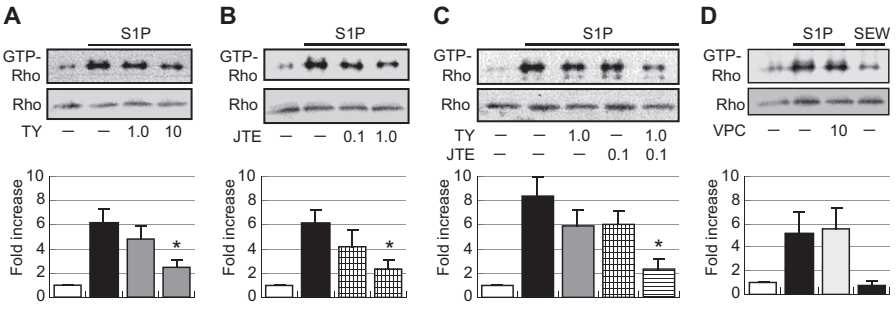

Fig. 5. Effects of S1P receptor antagonist on S1P-induced Rho activation in HCASMCs. HCASMCs were pretreated with vehicle or the indicated concentration (micromolar) of test the drug for 10 min, and further treated with vehicle, S1P (1.0 μM) or SEW2871 (SEW) (10 μM) for 3 min. The data obtained from three (A, B, and D) or four (C) independent experiments were quantitatively analyzed (bottom). *, $P < 0.05$, versus S1P alone (Dunnett's test).

TY-52156 might become a potent probe for assessing $S1P_3$ receptor-dependent signal in vivo. FTY-720 binds to all S1P receptors except for $S1P_2$ receptor (Huwiler and Pfeilschifter, 2008). Although FTY-720-induced bradycardia is mainly caused by the $S1P_3$ receptor-mediated activation of cardiac G protein-gated potassium channel (Ik_{Ach}), it has been reported to be associated with Ik_{Ach}-independent mechanisms through FTY-720 induction (Himmel et al., 2000; Forrest et al., 2004; Koyrakh et al., 2005). Based on the study of Ik_{Ach}-deficient mice, other pacing-related currents such as the hyperpolarization-activated inward current and the voltage-gated calcium current may be involved in the bradycardia with FTY-720 (Koyrakh et al., 2005). In addition, despite the lack of $S1P_3$ receptor agonism, a recent clinical study has reported that a selective $S1P_1/S1P_5$ receptor agonist (BAF-312, the structure of which has not been disclosed) decreased the heart rate in healthy subjects (Gergely et al., 2009). Our result showed that FTY-720 (intravenous) decreased HR and elevated MBP in rats. Pretreatment with TY-52156 before FTY-720 partially restored the FTY-720-induced HR reduction but did not attenuate the elevation of MBP. Because the oral administration of TY-52156 alone did not affect HR or SBP in conscious rats, this result probably means that there is no effect on hyperpolarization-activated inward current to modulate HR. We also found that TY-52156 did not affect voltage-gated calcium current in guinea pig ventricular myocytes (Supplemental Table). Thus, these results indicated that FTY-720-induced bradycardia may be involved in the mechanism, except through $S1P_3$ receptor. Although FTY-720 induced an elevation of MBP in a clinical study, the mechanism was not clear (Kappos et al., 2006). Therefore, TY-52156 inhibits $S1P_3$ receptor-dependent HR reduction in vivo and thus is

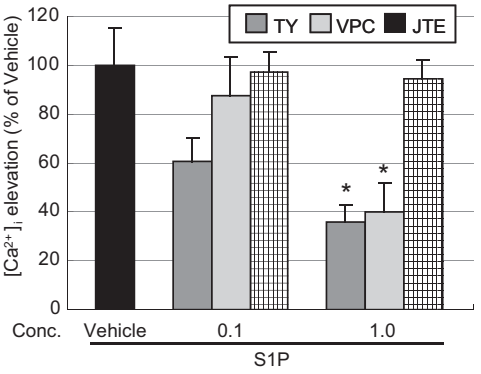

Fig. 6. Effects of S1P receptor antagonist on the S1P-induced increase in $[Ca^{2+}]_i$ in HCASMCs. HCASMCs were pretreated with vehicle or the indicated concentration (micromolar) of the test drug for 20 min, and further treated with vehicle or S1P (0.01 μM). Results are representative of three or four independent experiments. The relative percentage compared with the vehicle was calculated and expressed as mean ± S.E.M. ∗, $P < 0.05$, versus S1P alone (Dunnett's test).

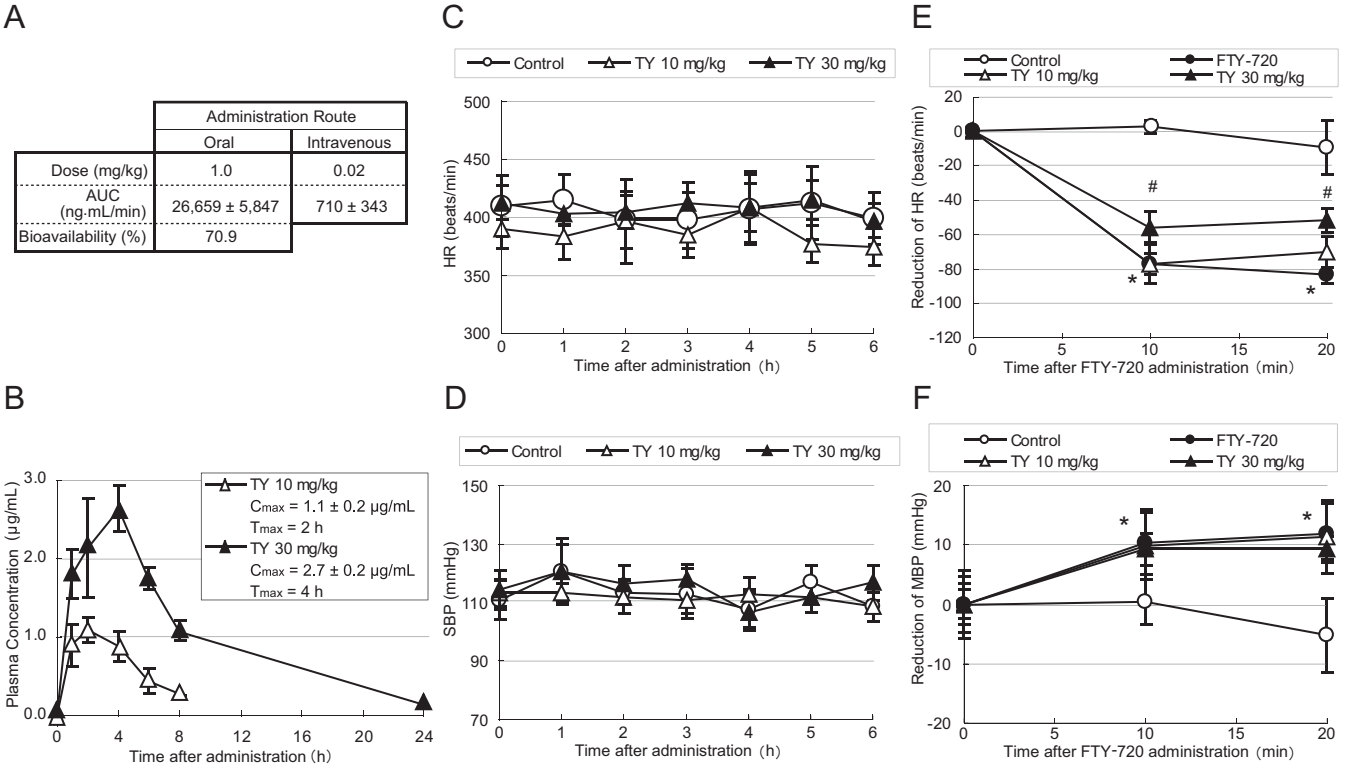

Fig. 7. Effects of TY-52156 on FTY-720-induced bradycardia and hypertension in SD rat. A, the oral bioavailability of TY-52156 was studied in SD rats. The plasma concentration of TY-52156 was determined by LC/MS/MS. Area under the blood concentration curve (AUC) from 0 to 360 min was calculated using a trapezoidal rule. The results are expressed as mean ± S.D. B, the plasma concentration of TY-52156 after oral administration was determined by LC/MS/MS. The dose of TY-52156 (TY) was 10 (△) or 30 mg/kg (▲). The results are expressed as mean ± S.D. for three SD rats. C and D, changes in HR (C) and SBP (D) were recorded after the oral administration of TY-52156 (TY) (10 mg/kg, △; or 30 mg/kg, ▲) or control (○) in conscious rats. Results are representative of five independent experiments. E and F, changes in HR (E) and MBP (F) were recorded after the administration of FTY-720 (1.0 mg/kg i.v.; ●) or control (○) in anesthetized rats. TY-52156 (TY) (10 mg/kg, △; or 30 mg/kg, ▲) was administered orally at 4 h before FTY-720 injection. Results are representative of six independent experiments. ∗, $P < 0.05$ versus control (unpaired Student's t test). #, $P < 0.05$ versus FTY-720 alone (Dunnett's test).

a potent probe for elucidating the role of $S1P_3$ receptor in animal models.

In conclusion, TY-52156 is a potent and selective antagonist of $S1P_3$ receptor. This compound may help us to distinguish $S1P_3$ receptor-dependent signals from $S1P_1$ and $S1P_2$ receptor-dependent signals in vitro and in vivo. $S1P_3$ receptor is responsible for the S1P-induced decrease in CF, and an $S1P_3$ receptor antagonist may be useful for the treatment of S1P-induced vascular diseases, including vasospasm.

Acknowledgments

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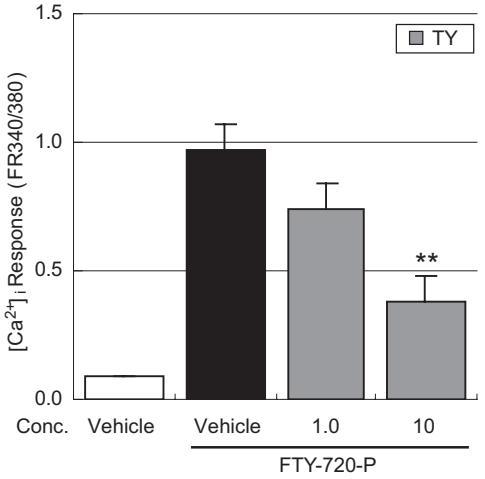

Fig. 8. Effects of TY-52156 on the FTY-720-P-induced increase in $[Ca^{2+}]_i$ in $S1P_3$-CHO. $S1P_3$-CHO were pretreated with vehicle or the indicated concentration (micromolar) of TY-52156 (TY) for 20 min and further treated with vehicle or FTY-720-P (0.5 μM). The results are representative of five independent experiments. The ratio of the fluorescence intensity was calculated. The relative percentage compared with the vehicle was calculated and expressed as mean ± S.E.M. *, $P < 0.05$, versus FTY-720-P alone (Dunnett's test).

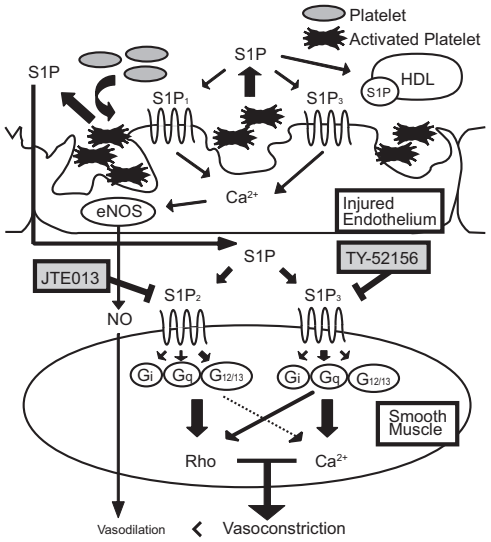

Fig. 9. Summary of signaling pathways for S1P-induced vasoconstriction. S1P causes vasoconstriction in smooth muscle cells via $S1P_2$ and $S1P_3$ receptors. Inhibition of both the increase in $[Ca^{2+}]_i$ and Rho activation contributes to the efficacy of TY-52156 against S1P-induced vasoconstriction.

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