confluence, cells were pretreated with vehicle or the indicated concentration (μM) 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) 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 (TM) LC/MS/MS System (Applied Biosystems/MDS SCIEX, Ontario, Canada). 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 ver. 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 (MK2000, Muromachi-kikai). In another experiment, SD rats were anesthetized by the injection of pentobarbital (50 mg/kg i.p.) and cannulas were placed in a carotid artery and femoral vein. Mean blood pressure (MBP) was measured with a

pressure transducer (DX-360) that was connected to the cannula placed in a carotid artery. HR was measured with a tachometer (1321; NEC-San-ei). 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 *t*-test or ANOVA 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 (7,500 compounds) by a Ca^{2+} fluorescent assay using $S1P_3$-CHO (Supplementary Methods). The hit criterion was defined as more than 50% inhibition of the S1P (0.01 uM)-induced increase in $[Ca^{2+}]_i$ at 10 uM. 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 *Ki* 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. 2). Furthermore, the combination of TY-52156 and VPC23019 showed greater inhibitory activity than either compound alone (Supplementary Fig. 2). 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 (a $S1P_1$ receptor-specific agonist)-induced increase in $[Ca^{2+}]_i$ in HUVECs was evaluated with or without TY-52156. Pretreatment with VPC23019 (a $S1P_1$ 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 sub-micromolar potency and a high degree of selectivity for $S1P_3$ receptor (Fig. 2B).

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. 2C and 2D). While 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 % 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 thought 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 prior to 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 analogue of thromboxane A_2 (TXA_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 $S1P_3$ 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 (eNOS). Vasoconstriction is presumably regulated by S1P-induced Rho activation and the increase in $[Ca^{2+}]_i$ in vascular smooth muscle cells. Since 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 $S1P_3$ 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 prior to FTY-720 injection, the plasma concentrations of TY-52156 were measured (Fig. 7B). We also confirmed that oral administration (p.o.) of TY-52156 did not affect either HR or SBP in conscious rats (Fig. 7C and 7D).

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 (i.v.)-induced sinus bradycardia was observed from 10 to 20 min (Fig. 7E). Pretreatment with TY-52156 (p.o.) partially but significantly attenuated FTY-720-induced bradycardia, but did not affect the FTY-720-induced elevation of MBP in unconscious rats (Fig. 7E and 7F).

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.

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 a Eu-GTP binding assay, TY-52156 was about 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 pKb 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 uM) did not inhibit p44/p42 MAPK phosphorylation in $S1P_3$-CHO, dose-dependent inhibition was observed at higher concentrations (30 to 100 μM) (Supplementary Fig. 3A). On the other hand, VPC23019 dose-dependently inhibited Eu-GTP binding to membranes ($S1P_3$) at 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. Additionally, since 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 CHO-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, TXA_2, and platelet-derived growth factor from activated platelets and by narrowing the lumen of the coronary arteries, respectively (Heldin and Westermark, 1999; Pomposiello et al., 1997). 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. Since $S1P_3$ 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, since 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 (Dantas et al., 2002; Igarashi and Michel, 2000; 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 (Davignon and Ganz, 2004; Kawano and Ogawa, 2004; Kugiyama et al., 1996). 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.

TY-52156 might become a potent probe for assessing $S1P_3$ receptor-dependent signal in vivo. FTY-720 binds to all S1P receptors except $S1P_2$ receptor (Huwiler and Pfeilschifter, 2008). While 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 (If) and the voltage-gated calcium current (ICa,L) 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) decreased the heart rate in healthy subjects (Gergely et al., 2009). Our result showed that FTY-720 (i.v.) decreased HR and elevated MBP in rats. Pretreatment with TY-52156 prior to FTY-720 partially restored the FTY-720-induced HR reduction, but did not attenuate the elevation of MBP. Since 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 If current to modulate HR. We also found that TY-52156 did not affect ICa,L 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. While 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 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.

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