Experimental eye research (2014.Apr) 121:94～101.

Role of Ca2+ -dependent and Ca2+ -sensitive mechanisms in sphingosine 1-phosphate-induced constriction of isolated porcine retinal arterioles in vitro.

Takayuki Kamiya, Taiji Nagaoka, Tsuneaki Omae, Takafumi Yoshioka, Shinji Ono, Ichiro Tanano, Akitoshi Yoshida

Role of Ca^{2+}-Dependent and Ca^{2+}-Sensitive Mechanisms in Sphingosine 1-Phosphate-Induced Constriction of Isolated Porcine Retinal Arterioles in Vitro

Takayuki Kamiya, Taiji Nagaoka*, Tsuneaki Omae, Takafumi Yoshioka, Shinji Ono, Ichiro Tanano, Akitoshi Yoshida
Department of Ophthalmology, Asahikawa Medical University, Asahikawa, Japan.

*Corresponding author: Department of Ophthalmology, Asahikawa Medical University, Midorigaoka Higashi 2-1-1-1, Asahikawa, 078-8510, Japan; Tel: +81-166-68-2543; fax: +81-166-68-2549.

E-mail address: nagaoka@asahikawa-med.ac.jp.

Supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Tokyo, Japan (B) 25293352 and (C)18591904 (to T. Nagaoka).

Disclosures: None

Word count: 3,933 words; figures: 7 (+ 4 supplementary); tables: 1

Abstract word count: 219

Abstract

Although sphingosine 1-phosphate (S1P), a bioactive lipid derived from activated platelets, has a variety of physiologic effects on vessels, no reports have described the effect of S1P on the retinal circulation. We examined the effect and underlying mechanism of the vasomotor action of S1P on porcine retinal arterioles. The porcine retinal arterioles were isolated, cannulated, and pressurized without flow for in vitro study. S1P-induced diameter changes were recorded using videomicroscopic techniques. S1P elicited concentration-dependent (1 nM-10 μM) vasoconstriction of the retinal arterioles that was abolished by the S1P receptor 2 (S1PR2) antagonist JTE-013. S1P-induced vasoconstriction was abolished by the Rho kinase (ROCK) inhibitor H-1152 and was inhibited partly by the protein kinase C (PKC) inhibitor Gö-6983. The inhibition of phospholipase C by U73122 and L-type voltage-operated calcium channels (L-VOCCs) by nifedipine inhibited S1P-induced vasoconstriction; a combination of both inhibitors abolished S1P-induced vasoconstriction. Furthermore, inhibition of myosin light chain kinase (MLCK) by ML-9 significantly blocked S1P-induced vasoconstriction; further coadministration of ML-9 with H-1152 or Gö-6983 abolished S1P-induced vasoconstriction. The current data suggest that S1P elicits vasoconstriction of the retinal arterioles via S1PR2 in vascular smooth muscle cells and this vasoconstriction may be mediated by the Ca^{2+}-sensitive pathway via activation of PKC leading to activation of ROCK and the Ca^{2+}-dependent pathway via activation of L-VOCCs resulting in activation of MLCK.

Keywords: sphingosine 1-phosphate, S1P, S1P receptor 2, porcine retinal arterioles, protein kinase C, vasoconstriction

Abbreviations

RBF: retinal blood flow; S1P: sphingosine 1-phosphate; S1PR: S1P receptor; ROCK: rho-associated coiled-coil-forming protein kinase; PKC: protein kinase C; PLC: phospholipase C; L-VOCCs: L-type voltage-operated calcium channels; MLCK: myosin light chain kinase

1. Introduction

Sphingosine 1-phosphate (S1P), a member of a large family of lipid metabolites called sphingolipids, induces a variety of biologic activities, such as immune responses (Spiegel and Milstien, 2011), inflammatory processes (van der Giet et al., 2008), organ perfusion (Bischoff et al., 2000b; Sumida and Stamer, 2010), and vascular development/maturation (Allende and Proia, 2002) in different organs (Schuchardt et al., 2011) through various high-affinity G-protein-coupled receptors (S1PR). A previous clinical study reported that S1P is a very strong and robust predictor of the occurrence and severity of coronary stenosis (Deutschman et al., 2003). Although a previous clinical study showed that the plasma concentration of sphingosine, which is the precursor of S1P, was elevated in patients with type 2 diabetes (Gorska et al., 2005), it has not been clarified fully if S1P is associated with retinal vascular disorders, especially diabetic retinopathy, in which we previously found that retinal blood flow (RBF) is impaired (Nagaoka et al., 2010; Nagaoka and Yoshida, 2013).

S1P also can modulate vascular tone through vasodilator and vasoconstrictor pathways mediated by S1PR expressed in vascular endothelium and smooth muscle cells, as shown in animal models (Bischoff et al., 2000a; Daum et al., 2009; Hemmings et al., 2004). However, the expression profiles of the different S1P receptor subtypes may vary among different vessels (Alewijnse et al., 2004). Although some investigators have reported that S1P caused vasoconstriction in isolated rat mesenteric and intrarenal arterioles in vitro (Bischoff et al., 2000a) and reduced renal and mesenteric blood flow in anaesthetized rats (Bischoff et al., 2000b), S1P induced constriction of isolated cerebral arteries, whereas S1P did not produce constriction in rat aorta or peripheral arteries,

including the carotid and femoral arteries (Coussin et al., 2002; Salomone et al., 2003). Moreover, some previous studies have reported that S1P caused vasodilation of the aorta and mesenteric arterioles in rats (Dantas et al., 2003; Roviezzo et al., 2006) via production of nitric oxide, suggesting that the effects of S1P on vascular regulation vary greatly. In addition, previous animal experiments have shown that Ca^{2+}-sensitization mechanisms such as the Rho kinase (ROCK) pathway via S1P receptor 2 (S1PR2) was involved with S1P-induced vasoconstriction in human coronary artery smooth muscle cells (Ohmori et al., 2003) and rat cerebral arteries but not aorta (Coussin et al., 2002). Taken together, these previous data suggested that the ability of S1P to act as a vasoactive mediator depends on the activation of associated signaling pathways and may vary in different organs.

It is worth noting that there was no study to examine the effect of S1P on the retinal microcirculation. Moreover, the cellular signaling pathway responsible for the direct impact of S1P on vascular tone in the retinal microcirculation has not been examined. In the current study, we evaluated the effect of S1P on the retinal arterioles and the underlying signaling mechanisms involved in this vasomotor activity using isolated vessels.

2. Materials and Methods

2.1 Animal preparation

The Animal Care Committee of Asahikawa Medical University approved all animal procedures, which were performed according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The eyes of pigs (age, 16-24 weeks; weight, 15-25 kg) of either sex were enucleated immediately after the animals were killed in a local abattoir and transported to the

laboratory in a moist chamber on ice. The procedure used for harvesting eyes has been described previously (Omae et al., 2012).

2.2 Isolation and cannulation of microvessels

The techniques used to identify, isolate, cannulate, pressurize, and visualize the retinal microvessels have been described previously (Hein et al., 2009). Briefly, the isolated retinal arterioles (~100 μm in situ) were cannulated with a pair of glass micropipettes and pressurized to 55 cmH_2O intraluminal pressure without flow using two independent pressure reservoir systems (Kuo et al., 1990). The vasomotor activity of the isolated vessels was recorded continuously throughout the experiments using videomicroscopic techniques (Hein et al., 2005). Our immunohistochemical analysis suggested that our preparation did not retain neuroglial tissue surrounding the VSMCs (data not shown).

2.3 Experimental protocols

2.3.1 Control experiment

Cannulated arterioles were bathed in physiologic saline solution (PSS) (in mM, NaCl 145.0, KCl 4.7, $CaCl_2$ 2.0, $MgSO_4$ 1.17, NaH_2PO_4 1.2, glucose 5.0, pyruvate 2.0, EDTA 0.02, and MOPS [3-(*N*-morpholino)propanesulfonic acid] 3.0) at 36°C to 37°C to allow development (~30-40 minutes) of basal tone. In one series of studies, the vasomotor response to cumulative extraluminal administration of S1P (1 nM-10 μM) (Hoefer et al., 2010) then was constructed based on evidence that the S1P concentrations in the plasma have been reported in the high nanomolar range (Alewijnse et al., 2004; Yatomi et al., 1997). The vessels were exposed to each concentration of agonist for 3 to 5 minutes until a stable diameter was established. As a result of the responses to the concentration of each agonist, the concentration-response curve was obtained. After measurement of the control

concentration response of S1P without drugs, the vessels were washed with PSS to allow the basal tone to redevelop. The vasoconstriction elicited by S1P was re-examined after 30 minutes to confirm the response reproducibility (n=8).

To elucidate the signaling mechanisms involved in the retinal arteriolar constriction induced by S1P, we performed the following series of experiments. We evaluated the relative roles of S1PR 1-3 in the retinal arteriolar response to S1P after the vessels were incubated with respective antagonists sodium 4-[(4-butoxyphenyl)thio]-2′ -[{4-[(heptylthio)methyl]-2-hydroxyphenyl}(hydroxy)methyl]biphenyl-3-sulfonate (compound 5, 1 μM) (Yonesu et al., 2010), JTE-013 (1 μM) (Hoefer et al., 2010), and suramin (100 μM) (Hedemann et al., 2004) before S1P was added. In another series of studies, we examined the involvement of L-type voltage-operated calcium channels (L-VOCCs) and the activation of ROCK, protein kinase C (PKC), phospholipase C (PLC), and myosin light chain kinase (MLCK) as signaling molecules in the S1P-induced vasoconstriction. The arterioles with tone were incubated in normal PSS-albumin containing the dihydropyridine L-VOCC blocker nifedipine (1 μM) (Hong et al., 2004), the ROCK inhibitor H-1152 (3 μM) (Potts et al., 2012), the PKC inhibitor Gö-6983 (3 μM) (Potts et al., 2012), the PLC inhibitor U73122 (1 μM) (Coussin et al., 2002), and the MLCK inhibitor ML-9 (10 μM) (Zhou and Murthy, 2004) before administration of S1P. Because H-1152 (3 μM), nifedipine (1 μM), and ML-9 (10 μM) reduced the vascular tone, we performed control experiments to adjust the vascular tone to the dilated vessels after H-1152, nifedipine, and ML-9 with sodium nitroprusside (SNP) as the control. All vessels were pretreated with antagonists or inhibitors extraluminally for at least 30 minutes.

2.3.2 Endothelial denudation

The role of the endothelium in S1P-induced constriction was evaluated by comparing the response before and after removal of the endothelium by luminal perfusion with the non-ionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1 propane sulfonate (CHAPS, 0.4%) (Sigma-Aldrich, St. Louis, MO) as described previously (Omae et al., 2011).

2.3.3 Immunohistochemical analysis

The immunohistochemical detection of S1PR2 in the vascular wall was performed after the cryomicrotome sections of the retinal arterioles were prepared. We previously described the techniques for immunohistochemical staining of the isolated retinal arterioles (Omae et al., 2011). We used the following primary antibodies: an anti-S1P receptor EDG-5 antibody (catalogue no. sc-25491, dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA), an anti-eNOS antibody (dilution 1:100, BD Biosciences, San Diego, CA), or an anti-α-smooth muscle actin antibody (dilution 1:600, Sigma-Aldrich, St. Louis, MO). The secondary antibodies included fluorescein isothiocyanate (FITC)-conjugated antibody (dilution 1:60, GE Healthcare Life Sciences, Piscataway, NJ) and Cy3-conjugated antibody (dilution 1:200, Abcam, Cambridge, MA). The slides were observed for green (FITC) and red (Cy3) images and analyzed with a confocal microscope (Fluoview FV 1000, Olympus Tokyo, Japan). Merged images were created with Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

2.4 Chemicals

Compound 5 was obtained from Daiichi Sankyo Co. (Tokyo, Japan). H-1152 was obtained from Calbiochem (San Diego, CA). Other drugs were obtained from

Sigma-Aldrich. JTE-013 and Gö-6983 was dissolved in dimethyl sulfoxide (DMSO). U73122, nifedipine, and ML-9 were dissolved in ethanol; other drugs were dissolved in PSS. All subsequent dilutions of drugs for use in experiments were performed using PSS. The final concentrations of ethanol and DMSO in the vessel bath were less than 0.1% (Hein et al., 2006). Vehicle control studies indicated that these final concentrations of solvents did not affect the arteriolar functions.

2.5 Data analysis

At the end of each experiment, the vessel was relaxed in EDTA (1 mM) calcium-free PSS to obtain the maximal diameter at 55 cmH_2O intraluminal pressure (Nagaoka et al., 2007). The diameter changes in response to S1P were normalized to the resting diameters and expressed as percentage changes in diameter (Hein et al., 2009). Data are reported as the mean ± standard error of the mean; *n* represents the number of vessels studied. The statistical analyses were performed on the original data after the normal distribution was confirmed using the Kolmogorov-Smirnov test. Statistical comparisons of the changes in resting tone by antagonists were performed using the Student's *t*-test. Two-way analysis of variance, followed by the Bonferroni multiple-range test, was used to determine the significance of the difference between the control and the experimental interventions. $P < 0.05$ was considered significant.

3. Results

3.1 Constriction of retinal arterioles induced by S1P

The basal tone in all vessels ($n = 87$) ranged from 40% to 60% (average, ~51.9% ± 2.0%) of the maximal diameter. The average resting and maximal vessel diameters were

45.2 ± 1.5 and 87.3 ± 5.0 μm, respectively. S1P induced concentration-dependent maximal constriction of the retinal arterioles within about 5 minutes. The threshold concentration for vasoconstriction was 0.1 μM, and the highest concentration (10 μM) of S1P caused a 30% reduction in the resting diameter (Fig. 1). Further study showed that S1P-induced constriction was reproducible and did not deteriorate after repeated application (Fig. 1D).

3.2 Role of S1P receptors and the endothelium in vasoconstriction in response to S1P

Blockade of S1PR2 by JTE-013 but not blockade of S1PR1 or S1PR3 by compound 5 and suramin, respectively, abolished the vasoconstrictive response to S1P (Fig. 2A). In another series of experiments, 10 vessels were subjected to the denudation protocol. After perfusion with CHAPS, three of the 10 vessels lost basal tone and two showed partial inhibition resulting from the endothelium-dependent vasodilator bradykinin. These apparently damaged or partially denuded vessels were excluded from further study. The remaining five vessels maintained basal tone (control, 52.9 ± 0.1% vs. denudation, 53.9 ± 4.5%; $P = 0.65$), and the bradykinin-induced vasodilation (10 nM) was abolished (control, 87.7% ± 0.9% vs. denudation, 1.0% ± 0.5%). In addition, these vessels exhibited normal vasodilation in response to SNP (Table). In these accepted denuded vessels, the constrictive response to S1P was not attenuated ($P = 0.729$) (Fig. 2B). Furthermore, after incubation of the denuded vessels with JTE-013, the constrictive response of the denuded vessels to S1P was comparable to the response to S1P before denudation (Fig. 2B).

3.3 Role of ROCK and PKC in vasoconstriction in response to S1P

Inhibition of ROCK by H-1152 (3 μM) and coadministration of H-1152 with the PKC inhibitor Gö-6983 (3 μM) abolished the S1P-induced vasoconstriction of the retinal arterioles (Fig. 3A). Because H-1152 (3 μM) reduced the vascular tone, we first added SNP

(300 μM) to the control vessels to adjust the vascular tone (Table). Inhibition of PKC by Gö-6983 (3 μM) partially but significantly ($p<0.05$) inhibited the S1P-induced vasoconstriction of the retinal arterioles (Fig. 3B).

3.4 Role of PLC and L-VOCCs in vasoconstriction in response to S1P

Inhibition of PLC by U73122 partly but significantly ($p<0.05$) inhibited the S1P-induced vasoconstriction (Fig. 4A). Because nifedipine reduced the vascular tone, we added SNP (30 μM) to the control vessels to adjust the vascular tone (Table). In our experiments, S1P-induced vasoconstriction of the retinal arterioles was reduced partly by pretreatment of nifedipine and almost abolished by subsequent administration of U73122 (Fig. 4B).

3.5 Role of MLCK in vasoconstriction in response to S1P

Inhibition of MLCK by ML-9 partly but significantly ($p<0.05$) inhibited the S1P-induced vasoconstriction (Fig. 5A). Coadministration of ML-9 and H-1152 or Gö-6983 almost abolished S1P-induced vasoconstriction of the retinal arterioles (Fig. 5A, B). Because ML-9 (10 μM) reduced the vascular tone, we first added SNP (0.3 μM) to the control vessels to adjust the vascular tone (Table). .

3.6 S1PR2 expression in retinal arterioles

In the retinal arterioles, S1PR2 was expressed in the vascular endothelium and smooth muscle (Fig. 6).

3.7 Response to SNP

Various interventions did not affect the SNP-induced dilation of the retinal arterioles (Table), suggesting that the vascular smooth muscle function was unaltered by these interventions.

4. Discussion

The current study showed for the first time that S1P induces concentration-dependent vasoconstriction of the retinal arterioles with about 30% constriction at high concentrations (1 nM-10 μM) (Fig. 1D). Previous studies have reported that S1P induces vasoconstriction in resistance vessels such as the mesenteric (Bischoff et al., 2000a; Hemmings et al., 2004), cerebral (Coussin et al., 2002; Salomone et al., 2008; Salomone et al., 2003), coronary (Ohmori et al., 2003; Salomone et al., 2003), and renal arteries (Bischoff et al., 2000a) but has little or no effect on conduit vessels such as the aorta (Coussin et al., 2002) and carotid (Salomone et al., 2003) and femoral arteries (Salomone et al., 2003), or causes vasodilation in the aorta (Roviezzo et al., 2006) and mesenteric arteries (Dantas et al., 2003). Our finding seems to agree with the former results. Furthermore, it was reported that the plasma concentrations of sphingosine, a precursor of S1P, was elevated in patients with type 2 diabetes (Gorska et al., 2005). Although we did not measure the concentration of S1P in patients with type 2 diabetes, further clinical study is needed to examine whether an elevated S1P concentration may be associated with impaired RBF in patients with type 2 diabetes (Nagaoka et al., 2010).

S1P activates G-protein coupled receptors S1PR1-5 (Sanchez and Hla, 2004; van Koppen et al., 1996), whereas only S1PR1-3 is present in vascular cells (Alewijnse et al., 2004; Peters and Alewijnse, 2007). Moreover, previous studies have shown that S1P-induced vasoconstriction depends on S1PR2 in the human coronary artery smooth muscle cells (Ohmori et al., 2003), and murine pulmonary vasculature (Szczepaniak et al., 2010) and spiral modiolar arteries (Kono et al., 2007), whereas other studies showed that

S1P-induced vasoconstriction depends on S1PR3 in rat mesenteric arteries (Hedemann et al., 2004) and cerebral arteries (Salomone et al., 2003), canine cerebral arteries (Murakami et al., 2010), and murine cerebral arteries (Salomone et al., 2008). In the current study, blockade of S1PR2 with JTE-013 but not of S1PR1 and S1PR3 with compound 5 and suramin inhibited S1P-induced vasoconstriction of the retinal arterioles (Fig. 2A), suggesting that only S1PR2 may be involved in the constriction of the retinal arterioles in response to S1P.

A previous report has shown that the vasoconstrictions of the isolated mouse basilar artery produced by KCl, U46619, and endothelin-1 were suppressed by JTE-013 (Salomone et al., 2008). However, in our preliminary studies, JTE-013 had no effect on the vasoconstriction response of porcine retinal arterioles to KCl, U46619, and endothelin-1 (Supplementary Fig. S1). Furthermore, non-specificity was reported at the 10-μM concentration (Salomone and Waeber, 2011), which was higher than that in the current study. Taken together, it is likely that the specificity and efficacy of JTE-013 at a concentration of 1 μM to block the effects of S1PR2 were validated in the current study.

It has been reported that S1PR2 is expressed in the vascular endothelium (Sanchez et al., 2007; Skoura et al., 2007) and smooth muscle (Daum et al., 2009; Sanchez and Hla, 2004). Although our immunofluorescence data showed the expression of S1PR2 in both endothelium (Fig. 6A) and smooth muscle of the retinal arterioles (Fig. 6B), removing the vascular endothelium did not affect the inhibitory activity of JTE-013 on S1P-induced vasoconstriction in the retinal arterioles (Fig. 2B), suggesting that S1PR2 in the vascular smooth muscle but not the endothelium is involved with the S1P-induced vasoconstriction of retinal arterioles. This seems to be consistent with the previous finding that inhibition of

S1PR2 by JTE-013 suppressed S1P-induced constriction in human coronary artery smooth muscle cells (Ohmori et al., 2003). Furthermore, we administered S1P intraluminally to confirm the effect of S1P on the vascular endothelium and found that intraluminal and extraluminal administration of S1P induced vasoconstriction of the retinal arterioles to a similar extent (extraluminally, 31.1% ± 1.3% vs. intraluminally, 28.2% ± 1.8% at a final S1P concentration of 10 μM), indicating that S1P may reach the vascular endothelium by extraluminal administration.

It is known that smooth muscle contractility occurs through increased intracellular Ca^{2+}-dependent phosphorylation of the myosin light chain (MLC) and Ca^{2+}-sensitization mechanisms under constant Ca^{2+} levels. Inhibition of MLC phosphatase activity by ROCK and PKC contributes to vasoconstriction regarding the Ca^{2+}-sensitization mechanisms (Somlyo and Somlyo, 2003; Zemlickova et al., 2004). It is worth noting that S1P mediated vasoconstriction via the ROCK pathway in the various vessels (Coussin et al., 2002; Szczepaniak et al., 2010). In the current study, blockade of ROCK by H-1152 (3 μM) almost abolished the S1P-induced vasoconstriction of the retinal arterioles (Fig. 3A). Taken together, it is likely that S1P may cause vasoconstriction of the retinal arterioles through Ca^{2+}-sensitization mechanisms via activation of ROCK.

We also found that pretreatment of the PKC inhibitor Gö-6983 reduced S1P-induced vasoconstriction of the retinal arterioles (Fig. 3B), suggesting that PKC plays an important role in S1P-induced vasoconstriction of the retinal arterioles. Our results are supported by numerous reports that S1P induced contraction via the PKC pathway in smooth muscle cells (Chung et al., 2008; Song et al., 2006; Zhou and Murthy, 2004). Interestingly, a previous study using the same isolated retinal vessel technique as ours showed that a PKC

activator phorbol-12,13-dibutyrate (PDBu) caused the constriction of the isolated retinal arterioles via the activation of ROCK (Potts et al., 2012). Taken together, we speculated that S1P may constrict the retinal arterioles via activation of ROCK through a PKC-dependent mechanism.

Besides Ca^{2+}-sensitization mechanisms, elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) with influx of extracellular Ca^{2+} and the release of Ca^{2+} from the intracellular calcium stores may be a major vasoconstrictive mechanism (Bischoff et al., 2000a; Ghosh et al., 1990; Hopson et al., 2011; Watterson et al., 2005). Moreover, a transient increase in $[Ca^{2+}]_i$ via the release of Ca^{2+} from the intracellular calcium stores reflects inositol trisphosphate ($InsP_3$) generation by stimulation of PLC activity (Coussin et al., 2002; Putney, 1999). The current data showed that inhibition of PLC with U73122 attenuated retinal arteriolar constriction in response to S1P (Fig. 4A), suggesting that PLC activation may be involved in S1P-induced vasoconstriction in the retinal arterioles. This result is in agreement with previous findings that S1P constricts smooth muscle via PLC activation (Chung et al., 2008; Zhou and Murthy, 2004). It has also been reported that PLC generated second messengers such as $InsP_3$ (Berridge, 1993) and diacylglycerol, leading to PKC activation (Dempsey et al., 2000; Nishizuka, 1988). Further, S1P-induced activation of extracellular signal-regulated kinases, critical to S1P-induced esophageal muscle cell contraction (Song et al., 2006), can be attenuated in the presence of U73122 and the PKC inhibitor chelerythrine (Chung et al., 2008). Overall, it appears that S1P-induced vasoconstriction of the retinal arterioles may be associated with activation of the PLC/PKC pathway.

It has been reported that S1P elevated $[Ca^{2+}]_i$ from the entry of extracellular calcium

via the calcium channel such as the L-VOCCs (Bischoff et al., 2000a). Indeed, L-VOCC blocker nifedipine abolished S1P-induced vasoconstriction in intrarenal arterioles (Bischoff et al., 2001). These findings seem to support our results that S1P-induced vasoconstriction of the retinal arterioles was reduced by pretreatment of nifedipine (Fig. 4B). Since residual vasoconstriction in the presence of nifedipine was almost abolished with subsequent U73122 treatment (Fig. 4B), it is speculated that both PLC and L-VOCCs may play some roles in the S1P-induced vasoconstriction in the retinal arterioles.

The increase in $[Ca^{2+}]_i$ subsequently was reported to activate MLCK by formation of a Ca^{2+}-calmodulin complex to initiate constriction (He et al., 2011; Kamm and Stull, 2001). A previous finding that S1P-induced contraction and MLC phosphorylation were abolished by the MLCK inhibitor ML-9 in rabbit gastric smooth muscle cells (Zhou and Murthy, 2004) seems to support our results that pretreatment with ML-9 inhibited S1P-induced vasoconstriction in porcine retinal arterioles (Fig. 5A). Furthermore, residual constriction in the presence of ML-9 decreased further with subsequent H-1152 or Gö-6983 treatment (Fig. 5A, B). Taken together, our findings suggest that S1P-induced vasoconstriction in the retinal arterioles may be mediated by a Ca^{2+}-dependent mechanism regulating MLCK activity, probably via elevation of $[Ca^{2+}]_i$ through the L-VOCCs, and a Ca^{2+}-sensitization mechanism via activation of ROCK and PKC.

Our study had some limitations. First, as shown in Table, H-1152 (3 μM), nifedipine (1 μM), and ML-9 (10 μM) resulted in a reduction of some basal tone because ROCK, extracellular Ca^{2+} entry via the L-VOCCs, and MLCK are thought to be involved with in the maintenance of spontaneous myogenic tones in the porcine retinal arterioles (Potts et al., 2012). Although we used SNP to adjust the basal tone of the control vessels to that of the

H-1152, nifedipine, and ML-9-treated vessels (Figs. 3A, 4B, 5A, 5B), these changes in the basal tone might have had some effects on our results. Second, careful attention should be paid to the selectivity of the inhibitors and antagonists used in the current study. It has reported that the ROCK inhibitor H-1152 may be involved in inhibiting Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) (Tamura et al., 2005), which has been suggested to play a role in vascular smooth muscle contraction (Kim et al., 2000; Rokolya and Singer, 2000). In our preliminary study, pre-incubation of vessels with the CaMKII inhibitor KN-93 (3 μM) had no effect on S1P-induced retinal vasoconstriction (Supplementary Fig. S2), indicating that H-1152 may not play a role in inhibition of S1P-induced vasoconstriction through inhibition of CaMKII. In addition, the PKC inhibitor Gö-6983 also has been reported to affect L-VOCCs (Welling et al., 2005). However, in our preliminary study, we found that Gö-6983 (3 μM) abolished the constrictive response to PKC activator PDBu (at 0.1 μM control, 50.1% ± 2.2% vs. Gö-6983 pre-incubation, -7.3% ± 2.8%; $P < 0.01$) (Supplementary Fig. S3) but had no significant effect on the constriction in response to L-VOCCs activator Bay K 8644 (at 1 μM control, 40.2% ± 3.0% vs. Gö-6983 pre-incubation, 42.8% ± 2.5%; $P = 0.56$) (Supplementary Fig. S4). We also found that Gö-6983 (3 μM) did not alter the basal tone of the isolated retinal arterioles, which was reduced by L-VOCC blocker nifedipine (Table). Taken together, it is unlikely that Gö-6983 (3 μM) inhibited L-VOCCs in the isolated retinal arterioles in the current study. Third, S1P may stimulate the transient receptor potential cation (TRPC) channels (Beech et al., 2009). Because the specific TRPC channel inhibitors were not commercially available, we could not investigate if the TRPC channels play a role in S1P-induced vasoconstriction in the current study. Finally, although our data suggested that the concentration of Ca^{2+} plays an

important role in S1P-induced vasoconstriction in the retinal arterioles, we could not measure the concentration of intracellular Ca^{2+} in the current study due to the limitations of our technique.

5. Conclusions

In summary, we first showed that S1P elicited potent vasoconstriction of the retinal arterioles via S1PR2 in vascular smooth muscle cells. The current findings suggest that S1P-induced vasoconstriction may be mediated by a combination of Ca^{2+}-dependent pathway via activation of L-VOCCs and consequent activation of the MLCK and Ca^{2+}-sensitization pathway via activation of the PKC/ROCK pathway (Fig. 7). A better understanding of the S1P system in the retinal circulation could lead to new potential therapies for retinal vascular diseases.

Acknowledgements

The authors thank Lih Kuo and Travis W. Hein for technical support and Lynda Charters for reviewing the manuscript.

References

Alewijnse, A.E., Peters, S.L., Michel, M.C., 2004. Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites. Br. J. Pharmacol. 143, 666-684.

Allende, M.L., Proia, R.L., 2002. Sphingosine-1-phosphate receptors and the development of the vascular system. Biochim. Biophys. Acta 1582, 222-227.

Beech, D.J., Bahnasi, Y.M., Dedman, A.M., Al-Shawaf, E., 2009. TRPC channel lipid specificity and mechanisms of lipid regulation. Cell Calcium 45, 583-588.

Berridge, M.J., 1993. Cell signalling. A tale of two messengers. Nature 365, 388-389.

Bischoff, A., Czyborra, P., Fetscher, C., Meyer Zu Heringdorf, D., Jakobs, K.H., Michel, M.C., 2000a. Sphingosine-1-phosphate and sphingosylphosphorylcholine constrict renal and mesenteric microvessels in vitro. Br. J. Pharmacol. 130, 1871-1877.

Bischoff, A., Czyborra, P., Meyer Zu Heringdorf, D., Jakobs, K.H., Michel, M.C., 2000b. Sphingosine-1-phosphate reduces rat renal and mesenteric blood flow in vivo in a pertussis toxin-sensitive manner. Br. J. Pharmacol. 130, 1878-1883.

Bischoff, A., Finger, J., Michel, M.C., 2001. Nifedipine inhibits sphinogosine-1-phosphate-induced renovascular contraction in vitro and in vivo. Naunyn. Schmiedebergs Arch. Pharmacol. 364, 179-182.

Chung, F.Y., Song, H.J., Park, S.Y., Jang, H.S., Kim, D.S., Sim, S.S., Sohn, U.D., 2008. Signaling mechanisms of sphingosine 1-phosphate-induced ERK1/2 activation in cultured feline esophageal smooth muscle cells. Arch. Pharm. Res. 31, 1437-1445.

Coussin, F., Scott, R.H., Wise, A., Nixon, G.F., 2002. Comparison of sphingosine 1-phosphate-induced intracellular signaling pathways in vascular smooth muscles: differential role in vasoconstriction. Circ. Res. 91, 151-157.

Dantas, A.P., Igarashi, J., Michel, T., 2003. Sphingosine 1-phosphate and control of vascular tone. Am. J. Physiol. Heart Circ. Physiol. 284, H2045-2052.

Daum, G., Grabski, A., Reidy, M.A., 2009. Sphingosine 1-phosphate: a regulator of arterial lesions. Arterioscler. Thromb. Vasc. Biol. 29, 1439-1443.

Dempsey, E.C., Newton, A.C., Mochly-Rosen, D., Fields, A.P., Reyland, M.E., Insel, P.A., Messing, R.O., 2000. Protein kinase C isozymes and the regulation of diverse cell responses. Am. J. Physiol. Lung Cell. Mol. Physiol. 279, L429-438.

Deutschman, D.H., Carstens, J.S., Klepper, R.L., Smith, W.S., Page, M.T., Young, T.R., Gleason, L.A., Nakajima, N., Sabbadini, R.A., 2003. Predicting obstructive coronary artery disease with serum sphingosine-1-phosphate. Am. Heart J. 146, 62-68.

Ghosh, T.K., Bian, J., Gill, D.L., 1990. Intracellular calcium release mediated by sphingosine

derivatives generated in cells. Science 248, 1653-1656.

Gorska, M., Dobrzyn, A., Baranowski, M., 2005. Concentrations of sphingosine and sphinganine in plasma of patients with type 2 diabetes. Med. Sci. Monitor. 11, CR35-38.

He, W.Q., Qiao, Y.N., Zhang, C.H., Peng, Y.J., Chen, C., Wang, P., Gao, Y.Q., Chen, C., Chen, X., Tao, T., Su, X.H., Li, C.J., Kamm, K.E., Stull, J.T., Zhu, M.S., 2011. Role of myosin light chain kinase in regulation of basal blood pressure and maintenance of salt-induced hypertension. Am. J. Physiol. Heart Circ. Physiol. 301, H584-591.

Hedemann, J., Fetscher, C., Michel, M.C., 2004. Comparison of noradrenaline and lysosphingolipid-induced vasoconstriction in mouse and rat small mesenteric arteries. Auton. Autacoid Pharmacol. 24, 77-85.

Hein, T.W., Ren, Y., Yuan, Z., Xu, W., Somvanshi, S., Nagaoka, T., Yoshida, A., Kuo, L., 2009. Functional and molecular characterization of the endothelin system in retinal arterioles. Invest. Ophthalmol. Vis. Sci. 50, 3329-3336.

Hein, T.W., Xu, W., Kuo, L., 2006. Dilation of retinal arterioles in response to lactate: role of nitric oxide, guanylyl cyclase, and ATP-sensitive potassium channels. Invest. Ophthalmol. Vis. Sci. 47, 693-699.

Hein, T.W., Yuan, Z., Rosa, R.H., Jr., Kuo, L., 2005. Requisite roles of A2A receptors, nitric oxide, and KATP channels in retinal arteriolar dilation in response to adenosine. Invest. Ophthalmol. Vis. Sci. 46, 2113-2119.

Hemmings, D.G., Xu, Y., Davidge, S.T., 2004. Sphingosine 1-phosphate-induced vasoconstriction is elevated in mesenteric resistance arteries from aged female rats. Br. J. Pharmacol. 143, 276-284.

Hoefer, J., Azam, M.A., Kroetsch, J.T., Leong-Poi, H., Momen, M.A., Voigtlaender-Bolz, J., Scherer, E.Q., Meissner, A., Bolz, S.S., Husain, M., 2010. Sphingosine-1-phosphate-dependent activation of p38 MAPK maintains elevated peripheral resistance in heart failure through increased myogenic vasoconstriction. Circ. Res. 107, 923-933.

Hong, Z., Olschewski, A., Reeve, H.L., Nelson, D.P., Hong, F., Weir, E.K., 2004. Nordexfenfluramine causes more severe pulmonary vasoconstriction than dexfenfluramine. Am. J. Physiol. Lung Cell. Mol. Physiol. 286, L531-538.

Hopson, K.P., Truelove, J., Chun, J., Wang, Y., Waeber, C., 2011. S1P activates store-operated calcium entry via receptor- and non-receptor-mediated pathways in vascular smooth muscle cells. Am. J. Physiol. Cell Physiol. 300, C919-926.

Kamm, K.E., Stull, J.T., 2001. Dedicated myosin light chain kinases with diverse cellular functions. J. Biol. Chem. 276, 4527-4530.

Kim, I., Je, H.D., Gallant, C., Zhan, Q., Riper, D.V., Badwey, J.A., Singer, H.A., Morgan, K.G., 2000. Ca2+-calmodulin-dependent protein kinase II-dependent activation of contractility in ferret aorta. J. Physiol. 526 Pt 2, 367-374.

Kono, M., Belyantseva, I.A., Skoura, A., Frolenkov, G.I., Starost, M.F., Dreier, J.L., Lidington, D., Bolz, S.S., Friedman, T.B., Hla, T., Proia, R.L., 2007. Deafness and stria vascularis defects in S1P2 receptor-null mice. J. Biol. Chem. 282, 10690-10696.

Kuo, L., Davis, M.J., Chilian, W.M., 1990. Endothelium-dependent, flow-induced dilation of isolated coronary arterioles. Am. J. Physiol. 259, H1063-1070.

Murakami, A., Takasugi, H., Ohnuma, S., Koide, Y., Sakurai, A., Takeda, S., Hasegawa, T., Sasamori, J., Konno, T., Hayashi, K., Watanabe, Y., Mori, K., Sato, Y., Takahashi, A., Mochizuki, N., Takakura, N., 2010. Sphingosine 1-phosphate (S1P) regulates vascular contraction via S1P3 receptor: investigation based on a new S1P3 receptor antagonist. Mol. Pharmacol. 77, 704-713.

Nagaoka, T., Hein, T.W., Yoshida, A., Kuo, L., 2007. Simvastatin elicits dilation of isolated porcine retinal arterioles: role of nitric oxide and mevalonate-rho kinase pathways. Invest. Ophthalmol. Vis. Sci. 48, 825-832.

Nagaoka, T., Sato, E., Takahashi, A., Yokota, H., Sogawa, K., Yoshida, A., 2010. Impaired retinal circulation in patients with type 2 diabetes mellitus: retinal laser Doppler velocimetry study. Invest. Ophthalmol. Vis. Sci. 51, 6729-6734.

Nagaoka, T., Yoshida, A., 2013. Relationship between retinal blood flow and renal function in patients with type 2 diabetes and chronic kidney disease. Diabetes Care 36, 957-961.

Nishizuka, Y., 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334, 661-665.

Ohmori, T., Yatomi, Y., Osada, M., Kazama, F., Takafuta, T., Ikeda, H., Ozaki, Y., 2003. Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via S1P2. Cardiovasc. Res. 58, 170-177.

Omae, T., Nagaoka, T., Tanano, I., Kamiya, T., Yoshida, A., 2012. Fenofibrate, an anti-dyslipidemia drug, elicits the dilation of isolated porcine retinal arterioles: role of nitric oxide and AMP-activated protein kinase. Invest. Ophthalmol. Vis. Sci. 53, 2880-2886.

Omae, T., Nagaoka, T., Tanano, I., Yoshida, A., 2011. Pioglitazone, a peroxisome proliferator-activated receptor-gamma agonist, induces dilation of isolated porcine retinal arterioles: role of nitric oxide and potassium channels. Invest. Ophthalmol. Vis. Sci. 52, 6749-6756.

Peters, S.L., Alewijnse, A.E., 2007. Sphingosine-1-phosphate signaling in the cardiovascular

system. Curr. Opin. Pharmacol. 7, 186-192.

Potts, L.B., Ren, Y., Lu, G., Kuo, E., Ngo, E., Kuo, L., Hein, T.W., 2012. Constriction of retinal arterioles to endothelin-1: requisite role of rho kinase independent of protein kinase C and L-type calcium channels. Invest. Ophthalmol. Vis. Sci. 53, 2904-2912.

Putney, J.W., Jr., 1999. TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium entry. Proc. Natl. Acad. Sci. U. S. A. 96, 14669-14671.

Rokolya, A., Singer, H.A., 2000. Inhibition of CaM kinase II activation and force maintenance by KN-93 in arterial smooth muscle. Am. J. Physiol. Cell Physiol. 278, C537-545.

Roviezzo, F., Bucci, M., Delisle, C., Brancaleone, V., Di Lorenzo, A., Mayo, I.P., Fiorucci, S., Fontana, A., Gratton, J.P., Cirino, G., 2006. Essential requirement for sphingosine kinase activity in eNOS-dependent NO release and vasorelaxation. FASEB J. 20, 340-342.

Salomone, S., Potts, E.M., Tyndall, S., Ip, P.C., Chun, J., Brinkmann, V., Waeber, C., 2008. Analysis of sphingosine 1-phosphate receptors involved in constriction of isolated cerebral arteries with receptor null mice and pharmacological tools. Br. J. Pharmacol. 153, 140-147.

Salomone, S., Waeber, C., 2011. Selectivity and specificity of sphingosine-1-phosphate receptor ligands: caveats and critical thinking in characterizing receptor-mediated effects. Frontiers in Pharmacology 2, 9.

Salomone, S., Yoshimura, S., Reuter, U., Foley, M., Thomas, S.S., Moskowitz, M.A., Waeber, C., 2003. S1P3 receptors mediate the potent constriction of cerebral arteries by sphingosine-1-phosphate. Eur. J. Pharmacol. 469, 125-134.

Sanchez, T., Hla, T., 2004. Structural and functional characteristics of S1P receptors. J. Cell. Biochem. 92, 913-922.

Sanchez, T., Skoura, A., Wu, M.T., Casserly, B., Harrington, E.O., Hla, T., 2007. Induction of vascular permeability by the sphingosine-1-phosphate receptor-2 (S1P2R) and its downstream effectors ROCK and PTEN. Arterioscler. Thromb. Vasc. Biol. 27, 1312-1318.

Schuchardt, M., Tolle, M., Prufer, J., van der Giet, M., 2011. Pharmacological relevance and potential of sphingosine 1-phosphate in the vascular system. Br. J. Pharmacol. 163, 1140-1162.

Skoura, A., Sanchez, T., Claffey, K., Mandala, S.M., Proia, R.L., Hla, T., 2007. Essential role of sphingosine 1-phosphate receptor 2 in pathological angiogenesis of the mouse retina. J. Clin. Invest. 117, 2506-2516.

Somlyo, A.P., Somlyo, A.V., 2003. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol. Rev. 83, 1325-1358.

Song, H.J., Choi, T.S., Chung, F.Y., Park, S.Y., Ryu, J.S., Woo, J.G., Min, Y.S., Shin, C.Y., Sohn, U.D., 2006. Sphingosine 1-phosphate-induced signal transduction in cat esophagus smooth muscle cells. Mol. Cells 21, 42-51.

Spiegel, S., Milstien, S., 2011. The outs and the ins of sphingosine-1-phosphate in immunity. Nature reviews. Immunology 11, 403-415.

Sumida, G.M., Stamer, W.D., 2010. Sphingosine-1-phosphate enhancement of cortical actomyosin organization in cultured human Schlemm's canal endothelial cell monolayers. Invest. Ophthalmol. Vis. Sci. 51, 6633-6638.

Szczepaniak, W.S., Pitt, B.R., McVerry, B.J., 2010. S1P2 receptor-dependent Rho-kinase activation mediates vasoconstriction in the murine pulmonary circulation induced by sphingosine 1-phosphate. Am. J. Physiol. Lung Cell. Mol. Physiol. 299, L137-145.

Tamura, M., Nakao, H., Yoshizaki, H., Shiratsuchi, M., Shigyo, H., Yamada, H., Ozawa, T., Totsuka, J., Hidaka, H., 2005. Development of specific Rho-kinase inhibitors and their clinical application. Biochim. Biophys. Acta 1754, 245-252.

van der Giet, M., Tolle, M., Kleuser, B., 2008. Relevance and potential of sphingosine-1-phosphate in vascular inflammatory disease. Biol. Chem. 389, 1381-1390.

van Koppen, C., Meyer zu Heringdorf, M., Laser, K.T., Zhang, C., Jakobs, K.H., Bunemann, M., Pott, L., 1996. Activation of a high affinity Gi protein-coupled plasma membrane receptor by sphingosine-1-phosphate. J. Biol. Chem. 271, 2082-2087.

Watterson, K.R., Ratz, P.H., Spiegel, S., 2005. The role of sphingosine-1-phosphate in smooth muscle contraction. Cell. Signal. 17, 289-298.

Welling, A., Hofmann, F., Wegener, J.W., 2005. Inhibition of L-type Cav1.2 Ca2+ channels by 2,(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) and 2-[1-(3-dimethyl-aminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide (Go6983). Mol. Pharmacol. 67, 541-544.

Yatomi, Y., Igarashi, Y., Yang, L., Hisano, N., Qi, R., Asazuma, N., Satoh, K., Ozaki, Y., Kume, S., 1997. Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. J Biochem 121, 969-973.

Yonesu, K., Nakamura, T., Mizuno, Y., Suzuki, C., Nagayama, T., Satoh, S., Nara, F., 2010. A novel sphingosine-1-phosphate receptor 1 antagonist prevents the proliferation and relaxation of vascular endothelial cells by sphingosine-1-phosphate. Biol. Pharm. Bull. 33, 1500-1505.

Zemlickova, E., Johannes, F.J., Aitken, A., Dubois, T., 2004. Association of CPI-17 with protein kinase C and casein kinase I. Biochem. Biophys. Res. Commun. 316, 39-47.

Zhou, H., Murthy, K.S., 2004. Distinctive G protein-dependent signaling in smooth muscle by sphingosine 1-phosphate receptors S1P1 and S1P2. Am. J. Physiol. Cell Physiol. 286, C1130-1138.

Legends

Fig. 1. Images of an isolated porcine retinal arteriole. (**A**) The vessel was transferred to the stage of an inverted microscope and allowed to develop resting basal tone (45-μm internal diameter) at 55 cm H_2O intraluminal pressure. (**B**) The diameter (31-μm internal diameter) of the vessel constricted with S1P (10 μM). (**C**) A representative tracing shows S1P (10 μM)-induced constriction of retinal arterioles initiated within 1 minute. The diameter returned to the baseline level after washout of the drug. **(D)** The response of isolated retinal arterioles to S1P. The dose-dependent vasoconstrictive effect of S1P (first trial, resting diameter, 45.8 ± 4.8 μm; maximal diameter, 85.4 ± 5.1 μm; n=8). The trial was repeated after a 30-minute washout period (second trial, resting diameter, 45.1 ± 4.7 μm; maximal diameter, 85.4 ± 5.1 μm; n=8). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus baseline.

Fig. 2. (A) The role of S1PR in the vasoconstrictive response to S1P. The dose-dependent vasoconstrictive response to S1P is examined before and after incubation with S1PR1 antagonist compound 5 (1 μM), S1PR2 antagonist JTE-013 (1 μM), and S1PR3 antagonist suramin (100 μM). **(B)** The effect of the removal of the endothelium by perfusion with 0.4% CHAPS and the effect of incubation with the S1PR2 antagonist JTE-013 after denudation. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control.

Fig. 3. (A) The effect of incubation with the ROCK inhibitor H-1152 (3 μM) after adjustment of the vessel tone. **(B)** The effect of incubation with the PKC inhibitor Gö-6983 (3 μM). R, resting diameter of vessels. $^{*}P < 0.05$ versus percent resting diameter at R. $^{\dagger}P$

< 0.05, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ versus control.

Fig. 4. (A) The effect of incubation with the PLC inhibitor U73122 (1 μM). **(B)** The effect of incubation with the L-VOCC blocker nifedipine (1 μM) after adjustment of the vessel tone. R, resting diameter of vessels. $^{*}P < 0.05$ versus percent resting diameter at R. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ versus control. $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$, and $^{\ddagger\ddagger\ddagger}P < 0.001$ versus nifedipine.

Fig. 5. (A) The effect of incubation with the MLCK inhibitor ML-9 (10 μM). Residual constriction in the presence of ML-9 also was examined after co-incubation with Gö-6983 (3 μM) after adjustment of the vessel tone. **(B)** The effect of co-incubation with ML-9 and H-1152 after adjustment of the vessel tone. R, resting diameter of vessels. $^{*}P < 0.05$ versus percent resting diameter at R. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ versus control. $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$, and $^{\ddagger\ddagger\ddagger}P < 0.001$ versus ML-9.

Fig. 6. Immunohistochemical analysis of S1PR2 in the retinal arterioles. **(A)** Staining with anti-EDG5 (*green*) and anti-eNOS (*red*) antibodies shows expression of S1PR2 and eNOS. The merged images show overlap staining (*yellow*) of S1PR2 with eNOS. **(B)** Staining with anti-EDG5 (*green*) and anti- α -smooth muscle actin (SMA, *red*) antibodies shows expression of S1PR2 and SMA. The merged images show overlap staining (*yellow*) of S1PR2 with SMA. The images are representative of three separate experiments.

Fig. 7. Schematic illustration of proposed signaling mechanisms involved in retinal

arteriolar constriction in response to S1P. Inhibition of these signaling pathways by their respective inhibitors is indicated by the vertical lines in reference to the direction of the straight line.

Supplementary Fig. S1. The effect of S1PR2 antagonist JTE-013 on the vasoconstriction response of porcine retinal arterioles to KCl (100 mM), U46619 (1 μM), and ET-1 (10 nM).

Supplementary Fig. S2. The effect of some protein kinases on the S1P (10 μM) -induced vasoconstriction of retinal arterioles.

Supplementary Fig. S3. The effect of Gö-6983 (3 μM) on the constrictive response to the PKC activator phorbol-12,13-dibutyrate (PDBu) (0.1 μM). $^{*}P < 0.05$ versus control (PDBu).

Supplementary Fig. S4. The effect of Gö-6983 (3 μM) on the constrictive response to the L-VOCCs activator Bay K 8644 (1 μM).

Figure 1.

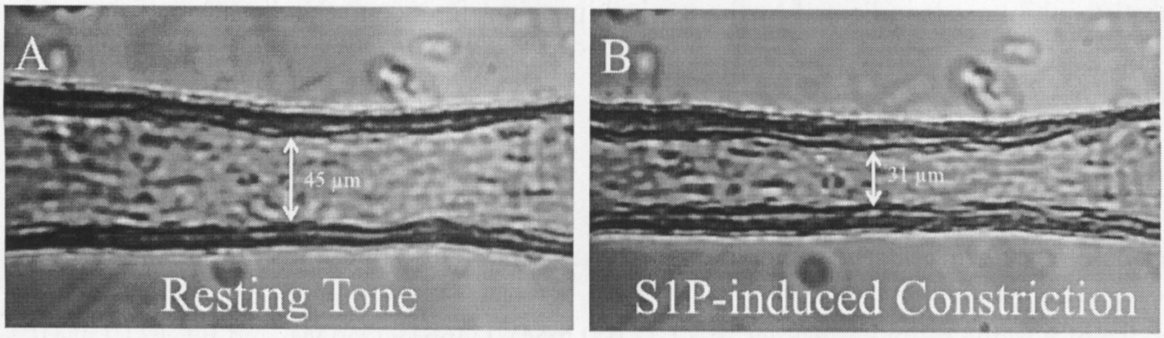


Figure 1 C

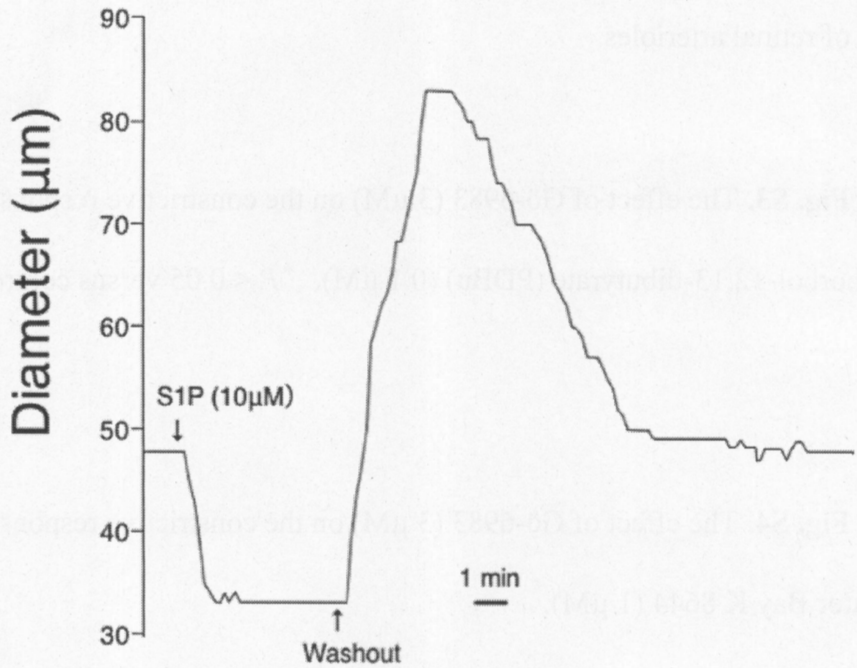


Figure 1 D

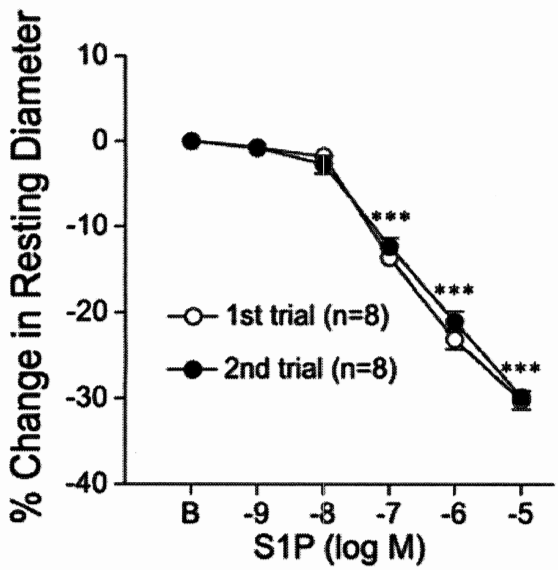


Figure 2 A

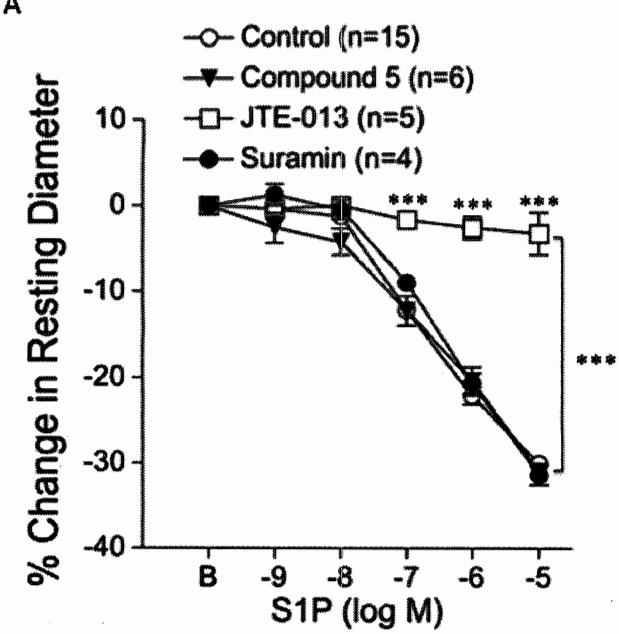


Figure 2 B

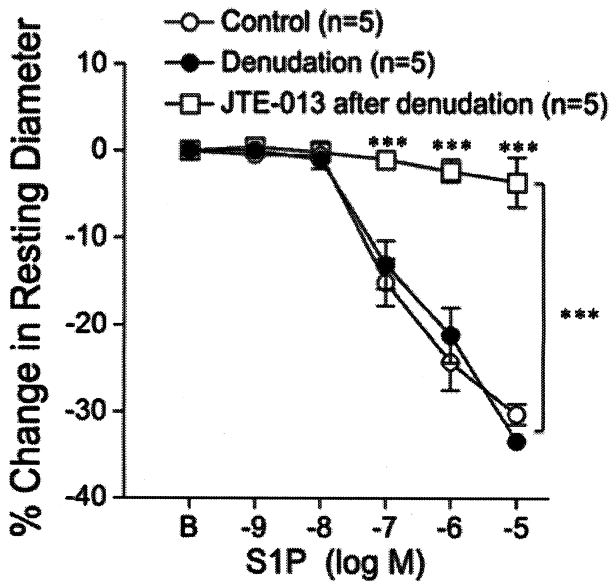

Control (n=5)
Denudation (n=5)
JTE-013 after denudation (n=5)
% Change in Resting Diameter
10
0
-10
-20
-30
-40
***
***
***
***
B
-9
-8
-7
-6
-5
S1P (log M)

Figure 3 A

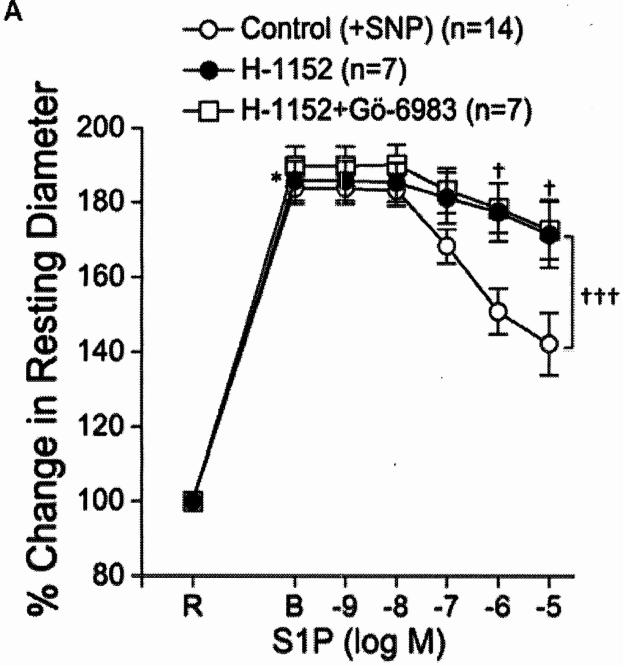


Figure 3 B

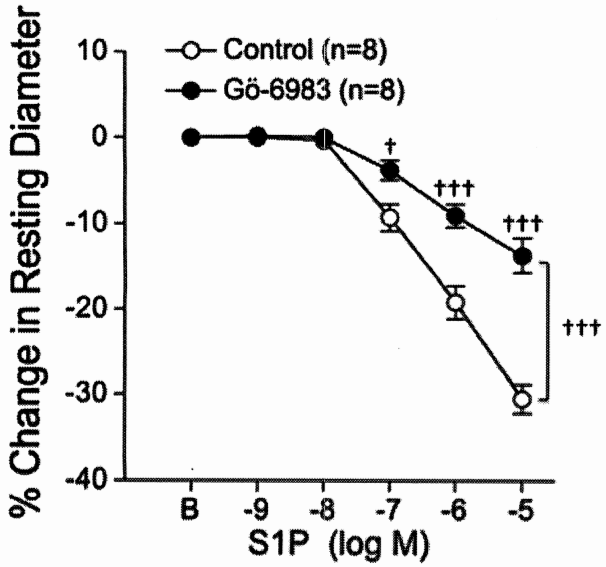


Figure 4 A

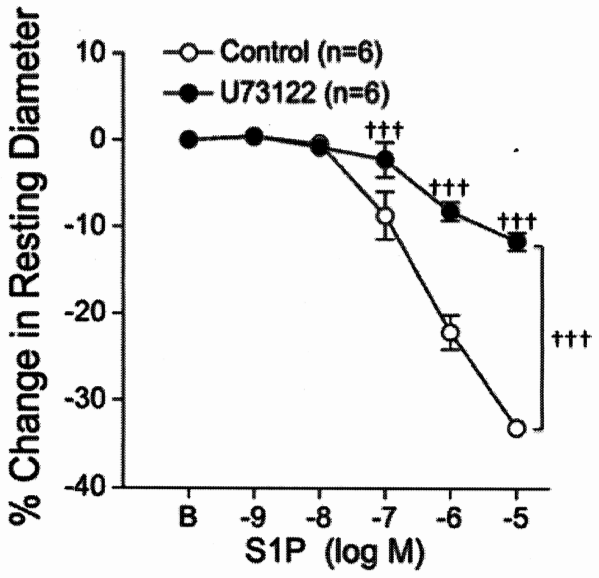


Figure 4 B

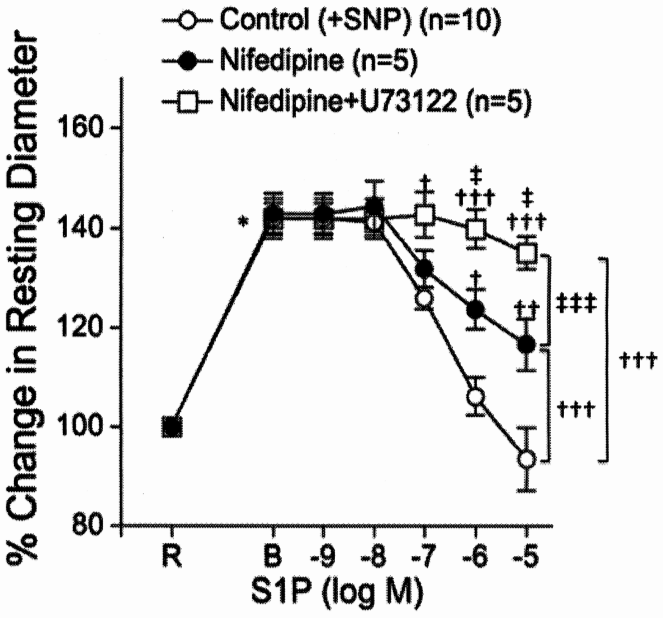


Figure 5 A

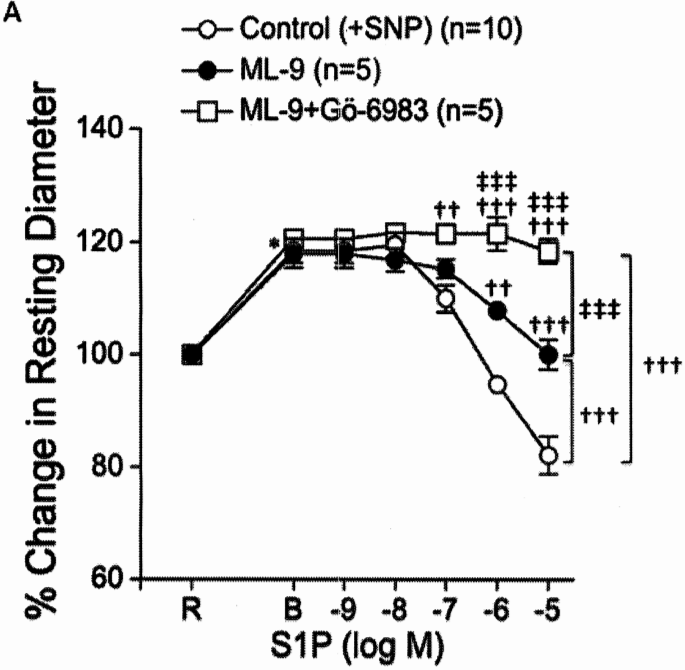


Figure 5 B

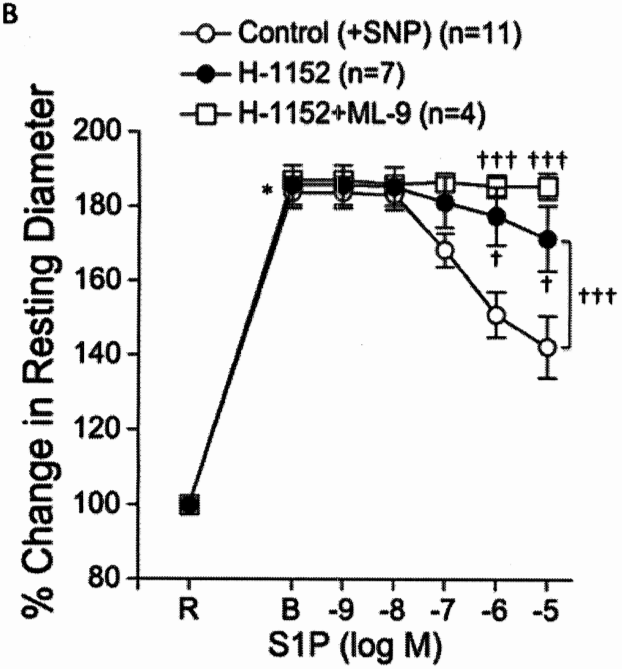
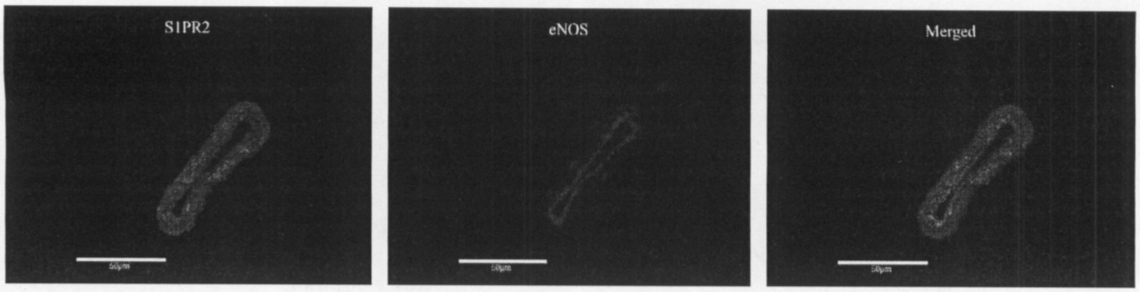


Figure 6

A. S1PR2+eNOS



B. S1PR2+SMA

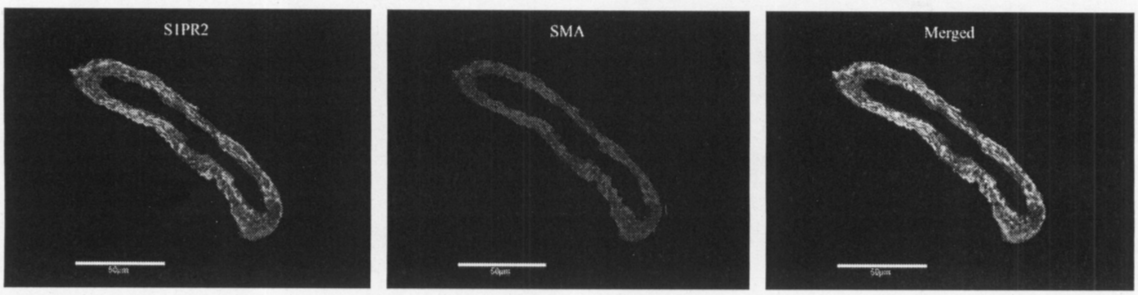


Figure 7

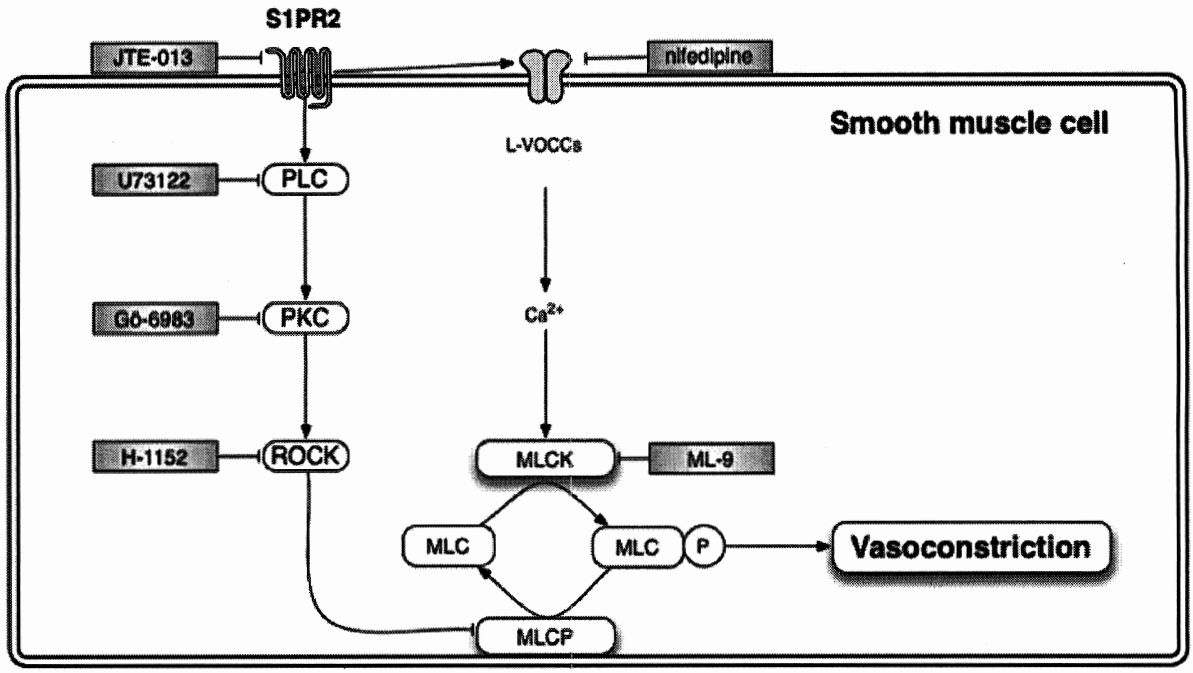

S1PR2
JTE-013
nifedipine
Smooth muscle cell
L-VOCCs
U73122
PLC
Gö-6983
PKC
Ca^{2+}
H-1152
ROCK
MLCK
ML-9
MLC
MLC
P
Vasoconstriction
MLCP

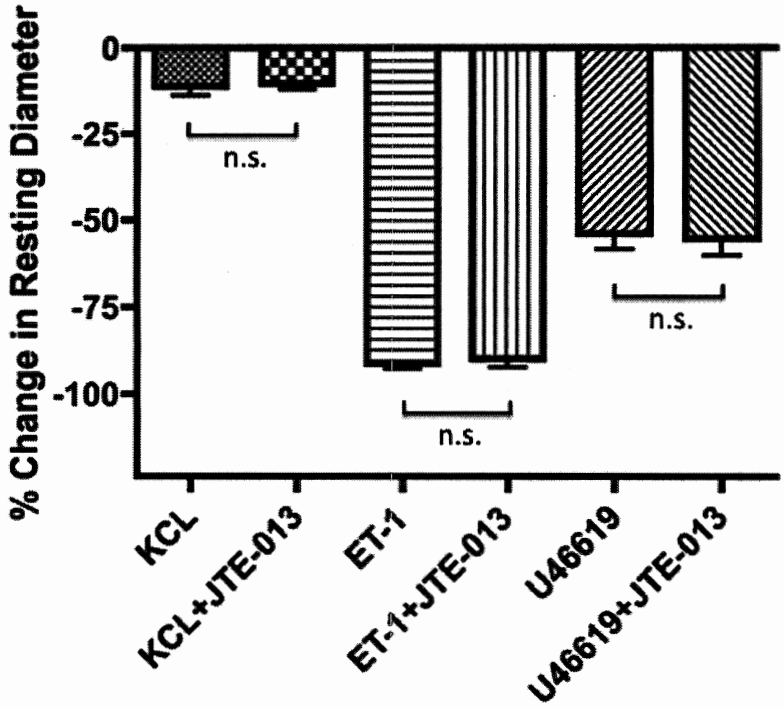
Table. Resting Diameters and Diameter Responses of Retinal Arterioles to SNP

			Sodium Nitroprusside (μM)			
	n	Resting diameter	0.1	1	10	100
Control	10	52.8 ± 6.5	7.4 ± 1.1	27.2 ± 2.9	51.6 ± 4.0	82.3 ± 1.7
Control + SNP (0.3 μM)	5	59.5 ± 1.5 *	14.4 ± 4.8	32.9 ± 5.6	57.2 ± 2.6	80.4 ± 2.5
Control + SNP (30 μM)	10	78.2 ± 1.6 *	14.8 ± 3.6	31.3 ± 4.0	46.5 ± 5.2	79.0 ± 2.2
Control + SNP (300 μM)	6	95.0 ± 0.1 *	10.2 ± 1.9	26.4 ± 4.0	50.4 ± 5.0	82.7 ± 2.6
Denudation	5	53.9 ± 4.5	8.0 ± 1.8	24.5 ± 5.0	54.9 ± 6.4	81.5 ± 2.0
Denudation + JTE-013	5	52.6 ± 2.5	10.6 ± 3.2	29.6 ± 7.6	56.0 ± 7.3	84.2 ± 2.6
Compound 5	6	50.7 ± 2.7	8.1 ± 1.4	26.6 ± 4.6	55.8 ± 5.3	82.2 ± 1.8
JTE-013	5	53.6 ± 2.4	7.3 ± 0.8	27.4 ± 3.1	51.7 ± 6.1	84.3 ± 1.7
Suramin	4	49.6 ± 2.0	9.9 ± 2.0	23.1 ± 5.2	47.2 ± 2.9	80.0 ± 4.6
H-1152	6	92.3 ± 2.4 *	11.0 ± 1.7	29.8 ± 3.6	52.8 ± 4.6	83.1 ± 2.7
H-1152 + Gö-6983	6	93.3 ± 2.6 *	9.9 ± 2.0	25.8 ± 4.1	50.3 ± 5.0	82.6 ± 2.5
Gö-6983	6	58.8 ± 0.6	11.9 ± 1.7	31.3 ± 3.8	56.8 ± 5.1	83.7 ± 2.8
U73122	4	52.5 ± 1.3	10.3 ± 4.0	31.4 ± 7.3	54.6 ± 7.0	81.9 ± 2.2
Nifedipine	5	78.6 ± 2.1 *	9.4 ± 2.2	31.0 ± 8.1	44.4 ± 9.4	83.0 ± 3.1
Nifedipine + U73122	5	79.0 ± 1.6 *	8.3 ± 2.6	30.2 ± 8.5	45.4 ± 9.1	79.2 ± 4.7
ML-9	5	59.0 ± 1.4 *	10.8 ± 2.9	33.0 ± 5.7	49.0 ± 3.7	81.6 ± 3.7
ML-9 + Gö-6983	5	61.3 ± 2.7 *	10.3 ± 2.9	27.0 ± 6.2	41.4 ± 7.4	81.8 ± 2.5
ML-9 + H-1152	4	96.0 ± 1.5 *	10.9 ± 3.0	31.3 ± 5.1	56.5 ± 3.6	84.5 ± 1.6

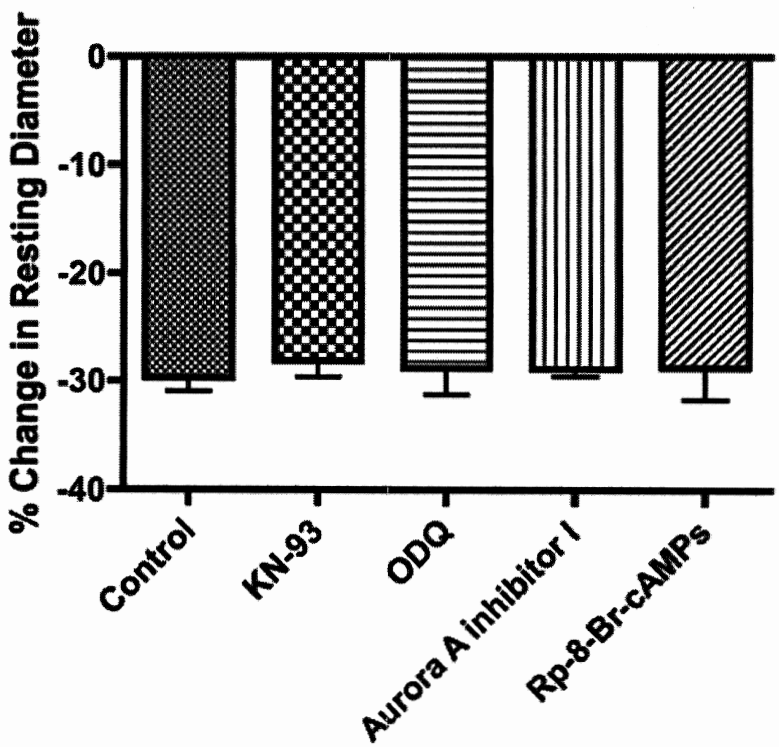
Data are expressed as the mean percentage of maximal dilation ± the standard error of the mean. n, number of vessels.

Based on the unpaired *t*-test, compared with controls, H-1152, nifedipine, and ML-9 decreased the resting tone.* $P < 0.05$ versus control. Based on two-way analysis of variance, compared with controls, the responses to SNP are unaffected by any perturbations.

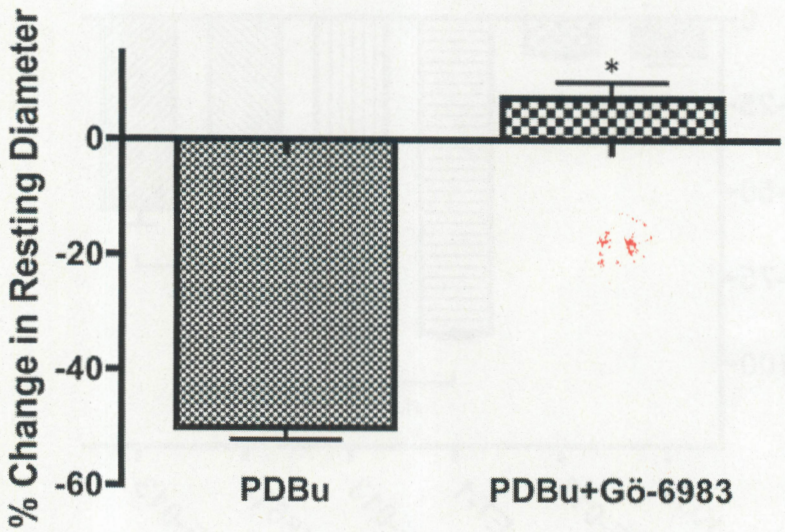
Supplementary Figure S 1



Supplementary Figure S 2



Supplementary Figure S 3



Supplementary Figure S 4