S1P activates store-operated calcium entry via receptor- and non-receptor-mediated pathways in vascular smooth muscle cells

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Am J Physiol Cell Physiol 300: C919–C926, 2011. First published January 26, 2011; doi:10.1152/ajpcell.00350.2010.—Sphingosine-1-phosphate (S1P) has been shown to modulate intracellular Ca2+ through both G protein-coupled receptors and intracellular second messenger pathways. The precise mechanism by which S1P activates store-operated calcium entry (SOCE) in vascular smooth muscle cells (VSMCs) has not been fully characterized. Because sphingolipids and Ca2+ modulate proliferation and constriction in VSMCs, characterizing the connection between S1P and SOCE may provide novel therapeutic targets for vascular diseases. We found that S1P triggered STIM1 puncta formation and SOCE in VSMCs. S1P-activated SOCE was inhibited by 2-aminoethoxydiphenyl borate (2-APB), diethylstilbestrol (DES), and gadolinium (Gd3+). SOCE was observed in VSMCs lacking either S1P2 or S1P3 receptors, suggesting that S1P acts via multiple signaling pathways. Indeed, both extracellular and intracellular S1P application increased the total internal reflection fluorescence signal in VSMCs cells transfected with STIM1-yellow fluorescent protein in a 2-APB-sensitive manner. These data, and the fact that 2-APB, DES, and Gd3+ all inhibited S1P-induced cerebral artery constriction, suggest that SOCE modulates vascular diseases associated with proliferation. From our data, we conclude that S1P triggers Ca2+ entry in VSMCs; S1P activating STIM1 and that this pathway plays a role in vessel constriction and pathological VSMC proliferation. From our data, we conclude that S1P activates SOCE in VSMCs; S1P can act extracellularly through S1P receptors or intracellularly to trigger STIM1 puncta formation and SOCE. We found increased S1P-induced SOCE in proliferative VSMCs, which correlated with increased expression levels of relevant genes. These findings suggest that S1P-induced SOCE signaling may play a role in vascular diseases associated with proliferation.

EXPERIMENTAL PROCEDURES

Materials. S1P (Avanti Polar Lipids, Alabaster, AL) was solubilized as a millimolar stock solution in a buffer containing 100 mM Tris, 145 mM NaCl, and 4 mg/ml fatty acid-free BSA, pH 9.0, for

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vessel experiments. For cell culture studies, S1P was added to a methanol/water (95:5) mixture in a glass vial, heated, and sonicated. When in solution, the remaining solvent was removed using a stream of nitrogen. A 1 mM S1P stock was prepared in 4 mg/ml fatty acid-free BSA. All other pharmacologic agents were purchased from Sigma (Sigma-Aldrich, St. Louis, MO) unless noted otherwise.

Animals and isolation of aortic VSMCs. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996), and were approved by the Institutional Animal Care and Use Committee. S1P3 receptor null mice (54) were bred and housed in our animal facility. Animals had free access to water and food. All experiments were performed using 20–25 g male mice, and C57BL/6 mice (Charles River Laboratories) were used for wild-type controls. Aortas were dissected from the animal and placed in ice-cold PBS, where they were cleaned of connective tissue and opened lengthwise, and endothelial cells were removed with a cotton swab. The aorta was then placed in 0.5 mg/ml elastase-2.0 mg/ml collagenase DMEM in the incubator; cells were dispersed at 15-min intervals until all tissue was dissociated. Digestion medium containing VSMCs was spun down at 500 g for 10 min, supernatant was removed, and cells were resuspended in complete media and plated on appropriate dishes for the desired experiment. To obtain contractile VSMCs, cells were grown for 2 days in 0.1% FBS-DMEM (supplemented with 200 U/ml penicillin, 0.2 mg/ml streptomycin). To obtain proliferative VSMCs, cells were grown for 7 days in 1.0% FBS-DMEM. All culture reagents were obtained from GIBCO (Invitrogen). Smooth muscle cell phenotype was confirmed by staining with anti-α-smooth muscle (α-SMC) actin (39): contractile VSMCs showed strong staining and retained a spindle-like shape (Supplemental Fig. S1A; Supplemental Material for this article is available online at the Journal website). Proliferative VSMCs expressed very little α-SMC actin, and their morphology had changed to that of proliferative SMCs (Supplemental Fig. S1B).

Functional studies in cerebral arteries. Adult male C57BL/6 mice were anesthetized using chloroform and killed by decapitation. The brain was removed and placed in a dissection dish filled with cold physiologic salt solution (PSS; composition in mM: 141 NaCl, 4.6 KCl, 1.7 MgSO4, 0.51 EDTA, 2.7 CaCl2, 10.0 HEPES, 1.0 MgCl2, 0.1 EGTA, 2.0 CaCl2, pH 7.4). Posterior cerebral arteries were dissected free from connective tissue. A first-order segment of the posterior cerebral artery was cannulated, pressurized, and mounted in an arteriograph (Living Systems Instrumentation, Burlington, VT) that contained PSS (37°C, pH 7.4) for 30-min equilibration. The arterial diameter was recorded using the Video Dimension analysis system, and transmural pressure was measured and controlled using a pressure servomechanism (fig. 1A), using three widely accepted SOCE inhibitors to test the hypothesis that S1P is acting through SOCE to induce Ca2+ entry: diethylaminobutyric acid (DEA-B) (13, 26, 30, 59), and Gd3+ (composition in mM: 140 NaCl, 10 HEPES, 0.09 MgCl2, 0.1 EGTA). Inhibitors were added at concentrations indicated, and Ca2+ (2 mM) was added to measure store-operated calcium influx. For Fura-2AM experiments, VSMCs were examined with an inverted microscope (Eclipse TE200-U; Nikon, Tokyo, Japan) equipped with an Epi-flatt (Nikon) and CoolSpan ER monochromator (Roper Scientific, Tucson, AZ). Image processing software (Photomatrix, HDRsoft, Montpellier, France) was used to acquire images that were taken every 8 s, monitored online, and analyzed with IPLab 3.7 software (Scanalytics, Rockville, MD). Intracellular calcium concentration ([Ca2+]i) was measured fluorometrically as absorbance of ratio at 340 and 380 nm (ΔF340/F380), with 510 nm emission. For Flu-4 experiments, VSMCs were examined with a Nikon Eclipse TiE inverted epifluorescence microscope (Nikon, Melville, NY) equipped with a ×60 oil immersion objective with 1.49 numerical aperture and Andor iXON EMCCD Camera. Flu-4 was excited at 488 nm, and emission was recorded at 510 nm; images were taken every second. Intracellular [Ca2+]i, was quantified using NIS elements.

Statistical analysis. The data were analyzed with the Student’s t-test when comparing means between two groups. P < 0.05 was considered statistically significant.

RESULTS

SIP induces SOCE in VSMCs. S1P modulates Ca2+ in VSMCs (53) and constricts brain arteries (42, 43, 48), but the precise mechanism involved in this process is not known. To test the hypothesis that S1P is acting through SOCE to induce Ca2+ entry and cause vessel constriction, we examined S1P-induced SOCE in primary aortic VSMCs. We first characterized pharmacologically the Ca2+ response in primary VSMCs (Fig. 1A), using three widely accepted SOCE inhibitors to assess the role of SOCE in S1P-induced Ca2+ entry: diethylstilbestrol (DES) (10, 55), 2-aminoethoxydiphenylborate (2-APB) (13, 26, 30, 59), and Gd3+ (10, 14, 49). S1P induced a large increase in SOCE when Ca2+ was added to the bath that was not observed when vehicle was applied followed by Ca2+ readdition. As seen in Fig. 1B, 1 μM and 10 μM DES inhibited...
S1P TRIGGERS STORE-OPERATED CALCIUM ENTRY

Fig. 1. Sphingosine-1-phosphate (S1P) induces store-operated calcium entry in vascular smooth muscle cells (VSMCs). A: representative trace of changes in Fura ratio (R) in VSMCs treated with S1P, followed by addition of Ca^{2+}. B: summary data of inhibitory effects of diethylstilbestrol (DES; 1 and 10 μM), 2-aminoethoxydiphenyl borate (2-APB; 7.5 and 75 μM), and gadolinium (Gd^{3+}; 6 μM). Veh, vehicle. C: S1P (1 μM) application after thapsigargin (SIP/TG, 5 μM) does not result in a further increase in intracellular calcium concentration ([Ca^{2+}]). Whereas TG application after S1P-induced Ca^{2+} release (TG/S1P) does cause further increase in [Ca^{2+}]. D: inhibition of L-type Ca^{2+} channels by 1 μM nifedipine does not reduce TG- or S1P-induced store-operated calcium entry (SOCE). Averages represent n = 17–28 cells per experiment from 3 individual experiments. *P < 0.05, **P < 0.01.

Role of the S1P1 receptor in S1P-induced Ca^{2+} release and wild-type VSMCs (Fig. 2, A). However, S1P-induced SOCE was the same in both receptor knockouts and wild-type VSMCs (Fig. 2, A and C). No significant differences were observed between VSMCs from S1P2 and S1P3 receptor-null mice and wild-type controls. As expected, the initial Ca^{2+} release caused by S1P application was significantly reduced in both the S1P2 and S1P3 null aortic VSMCs (Fig. 2, A and C). However, S1P-induced SOCE was the same in both receptor knockouts and wild-type VSMCs (Fig. 2, A and C). To examine the role of the S1P1 receptor in S1P-induced Ca^{2+} release and SOCE, we used VPC23019/1, an antagonist of the S1P1 receptor, with limited ability (about 30-fold less) to block S1P1 receptors; these experiments were therefore performed in S1P2–/– and S1P3–/– VSMCs to specifically examine S1P1 inhibition. S1P-induced Ca^{2+} release was completely absent when S1P1 and S1P3 receptors were blocked and knocked out, respectively (Fig. 2E), but S1P-induced SOCE remained intact (Fig. 2F).

Given that S1P-induced SOCE was intact in both S1P2 and S1P3 receptor-null VSMCs and when S1P1 was inhibited, we hypothesized that S1P was able to activate SOCE in a receptor-independent manner. We performed TIRF microscopy on VSMCs transfected with STIM1-YFP (a representative trace is shown in Fig. 3A). Application of extracellular S1P triggered an increase in STIM1-YFP that was comparable to that caused by TG application (Fig. 3B). We then loaded the cells with a photolyzable (caged) derivative of S1P (24, 38) to test the effect of intracellular S1P on STIM1 localization. A 6-s exposure to UV light resulted in an increase in STIM1-YFP TIRF after photolysis of caged S1P (Fig. 3B). Both extracellular and intracellular S1P as well as TG-induced increases in STIM1-YFP TIRF were strongly inhibited by 2-APB (75 μM, Fig. 3B) (9). Similar experiments were performed in COS-7 cells, and both intracellular and extracellular application of S1P triggered increases in TIRF of STIM1-YFP (supplemental Fig. S2). SOCE contributes to S1P-induced constriction in isolated vessels. To examine the potential functional significance of S1P-induced SOCE in VSMCs, the effect of pharmacological SOCE inhibition was examined on S1P-induced constriction of the posterior cerebral artery. 2-APB dose dependently inhibited S1P-induced constrictions (7.5 and 75 μM, Fig. 4A). Gd^{3+} (100 μM) (14, 40) and DES (10 μM) both significantly inhibited S1P-induced constriction as well. Interestingly, 2-APB also inhibited phenylephrine-induced constriction but had no effect on the constriction caused by the thromboxane A2 receptor agonist U46619 (1 μM), indicating that SOCE mediates constriction to some, but not other, constrictors (supplemental Fig. S3).

Cerebral arteries constrict to S1P, whereas aorta does not (43). However, our Ca^{2+} imaging experiments performed in VSMCs from aortic cells show that S1P induces SOCE in these cells. To examine whether different levels of STIM1, Orai, and S1P receptor expression might underlie the different response of cerebral arteries vs. aorta, we performed quantitative RT-PCR in freshly isolated arteries. STIM1 expression was 5.4-fold higher in cerebral arteries than in the aorta, and both Orai2 and Orai3 expression was higher in cerebral arteries. 2.9- and 2.4-fold, respectively (Fig. 4). Expression levels of S1P3 and S1P1 receptors were 27-fold and 12-fold higher in cerebral vessels than aortas, respectively (Fig. 4). S1P2 receptor expression was 3-fold higher in cerebral versus aortic tissue.

S1P-induced SOCE is increased in proliferative VSMCs. SOCE is known to be altered when VSMCs switch from a contractile to a proliferative phenotype (3, 32). To test the hypothesis that S1P-induced SOCE might differ in contractile
and proliferative VSMCs, we selected culture conditions to obtain either phenotype. VSMCs grown for 2 days in 0.1% FBS showed high \(/H9251/-smooth muscle actin staining and retained a spindle-like structure, whereas VSMCs grown for 7 days in 1.0% FBS showed very low \(/H9251/-smooth muscle actin staining and displayed a more fibroblast-like morphology (supplemental results, Fig. S1). While S1P-induced SOCE was significantly smaller than TG-induced SOCE in contractile VSMCs, both agents induced similar (and higher) SOCE responses in proliferative VSMCs (Fig. 5A).

Fig. 2. S1P-induced SOCE is intact when S1P receptors are genetically absent and pharmacologically inhibited in mouse aortic VSMCs. VSMCs were cultured from S1P2 and S1P3 receptor-knockout mice (KO), and SOCE in response to TG (5 \(\mu\)M) and S1P (1 \(\mu\)M) was measured. A and B: the initial calcium peak was first measured after application of TG or S1P in S1P2\(^{-/-}\) VSMCs (A), and SOCE was measured after addition of 2 mM Ca\(^{2+}\) in response to the two drugs (B). C and D: the initial calcium peak was then measured in S1P3\(^{-/-}\) VSMCs after application of TG or S1P (C), and SOCE was measured after addition of 2 mM Ca\(^{2+}\) in response to the two drugs (D). E and F: finally, we used S1P3\(^{-/-}\) VSMCs treated with VPC23019 (10 \(\mu\)M, 30 min) to measure the initial calcium peak after application of TG or S1P (E) and SOCE after addition of 2 mM Ca\(^{2+}\) in response to the two drugs (F). WT, wild type. Averages represent \(n = 10–34\) cells from 3 individual experiments. *\(P < 0.05\), **\(P < 0.01\).

Fig. 3. Extracellular and intracellular S1P applications induce formation of STIM1-yellow fluorescent protein (YFP) puncta. VSMCs were transfected with STIM1-YFP. A: representative trace of total internal reflection fluorescence (TIRF) of STIM1-YFP. S1P (1 \(\mu\)M) application was followed by addition of 2-APB (75 \(\mu\)M). B: average increase in intensity and decrease after 2-APB application: stimulated (time \(t = 180\) s minus basal \(t = 0\)) is shown for each condition [TG, 5 \(\mu\)M; S1P, 1 \(\mu\)M; caged-S1P (c-S1P), 1 \(\mu\)M]. C: representative images of STIM1-YFP puncta formation triggered by extracellular S1P (1 \(\mu\)M) application as seen in TIRF images; 2-APB (75 \(\mu\)M) reverses the accumulation of STIM1 at the plasma membrane. Cells were stimulated at \(t = 30\) s with either extracellular application of TG or S1P, or a 6-s UV flash to uncage S1P. Averages represent \(n = 4–15\) cells from 3 individual experiments. *\(P < 0.05\), **\(P < 0.01\).
Because increased STIM1 and Orai1 levels have been reported to coincide with VSMC switch from a contractile to a proliferative phenotype and associated SOCE alterations (32), we next examined whether the increased SOCE in response to S1P in proliferative VSMCs was associated with altered expression of STIM1 and Orai (Fig. 5). STIM1 expression was significantly increased (2.6-fold) in primary aortic VSMCs grown under proliferative conditions compared with those grown under contractile conditions. Orai1 and Orai2 expression was also increased (1.8- and 2-fold, respectively) in VSMCs grown under proliferative conditions compared with those grown under contractile conditions. Orai1 and Orai2 expression was also increased (1.8- and 2-fold, respectively) in VSMCs grown under proliferative conditions compared with those grown under contractile conditions. Because the increased S1P-induced SOCE response in proliferative VSMCs suggested S1P receptor upregulation, we compared the expression levels of the three S1P receptors potentially involved in VSMC signaling (17, 50): S1P1 (4.9-fold) and S1P3 (2.3-fold) receptor expression were both significantly increased in VSMCs grown in proliferative culture conditions (Fig. 5). Expression of S1P2 receptor was not significantly different between the two culture conditions.

**DISCUSSION**

This study shows that S1P can act through receptor-dependent or -independent mechanisms to activate STIM1 and SOCE in vascular smooth muscle cells. We found that this pathway contributes to constriction of cerebral arteries and is upregulated in proliferative VSMCs. These findings emphasize the physiologic relevance of this pathway and suggest a novel signaling mechanism for potential therapeutic intervention in vascular disease associated with VSMC proliferation.

We observed S1P-induced STIM1 puncta formation using TIRF microscopy, indicating that S1P can trigger activation of SOCE. While puncta formation has been documented in cells lines using thapsigargin (36), our observation is important because it is made in primary VSMCs, using an agent, S1P, known to play a major role in the cardiovascular system under both physiological (6) and pathological conditions (11, 15). Although S1P has been shown to modulate intracellular Ca$^{2+}$ stores in VSMCs (6, 8, 23), this is the first pharmacological characterization of S1P-induced Ca$^{2+}$ entry and of direct intracellular effects of S1P in these cells. Our direct observation of STIM1 activation in VSMCs is important because STIM1 is now known to be involved in other processes than Ca$^{2+}$ signaling (19). Three inhibitors of SOCE (2-APB, DES, Gd$^{3+}$) blocked S1P-induced Ca$^{2+}$ entry in primary VSMCs. Although these inhibitors can have nonspecific effects, they are routinely used to study
SOCE (as reviewed in Refs. 10, 13, 20, and 37). Because there is still debate in the literature on the coupling of STIM1 with TRPC and Orai channel, further experiments are needed to definitely show whether STIM1 only associated with Orai in VSMCs to trigger SOCE. The finding that 2-APB significantly inhibited S1P-induced SOCE at low (7.5 μM) but not at high, concentrations (75 μM) could reflect the known ability of 2-APB to activate Orai3 channels at higher (50 μM) concentrations (9, 59). Our data in primary VSMC demonstrate that 2-APB can completely reverse STIM1 puncta formation. Similar findings have been published by other laboratories, and it is hypothesized that 2-APB not only has direct actions on channel inhibition but also uncouples STIM1 from Orai and reverses STIM1 oligomerization (9, 47). S1P-induced puncta formation of STIM1, taken together with the consistent effect of three different inhibitors, strongly indicates that S1P activates SOCE in VSMCs. Our data are in agreement with the literature demonstrating a role for SOCE in refilling Ca\(^{2+}\) stores in VSMCs and in the regulation of vascular reactivity (30, 46, 52, 56, 58).

As extensively documented, S1P1-3 are expressed in VSMCs while S1P4 and S1P5 are absent (for review see Ref. 25). Although signaling through S1P2 and S1P3 receptors has been shown to contribute to S1P-induced vasoconstriction (22, 42, 43), the present results obtained in S1P2 and S1P3 receptor-null aortic VSMCs and in VSMCs in which S1P1 was inhibited with VPC23019 demonstrate that S1P can signal either via other receptors or via intracellular mechanisms to induce SOCE. To test this hypothesis, we used a previously characterized photolyzable S1P derivative to release S1P inside VSMCs (24). Caged S1P has been shown to release Ca\(^{2+}\) from endoplasmic reticulum Ca\(^{2+}\) stores (24), but downstream SOCE activation has not been reported. Here we show that caged S1P activates SOCE by visualizing STIM1 recruitment to the plasma membrane. It might be argued that S1P generated from photolysis of intracellular caged S1P might leech out and activate G protein-coupled receptor, but this possibility was ruled out in a previous study using caged S1P (24). Therefore our results strongly suggest that intracellular S1P can activate SOCE. The exact mechanisms of this novel signaling pathway have yet to be characterized.
We then set out to highlight the physiologic relevance of this pathway under physiological (cerebral vessel constriction) and potentially pathological conditions (VSMC proliferation). The functional significance of primary culture data was established in intact vessels by demonstrating the inhibition of S1P-induced constriction of the posterior cerebral artery by 2-APB, Gd³⁺, and DES. While inhibition by 2-APB of phenylephrine-induced cerebral artery constrictions has been previously reported (30), the lack of effect of 2-APB on thromboxane A₂ receptor-induced constriction (U46619) suggests possible differences in the signaling pathways of S1P (or phenylephrine) and thromboxane A₂ and their differential ability to activate SOCE.

S1P-induced SOCE was demonstrated in aortic VSMCs, and the posterior cerebral artery was used to assess the role of SOCE in S1P-induced vessel constriction; we hypothesized that differences in expression patterns of STIM1, Orai, and S1P receptors in the two vascular beds could explain why cerebral vessels constrict to S1P while aorta does not. S1P receptor expression studies have been previously compared in different vessels (6), leading to the suggestion that higher expression of S1P receptors in cerebral arteries could account for the fact that S1P elicits a much stronger constriction in cerebral arteries than in the aorta. Based on our finding that all components of the SOCE pathway except Orai are more highly expressed in cerebral arteries than in aorta, and S1P receptors are more highly expressed in the cerebral vessels as well, we hypothesize that differential expression of proteins of the SOCE pathway might also play a key role in the vessel specific constriction to S1P.

VSMC proliferation is a critical component of vascular dysfunction such as diabetes and atherosclerosis. Because SOCE is upregulated in proliferative VSMCs (32), we hypothesized that S1P-induced SOCE might be specifically upregulated in proliferative VSMCs, which was indeed the case. The finding of increased SOCE in response to S1P is particularly interesting in light of proliferative vascular diseases in which S1P is known to play a role (6, 7). It is tempting to speculate that STIM1 and Orai upregulation is the cause of the increase in SOCE that we see in proliferative VSMCs. In addition, proliferative VSMCs also showed increased expression of S1P receptor mRNAs. This may explain why S1P-induced SOCE was comparable to that of TG-induced SOCE in proliferative VSMCs, while both the S1P and TG responses were increased in these cells.

Emphasizing the relevance of our findings to proliferative VSMC diseases, changes in S1P receptors in response to acute balloon injury were found in the rat carotid artery, leading to the conclusion that S1P₁/S1P₃ receptors promote proliferation (51). Dysregulation of SOCE has been implicated in diabetes, atherosclerosis and hypertension (12, 14, 57) and SOCE has also been shown to phosphorylate cyclic AMP response element-binding (CREB) protein and upregulate proliferative genes (34, 35). With the new knowledge that S1P can activate SOCE and that S1P receptors, STIM1, Orai, and SOCE are all upregulated in proliferative VSMCs, it will be interesting to study the link between S1P and SOCE in proliferative vascular diseases. It is likely that inhibition of this pathway might reduce vascular dysfunction (increased contractility/proliferation) in these conditions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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