Sphingosine 1-Phosphate-induced Mobilization of Intracellular Ca$^{2+}$ Mediates Rac Activation and Adherens Junction Assembly in Endothelial Cells*

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Sphingosine 1-phosphate (S1P) ligation of endothelial differentiation gene-1 receptor coupled to the heterotrimeric G protein, G$i$, promotes endothelial barrier strengthening via Rac-dependent assembly of adherens junctions (AJs). However, the mechanism of Rac activation induced by S1P stimulation remains unclear. In live endothelial cells expressing GFP-Rac, we observed that S1P induced the translocation of Rac to intercellular junctions, resulting in junctional sealing. We investigated the role of intracellular Ca$^{2+}$ in signaling Rac activation and the enhancement of endothelial barrier function. We observed that S1P activated the release of Ca$^{2+}$ from endoplasmic reticulum stores, and subsequent Ca$^{2+}$ entry via lanthanum-sensitive store-operated Ca$^{2+}$ channels (SOC) after store depletion. Inhibition of G$i$, phospholipase C, or inositol triphosphate receptor prevented the S1P-activated increase in intracellular Ca$^{2+}$ as well as Rac activation, AJ assembly, and enhancement of endothelial barrier. Chelation of intracellular Ca$^{2+}$ with BAPTA blocked S1P-induced Rac activation, indicating the requirement for Ca$^{2+}$ in the response. Inhibition of SOC by lanthanum or transient receptor potential channel 1 (TRPC1), a SOC constituent, by TRPC1 antibody, failed to prevent S1P-induced Rac translocation to junctions and AJ assembly. Thus, our results demonstrate that S1P promotes endothelial junctional integrity by activating the release of endoplasmic reticulum-Ca$^{2+}$, which induces Rac activation and promotes AJ annealing.

The vascular endothelium forms the barrier between intravascular and extravascular compartments. Vascular endothelial (VE)-cadherin$^1$ and its associated proteins, catenins, are

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$^*$ The abbreviations used are: VE-cadherin, vascular endothelial cadherin; S1P, sphingosine 1-phosphate; Edg, endothelial differentiation gene; TER, transendothelial electrical resistance; AJs, adherens junctions; IP$_3$, inositol triphosphate; 2-APB, 2-aminoethoxydiphenyl borate; SOCs, store operated Ca$^{2+}$ channels; TRPC, transient receptor potential channel; ER, endoplasmic reticulum; HPAE cell, human pulmonary arterial endothelial cell; HUVE cell, human umbilical venular endothelial cell; HBSS, Hanks’ balance salt solution; [Ca$^{2+}$]$i$, intracellular calcium concentration; Abs, antibodies; EGFP, enhanced green fluorescent protein; PTX, pertussis toxin; PLC, phospholipase C; SPH, the structural units of adherens junctions (AJs), having both endothelial barrier-stabilizing and signaling functions (1). VECadherins are transmembrane glycoproteins that adhere homotypically in a Ca$^{2+}$-dependent manner through their ectodomains. Cadherin cytoplasmic tail binds b-catenin, which links it to the actin cytoskeleton via a-catenin (1). Catenin-dependent interaction of cadherin with actin cytoskeleton enables cadherins to provide the tensile strength required to maintain endothelial barrier integrity (2). Mechanisms promoting instability and weakening of AJs increase endothelial permeability and induce protein-rich exude characteristics of tissue inflammation (1, 3, 4).

Sphingosine 1-phosphate (S1P), the lipid mediator released from activated platelets, has recently been shown to have a potent vascular endothelial barrier protective effect (5–7). S1P binds to endothelial differentiation gene-1 (Edg-1) receptor leading to activation of heterotrimeric G proteins of the G$i$ class (6, 8, 9), which signals enhancement of endothelial barrier function (6, 7, 9, 10). Studies have implicated the monomeric GTPase Rac as the downstream effector mediating increased endothelial barrier function (6, 9, 11, 12). Although the precise mechanisms are unclear, Rac may function by inducing formation of the cortical actin band and assembly of AJ proteins (6, 9, 11–13).

Studies showed that S1P induces an increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$i$) in various cell types, including endothelial cells (14–16). The increase in [Ca$^{2+}$]$i$, occurred because of release of Ca$^{2+}$ from endoplasmic reticulum (ER) stores through inositol 1,4,5-trisphosphate (IP$_3$)-sensitive channels as well as activation of plasma membrane non-selective Ca$^{2+}$ channels (17–19). Although the S1P-induced increase in [Ca$^{2+}$]$i$, appears to be mediated via a G$i$-dependent pathway (15, 16), it is not known whether the rise in [Ca$^{2+}$]$i$, contributes to the S1P-induced increase in endothelial barrier function. Recent evidence indicates that activation and translocation of Rac requires intracellular Ca$^{2+}$ (20). We therefore surmised that S1P induces an increase in [Ca$^{2+}$]$i$, leading to Rac activation, thereby serving as a critical intermediate signal by which G$i$ promotes junctional stability.

EXPERIMENTAL PROCEDURES

Materials—S1P and sphingosine (SPH) were purchased from Avanti Polar Lipids (Alabaster, AL). Human pulmonary arterial endothelial (HPAE), human umbilical venular (HUVE) cells, and endothelial growth media were from Clonetics (San Diego, CA). Fura 2-AM, BAPTA-AM (a high-affinity calcium chelator), and Alexa-labeled secondary antibodies (Abs) were purchased from Molecular Probes (Eugene, OR). Pertussis toxin was purchased from Sigma and 2-aminoethoxydiphenyl borate (2-APB) (IP$_3$ receptor antagonist) and U73122 sphingosine; GDI, guanine nucleotide dissociation inhibitor; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid.

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**Fig. 1. Effects of S1P on intracellular Ca\(^{2+}\) concentration, Rac activity, and endothelial barrier function.** A, Western blot showing expression of Edg-1 in HPAE (HP) and HUVE (HU) cells. Cells were lysed with SDS sample buffer, separated on a 10% gel, and Western blotted with Edg-1 Ab (top) or actin Ab (bottom) to show equal protein loading. Results are representative of at least two experiments. B, Ca\(^{2+}\) transients in HPAE cells. Cells were loaded with fura 2-AM after which they were stimulated with the indicated concentrations of S1P to measure the increase in cytosolic Ca\(^{2+}\) concentration. As results from the experiments were similar, data from a representative experiment are shown (n = 2). C and D, Rac activity in response to S1P. Cells stimulated with S1P were lysed to assay Rac activity as described under “Experimental Procedures.” Rac activity is evident by the increased amount of GTP-bound Rac (C and D, bottom) compared with the amount of Rac in whole cell lysates (C and D, bottom). In panel D, Rac activity was determined 5 min after stimulation with S1P. Results are representative of two to three independent experiments. E, S1P enhances endothelial barrier function. HPAE cells plated on gold electrodes were stimulated with the indicated concentrations of S1P or SPH to determine TER (a measure of endothelial barrier function) across the monolayer.

(phospholipase C (PLC) inhibitor) were from Calbiochem (La Jolla, CA). Anti-goat VE-cadherin Ab and Edg-1 were from Santa Cruz Biotechnology (San Diego, CA); anti-mouse β-catenin, anti-mouse p120-catenin, and anti-mouse Rac Abs were purchased from BD Biosciences (San Diego, CA); anti-mouse actin was from Sigma; and horseradish peroxidase secondary Abs were purchased from Jackson Immunoresearch Labs (West Grove, PA). Electrodes for transendothelial electrical resistance measurements were purchased from Applied Biosciences (Troy, NY). Electroporation equipment and 0.4-mm cuvettes for electroporating eukaryotic cells were from Bio-Rad. The 4-well LabTek chambers for live cell confocal imaging were purchased from Nalge (Fisher, IL).

**Endothelial Cell Cultures—**HPAE or HUVE cells were cultured in a T-75 flask coated with 0.1% gelatin in EBM-2 medium supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO\(_2\) and 95% air until they formed a confluent monolayer. Cells from each of the primary flasks were dehumidified atmosphere of 5% CO\(_2\) and 95% air until they formed a confluent monolayer. Cells from each of the primary flasks were detached with 0.025% trypsin/EDTA and plated on 100-mm dishes for the Rac pull-down assay, 5-well electrodes for electrical resistance measurements, LabTek chambers for live cell confocal imaging, or 12-mm coverslips for immunofluorescence microscopy as described below. In all experiments, the monolayer of HPAE was incubated for 1–2 h in MCDB 131 serum-free medium before treatment with inhibitors or agonists. In all experiments, cells were used between passages 4 and 8.

**Cell Transfection—**The EGFP vector containing wild-type Rac was a generous gift from Dr. M. Phillips (New York University School of Medicine, New York), pCMV5 vector and vectors containing dominant negative Rho mutant (N19Rho) or dominant negative Rac mutant (N17Rac) were kindly provided by Dr. T. Kozasa (University of Illinois, Chicago, IL). The DNA was purified using the Endo-free Qiagen kit. For cell transfection, HPAE cells were trypsinized, mixed with DNA of interest and salmon sperm DNA in 0.4-cm cuvettes, followed by electroporation at 950 microfarads and 180 mV using the Bio-Rad electroporator. The cells were used at 24 h after transfection when there was evidence of the expression of protein.

**Immunofluorescence and Live Cell Imaging—**Cells were stimulated with S1P (1 μM) for the indicated times, rinsed quickly with ice-cold HBSS, and fixed with 2% paraformaldehyde. Cells were permeabilized for 3 min with 0.1% Triton X-100 in HBSS followed by incubation for 20 min with 1% ovalbumin. Cells were then incubated with anti-VE-cadherin, anti-β-catenin, or anti-p120-catenin Abs. Following incubation, cells were rinsed and incubated with appropriate Alexa-labeled secondary Abs. Cells were then rinsed 6 times with HBSS and mounted with anti-fade media. Cells were viewed using a 63 × 1.2 NA objective Zeiss LSM-510 META confocal microscope using appropriate filters.

For live cell imaging, cells transfected with EGFP-Rac were washed with HBSS supplemented with 10 mM HEPES and placed on confocal microscope. A series of time-lapse confocal images were acquired at 45-s intervals following S1P stimulation using EGFP excitation laser lines (21). Only still images of this movie at the indicated times are shown in Figs. 2A and 2B.

**Cytosolic Ca\(^{2+}\) Measurement—**Increase in intracellular Ca\(^{2+}\) was measured using the Ca\(^{2+}\)-sensitive fluorescent dye fura 2-AM (21, 22). For loading cells with fura 2-AM, cells grown on 25-mm coverslips were incubated with 3 μM fura for 15 min at 37 °C. Cells were then washed 2 times with HBSS and imaged using an Attofluor Ratio Vision digital fluorescence microscopy system (Atto Instruments, Rockville, MD) equipped with a Zeiss Axiovert S100 inverted microscope and a F-Fluar ×40, 1.3 NA oil immersion objective. Regions of interest in individual cells were marked and excited at 334 and 380 nm with emission at 520 nm. The 334/380-excitation ratio, which increased as a function of intracellular Ca\(^{2+}\) concentration, was captured at 5-s intervals.

**Whole Cell Patch Clamp Recording in Endothelial Cells—**Patch clamp recording in the whole cell configuration was performed as described on HPAE cells attached to a coverslip (17, 22, 23). Standard extracellular and pipette solutions (22) were provided. SOC currents were measured at the holding potential (−50 mV) following application of S1P. All experiments were performed at room temperature.

**Western Blotting—**HPAE or HUVE monolayers were washed with 1× phosphate-buffered saline and lysed with SDS sample buffer. Proteins from each lysate were separated by electrophoresis on a 15% polyacrylamide gel, and transferred to nitrocellulose membrane for Western blotting with Edg-1 or actin Abs.

**Rac Activity Assay—**pGEX-2T containing PAK binding domain was kindly provided by Dr. I. Lopez (University of Illinois, Chicago, IL). Bacterial expressed glutathione S-transferase-PAK binding domain was purified from isopropyl-1-thio-D-galactopyranoside (1 mM)-induced DH5α cells previously transformed with the appropriate plasmid as described (24). Confluent HPAE cells grown in 100-mm dishes were stimulated for the indicated times with 0.1 to 1 μM S1P. Cells were then quickly washed with ice-cold Tris-buffered saline and lysed in buffer.
RESULTS

S1P-induced Increase in Intracellular Ca\(^{2+}\) Is Associated with Increased Rac Activity and Enhancement of Endothelial Barrier Function—Studies show that S1P enhances barrier function via Edg-1 receptor-mediated activation of G\(_i\) signaling (6–9). In other studies, S1P has been shown to increase intracellular Ca\(^{2+}\) (14–16). We therefore determined the effects of S1P on intracellular Ca\(^{2+}\), Rac activity, and barrier function in HPAE cells. As shown in Fig. 1A, Edg-1 receptor is expressed in HPAE cells. In fura 2-loaded HPAE cells, S1P increased intracellular Ca\(^{2+}\) in a dose-dependent manner (Fig. 1B). We show using the glutathione S-transferase-PAK binding domain that S1P induced the activation of Rac within 2 min, in agreement with previous findings (6, 9, 13, 26), and the response was sustained for up to 20 min (Fig. 1C). Maximum increase in Rac activity was observed at the S1P concentration of 1 \(\mu\)M S1P in a dose-dependent manner rapidly enhanced endothelial barrier function as reflected by an increase in TER (Fig. 1E). The possibility that the effect of S1P on barrier stabilization is not the result of S1P metabolites such as SPH, we determined the effect of S1P on barrier stabilization.

S1P-induced Ca\(^{2+}\) Signaling Regulates Rac Activation

(50 mM Tris, pH 7.5, 10 mM MgCl\(_2\), 0.3 M NaCl, 2% Igepal, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 14,000 \(\times\) g at 4 °C for 2 min and equal volumes of cell lysates were incubated with glutathione S-transferase-PBD beads at 4 °C for 1 h. The beads were washed three times with wash buffer (25 mM Tris, pH 7.5, 30 mM MgCl\(_2\), 40 mM NaCl, 10 \(\mu\)g/ml each of aprotenin and leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride). Rac bound to beads was eluted by boiling each sample in Laemml sample buffer. Eluted samples from beads and total cell lysate were then electrophoresed on 15% SDS-PAGE gels, transferred to nitrocellulose, blocked with 5% nonfat milk, and analyzed by Western blotting using a monoclonal anti-Rac Ab. In addition, cell lysate from each sample was Western blotted with anti-Rac Ab to confirm equal protein loading in each lane.

Transendothelial Electrical Resistance Measurement—The time course of endothelial cell retraction, a measure of increased endothelial permeability, was assessed by recording transendothelial electrical resistance (TER) as described (25). Briefly, HPAE cells grown on the electrodes were serum-deprived for 1 h and then stimulated with the indicated concentrations of S1P to determine the TER across the monolayer. The small electrode and larger counterelectrode were connected to a phase-sensitive lock-in amplifier. A constant current of 1 mA was supplied by a 10-V 4 KHz alternating current connected serially to a 1 M SPH slowly increased TER, which at 12 min was only 17% of 1 \(\mu\)M S1P-induced TER (Fig. 1E). Thus, the S1P-induced increase in endothelial barrier function is not the result of S1P metabolism into SPH. As the maximum increase in intracellular Ca\(^{2+}\), Rac activity, and barrier function in HPAE cells was observed at the S1P concentration of 1 \(\mu\)M S1P in a dose-dependent manner rapidly enhanced endothelial barrier function as reflected by an increase in TER (Fig. 1E). The possibility that the effect of S1P on barrier stabilization is not the result of S1P metabolites such as SPH, we determined the effect of S1P on barrier stabilization.

S1P Induces Rac-dependent Assembly of Endothelial Junctions—We investigated the dynamics of Rac using time-lapse imaging of GFP-Rac to determine...
the effects of S1P in translocating Rac to the inter-endothelial junctions and junctional sealing. We used HPAE cells expressing low levels of GFP-Rac for the imaging studies. In unstimulated cells, Rac was primarily localized near the perinuclear area with some present at the cell contact sites (Fig. 2A). Application of S1P resulted in redistribution of Rac to cell junctions within minutes, in association with forming intercellular junctions (arrow shows the loss of interjunctional gaps following addition of S1P) (Fig. 2A). We next determined the effects of S1P in inducing adherens junction assembly. Cells were stimulated for 30 min with S1P and then they were fixed and stained with β-catenin or VE-cadherin Abs. As shown in Fig. 2B, S1P promoted the assembly of β-catenin and VE-cadherin in junctions in the form of a thick band, which effectively sealed the junctions. We contrasted the roles of Rho and Rac in S1P-induced enhancement of the endothelial barrier by determining TER. HPAE cells transfected with vector, N17Rac (dominant negative Rac), or N19Rho (dominant negative Rho) were seeded onto gold-plated electrodes, and then stimulated with S1P. S1P failed to promote the endothelial barrier in cells overexpressing dominant negative Rac mutant (Fig. 2C and D). However, S1P-induced barrier enhancement was not affected in cells expressing dominant negative Rho mutant (Fig. 2C and D). Altogether, these findings indicate that Rac is involved in signaling S1P-induced endothelial barrier enhancement by promoting assembly of AJs.

**Role of Ca\textsuperscript{2+} Signaling in S1P-induced Endothelial Barrier Enhancement—**Studies show that S1P induces an increase in intracellular Ca\textsuperscript{2+} by a G\textsubscript{i}-PLC-mediated pathway (14–16). We therefore addressed the possibility that a rise in intracellular Ca\textsuperscript{2+} is responsible for signaling Rac activation, and thus promotes endothelial barrier enhancement in response to S1P. We pretreated HPAE cells with pertussis toxin (PTX), to block G\textsubscript{i} activation, and stimulated cells with S1P and determined the changes in TER, cytosolic Ca\textsuperscript{2+}, and Rac activity. S1P failed to increase intracellular Ca\textsuperscript{2+} in PTX-pretreated cells (Fig. 3, A and B). Also inhibition of G\textsubscript{i} prevented the increase in Rac activity and TER in response to S1P (Fig. 3, C and E). To determine the role of PLC in mediating the S1P response, we used U73122, an inhibitor of PLC (27). We found that pretreatment with U73122 prevented the S1P-induced rise in intracellular Ca\textsuperscript{2+} (Fig. 3, A and B). PLC inhibition also prevented Rac activation in response to S1P (Fig. 3D) and barrier function enhancement (Fig. 3F). We also observed that the basal activity of Rac was reduced in HPAE cells pretreated with either PTX or U73122 (Fig. 3, C and D), indicating that normal G\textsubscript{i} and PLC activity is required for maintaining Rac activity under basal conditions. Thus, S1P activates endothelial barrier enhancement via a pathway involving G\textsubscript{i}, PLC, increase in intracellular Ca\textsuperscript{2+}, and this in turn leads to Rac activation. To determine the possibility that S1P-activated Ca\textsuperscript{2+} mobilization and Rac-mediated enhancement of barrier function are casually related, we used the membrane permeant Ca\textsuperscript{2+} chelator BAPTA-2AM. Fig. 4, A and B, shows the effects of BAPTA-AM on S1P-induced Rac activation and adherens junction assembly. Pretreatment with BAPTA-AM alone interfered with Rac activity and disassembly of adherens junctions. S1P failed to induce Rac activation and assembly of AJs in this experiment (Fig. 4A). Simultaneous measurement of intracellular Ca\textsuperscript{2+} confirmed that BAPTA-AM effectively blocked the S1P-induced intracellular Ca\textsuperscript{2+} rise (Fig. 4, C and D). Thus, the results show that intracellular Ca\textsuperscript{2+} is required...
for S1P-mediated Rac activation and enhancement of endothelial barrier function.

**Ca\textsuperscript{2+}** Release from Stores Is a Requirement for S1P-induced Endothelial Barrier Enhancement—S1P may increase intracellular Ca\textsuperscript{2+} concentration by mobilizing the release of Ca\textsuperscript{2+} from ER stores and subsequently activating membrane Ca\textsuperscript{2+} entry through non-selective cation channels (14–16); thus, we addressed the component of Ca\textsuperscript{2+} transient responsible for enhancing the endothelial barrier. We used various strategies to determine the contribution of ER-Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} entry mediated by plasmalemmal Ca\textsuperscript{2+} channels. The two phases of Ca\textsuperscript{2+} transient were separated by the “add-back protocol” (Fig. 5A). Under Ca\textsuperscript{2+}-free bath conditions, S1P elicited the Ca\textsuperscript{2+} transient because of release of sequestered Ca\textsuperscript{2+} into cytoplasm. The sustained component of Ca\textsuperscript{2+} transient appeared when extracellular Ca\textsuperscript{2+} was restored to the bath medium, signifying entry of extracellular Ca\textsuperscript{2+} (Fig. 5A). To determine the contribution of non-selective SOC plasma membrane channels in regulating S1P-mediated Ca\textsuperscript{2+} response, we used the whole cell patch clamp technique. After the whole cell configuration was achieved, we applied S1P to cells, and measured the resulting SOC that we operationally defined as the La\textsuperscript{3+}-sensitive inward current at −50 mV. Continuous current recordings show the rapid development of a La\textsuperscript{3+}-sensitive inward current induced by S1P (Fig. 5, B and D). There was no current in the absence of S1P, indicating that the current was not the result of a nonspecific leak (Fig. 5, C and D). When cells were pretreated with IP3 receptor antagonist 2-APB, the release and store-dependent Ca\textsuperscript{2+} entry were abolished (Fig. 6, A–C). 2-APB pretreatment not only reduced basal Rac activity and disrupted adherens junction but also prevented S1P-induced Rac activation (Fig. 6D) and AJs assembly (Fig. 6E); thus, indicating the key role of the release of ER-Ca\textsuperscript{2+} in the mechanism of SIP-induced Rac activation and endothelial barrier enhancement.

In another experiment, we used the trivalent cation La\textsuperscript{3+}, a known blocker of non-selective Ca\textsuperscript{2+} channels, to address the role of SOCs in S1P responses. As TRPC1 plays a major role in regulating SOC-induced Ca\textsuperscript{2+} entry in human endothelial cells (22, 28, 29), we used a specific TRPC1 Ab raised against the extracellular epitope of TRPC1 (22) to determine the role of TRPC1 in the mechanism of SIP-induced barrier enhancement. Fig. 7A shows that addition of La\textsuperscript{3+} rapidly abolished the Ca\textsuperscript{2+} entry phase, indicating that Ca\textsuperscript{2+} entry is mediated via SOCs. However, in the presence of La\textsuperscript{3+}, AJ assembly induced by SIP persisted (Fig. 7B). Pretreatment with TRPC1 Ab prevented the influx of Ca\textsuperscript{2+} following Ca\textsuperscript{2+} store depletion (Fig. 8A), whereas control IgG had no effect. In these experiments, TRPC1 Ab did not significantly reduce the ER-Ca\textsuperscript{2+} release (Fig. 8A). These findings indicate that TRPC1 in endothelial cells is required for Ca\textsuperscript{2+} entry via SOCs in response to S1P. However, inhibition of TRPC1 function affected neither S1P-
FIG. 5. S1P activates store-operated Ca\(^{2+}\) entry. A, cells were loaded with fura 2-AM, and stimulated with S1P in the absence of extracellular Ca\(^{2+}\) to deplete ER Ca\(^{2+}\). This was followed by repletion of \([\text{Ca}^{2+}]_o \) (1.5 mM) to determine Ca\(^{2+}\) entry. B and D, S1P-induced activation of lanthanum-sensitive store-operated currents recorded continuously at the holding potential (~50 mV). B shows a representative trace of S1P-induced inward currents as reflected by downward deflection in the current trace. C shows background current in untreated cells. D, data shown as mean ± S.E. of maximum steady-state current density (pA/pF) above basal levels in the conditions indicated (n = 4). In B and C, the dotted line marks zero current level.

FIG. 6. Inhibition of IP\(_3\) receptor prevents S1P-induced increase in intracellular Ca\(^{2+}\) concentration, Rac activation, and adherens junction assembly. A, cells were pretreated without or with 100 \(\mu\)M APB for 15 min. Cells were loaded with fura 2-AM, and stimulated with S1P in the absence of extracellular Ca\(^{2+}\) to deplete ER Ca\(^{2+}\). This was followed by repletion of \([\text{Ca}^{2+}]_o \) (1.5 mM) to determine Ca\(^{2+}\) entry. B, plot shows mean ± S.E. for the S1P-induced increase in Ca\(^{2+}\) from multiple experiments calculated as the maximum increase over the basal value under various experimental conditions (n = 3). * indicates significant reduction in Ca\(^{2+}\) release from stores as well as Ca\(^{2+}\) entry in cells treated with 2-APB. C, cells pretreated without or with 100 \(\mu\)M APB for 15 min were stimulated with 1 \(\mu\)M S1P to assay Rac activity at the indicated times. Rac activity is evident by the increased amount of GTP-bound Rac (top) compared with the amount of Rac in whole cell lysates (bottom). Data are representative of three separate experiments. D, HPAE cells plated on glass coverslips were left untreated or were treated with 100 \(\mu\)M APB for 15 min. Cells were then stimulated with 1 \(\mu\)M S1P for 30 min, fixed, and stained with anti-p120 catenin Ab after which they were viewed under confocal microscope using appropriate filters. Bar represents 10 \(\mu\)m.
The increase in intracellular Ca\textsuperscript{2+} concentration secondary to release from ER stores was a critical factor in mediating the S1P-induced Rac activation and AJ assembly because blocking ER Ca\textsuperscript{2+} release using 2-ABP prevented these responses. Thus, our data show that S1P induces endothelial barrier enhancement by activation of G\textsubscript{i}, thereby mediating PLC-dependent release of Ca\textsuperscript{2+} from ER stores via IP\textsubscript{3}-sensitive channels. Increased intracellular Ca\textsuperscript{2+} in turn promotes endothelial barrier function by activating the Rac-dependent assembly of AJs.

Several studies have shown that S1P acts through various pathways to mediate PLC-dependent release of intracellular Ca\textsuperscript{2+} in endothelial cells (17, 19, 21, 22, 29). Increased intracellular Ca\textsuperscript{2+} is required to maintain normal Rac function, integrity of AJs, and endothelial barrier function.

Our findings reinforce and amplify previous evidence that Ca\textsuperscript{2+} signaling is an important determinant of Rac activation (20); however, the mechanism for Ca\textsuperscript{2+}-signaling-dependent Rac activation is unknown. Rac activation requires upstream effectors that convert Rac-GDP (inactive state) to Rac-GTP (active state) (30). Rac is held inactive by guanine nucleotide dissociation inhibitor (GDI)-1. Dissociation of GDI-1 from the Rac-GDP-GDI complex is required for GTP exchange by guanine nucleotide exchange factors such as Tiam-1 and Vav2 (31, 32). Evidence suggests that Ca\textsuperscript{2+} signaling can activate Rac by stimulating dissociation of GDI-1 from the Rac-GDI complex (20). In addition, Tiam-1 activation may occur by a Ca\textsuperscript{2+}-dependent pathway (33), which may lead to Rac activation.

A rise in intracellular Ca\textsuperscript{2+} is an important signal mediating the increase in endothelial permeability in response to agonists, thrombin, vascular endothelial growth factor, and hystamine (17, 19, 21, 22, 29, 34). Increased intracellular Ca\textsuperscript{2+} activates myosin light chain kinase, which phosphorylates myosin light chain resulting in actinomysin-driven endothelial contraction (4). RhoA GTPase, which is activated by the Ca\textsuperscript{2+}-dependent protein kinase C-\alpha pathway, also increases myosin light chain phosphorylation via RhoA kinase-mediated inhibition of myosin light chain phosphatase (4, 35). Contraction of endothelial cells leads to inter-endothelial gap formation by disruption of intercellular junctions (1, 4). Thus, we addressed the contribution of SOCs in the mechanism of S1P-induced barrier enhancement. We showed that S1P-induced store depletion in regulating Rac activation, AJ assembly, and barrier enhancement in response to S1P. Altogether, the results demonstrate that S1P-induced release of Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive ER stores is a critical requirement for Rac activation, AJ assembly, and increased barrier function.

**DISCUSSION**

S1P has been shown to increase endothelial barrier function by promoting the formation of the cortical actin band that promotes the apposition of AJs and junctional annealing (6, 7, 9, 10, 12). As intracellular Ca\textsuperscript{2+} signaling is a key determinant of endothelial permeability (17, 19, 21, 22, 29), we addressed the possible role of S1P-activated rise in intracellular Ca\textsuperscript{2+} in the mechanism of Rac activation and enhancement of endothelial barrier. Our results demonstrate the essential role of release of Ca\textsuperscript{2+} from ER stores in signaling Rac-mediated junctional assembly and barrier integrity. We showed that S1P via activation of the heterotrimeric G protein, G\textsubscript{i}, triggered release of Ca\textsuperscript{2+} from ER stores as well as Ca\textsuperscript{2+} entry after store depletion through SOCs. We also observed that S1P induced the translocation of Rac within 2 min to the cell-cell contact sites resulting in formation of intercellular junctions. The enhancement of endothelial barrier function required normal Rac function because these responses were not seen in cells expressing the dominant negative Rac mutant (N17Rac). S1P was shown to strengthen AJs by increasing the assembly of the VE-cadherin and \(\beta\)-catenin complex. The increase in intracellular Ca\textsuperscript{2+} concentration secondary to release from ER stores was a critical factor in mediating the S1P-induced Rac activation and AJ assembly because blocking ER Ca\textsuperscript{2+} release using 2-ABP prevented these responses. Thus, our data show that...
The development of a La3+-sensitive inward current. Endothelial SOCs are composed of transient receptor potential canonical (TRPC) channels that mediate Ca2+ entry after store depletion (17, 19, 22, 29). TRPC1 is expressed in several cell types, including human endothelial cells (22, 28, 29), vascular smooth muscle cells (36), salivary gland cells (37), human platelets (38), and airway smooth muscle cells (39). In this study, we focused on TRPC1 because human endothelial cells predominantly express this isoform (17, 19, 22, 29). It has been shown that SOC is inhibited by extracellular addition of TRPC1 antibody recognizing the sequence TRPC1557–571 (22, 37, 38). We showed that pretreatment of endothelial cells with anti-TRPC1 Ab inhibited SOC-induced Ca2+ entry in endothelial cells, indicating that TRPC1 is a critical protein constituent of functional SOCs. Interestingly, SIP-induced junctional assembly and Rac activity were not altered when the Ca2+ entry component of the Ca2+ transient was blocked using either La3+ or TRPC1 Ab. These findings preclude the role of SOCs in the mechanism of Rac activation and AJ assembly in response to SIP. However, the increase in intracellular Ca2+ concentration secondary to release from ER stores was a critical factor mediating the SIP-induced Rac activation and AJ assembly because blocking ER Ca2+ release using 2-ABP prevented these responses.

It is not clear how the increase in intracellular Ca2+ concentration resulting from Ca2+ entry in response to agonists such as thrombin results in increased endothelial permeability, although release of Ca2+ from ER stores in response to SIP enhances the endothelial barrier. It is possible that intracellular Ca2+ can produce two opposing effects on endothelial barrier function by activating a different set of effector proteins. Thrombin-induced increase in intracellular Ca2+ disrupts barrier function by activating the Rho pathway (21, 35, 40), whereas SIP-induced intracellular Ca2+ signals Rac activation that increases barrier function. Future investigations of the differential activation of Ca2+ signaling pathways induced by thrombin versus SIP will help to clarify these distinct roles of Ca2+ signaling by either increasing endothelial permeability or, in specific circumstances, enhancing the endothelial barrier.

In conclusion, we demonstrate that SIP induces Rac activation by causing the release of Ca2+ from ER stores. Rac upon activation translocates to intercellular junctions and increases intercellular adhesion, thereby promoting endothelial barrier function. Thus, the present study identifies a novel role of Ca2+ released from intracellular stores in signaling the SIP-induced increase in endothelial barrier function.

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SIP-induced Ca\(^{2+}\) Signaling Regulates Rac Activation

Sphingosine 1-Phosphate-induced Mobilization of Intracellular Ca\(^{2+}\) Mediates Rac Activation and Adherens Junction Assembly in Endothelial Cells
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