Microtubule Motors Regulate $I_{SOC}$ Activation Necessary to Increase Endothelial Cell Permeability

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Calcium store depletion activates multiple ion channels, including calcium-selective and nonselective channels. Endothelial cells express TRPC1 and TRPC4 proteins that contribute to a calcium-selective store-operated current, $I_{SOC}$. Whereas thapsigargin activates the $I_{SOC}$ in pulmonary artery endothelial cells (PAECs), it does not activate $I_{SOC}$ in pulmonary microvascular endothelial cells (PMVECs), despite inducing a significant rise in global cytosolic calcium. Endoplasmic reticulum exhibits retrograde distribution in PMVECs when compared with PAECs. We therefore sought to determine whether endoplasmic reticulum-to-plasma membrane coupling represents an important determinant of $I_{SOC}$ activation in PAECs and PMVECs. Endoplasmic reticulum organization is controlled by microtubules, because nocodozole induced microtubule disassembly and caused retrograde endoplasmic reticulum collapse in PMVECs. In PMVECs, rolipram treatment produced anterograde endoplasmic reticulum distribution and revealed a thapsigargin-activated $I_{SOC}$ that was abolished by nocodozole and taxol. Microtubule motors control organelle distribution along microtubule tracks, with the dynein motor causing retrograde movement and the kinesin motor causing anterograde movement. Dynamin expression reduces dynein motor function inducing anterograde endoplasmic reticulum transport, which allows for direct activation of $I_{SOC}$ by thapsigargin in PMVECs. In contrast, expression of dominant negative kinesin light chain reduces kinesin motor function and induces retrograde endoplasmic reticulum transport; dominant negative kinesin light chain expression prevented the direct activation of $I_{SOC}$ by thapsigargin in PAECs. $I_{SOC}$ activation is an important step leading to disruption of cell–cell adhesion and increased macromolecular permeability. Thus, microtubule motor function plays an essential role in activating cytosolic calcium transitions through the membrane $I_{SOC}$ channel leading to endothelial barrier disruption.

Endothelial cells form a semi-permeable interface between blood and tissue that restricts the access of macromolecules, solutes, and water to interstitium. During inflammation, neurohumoral inflammatory agonists increase endothelial cell cytosolic calcium ([Ca$^{2+}$]), and this rise in [Ca$^{2+}$], reorganizes the cytoskeleton, decreases cell–cell and cell–matrix adhesion, and increases centripetally directed tension, all of which contribute to intercellular gap formation that allows for an increase in paracellular permeability (1–4). Neurohumoral inflammatory agonists activate G$_q$ proteins and may promote calcium entry through either receptor-operated or store-operated calcium entry channels; however, it is the activation of store-operated calcium (SOC)$^3$ entry channels that is most important for increasing endothelial cell permeability (5–7).

Thapsigargin inhibits the endoplasmic/sarcoplasmic reticulum calcium ATPase and prevents calcium reuptake into intracellular stores (8). Consequently, thapsigargin activates SOC entry without requiring G protein signaling. As with G$_q$ agonists, thapsigargin increases endothelial cell permeability in the intact pulmonary circulation and in cultured pulmonary artery endothelial cells. Interestingly, not all endothelial cell phenotypes respond equally to thapsigargin. Pulmonary artery endothelial cells (PAECs), in particular, respond to thapsigargin with a larger [Ca$^{2+}$], rise than do pulmonary microvascular endothelial cells (PMVECs) (9). This finding is also true in recalciﬁcation experiments, where thapsigargin is first applied to cells incubated in nominal extracellular calcium, followed by the readdition of physiological calcium concentrations to measure the [Ca$^{2+}$], response to entry through maximally activated SOC entry channels. In these experiments, calcium release is similar in PAECs and PMVECs, yet the [Ca$^{2+}$], response to SOC entry is greater in PAECs. One key difference between these cell types is that thapsigargin activates a calcium-selective, store-operated calcium entry current, $I_{SOC}$ in PAECs, and it does not directly activate this current in PMVECs (9). Moreover, $I_{SOC}$ activation is important for thapsigargin to induce interendothelial cell gap formation and increase paracellular permeability.

Both PAECs and PMVECs express TRPC1 and TRPC4 proteins. These two proteins likely comprise, in some as yet undefined oligomeric state, the molecular basis of the endothe-
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The reason that thapsigargin is able to directly activate $I_{\text{SOC}}$ in PAECs and not directly activate $I_{\text{SOC}}$ in PMVECs is unknown. However, proper coupling between the endoplasmic reticulum and the plasma membrane is important for activation of SOC entry pathways (14). Microtubule motors dynamically traffic endoplasmic reticulum (15–21). Kinesin motors move organelle and vesicular cargo in anterograde fashion, toward the plus end of microtubules at the cell periphery, whereas dynein motors move organelle and vesicular cargo in retrograde fashion, toward the minus end of microtubules near the microtubule-organizing center. The endoplasmic reticulum resides in closer proximity to the plasma membrane in PAECs than in PMVECs, particularly at sites of cell-cell contacts (22), suggesting that the kinesin motor function may predomi-nate in PAECs. We therefore sought to determine whether microtubule motor function is an important determin-ant of $I_{\text{SOC}}$ activation in PAECs and PMVECs. Our findings indicate that inhibiting kinesin activity prevents thapsigargin from activating $I_{\text{SOC}}$ in PAECs, whereas inhibiting dynein activity allows thapsigargin to directly activate $I_{\text{SOC}}$ in PMVECs. Moreover, $I_{\text{SOC}}$ activation is an important determinant of endothelial cell barrier function, because calcium permeation through this channel is sufficient to increase endothelial cell permeability. Thus, we report that microtubule motor function critically regulates endoplasmic reticulum coupling to the plasma membrane necessary to activate the $I_{\text{SOC}}$ and disrupt the endothelial cell barrier.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Pulmonary Endothelial Cells—Primary cultures of rat PAECs and PMVECs were performed using methods detailed by Stevens and co-workers (22). The phenotype of each cell population was verified by lectin binding as detailed previously (22), and the cells were passaged by trypsinization. Low passage cultures were used for all of the experiments. For some studies, the cells were treated with either thapsigargin alone (1 μmol/liter) for 15 min, rolipram (10 μmol/liter) for 30 min, or thapsigargin followed by rolipram prior to analysis. The microtubules were disrupted by treatment with either nocodazole (10 μmol/liter) or taxol (5 μmol/liter) for 90 min.

Electron Microscopy—PAECs and PMVECs were grown to confluence on membrane filters and then fixed and processed for electron microscopy using methods that were detailed previously (22). The sections were photographed, the images were printed, and then the distance between the plasma membrane and the region of the endoplasmic reticulum nearest the plasma membrane was recorded for each sample.

Fluorescence Microscopy—The cells were plated on glass coverslips and then cultured. For analyzing microtubule distributions, the cells were either fixed immediately or were pretreated with rolipram, thapsigargin, rolipram and thapsigargin, nocodazole, or taxol prior to fixation. Fixation was performed by immersing the coverslips in −20 °C MeOH for 6–8 min, and then the microtubules were labeled using previously reported procedures (23–25). Commercially available antibodies against α-tubulin (Sigma-Aldrich) and fluorescein isothiocyanate-labeled anti-mouse IgG/IgM (Roche Applied Science) were used.

To image endoplasmic reticulum distributions in cultured cells, ER Tracker (Molecular Probes, Eugene, OR) was added to culture medium at 1 μmol/liter for 20 min, and then the cells were observed by epifluorescence microscopy without fixation.

Immunoblot Analysis—Immunoblotting using antibody against α-tubulin was used to measure tubulin monomer and polymer levels in cultured PAECs and PMVECs. These studies were performed essentially as outlined previously by Minotti et al. (26) and Marklund et al. (27). Briefly, the cells were plated and cultured as detailed above, and then thapsigargin, rolipram, or both compounds were added. The cells then were rinsed, and then 200 μl of buffer containing 80 mmol/liter 1,4-piperazinediethanesulfonic acid, pH 6.8, 1 mmol/liter MgCl₂, 1 mmol/liter EGTA, 0.5% Triton X-100, 5 μg/ml taxol, and 0.5 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin was added to permeabilize the cells. After 5 min, the supernatant containing the soluble tubulin was collected, and the cells then were rinsed with an additional 200 μl of the extraction buffer. The two extracts were pooled, and 2× SDS-PAGE sample buffer was added. The tubulin polymer fraction then was collected by adding 800 μl of 1× SDS-PAGE sample buffer to the culture dishes containing the residual cell ghosts. The polymer and monomer tubulin fractions then were analyzed by immunoblot analysis using previously reported procedures (28), and the blots were developed using chemiluminescence procedures.

Production of Viral Constructs—Overexpression of dynamitin was used to inhibit dynein activity in PMVECs. A previously characterized cDNA encoding dynamitin (29) was obtained from Dr. Trina Schroer (Johns Hopkins University). The cDNA was excised and subcloned in-frame into an adenovirus shuttle vector, a derivative of pDC512 (Microbiob Systems, Toronto, Canada) containing a GFP tag to produce an enhanced GFP-dynamitin fusion protein. The resulting plasmid was introduced into HEK293 cells by co-transfection with pBGHftrdE1,3FLP. The recombinant adeno-viral progeny were amplified in HEK293 cells and purified by banding in a gradient of CsCl. Infection of PMVECs at a multiplicity of infection of 50 gave an infection rate of >99% as judged by GFP expression. PMVECs were infected and cultured 72 h prior to use in patch clamp studies. The cells that were infected with adenovirus expressing GFP alone were used as controls. Dominant negative kinesin light chain (KLC) constructs were produced in revert-irous vectors for inhibiting kinesin in PAECs. Isolation of the heptad repeat region of the KLC cDNA was achieved using a strategy originally outlined by Verhey et al. (30). Briefly, total RNA was isolated from rat PAECs using TRizol reagent (Invitrogen), and the heptad repeat region of the KLC was amplified by reverse transcription-PCR (23) using the forward primer ggaattcgcaccatgtccacatcttataaaaacaaag and the reverse primer gggaattccgacacgcttgctagcg. The resulting PCR product was ligated into the TOPO-TA vector (Invitrogen). The inserts were released by digestion with BamHI and EcoRI, and the cDNA encoding the KLC fragment then was inserted into appropriately digested pBABE. Retrovi-ral supernatants were produced in Phoenix Amphi cells (kindly provided by Dr. Gary Nolan, Stanford, CA) by CaPO₄-mediated transfection. The supernatants were sterilized and used to infect PMVECs. Transfectants were selected with hygromycin.
(200 μg/ml), and transfected cells (i.e. KLCdn-expressing PAECs) were used for patch clamp analyses and time lapse microscopy and intercellular gap determination.

Patch Clamp Electrophysiology—Conventional whole cell voltage clamp configuration was performed to measure transmembrane currents in single rat PAECs or PMVECs by the standard giga-seal patch clamp technique, as described by our prior work. Confluent cells were enzyme dispersed, seeded onto 35-mm plastic culture dishes, and then allowed to reattach for at least 24 h before patch clamp experiments were performed. Patch clamp recordings were obtained from single (electrically isolated) rat PAECs or PMVECs exhibiting the morphology consistent with the cells from a confluent monolayer. Recording pipettes were heat polished to produce a tip resistance in the range of 3–5 megaohms in the internal solution. To examine calcium currents, the pipette solution contained (in mmol/liter) 130 N-methyl-d-glucamine, 10 Hepes, 2 EGTA, 1 Ca^{2+}, 2 Mg^{2+}-ATP, 1 N-phenylantranilic acid, 0.1 5-nitro-2(3-phenylpropylylamino benzoic acid) (pH 7.2, adjusted with methane sulfonic acid). The external (bath) solution contained (in mmol/liter) 120 aspartic acid, 5 Ca(OH)_{2}, 5 CaCl_{2}, 10 Hepes, 0.5 3,4-diaminopyridine (pH 7.4, adjusted with tetraethyiammonium hydroxide). All of the solutions were adjusted to 290–300 mOsm with sucrose. The currents were recorded with a computer-controlled EPC9 patch clamp amplifier (HEKA). Cell capacitance and series resistance were calculated with the software-supported internal routines of the EPC9 and compensated before each experiment. The voltage pulses were applied from −100 to +60 mV in 20-mV increments after the whole cell configuration was achieved, with 200 ms of duration during each voltage step and 2-s intervals between steps. The holding potential between each step was 0 mV. Data acquisition and analysis were performed with Pulse/PulseFit software (HEKA) and filtered at 2.9 kHz.

Time Lapse Microscopy and Intercellular Gap Determination—KLCdn-expressing PAECs were grown to confluence on 25-mm glass coverslips, placed on the microscope stage, and imaged (40× oil immersion) at 1-min intervals for 60 min using MetaMorph (Molecular Devices, Downingtown, PA) and Spot Software (Diagnostic Instruments, Sterling Heights, MI). Thapsigargin (1 μmol/liter) was added at the beginning (time 0) of the experiments.

RESULTS

Initial studies established the parameters required to disrupt microtubule patterns in PMVECs. Cultured cells were treated with various concentrations of either nocodazole (0.05–10.0 μmol/liter) or taxol (0.5–10 μmol/liter) for times ranging from 10 min to 3 h. The cells were then fixed and processed for anti-tubulin immunofluorescence microscopy. As shown in Fig. 1, endothelial cells have elaborate microtubule networks, and the microtubules are completely disassembled by 60 min of treatment with nocodazole (10 μmol/liter). In contrast, taxol (5 μmol/liter) induced the formation of additional microtubules that appeared to be organized into bundles within 60 min.

Thapsigargin activates calcium nonselective and selective (e.g. $I_{SOC}$) calcium currents in PAECs but is not sufficient to activate $I_{SOC}$ in PMVECs (9). However, inhibition of type 4 phosphodiesterase activity unmasks thapsigargin-stimulated $I_{SOC}$ in PMVECs (9), and these microvascular cells express the TRPC1 and TRPC4 subunits that comprise the molecular basis of the current (11). Studies were therefore designed to investigate why thapsigargin fails to directly activate $I_{SOC}$ in PMVECs. Published data demonstrated that endoplasmic reticulum-plasma membrane distance in PMVECs is two times greater than it is in PAECs; this increased endoplasmic reticulum-plasma membrane coupling distance is particularly prominent at the cell-cell border (22). Initial studies were performed to assess whether the endoplasmic reticulum-plasma membrane distance was altered when phosphodiesterase 4 activity was inhibited in PMVECs. Following treatment with rolipram to inhibit phosphodiesterase 4, PMVECs were fixed and processed for transmission electron microscopy analyses. As shown in Fig. 2, the endoplasmic reticulum was translocated toward the plasma membrane following rolipram treatment, suggesting that endoplasmic reticulum localization may be critical for $I_{SOC}$ activation. To determine whether microtubules regulate endoplasmic reticulum localization within PMVECs, organelle distribution was assayed in control and nocodazole-treated PMVECs using the endoplasmic reticulum-specific probe diOC_{6}. As shown in Fig. 2 (D and E), disruption of the PMVEC microtubule cytoskeleton caused the endoplasmic reticulum to collapse toward the cell center, demonstrating that microtubules control endoplasmic reticulum distribution in PMVECs.

We have previously reported that rolipram pretreatment is required for thapsigargin to activate calcium permeation through the calcium-selective current, $I_{SOC}$, in PMVECs (9). We confirmed this finding presently. Thapsigargin (1 μmol/liter) applied through the patch pipette to single cells did not elicit the $I_{SOC}$. However, pretreating cells with rolipram (10 μmol/liter) for 10 min before break-in was sufficient for thapsigargin to activate the current (Fig. 3A). As previously reported, the $I_{SOC}$ is an inward calcium current (40 pA at −100 mV, $−1−1.5$ pA/pF) with a reversal potential near +40 mV that is inwardly rectifying. Because the intracellular distribution of endoplasmic reticulum relies on transport by microtubule motors, we next sought to determine whether such endoplasmic reticulum realignment toward the plasma membrane was...
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required for \( I_{\text{SOC}} \) activation. PMVECs were therefore first pre-treated with either nocodazole (10 \( \mu \text{mol/liter} \) for 60 min) or taxol (5 \( \mu \text{mol/liter} \) for 60 min), and then we administered rolipram and thapsigargin (Fig. 3B). Both nocodazole and taxol prevented rolipram/thapsigargin from activating \( I_{\text{SOC}} \), suggesting that an intact microtubule network is necessary for rolipram to reveal the thapsigargin-activated \( I_{\text{SOC}} \).

These findings incriminate microtubule network organization as a key intermediate that controls endoplasmic reticulum coupling to the plasma membrane necessary for \( I_{\text{SOC}} \) activation. Two opposing microtubule motors determine the vector for transport along microtubules. Dynein moves bound vesicles toward the minus end of microtubules (toward the centrosome or microtubule organizing center), whereas kinesin moves bound vesicles toward the positive end of microtubules (toward the plasma membrane). Because endoplasmic reticulum interacts with microtubules and possesses a central distribution in PMVECs (more so than in PAECs), we reasoned that the dynein motor predominates in these cells and represents an important mechanism for controlling \( I_{\text{SOC}} \) activation. To test this idea, full-length dynamitin-GFP cDNA was expressed in PMVECs using adenovirus. The cells were selected to homogeneity using GFP fluorescence and trypsinized into single cell suspensions for electrophysiology recordings. Thapsigargin (1 \( \mu \text{mol/liter} \)) application induced the \( I_{\text{SOC}} \) current in PMVECs heterologously expressing dynamitin; \( I_{\text{SOC}} \) activation did not require rolipram pretreatment (Fig. 4). In contrast, in cells infected with adenovirus to express GFP, thapsigargin did not directly activate \( I_{\text{SOC}} \). Thus, the dynein molecular motor predominates in PMVECs and prevents thapsigargin from directly activating \( I_{\text{SOC}} \). Disrupting the activity of the dynein motor is sufficient to reveal thapsigargin-activated \( I_{\text{SOC}} \).

Unlike in PMVECs, endoplasmic reticulum is closely coupled to the plasma membrane in PAECs, and thapsigargin is sufficient to activate the \( I_{\text{SOC}} \) in these cells. Close endoplasmic reticulum-plasma membrane coupling in PAECs indicates that the kinesin motor predominates in control of the microtubule-

![Figure 2](image1.png)

**FIGURE 2.** Microtubules control endoplasmic reticulum disposition. **A**, transmission electron microscopic analyses of control PMVECs illustrates a lateral endoplasmic reticulum stack that is \( \sim 400 \text{ nm} \) from the basal plasma membrane and is separated from the membrane by filamentous actin. **B**, transmission electron microscopic analyses of PMVECs that were treated with rolipram (10 \( \mu \text{mol/liter} \) for 10 min) followed by thapsigargin (1 \( \mu \text{mol/liter} \) for 10 min) demonstrates that the endoplasmic reticulum (arrows) has moved to within 200 nm of the plasma membrane. Summary data from 23 rolipram and thapsigargin-treated PMVECs (155 \( \pm \) 14 nm) and 36 control PMVECs (248 \( \pm \) 15 nm) derived from three independent experiments are shown in C. *, \( p < 0.0001 \). **D** and **E**, microtubule organization determines endoplasmic reticulum disposition. Distribution was analyzed using the endoplasmic reticulum-specific probe diOC\(_6\) in untreated PMVECs (D) and in cells that were first treated with nocodazole (E; 10 \( \mu \text{mol/liter} \) for 60 min) prior to observation. The arrowheads in D show the cell borders, demonstrating that the endoplasmic reticulum has collapsed toward the cell center in cells without intact microtubules.

![Figure 3](image2.png)

**FIGURE 3.** Type 4 phosphodiesterase inhibition unmasked \( I_{\text{SOC}} \) in PMVECs, an effect that was abolished by either disrupting or stabilizing microtubules. **A**, single PMVECs were incubated with the type 4 phosphodiesterase inhibitor rolipram (10 \( \mu \text{mol/liter} \)), and macroscopic currents were recorded by the whole cell patch clamp configuration as described elsewhere. The currents were evoked by applying serial 200-ms pulses from \(-100 \text{ mV} \) to \(+60 \text{ mV} \) with 20-mV increments from holding potential of 0 mV. In rolipram-incubated PMVECs, intracellular infusion of thapsigargin (1 \( \mu \text{mol/liter} \), EC\(_{50}\) ) revealed the activation of \( I_{\text{SOC}} \). **Left panel**, representative current traces obtained in a rolipram-incubated PMVEC with intracellular thapsigargin infusion. **Right panel**, averaged current-voltage (I-V) relationship obtained from eight cells. **B**, single PMVECs were incubated with rolipram along with nocodazole (\( \bullet \)) or taxol (\( \bullet \)), and macroscopic currents were recorded. Both nocodazole and taxol abolished the \( I_{\text{SOC}} \), revealed in rolipram-incubated PMVECs with intracellular thapsigargin infusion. **Left panel**, representative current traces obtained in rolipram and nocodazole or rolipram and taxol preincubated PMVECs with intracellular thapsigargin infusion, respectively. **Right panel**, averaged I-V relationships obtained from cells incubated with rolipram plus nocodazole or taxol (\( n = 6 \) each). *, \( p < 0.005 \) rolipram-incubated PMVECs versus rolipram alone with nocodazole- or taxol-incubated PMVECs.
dependent endoplasmic reticulum organization. We therefore constructed a kinesin light chain dominant negative (KLCdn) construct that contained a hygromycin resistance cassette and placed this construct in retroviruses for expression. PAECs expressing KLCdn were selected to homogeneity using hygromycin and seeded at single cell density for electrophysiology recordings. Whereas thapsigargin directly activated the $I_{\text{SOC}}$ in control cells, it failed to initiate the calcium-selective current in PAECs expressing the KLCdn construct (Fig. 5). Because rolipram was sufficient to reveal a thapsigargin-activated $I_{\text{SOC}}$ in PMVECs, we determined whether this type 4 phosphodiesterase inhibitor could similarly reveal the $I_{\text{SOC}}$ in control PAECs (4). Indeed, rolipram treatment did not induce gap formation in PAECs expressing KLCdn (Fig. 7). This observation supports our prior work, which established that the $I_{\text{SOC}}$ in PAECs expressing KLCdn led to endothelial cell barrier disruption.

**DISCUSSION**

Thapsigargin activates both calcium-selective and nonselective SOC entry pathways in PAECs, but it does not activate the calcium-selective SOC entry pathway in PMVECs (11). Indeed, thapsigargin fails to directly activate the $I_{\text{SOC}}$ in microvascular cells despite the fact they express the TRPC1 and TRPC4 proteins that are responsible for the current. These observations suggest that a cell type-specific regulatory mechanism prevents the $I_{\text{SOC}}$ activation in PMVECs. Endoplasmic reticulum coupling to the plasma membrane is impaired in PMVECs, relative to PAECs (22). Endoplasmic reticulum tubules bind to motor protein complexes and are moved by kinesin and dynein motors along microtubule tracks in either anterograde (cell
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![Image](https://example.com/image1)

**FIGURE 6. Consequences of $I_{SOC}$ activation in PMVECs with disrupted kinesin activity and in PAECs with disrupted dynein activity.**

**A.** Averaged $I-V$ relationship obtained from each group of single PMVECs expressing KLCdn (square, $n=4$), PMVECs expressing dominant negative mutants of kinesin heavy chain (control, ■, $n=7$), rolipram (10 μmol/liter)-incubated PMVECs expressing KLCdn (△, $n=3$), and rolipram-incubated control PMVECs (△, $n=4$) with intracellular infusion of thapsigargin. The currents were evoked by applying serial 200-ms pulses from −100 mV to +60 mV with 20-mV increments from holding potential of 0 mV, *p < 0.05 rolipram-treated control PMVECs versus control PMVECs, $p < 0.005$ rolipram-transduced PAECs (dynamitin-transduced PAEC) with intracellular thapsigargin infusion. **B.** Left panel, representative current traces obtained in a PAEC heterologously expressing dynamitin (dynamitin-transduced PAEC) with intracellular thapsigargin infusion. Right panel, averaged $I-V$ relationship obtained from each group of dynamitin-transduced PAECs (■, $n=6$), GFP-transduced PAECs (control, ■, $n=6$), and rolipram-incubated dynamitin-transduced PAECs (△, $n=4$) with intracellular infusion of thapsigargin, *p < 0.005 dynamitin-transduced PAECs versus rolipram-incubated dynamitin-transduced PAECs. Note the difference in the y-axis scale in each figure.

![Image](https://example.com/image2)

**FIGURE 7. Activation of $I_{SOC}$ is necessary for thapsigargin to induce interendothelial cell gap formation.**

Upper panels, thapsigargin (1 μmol/liter) rapidly induces intercellular gap formation in confluent monolayers of PAECs (see on-line supplemental materials, Movie A). Lower panel, PAECs expressing KLCdn are unresponsive to thapsigargin (1 μmol/liter) and maintain a coherent monolayer (see on-line supplemental materials, Movie B). Images in both panels are representative of five experiments in each group. The arrowheads denote cell-cell border. The arrow denotes when thapsigargin was applied. Note that images taken beyond 40-min duration were omitted from this figure presentation.

periphery) or retrograde (microtubule organizing center) fashion, respectively (15–19, 21, 29–33). Evidence that endoplasmic reticulum in PMVECs possesses a centrally localized distribution indicates that the dynein motor predominates in these cells. We therefore sought to examine whether microtubule motor function is a critical determinant of $I_{SOC}$ activation.

We had previously observed that pretreating PMVECs with rolipram allowed for thapsigargin activation of the $I_{SOC}$ (9). If our hypothesis is correct, that endoplasmic reticulum-to-plasma membrane coupling is an important determinant of $I_{SOC}$ activation, then rolipram pretreatment should promote endoplasmic reticulum coupling to the plasma membrane. Rolipram pretreatment optimized coupling between the endoplasmic reticulum and the plasma membrane, as illustrated by transmission electron microscopy. As in our earlier studies, rolipram pretreatment revealed thapsigargin activation of $I_{SOC}$. Microtubule disruption using nocodazole not only collapsed the endoplasmic reticulum in retrograde fashion but prevented rolipram and thapsigargin from activating the current. These observations are reminiscent of those in melanocytes, where melanophores traffic along microtubule tracks (32–34). Melanophores are lysosome-like vesicular structures that contain melanin and are therefore responsible for pigmentation. Inhibiting phosphodiesterase activity, which increases cAMP, and increased cAMP inhibits dynein motor function and facilitates anterograde melanophore transport (35, 36). Rolipram increases PMVEC cAMP (37, 38), and thus it is likely that the resulting cAMP signal is responsible for optimizing the endoplasmic reticulum-to-plasma membrane coupling necessary for thapsigargin to activate the $I_{SOC}$.

Because it is motor function that controls vesicular transport along microtubules, we next determined whether inhibiting dynein would be sufficient to allow thapsigargin to directly activate the $I_{SOC}$ in PMVECs. Dynamitin is a 50-kDa subunit found within the dynactin complex that interacts with the dynein motor (20). The dynactin complex binds to ZW10, as part of a larger complex comprising syntaxin-18 (21). Syntaxin-18 is a membrane-associated SNAP receptor found on endoplasmic reticulum; thus, this multi-protein complex attaches endoplasmic reticulum to microtubules important for their retrograde
distribution in interphase cells. Dynamin overexpression disrupts the normal oligomeric state of the dynactin multi-protein complex and in so doing inhibits dynein function (20). Interphase cells that are overexpressing dynamin possess limited retrograde vesicular trafficking, and consequently organelles distribute in an anterograde fashion. In our studies, thapsigargin directly activated the I_{SOC} in PMVECs that were expressing dynamin, suggesting that an anterograde endoplasmic reticulum distribution is necessary for thapsigargin to activate the current. Thus, the dynein motor predominates in PMVECs, and inhibiting this motor function allows for thapsigargin I_{SOC} activation.

We next reasoned that if thapsigargin activation of the I_{SOC} requires intimate endoplasmic reticulum-to-plasma membrane coupling, then inhibiting kinesin activity in PAECs should prevent thapsigargin from directly activating the current. We introduced a KLCdn construct in PAECs to reduce anterograde transport of the tubules and observed that thapsigargin could no longer directly activate the I_{SOC}. Despite its inability to activate the I_{SOC}, thapsigargin elicited a rise in global cytosolic calcium in KLCdn-expressing cells, indicating that not all SOC entry channels are equally sensitive to disruption of the endoplasmic reticulum-to-plasma membrane coupling (data not shown). Indeed, these results are compatible with our earlier findings showing that the I_{SOC} contributes only a minor proportion of the global rise in cytosolic calcium (9). Rolipram pretreatment could not rescue the thapsigargin-activated I_{SOC} in PAECs expressing the KLCdn. If rolipram acts by increasing cAMP and inducing anterograde tubule transport, as in the melanosphere system (35, 36), then it should not have rescued the thapsigargin-activated current, because KLCdn prevents motor function. Altogether, these findings demonstrate that a kinesin motor predominates in PAECs and is responsible for the endoplasmic reticulum-to-plasma membrane coupling that is needed for I_{SOC} activation.

Although our findings illustrate the importance of microtubule motors in establishing proper coupling between the endoplasmic reticulum and membrane SOC entry channels, they do not specifically address the nature of this coupling. In our studies thapsigargin induced similar calcium release responses, irrespective of endoplasmic reticulum location (data not shown); neither dynamitin nor KLCdn influenced calcium release, per se. It is therefore likely that the influence of microtubule motors is to position endoplasmic reticulum within cellular domains that possess appropriate ion channels, in this case TRPC1 and TRPC4. Such refined coupling appears to be a requirement for activation of the I_{SOC}, more so than with nonselective cation currents. Such refined control of organelle distribution and ion channel function is not unprecedented. Similar results were observed in mast cells, in which nocodazole collapsed endoplasmic reticulum in retrograde fashion and prevented activation of calcium release-activated calcium (I_{CRAC}) channels without abolishing the global cytosolic calcium response to thapsigargin (39). Microtubule motors not only traffic endoplasmic reticulum but also other organelles such as mitochondria. Indeed, activation of I_{CRAC} in T lymphocytes induces kinesin-dependent anterograde transport of mitochondria (40). This transport allows for calcium uptake into mitochondria, which limits calcium-dependent feedback inhibition of the I_{CRAC}. These findings collectively indicate that intracellular organelle trafficking is important for fine tuning the calcium signal to appropriately respond to environmental demands.

We have reported that calcium permeation through the I_{SOC} is important for disrupting endothelial cell-cell adhesion and increasing permeability (9). This conclusion was based upon evidence that thapsigargin increased PAEC permeability but did not increase PMVEC permeability unless the microvascular cells were first treated with rolipram to allow for I_{SOC} activation. In the present study, we again tested this idea using PAECs expressing the KLCdn. In these cells, thapsigargin was no longer sufficient to increase PAEC permeability. Thus, this result supports our earlier conclusion that activation of the I_{SOC}, although contributing to only a small proportion of the global thapsigargin-induced cytosolic calcium response, is important for disrupting endothelial barrier function. Future studies will be required to determine how microtubule motors present the endoplasmic reticulum to the TRPC1 and TRPC4 channel within membrane domains that dynamically control endothelial cell-cell adhesion.

In summary, our findings have resolved that microtubule motor function is an important determinant of I_{SOC} activation. The dynein motor predominates in PMVECs and prevents thapsigargin from directly activating the current. In contrast, the kinesin motor predominates in PAECs and allows for endoplasmic reticulum-to-plasma membrane coupling that is sufficient for thapsigargin to activate the I_{SOC}. Moreover, the endoplasmic reticulum-to-plasma membrane coupling has important functional implications, because thapsigargin only increases endothelial cell permeability in cells in which the I_{SOC} is activated. Thus, we show for the first time that phenotypically distinct cell types dynamically influence ion channel activation through their control of endoplasmic reticulum distribution.

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