Store-operated calcium entry promotes shape change in pulmonary endothelial cells expressing Trp1

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Moore, Timothy M., George H. Brough, Paul Babal, John J. Kelly, Ming Li, and Troy Stevens. Store-operated calcium entry promotes shape change in pulmonary endothelial cells expressing Trp1. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L574–L582, 1998.—Activation of Ca 2+ entry is known to produce endothelial cell shape change, leading to increased permeability, leukocyte migration, and initiation of angiogenesis in conduit-vessel endothelial cells. The mode of Ca 2+ entry regulating cell shape is unknown. We hypothesized that activation of store-operated Ca 2+ channels (SOCs) is sufficient to promote cell shape change necessary for these processes. SOC activation in rat pulmonary arterial endothelial cells increased free cytosolic Ca 2+ that was dependent on a membrane current having a net inward component of 5.45 ± 0.90 pA/pF at −80 mV. Changes in endothelial cell shape accompanied SOC activation and were dependent on Ca 2+ entry-induced reconfiguration of peripheral (cortical) filamentous actin (F-actin). Because the identity of pulmonary endothelialSOCs is unknown, but mammalian homologues of the Drosophila melanogaster transient receptor potential (trp) gene have been proposed to form Ca 2+ entry channels in nonexcitable cells, we performed RT-PCR using Trp oligonucleotide primers in both rat and human pulmonary arterial endothelial cells. Both cell types were found to express Trp1, but neither expressed Trp3 nor Trp6. Our study indicates that 1) Ca 2+ entry in pulmonary endothelial cells through SOCs produces cell shape change that is dependent on site-specific rearrangement of the microfilamentous cytoskeleton and 2) Trp1 may be a component of pulmonary endothelial SOCs.

PULMONARY ENDOTHELIAL CELLS are a nonexcitable cell type in which humoral and neural signaling agents increase the free cytosolic Ca 2+ concentration ([Ca 2+]i) by inducing Ca 2+ release from intracellular stores and Ca 2+ entry across the cell membrane (4, 34). Increased [Ca 2+]i has been implicated in many endothelial-directed vascular responses including regulation of vascular tone and permeability (2, 23, 36), angiogenesis (20), and leukocyte trafficking (17). Activation of Ca 2+ entry appears essential for each of these processes, although many modes of Ca 2+ entry exist and a specific pathway regulating endothelial cell shape has yet to be identified.

It is widely accepted that endothelial cells possess capacitative, or store-operated, Ca 2+ entry pathways (8, 13, 31, 35, 41, 42). However, specific store-operated Ca 2+ channels (SOCs) responsible for Ca 2+ entry into nonexcitable cell types are largely unidentified. Recent cloning and expression of the transient receptor potential (trp) gene product from the Drosophila melanogaster retina reveal that this product forms a Ca 2+-permeant cation channel that mediates Ca 2+ entry after intracellular inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] is generated and Ca 2+ is liberated from intracellular stores (11, 15, 25). Six mammalian homologues of Drosophila Trp are known (5), and mRNA for these have been reported in bovine aortic endothelial cells (12). Although Trp3 and Trp6 are not SOCs (6, 46), Trp1 may form SOCs based on the following experimental evidence: 1) Trp1 and its splice variant TRPC1A increase store-operated Ca 2+ entry when expressed in COS cells (45, 47) and 2) expression of antisense trp sequences in murine L(tk−) cells greatly attenuates store-operated Ca 2+ entry evoked by Ins(1,4,5)P3 (45). Information concerning putative functions for Trp2, -4, and -5 is lacking in the literature.

Because activation of store-operated Ca 2+ entry is known to increase vascular permeability in isolated lungs (9, 18), thereby suggesting that pulmonary endothelial SOCs are important for regulation of endothelial barrier integrity, we designed studies to characterize the store-operated Ca 2+ entry pathway in rat (R) pulmonary arterial endothelial cells (PAECs). We hypothesized that a functional consequence of activating endothelial SOCs is a change in cell shape, leading to interendothelial gap formation and cytoskeletal rearrangement. To test this hypothesis, we challenged RPAECs with thapsigargin, a plant alkaloid that activates store-operated Ca 2+ entry independent of ligand-receptor-G protein-coupled processes (40, 43), and monitored the changes in endothelial cell shape and microfilamentous cytoskeletal arrangement. We then determined whether RPAECs express Trp1 in order to address the possible molecular basis for the pulmonary endothelial store-operated Ca 2+ entry pathway. Our data indicate that store-operated Ca 2+ entry promotes cell shape change in rat pulmonary endothelial cells expressing Trp1 and further suggest that Ca 2+ entry through SOCs involves site-specific rearrangement of the microfilamentous cytoskeleton.

METHODS

Isolation of RPAECs. Male Sprague-Dawley rats (CD strain, 350–400 g; Charles River) were euthanized by an intraperitoneal injection of 50 mg of pentobarbital sodium (Nembutal, Abbott Laboratories, Chicago, IL). After sternotomy, the heart and lungs were removed en bloc, and the pulmonary arterial segment between the heart and lung hilus was dis-
sected, split, and fixed onto a 35-mm plastic dish. Endothelial cells were obtained by gentle intimal scraping with a plastic cell lifter and were seeded into a 100-mm petri dish containing 10 ml of seeding medium (−1:1 DME-Ham's F-12 + 10% fetal bovine serum) (37). After incubation for 1 wk (21% O2-5% CO2-4% H2 at 37°C), smooth muscle cell contaminants were marked and then removed by pipette aspiration. Cells were verified as endothelial by positive factor VIII staining and uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetratramethylindocarbocyanine perchlorate (DiI)-labeled acetylated low-density lipoprotein. When the primary culture reached confluence, cells were passaged by trypsin digestion into T-75 culture flasks (Corning), and standard tissue culture techniques were followed until the cells were ready for experimentation (passages 6–20).

[Ca2+]i measurement by fura 2 fluorescence. RPAECs were seeded onto chambered glass coverslips (Nalge Nunc, Naperville, IL) and grown to confluence. [Ca2+]i was estimated with the Ca2+-sensitive fluorophore fura 2-AM (Molecular Probes, Eugene, OR) according to methods previously described by our laboratory (38). Because this is the first report of [Ca2+]i measurements in cultured RPAECs, a summary of the technique will be presented. RPAECs were washed with 2 ml of a HEPEs (Fisher Scientific, Atlanta, GA)-buffered physiological salt solution (PSS) containing (in g/l) 6.9 NaCl, 0.35 KCl, 0.16 KH2PO4, 0.141 MgSO4, 2.0 g-d-glucose, and 2.1 NaHCO3. The loading solution (1 ml) consisted of PSS plus 3 µM fura 2-AM, 3 µl of a 10% pluronid acid solution, and 2 mM or 100 nM CaCl2. RPAECs were reloaded for 20 min in a CO2 incubator at 37°C. After this loading period, the cells were again washed with PSS (2 ml) and treated with deesterification medium (PSS + 2 mM or 100 nM CaCl2) for an additional 20 min. After deesterification, [Ca2+]i was assayed with an Olympus IX70 inverted microscope at ×400 with a xenon arc lamp photomultiplier system (Photon Technologies, Monmouth Junction, NJ), and data were acquired and analyzed with PTI Felix software. Epifluorescence (signal averaged) was measured from three to four endothelial cells in a confluent monolayer, and the changes in [Ca2+]i, are expressed as the fluorescence ratio of the Ca2+-bound (340-nm) to Ca2+-unbound (380-nm) excitation wavelengths emitted at 510 nm.

Electrophysiological determination of store-operated Ca2+ entry. Whole cell patch clamp was utilized to measure transmembrane ion flux in thapsigargin-stimulated RPAECs. Confluent RPAECs 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. Single RPAECs exhibiting a flat, polyhedral morphology were studied. These cells were chosen for study because their morphology was consistent with RPAECs from a confluent monolayer. The extracellular and pipette solutions were composed of the following (in mM): 1) extracellular: 110 tetraethylammonium aspartate, 10 calcium aspartate, 10 HEPES, and 0.5 3,4-diaminopyridine; and 2) pipette: 130 N-methyl-D-glucamine, 1.15 EGTA, 10 HEPES, 1 Ca(OH)2, and 2 Mg2+-ATP. Both solutions were adjusted to 290–300 mosM with sucrose and pH 7.4 with methane sulfonic acid, and [Ca2+]i was estimated as 100 nM (10). The pipette resistance was 2–5 MΩ. Data were obtained with a HEKA EPC9 amplifier (Lambrecht/Pfaltz) and sampled online with Pulse + Pulsefit software (HEKA). All recordings were made at room temperature (22°C). To generate current-voltage (I-V) relationships, voltage pulses were applied from −100 to +100 mV in 20-mV increments, with a 200-ms duration during each voltage step and a 2-s interval between steps. The holding potential between each step was 0 mV.

Assessment of endothelial cell shape change. RPAECs were seeded onto 35-mm plastic culture dishes and grown to confluence. Growth medium was replaced with experimental buffer (same as that used for [Ca2+]i measurements but without fura 2), and the cells were subjected to one of the following protocols: 1) vehicle control (5 min) in 2 mM or 100 nM extracellular Ca2+ concentration [Ca2+]o; 2) thapsigargin (1 µM, 5 min) in 2 mM or 100 nM [Ca2+]o; or 3) thapsigargin in 100 nM [Ca2+]o + readdition of 2 mM CaCl2 (5 min). At the end of each experiment, the cell monolayers were fixed in 3% glutaraldehyde-PBS for 2 h. The cells were washed two times with 0.1 M cacodylate buffer, dehydrated by immersion in a series of ethanol dilutions, critical point dried in CO2, and covered with 20 nm of gold. Specimens were viewed at 10 kW at a 15° inclination. Scanning electron micrographs were taken of representative areas in the monolayer by a pathologist blinded to the experimental protocols.

Identification of filamentous actin arrangement. Experiments to determine filamentous actin (F-actin) arrangement were conducted in parallel with those assessing endothelial cell shape change. RPAECs were seeded onto glass coverslips, and F-actin was stained with Oregon Green-phalloidin (Molecular Probes) with a standard fixation and staining protocol. Cells were analyzed by confocal microscopy (excitation at 496 nm and emission at 520 nm). Micrographs were taken at multiple cellular depths (0.3-µm steps, 13–15 sections) and were used to account for the microfilamentous cytoskeleton configurations of the cells.

Identification of trp gene products in pulmonary endothelial cells. For RT-PCR cloning experiments, RPAECs and human (H) PAECs (Clonetics, San Diego, CA) were studied. Standard techniques for RT-PCR subdroning were followed. All chemical reagents used were of molecular biological grade. Briefly, total RNA was extracted from RPAECs and PAECs grown to confluence in 75-cm2 culture flasks (107 cells) with RNA Stat-60 (Tel-Test “B,” Friendswood, TX). First-strand synthesis was performed with reverse transcriptase and oligo(dT) primer (Gibco BRL) on 1 µg of DNase I-treated total RNA. PCR was then performed with the following sets of primers: 1) Trp1: 5′-TCG CCG AAC GAG GTG ATG G-3′ (sense) and 5′-GTT ATG GTA ACA GCA TTT CTC C-3′ (antisense); 2) Trp3: 5′-ACC TCT CAG GCC TAA GGG AG-3′ (sense) and 5′-CTT TCA GAA GTG TTC TCC TGC-3′ (antisense); and 3) Trp6: 5′-CA TCA ATG GCT CCA AG-3′ (sense) and 5′-CAC CAT ACA GAA CGT AGC CG-3′ (antisense). PCR products were ligated into pCR2.1 vectors (TA Cloning Kit, Invitrogen, San Diego, CA) and transformed into competent cells, and screened by PCR for proper inserts. Bacterial cultures were grown for 16–18 h, and the plasmids were purified with Promega Wizard Miniprep (Madison, WI). Sequencing was performed by an automated fluorescence sequencing (ABI370A), and deduced amino acid alignments were carried out with the Blast software program.

RESULTS

Thapsigargin activates store-operated Ca2+ entry in RPAECs. We monitored fura 2 epifluorescence, and as shown in Fig. 1A and summarized in Fig. 1C (open bars), RPAECs incubated in 2 mM [Ca2+]o, had baseline fluorescence ratios averaging 0.91 ± 0.02. Thapsigargin produced a gradual increase in [Ca2+]i to a peak level followed by a modest decline, producing a new steady state, or plateau, in [Ca2+]i. Figure 1, B (dashed line) and C (solid bars), illustrates that the thapsigargin-induced response was dependent on [Ca2+]o. When experiments were repeated in PSS containing 100 mM
Store-operated Ca\(^{2+}\) entry evokes endothelial cell shape change and F-actin cytoskeletal rearrangement in RPAECs. To determine a functional consequence of SOC activation in RPAECs, we assessed changes in endothelial cell shape and formation of intercellular gaps in thapsigargin-treated confluent RPAEC monolayers. Because we determined that SOC activation was apparent 3–5 min after thapsigargin treatment, we studied endothelial morphology at this fixed time point. Figure 3 shows scanning electron micrographs of RPAECs after different treatments. Untreated RPAECs (Fig. 3A) in 2 mM [Ca\(^{2+}\)]\(_o\) exhibited a characteristic “cobblestone” morphology essentially devoid of intercel-
lular gaps. Thapsigargin produced endothelial cell retraction and intercellular gap formation (Fig. 3B). The changes in endothelial cell morphology were dependent on \([\text{Ca}^{2+}]_o\), because RPAECs incubated in 100 nM \([\text{Ca}^{2+}]_o\) and challenged with thapsigargin displayed little change in morphology and a lack of interendothelial gaps (Fig. 3C). The subsequent readition of 2 mM \([\text{Ca}^{2+}]_o\) had a dramatic effect on endothelial cell shape, causing pronounced cell retraction and gap formation (Fig. 3D). Thus \(\text{Ca}^{2+}\) entry through activated SOCs sufficiently promoted endothelial cell shape alterations and interendothelial gap formation.

Because the actin cytoskeleton is pivotal for determining endothelial cell shape, we studied the arrangement of F-actin in control and thapsigargin-treated RPAECs. Figure 4A shows F-actin localization in untreated RPAECs incubated with 2 mM \([\text{Ca}^{2+}]_o\). Under these conditions, cells contained dense peripheral actin bands with apparent focal contact sites between cells. Some transcellular, centrally located filaments were also
seen. Figure 4B shows that incubation of RPAECs in low [Ca\(^{2+}\)]\(_o\) alone had an effect on F-actin configuration. Diffuse, punctate F-actin staining was observed centrally in the cell, whereas densely stained focal sites at the peripheral intercellular junctions were still obvious. Thapsigargin-treated RPAECs incubated in 2 mM [Ca\(^{2+}\)]\(_o\) (Fig. 5A) showed a decrease in peripheral F-actin density and an increase in the number and/or density of central transcellular F-actin filaments. Actin-containing projections could be seen spanning the interendothelial gaps. Thapsigargin administration to RPAECs incubated in low [Ca\(^{2+}\)]\(_o\) (Fig. 5B) produced only modest changes in F-actin arrangement compared with incubation in low [Ca\(^{2+}\)]\(_o\) alone. However, the subsequent readdition of 2 mM [Ca\(^{2+}\)]\(_o\) (Fig. 5C) produced the appearance of dense, transcellular fibers and a decrease in peripheral F-actin staining. Thus [Ca\(^{2+}\)]\(_o\) appears to affect the localization of intracellular F-actin, and Ca\(^{2+}\) influx through activated SOCs configures the microfilamentous cytoskeleton for the alteration of cell shape.

RT-PCR reveals the presence of Trp1 in RPAECs. We screened for three specific mammalian trp gene products, Trp1, Trp3, and Trp6, because all are associated with Ca\(^{2+}\) influx into nonexcitable cell lines, although only Trp1 appears to possess the functional capacity to

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**Fig. 4.** Effect of [Ca\(^{2+}\)]\(_o\) on F-actin distribution. Confocal microscopy was performed on 0.3-µm sections. Three micrographs/treatment are shown. A: unchallenged RPAEC monolayers in 2 mM [Ca\(^{2+}\)]\(_o\). B: RPAECs incubated in 100 nM [Ca\(^{2+}\)]\(_o\). I: cross section through tops of cells. In 2 mM [Ca\(^{2+}\)]\(_o\), staining appeared as a peripheral band with apparent cell-to-cell contact sites. In low [Ca\(^{2+}\)]\(_o\), diffuse punctate staining was observed throughout cells, but contact sites between cells were still obvious. II and III: cross sections through middle and lower aspects of cells, respectively. In 2 mM [Ca\(^{2+}\)]\(_o\), F-actin aligned in radiating strands, with obvious F-actin-containing focal contact sites. Low [Ca\(^{2+}\)]\(_o\) was characterized by diffuse staining throughout, with cell junction integrity still intact.

**Fig. 5.** Effect of thapsigargin on F-actin organization in presence of 2 mM (A), 100 nM (B), and 100 nM [Ca\(^{2+}\)]\(_o\), followed by restoration of [Ca\(^{2+}\)]\(_o\) to 2 mM (C). Confocal microscopy was performed on 0.3-µm sections. Three micrographs/treatment are shown. I: cross section through upper portion of cells. In presence of 2 mM [Ca\(^{2+}\)]\(_o\), diffuse perinuclear staining is evident. In low [Ca\(^{2+}\)]\(_o\), a peripheral actin band with cell-to-cell contact sites is prominent. This peripheral band retracted after Ca\(^{2+}\) was readded, and intercellular actin projections are discernible. II and III: cross sections of middle and lower portions of cells, respectively. In presence of 2 mM [Ca\(^{2+}\)]\(_o\), peripheral (cortical) actin band is absent, and F-actin appears to align in stress fibers. In low [Ca\(^{2+}\)]\(_o\), diffuse punctate staining is observed, but cortical actin band is still present. On readdition of [Ca\(^{2+}\)]\(_o\), stress fiber formation is obvious.
mediate store-operated Ca\textsuperscript{2+} entry. We did not amplify Trp3 or Trp6 products from confluent RPAECs. To determine whether this was a species-specific effect, we performed RT-PCR with HPAECs but likewise detected neither Trp3 nor Trp6 expression. However, both products could be amplified in rat brain, indicating that our primers were capable of amplifying these trp gene products (data not shown). In contrast, RT-PCR products for Trp1 were identified in both RPAECs and HPAECs. The RPAEC and HPAEC products were 96 and 100% homologous, respectively, to the reported nucleotide sequence for human Trp1 (Fig. 6A). The deduced amino acid alignments revealed 100% amino acid homology between both endothelial products and human Trp1 over the region studied (Fig. 6B). Thus Trp1 is present and may contribute to RPAEC SOC formation, whereas Trp3 and Trp6 likely are not expressed in the pulmonary endothelium.

**DISCUSSION**

Although activation of Ca\textsuperscript{2+} entry is sufficient to induce the interendothelial cell gap formation necessary for the transit of macromolecules and cells from blood into tissue, the mode of Ca\textsuperscript{2+} entry responsible for changing cell shape is unknown. Nonexcitable cells possessing store-operated Ca\textsuperscript{2+} entry pathways. Store-operated Ca\textsuperscript{2+} entry is activated in response to agonist-induced stimulation of membrane phospholipases, generation of Ins(1,4,5)P\textsubscript{3}, Ca\textsuperscript{2+} release from intracellular stores, and subsequent lowering of store Ca\textsuperscript{2+} concentrations (4, 8, 13, 16, 31, 34, 35, 41, 42). Presently, there are three prevailing questions regarding store-operated Ca\textsuperscript{2+} entry pathways. 1) What specific cellular functions are regulated by Ca\textsuperscript{2+} influx through this pathway? 2) What is the molecular identity of the membrane channels responsible for mediating store-operated Ca\textsuperscript{2+} entry? 3) What is the nature of the signal linking Ca\textsuperscript{2+} store depletion to store-operated Ca\textsuperscript{2+} entry? Our present study addressed the first two of these three important questions.

Thapsigargin was utilized to test store-operated Ca\textsuperscript{2+} entry pathways because this agent produces intracellular Ca\textsuperscript{2+} store depletion without the confounding influences of ligand-receptor-heterotrimeric G protein activation (40, 43, 47). Fura 2-loaded RPAEC monolayers exhibited an increased [Ca\textsuperscript{2+}] that was dependent on Ca\textsuperscript{2+} influx in response to thapsigargin, thereby indicating the presence of store-operated Ca\textsuperscript{2+} entry pathways. To begin elucidating the electrophysiological characteristics of RPAEC SOCs, we performed whole cell patch clamp in single cells. We designed intracellular and extracellular patch solutions to isolate thapsigargin-activated anion channels. It was possible that the total current measured in response to thapsigargin reflected coactivation of both a Ca\textsuperscript{2+}-selective cation channel and an anion channel because aspartate was utilized to replace Cl\textsuperscript{−} in the extracellular solution, and aspartate has recently been shown to be conducted through Ca\textsuperscript{2+}-and/or volume-activated Cl\textsuperscript{−} channels(29). In support of this idea, N-phenylanthranilic acid, a potent blocker of Ca\textsuperscript{2+}-activated anion channels (27), had little effect on the inward current observed at negative voltages but strongly attenuated the outward current at positive voltages (data not shown). Thus thapsigargin may activate an anion channel capable of conducting large organic anions as previously reported in bovine pulmonary endothelium (27, 29). When the anion conductance contribution to the total thapsigargin-stimulated current is then considered, a current analogous to I\textsubscript{CRAC} is apparent. Future electrophysiological studies, including ion-selectivity experiments and single-channel analysis, are necessary to fully characterize the thapsigargin-sensitive Ca\textsuperscript{2+}-permeable channels in RPAECs.

Activation of SOCs in RPAECs causes the appearance of intercellular gaps and rounding of endothelial cells. One intracellular target affected by SOC activation is plasmalemmal-associated and centrally located F-actin. It is accepted that changes in [Ca\textsuperscript{2+}] lead to reconfigurations of the microfilamentous cytoskeleton (21, 22, 30), although it has previously been unclear.

**Fig. 6.** A: sequence comparison (nucleotides 1–195) between human Trp1 (hTrp1) and RT-PCR products from human PAECs (HPAECs) and RPAECs. *Differences between RPAEC and HPAEC products.**
whether Ca\(^{2+}\) release from intracellular stores or Ca\(^{2+}\) influx is necessary to produce cytoskeletal changes leading to cell shape change.

Thapsigargin produced a loss of plasmalemmal F-actin staining concurrent with an increase in central F-actin staining. When store depletion alone was produced, i.e., thapsigargin in the absence of [Ca\(^{2+}\)]\(_{o}\), rearrangement of cortical actin fibers did not occur and less F-actin staining was observed centrally. Under these conditions, RPAECs did not respond to thapsigargin with a change in cell shape. The readdition of [Ca\(^{2+}\)]\(_{o}\) caused morphological changes in both the peripheral (loss of dense actin staining) and centrally located (increased actin staining and transcellular filament formation) F-actin pods, indicating that Ca\(^{2+}\) influx through SOCs is sufficient to adjust the microfilament system of the cells to produce interendothelial gap formation. It is presently unclear how Ca\(^{2+}\) influx through SOCs specifically regulates the endothelial F-actin cytoskeleton, although a possible mediator of the Ca\(^{2+}\) influx-induced cytoskeletal rearrangement is Rho, a small-molecular-weight monomeric G protein, the activity of which produces actin polymerization and stress fiber formation (1, 14).

Interestingly, incubation of RPAEC monolayers in low [Ca\(^{2+}\)]\(_{o}\) alone caused rearrangement of central F-actin but had no apparent effect on peripheral, or cortical, F-actin. Under these conditions, Ca\(^{2+}\) release could have been promoted because a more favorable electrochemical gradient for Ca\(^{2+}\) to leak from intracellular stores existed. Centrally located F-actin in close proximity to Ca\(^{2+}\) stores could have been affected by Ca\(^{2+}\) release but not in a manner sufficient to drive an active cell shape change. We speculate that these observations may allude to the mechanism(s) leading to plasmalemmal SOC activation, i.e., through Ca\(^{2+}\) release-induced cytoskeletal rearrangement coupled to activation of plasmalemmal SOCs. Another possibility to consider with respect to the F-actin rearrangement is that low [Ca\(^{2+}\)]\(_{o}\) provided less basal Ca\(^{2+}\) influx that was somehow setting the F-actin cytoskeletal architecture. Future studies will be required to address this novel observation of the ability of [Ca\(^{2+}\)]\(_{o}\) to regulate the endothelial cytoskeleton and to specifically address whether F-actin is a vital component of the SOC activation mechanism.

Although our data clearly demonstrate that activation of SOCs regulate endothelial cell shape via effects on the microfilamentous cytoskeleton, we were unable to perform antagonist studies to specifically block SOC activation and the resulting cell shape change. This is because only nonspecific antagonists of endothelial Ca\(^{2+}\) entry pathways exist and the molecular identity of SOCs is unknown. In fact, the collective data from several previous studies (7, 24, 28, 31, 33, 35, 39, 41, 44) that investigated the nature of Ca\(^{2+}\) entry pathways in endothelial cells indicate that multiple SOCs and receptor-operated channels may exist, each having specific electrophysiological profiles and modes of optimal activation. We did, however, begin to deduce the identity of pulmonary endothelial SOCs using RT-PCR. Several trp gene products (Trp1 and Trp3-6) have recently been identified in systemic endothelial cells (12), and our findings indicate that at least Trp1, but neither Trp3 nor Trp6, is expressed in pulmonary endothelial cells. It is uncertain how trp gene products may be organized in the membrane to form a functional channel, but it has been proposed that SOCs may be composed of trp homo- and/or heteromultimers (5). Because our data indicate that neither Trp3 nor Trp6 are present in rat or human pulmonary endothelial cells, the SOC is not composed of Trp1-Trp3 or Trp1-Trp6 heteromultimers.

What are the implications of the observation that SOC activation produces changes in PAEC shape? It is possible that endothelial SOCs are integral for regulating pulmonary vascular permeability responses to inflammatory mediators. Whole lung studies (9, 18) have shown that activation of SOCs alone is sufficient to produce increased vascular permeability as assessed by measures of the filtration coefficient. In addition, SOC activation promotes increased flux of macromolecules across RPAEC monolayers (19, 26). However, stimulation of the thapsigargin-sensitive store-operated Ca\(^{2+}\) entry pathway in rat pulmonary microvascular endothelial cells promotes neither increased macromolecular permeability nor changes in cell shape (19). These observations suggest that inflammatory processes involving endothelial SOC activation can produce pulmonary edema mediated by the appearance of large-vessel leak sites away from the gas-exchanging microcirculatory bed. Therefore, future studies are needed to determine whether 1) pulmonary conduit-vessel endothelium and microvascular endothelium represent distinct phenotypes having separate regulatory properties, 2) changes in conduit-vessel endothelial cell shape in situ lead to significant, function-compromising pulmonary edema, 3) the precipitating factors for increasing large-vessel and small-vessel (capillary) permeabilities are the same, and 4) interventions to alleviate pulmonary edema can be designed to selectively target conduit-vessel endothelial cells vs. microvascular endothelial cells.

The shape change elicited in response to SOC activation in RPAECs has additional importance for other endothelial-directed physiological processes such as angiogenesis and regulation of leukocyte movement. The angiogenic process requires migration of endothelial cells that, in turn, is dependent on the ability of cells to change shape and decrease their cell-to-cell and cell-to-matrix tethering (3). Inhibition of non-voltage-gated Ca\(^{2+}\) channels, presumably including SOCs, inhibits angiogenic factor-induced proliferation, migration, and tube formation of human umbilical venous endothelial cells (20), which are endothelial cells derived from conduit vessels. In addition, a study (17) has shown that human umbilical venous endothelial cell-directed regulation of leukocyte trafficking is [Ca\(^{2+}\)]\(_{i}\) dependent. Changes in endothelial cell shape and tethering that accompany neutrophil adhesion and migration require increased [Ca\(^{2+}\)]. How the increased [Ca\(^{2+}\)]\(_{i}\) occurs is not clear, but a transmembrane Ca\(^{2+}\) flux is required
for certain leukocyte secretory products to increase endothelial [Ca\textsuperscript{2+}]i (32), thereby suggesting a role for SOCE-mediated Ca\textsuperscript{2+} entry. Our data, in combination with these findings, suggest that initiation sites for angiogenesis and leukocyte diapedesis in vivo may be located in pulmonary vascular segments lined with endothelial cells possessing SOCs that regulate cell shape.

In summary, we have shown that RPAECs possess thapsigargin-activated SOCs that conduct current similar to the C\textsubscript{RAC}. RPAECs respond to this mode of Ca\textsuperscript{2+} entry with changes in cell shape, interendothelial gap formation, and rearrangement of the F-actin cytoskeleton. Cytoskeletal rearrangement may be differentially regulated by the extracellular and intracellular Ca\textsuperscript{2+} pools, with Ca\textsuperscript{2+} influx being necessary to produce a cytoskeleton configured for cell shape change. In addition, pulmonary endothelial cells from rats (and humans) express Trp1, which may be integral for forming native SOCs in these cell types. Finally, pulmonary conduit vessel-derived endothelial SOC activation leading to interendothelial gap formation may be the basis for some forms of pulmonary edema and/or a component of angiogenesis and regulation of leukocyte trafficking to and from the vasculature.

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