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Forum Review Article

TRPing on the Lung Endothelium: Calcium Channels That Regulate Barrier Function

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Abstract

Rises in cytosolic calcium are sufficient to initiate the retraction of endothelial cell borders and to increase macromolecular permeability. Although endothelial cell biologists have recognized the importance of shifts in cytosolic calcium for several decades, only recently have we gained a rudimentary understanding of the membrane calcium channels that change cell shape. Members of the transient receptor potential family (TRP) are chief among the molecular candidates for permeability-coupled calcium channels. Activation of calcium entry through store-operated calcium entry channels, most notably TRPC1 and TRPC4, increases lung endothelial cell permeability, as does activation of calcium entry through the TRPV4 channel. However, TRPC1 and TRPC4 channels appear to influence the lung extraalveolar endothelial barrier most prominently, whereas TRPV4 channels appear to influence the lung capillary endothelial barrier most prominently. Thus, phenotypic heterogeneity in ion channel expression and function exists within the lung endothelium, along the arterial–capillary-venous axis, and is coupled to discrete control of endothelial barrier function. *Antioxid. Redox Signal*. 11, 765–776.

Introduction

Endothelium forms a semipermeable barrier between the blood and the underlying tissue. In this capacity, the endothelium controls movement of gases (*e.g.*, oxygen and carbon dioxide), fluids, and macromolecules between the two compartments. Disruption of the endothelial barrier is a contributing factor in numerous pathologies, including acute lung injury. Mechanisms underlying endothelial barrier disruption remain incompletely understood, although a rise in cytosolic calcium resulting from calcium entry across the plasma membrane initiates the cytoskeletal reorganization that increases cell tension and disrupts cell junctions. Given the significance of calcium entry in endothelium, recent work has focused on identifying putative calcium entry mechanisms that govern the behavior of this cell type. In 1990, not a single endothelial cell calcium ion channel had been resolved (52–56). Presently, several candidate proteins are known to be expressed in endothelium and contribute subunits to functional channels. However, we still lack a suitable understanding of the molecular make-up of functional calcium channels in endothelium. In this review, we address how calcium influx across the plasma membrane impacts upon endothelial cell barrier function along the pulmonary vascular axis, and discuss the ion channel proteins known to contribute to calcium entry in these non-excitable cells.

Calcium Entry in the Endothelium

Endothelial and vascular biologists have recognized for many years that an increase in cytosolic calcium triggers retraction of endothelial cell borders. Cell retraction forms interendothelial cell gaps that generate a paracellular pathway for fluid exudation (26–28, 42–44, 46, 51, 72, 74, 77, 79). In many systemic and pulmonary inflammatory processes, neurohumoral inflammatory agonists act on specific membrane receptors to increase endothelial cell cytosolic calcium, and cause tissue edema. An approximate 20,000:1 calcium gradient exists across the plasma membrane, where extracellular calcium concentrations approximate 1.2 m*M* and intracellular calcium concentrations approximate 100 n*M*. Calcium gradients exist between intracellular organelles, such as the mitochondria (200 n*M*) (5, 9, 64, 65, 68) and endoplasmic reticulum (ER; 1 μM) (11, 47), and the cytosol as

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well. However, of the intracellular organelles, ER most dynamically controls calcium fluctuations between its lumen and the cytosol. The ER possesses a calcium ATPase that loads calcium into its lumen, and it possesses two receptor channels, the inositol 1,4,5-trisphosphate ($InsP_3$) receptor and ryanodine receptor channels, which release calcium into the cytosol. Thus, cytosolic calcium transitions occur from either calcium that is released from the ER, or calcium that enters the cell across the plasma membrane.

Many inflammatory agonists, such as bradykinin, substance P, ATP, histamine, and thrombin, bind membrane receptors that activate Gq coupled receptors and phospholipase C. Phospholipase C cleaves phosphotidyl inositol-4,5-bisphosphate into InP_3 and diacylglycerol. InP_3 diffuses through the cytosol to bind to its receptor on the endoplasmic reticulum, and initiates calcium release. Cytosolic calcium measurements detect this calcium release phase as an abrupt, but short-lived increase ("spike") in cytosolic calcium. If extracellular calcium is present, then a "plateau" in cytosolic calcium is observed. To resolve whether calcium release from the ER, or calcium entry across the plasma membrane, is required to disrupt the endothelial cell barrier, inflammatory agonists have been used to activate the endothelium in the absence of extracellular calcium (51). Under these experimental conditions, most studies find no significant increase in endothelial cell permeability, illustrating that calcium entry across the plasma membrane, and not calcium release from the ER, is required to disrupt the endothelial cell barrier, and highlighting the importance of resolving calcium entry pathways that fuel inflammation and tissue edema (51).

The Store-Operated Calcium Entry Paradigm

More than 20 years ago, Putney recognized the important association between calcium release from the ER, and calcium entry across the plasma membrane (63). Since Gq-linked agonists initiate ER calcium release that is followed closely by calcium entry across the plasma membrane, he reasoned a causal link between ER calcium release and calcium entry may exist. He referred to this relationship as "capacitative" or "store"-operated calcium entry. The idea emerged that calcium entry was necessary to refill the ER calcium store following its depletion by InsP3, as in a capacitor. We now know that while calcium entry is essential to maintain ER calcium concentrations, calcium entry across the plasma membrane is also necessary to regulate membrane-delimited enzymes such as adenylyl cyclase, and effectors such as the cadherin–catenin complex and actin-associated regulatory proteins.

In the early studies of Putney and others, it was not clear whether ER calcium release, or ER calcium depletion, was the critical stimulus for calcium entry across the plasma membrane. Two different experimental procedures were used to test this idea. In one experimental design, free ER calcium was buffered using a low affinity calcium buffer. Using this approach, ER calcium store "depletion" was sufficient to activate calcium entry across the plasma membrane (32). In a second experimental approach, inhibitors of the sarcoplasmic, endoplasmic reticulum calcium ATPase (SERCA) were used to deplete ER calcium (76). Using this approach, ER calcium store "depletion" was also sufficient to activate calcium entry across the plasma membrane (80). Thus, it is ER calcium depletion, and not calcium release *per se*, that is necessary to activate calcium entry. Based upon the necessary coupling between ER calcium concentrations and calcium entry across the plasma membrane, this process is referred to as store-operated calcium (SOC) entry.

SERCA inhibitors have been useful tools, allowing us to resolve the role that SOC entry plays in control of the endothelial cell barrier. Thapsigargin is a suicide SERCA inhibitor, which induces a slow calcium leak out of the ER into the cytosol, followed by calcium entry through so-called SOC entry channels (76). These phases can be separated using a recalcification protocol, where thapsigargin is first added to cells under low extracellular calcium conditions, followed by calcium replenishment to the media (Fig. 1) (6, 37, 50). This recalcification protocol illustrates that calcium release is not sufficient to increase permeability, whereas calcium entry immediately triggers cell border retraction and increased macromolecular flux (Fig. 1). Thus, activation of SOC entry increases endothelial cell permeability.

The nature of coupling between the ER and plasma membrane that underlies SOC entry is poorly understood. Several hypotheses have been advanced, including the idea that a soluble messenger is released from the ER, that the ER is

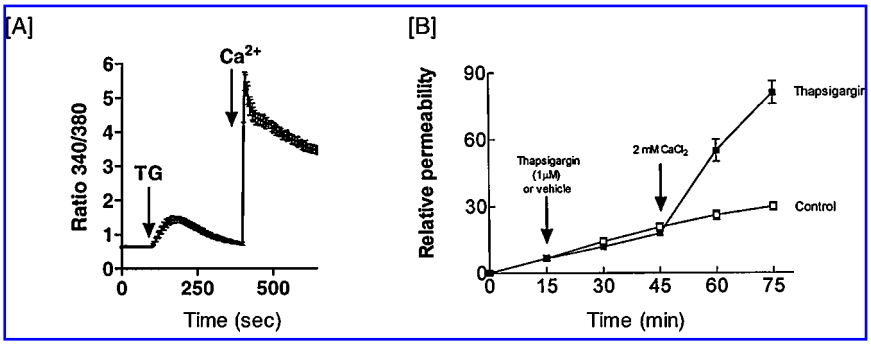

FIG. 1. Activation of SOC entry increases endothelial cell permeability. (**A**) Pulmonary artery endothelial cells grown to confluence were loaded with Fura2/AM, and cytosolic calcium measured using the recalcification protocol. Thapsigargin (TG; 1 μM) applied to cells incubated in nominal extracellular calcium (100 nM) induced a transient increase in cytosolic calcium, due to calcium release from the ER. Replenishing extracellular calcium (Ca^{2+}) to 2 mM resulted in the rapid influx of calcium through open SOC entry channels. For experimental details, see ref. 57. (**B**) Pulmonary artery endothelial cells were grown to confluence on transwell plates, and dextran permeability was measured across the monolayer over a 75-min time period. Application of thapsigargin to cells in the low extracellular calcium (100 nM) did not increase macromolecular permeability. However, replenishing extracellular calcium, to allow calcium entry through SOC entry channels, abruptly increased permeability. For experimental details, see (50).

physically coupled to SOC entry calcium channels, and that vesicles containing SOC entry channels are inserted into the plasma membrane after ER store depletion (7, 39, 41, 45, 59, 60, 66, 69, 70). While evidence has emerged supporting each of these models, there is little-to-no consensus regarding a universal mechanism linking store depletion to channel activation. It may be that each of these proposed mechanisms does in fact occur. As there are multiple types of SOC entry channels, it is possible that different regulatory mechanisms occur in a channel-type, or even a cell-type, specific manner.

The ER is closely apposed to the plasma membrane in endothelial cells (38, 85). We therefore examined whether a cytoskeletal structure links ER store depletion to activation of SOC entry. Spectrin is a principal component of the membrane skeleton; it is a large heterotetramer, comprised of α and β subunits that are organized in antiparallel fashion. Protein 4.1 binds to the amino-terminus of β-spectrin, and links spectrin to transmembrane proteins, including ion channels. Since spectrin can extend to nearly 250 nm and is highly flexible in nature, we reasoned that it links the ER to SOC entry channels. We microinjected antibodies to disrupt the spectrin-protein 4.1 interaction, and measured thapsigargin-induced SOC entry (Fig. 2) (87). Our findings revealed an approximate 30% inhibition of SOC entry. To understand more precisely how the spectrin–protein 4.1 interaction impacts SOC entry, we performed electrophysiological studies to separate calcium selective from calcium nonselective ionic currents. Whereas inhibition of the spectrin–protein 4.1 interaction abolished a store-operated (*e.g.*, thapsigargin activated) calcium selective current, I_{SOC}, it did not inhibit calcium nonselective currents. I_{SOC} has characteristics similar to the originally identified I_{CRAC} (calcium release activated calcium) in mast cells and T lymphocytes (21, 22, 34–36). I_{SOC} is a small, calcium-selective current (1–1.5 pA/pF) that is inwardly rectifying, exhibits a reversal potential near +40 mV and is blocked by lanthanum (10, 15, 16, 86, 87). While disruption of the spectrin–protein 4.1 interaction abolished I_{SOC} activation, it also decreased the magnitude of interendothelial cell gaps, indicating that calcium entering the cell through the I_{SOC} is critically linked to retraction of cell borders.

Evidence that disrupting the spectrin–protein 4.1 interaction inhibited a I_{SOC}, but not a nonselective calcium current, suggested multiple SOC entry pathways exist in endothelial cells, and allowed for screening candidate proteins that may contribute subunits to a SOC entry channel. Indeed, members of the transient receptor potential (TRP) superfamily of proteins have been implicated in the molecular make-up of SOC entry channels. The initial TRP was identified in the *Drosophila melanogaster* phototransduction system in the late 1980s and early 1990s (30, 48, 49, 61, 62).

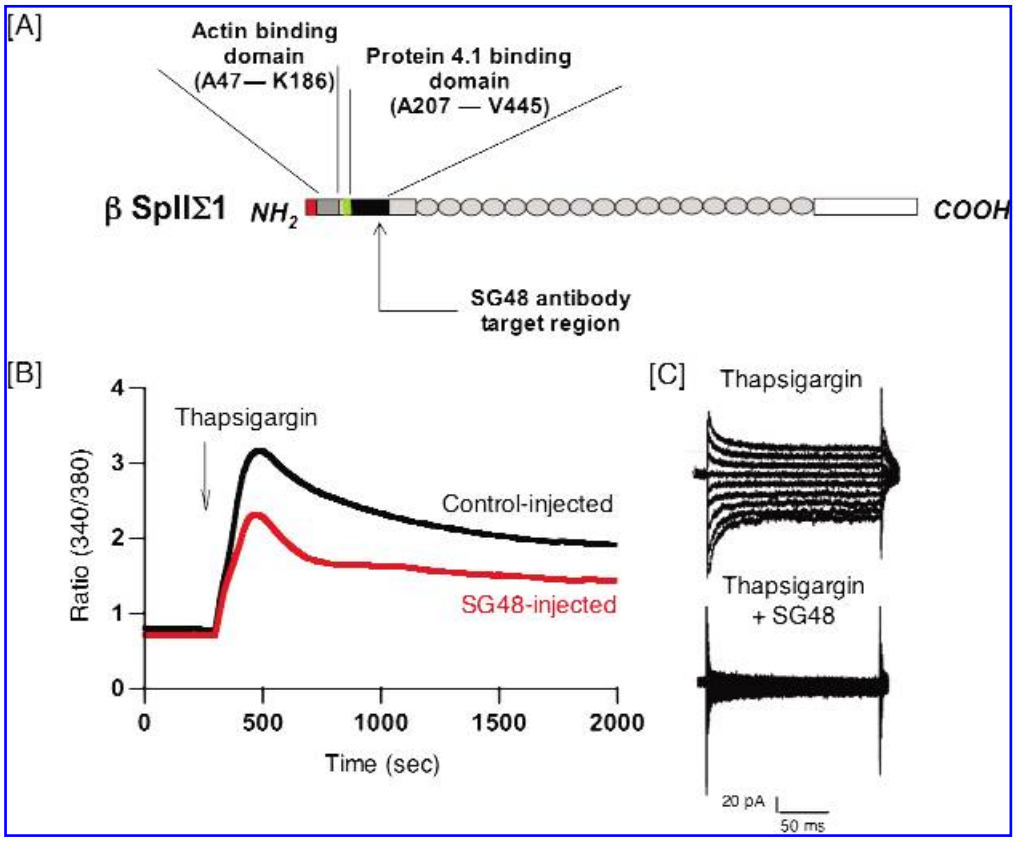

FIG. 2. A spectrin–protein 4.1 interaction is necessary for $I_{V\acute{U}\ddot{O}}$ activation. (**A**) Schematic representing the actin and protein 4.1 binding sites on nonerythroid β-spectrin. The SG48 antibody targets a distal region within the protein 4.1 binding domain, and disrupts the spectrin–protein 4.1 interaction. (**B**) Microinjection of SG48 into pulmonary artery endothelial cells decreases the global cytosolic calcium response to thapsigargin (1 μM). For experimental details, see ref. 87. (**C**) Microinjection of SG48 into single pulmonary artery endothelial cells abolishes thapsigargin (1 μM) activation of I_{SOC}. For experimental details, see ref. 87. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

TRP proteins are six transmembrane spanning structures that encode ion channels (Fig. 3). Functional TRP channels are believed to be tetramers in which the pore region is formed by coalescence of the 5/6 transmembrane domains of each of the four constituent subunits. All TRP channels (except TRPM4 and TRPM5) conduct calcium, although selectivity for calcium varies significantly even among members of the same subfamily (88). Members of the TRPC (canonical) subfamily are expressed in endothelial cells and contribute to calcium entry across the plasma membrane. The canonical proteins (TRPCs) most closely resemble the originally identified *Drosophila* TRP. There are seven known members of the TRPC subfamily; TRPCs 1, 3, 4, and 5 have been implicated as SOC entry channels (89, 90).

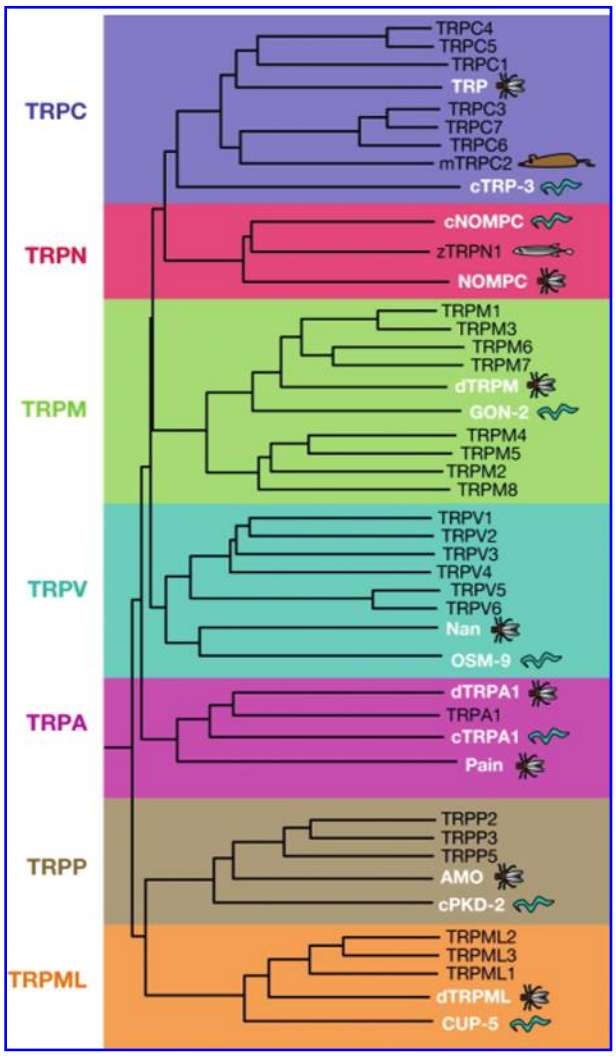

FIG. 3. Schematic representation of the TRP protein family tree. TRP proteins were first identified in *Drosophila melanogaster.* The canonical (TRPC) mammalian proteins possess the highest homology to *Drosophila* TRPs. Greater divergence is seen in the melastatin (TRPM) and vanilloid (TRPV) subfamilies. Adapted from ref. 82. Reprinted, with permission, from the Annual Review of Chemistry, Volume 76 ©2007 by Annual Reviews www.annualreviews.org (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

The endogenous oligomeric state and subunit stoichiometry has not been resolved for any TRP-containing channel. It may well be that the TRPC homologues can contribute to different types of calcium entry channels, especially if they heteromultimerize with different members of the subfamily. It is important to note, however, that most studies of TRPC homologues have been performed in heterologous expression systems. A limitation of this approach is that it is not quite clear whether the overexpressed proteins combine correctly with other channel subunits to form a channel reflective of the endogenous state. Studies of channels at the endogenous level, while often more difficult, offer the distinct advantage of representing the native channel accurately.

Two studies have directly addressed the role of endogenous TRPC1 and TRPC4 to SOC entry in endothelial cells and, more specifically, the role of these proteins in the I_{SOC}. In the first study, an antisense approach was utilized to determine whether TRPC1 contributes to endogenous lung endothelial SOC entry (10). Here it was observed that SOC entry and the calcium current through the I_{SOC} channel was reduced in human pulmonary artery endothelial cells (PAECs) in which TRPC1 expression was knocked down. The second study utilized a TRPC4 knockout mouse model to examine SOC entry and the I_{SOC} channel in aortic endothelial cells (25). Importantly, the calcium current through the I_{SOC} channel was abolished. Collectively, these studies suggest that at least TRPC1 and TRPC4 heteromultimerize to form the I_{SOC} channel. Prior studies have shown that TRPC1 and TRPC4 interact and, importantly, TRPC1 requires the presence of TRPC4 in order to localize in the plasma membrane (4, 29, 33, 67, 75).

Since TRPC1 and TRPC4 contribute subunits to the I_{SOC} channel, and since the spectrin–protein 4.1 interaction is necessary to activate I_{SOC}, we questioned whether protein 4.1 directly interacts with either of these two proteins. Sequence analysis of all TRPC proteins revealed that two of them, TRPC3 and TRPC4, possess a conserved protein 4.1 binding domain (16). Both TRPC4 and protein 4.1 were resolved in a pellet fraction of detergent extracted endothelial cells, and shifted from the pellet to the supernatant fraction upon exposure to low salt concentrations (15). Co-immunoprecipitation of TRPC4 and protein 4.1 from the salt-dissociated supernatant revealed they interact in endothelium (Fig. 4). Further, immunoblotting for TRPC1 demonstrated that it, too, is a part of this larger channel complex. Deletion of the protein 4.1 binding domain and an adjacent proline-rich region prevented I_{SOC} activation (15). Similarly, introduction of a competitive peptide to inhibit protein 4.1 binding to TRPC4 abolished I_{SOC} activation (15). Thus, protein 4.1 couples spectrin to a TRPC4-containing channel that underlies the I_{SOC}.

I_{SOC} and Lung Endothelial Cell Permeability

We initially sought to determine whether activation of SOC entry was sufficient to increase lung endothelial cell permeability. To address this question, permeability was measured in isolated perfused rat lungs, using the filtration coefficient (Kf) method. Thapsigargin increased Kf over a range of concentrations; the EC_{50} was 30 n*M*, equal to the EC_{50} for thapsigargin to increase cytosolic calcium in PAECs, and equal to the IC_{50} for thapsigargin to inhibit SERCA function (12, 13). The thapsigargin-induced increase in Kf required extracellular calcium, as reducing extracellular cal-

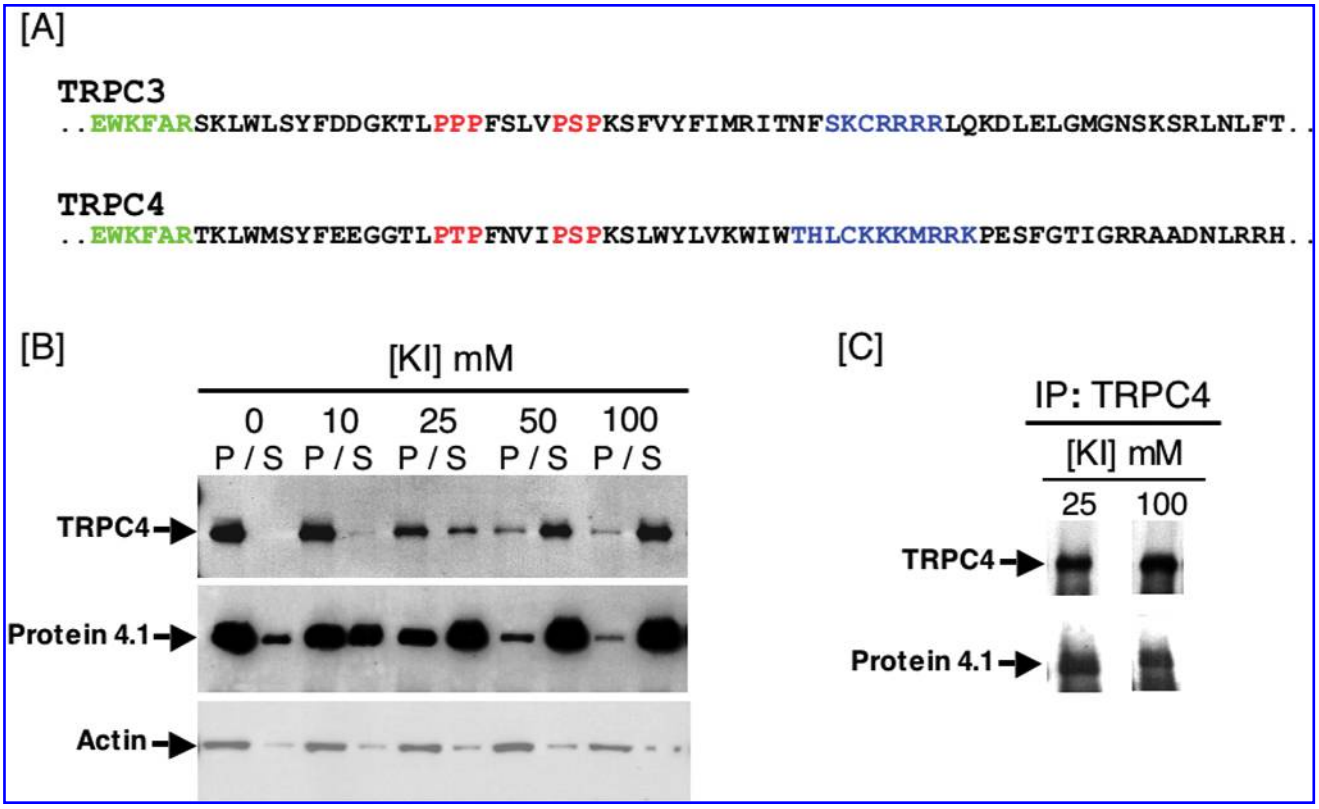

FIG. 4. TRPC4 and protein 4.1 interact in endothelial cells. (**A**) Conserved protein 4.1 binding domains (*blue*) were identified on the TRPC3 and TRPC4 carboxy-tails, downstream of the signature sequence that is conserved in all TRPC proteins (*green*) and a proline-rich region (*red*). (**B**) TRPC4 and protein 4.1 show similar salt-sensitivity in detergent extractions. Detergent extraction and immuno-blotting was performed over a range of salt (potassium iodide, KI) concentrations. Whereas both TRPC4 and protein 4.1 are resolved in the pellet fraction in the absence of salt, 100 m*M* KI is sufficient to dissociate TRPC4 and protein 4.1 into the supernatant fraction. For experimental details, see ref. 15. (**C**) TRPC4 and protein 4.1 co-immunoprecipitate in the salt-dissociated supernatant. For experimental details, see ref. 15. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

cium to 10 μM (from 2.2 m*M*) abolished the rise in permeability. Thus, these findings suggested that thapsigargin—the activation of SOC entry—was sufficient to increase lung endothelial cell permeability.

In addition to measuring permeability, lungs were examined by histology and electron microscopy. Thapsigargin induced large perivascular cuffs in both arteries and veins, but did not cause alveolar edema (12). One explanation for this finding was that thapsigargin increased lung microvascular endothelial cell permeability, and the transudate flowed by negative interstitial pressure into the interstitium surrounding larger vessels. However, electron micrographs revealed no direct evidence for increased permeability within the septal compartment, yet thickened and hydrated interstitial tortuosities were observed between smooth muscle cells within extra-alveolar vessels (Fig. 5) (12). Moreover, scanning electron micrographs revealed inter-endothelial cell gaps formed between adjacent cells in extra-alveolar vessels, but not within the capillary septal compartment (86). These findings suggested that the direct activation of SOC entry increased extra-alveolar, but not alveolar, endothelial cell permeability.

From both clinical and experimental observations, it is clear that isoproterenol and epinephrine exert diverse anti-inflammatory actions. These agonists increase endothelial cell cAMP, and in so doing, prevent or reverse gap formation and reduce permeability (51). cAMP is inactivated by type 4 phosphodiesterase. Indeed, the type 4 phosphodiesterase inhibitor, rolipram, also increases endothelial cell cAMP (20, 71), and prevents permeability responses in multiple experimental lung injury models (51). We therefore pretreated isolated rat lungs with rolipram, before administering thapsigargin (86). As predicted, Kf decreased significantly (Fig. 6). Consistent with this functional data, lung histology and electron micrographs revealed that rolipram prevented gap formation in endothelial cells in extra-alveolar vessels. However, new leak sites appeared in the capillary segments. These findings indicated that rolipram had not just prevented fluid leak across extra-alveolar segments, but had revealed new thapsigargin-activated leak sites across capillary endothelium.

To examine how rolipram enables thapsigargin to induce capillary leak, calcium permeation was examined in PAECs and pulmonary microvascular endothelial cells (PMVECs) (86). Thapsigargin activated a SOC entry pathway capable of conducting multiple divalent cations (calcium nonselective) in both cell types. In addition, thapsigargin activated a calcium-selective SOC entry pathway in PAECs; this calcium entry mode was absent in PMVECs. Consistent with this observation, patch clamp studies revealed that, whereas thapsigargin activated the I_{SOC} in PAECs, it did not activate the I_{SOC} in

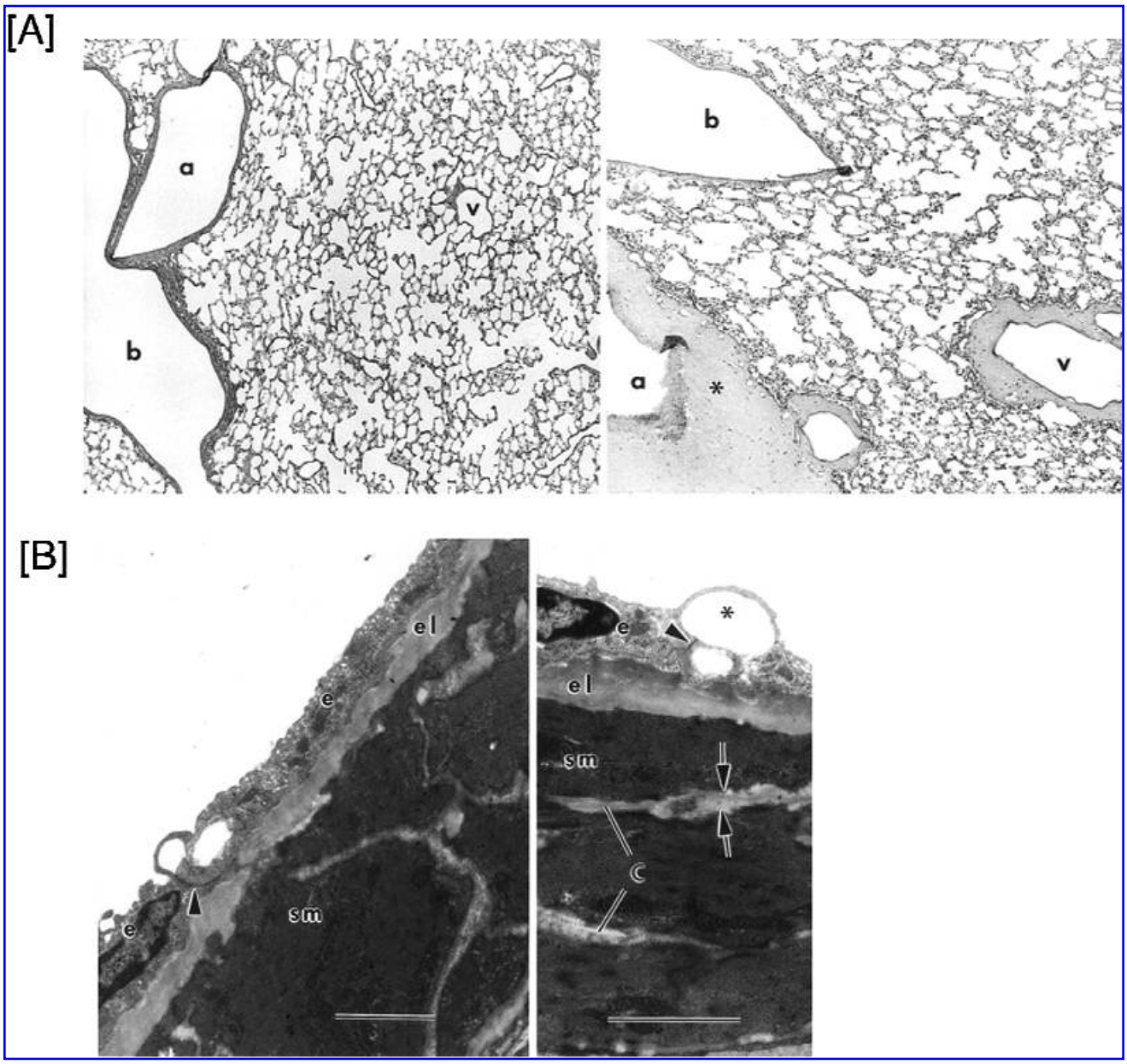

FIG. 5. Thapsigargin induces leak sites in extra-alveolar vessels. (**A**) Thapsigargin (100 n*M*) infusion into the circulation of isolated perfused lungs results in increased lung permeability, with abundant fluid accumulation in cuffs around arteries and veins. *Left panel* is a vehicle control lung section and the *right panel* is a thapsigargin-treated lung section. a, artery; b, bronchiole; v, vein. For experimental details, see (12). (**B**) Morphological assessment revealed that thapsigargin induces leak sites in extra-alveolar vessels. Transmission electron micrographs show subendothelial fluid accumulation with thickening of the subendothelial architecture and distention of collagen bundles between adjacent smooth muscle cells. C, collagen; el, elastic lamina; sm, smooth muscle; *arrowhead* denotes a partially preserved junction. *bleb, indicative of endothelial leak. For experimental details, see ref. 12.

PMVECs. Interestingly, pretreatment with rolipram abolished I_{SOC} in PAECs, and revealed I_{SOC} in PMVECs. Just as occurred in the intact pulmonary circulation, rolipram pretreatment prevented thapsigargin from inducing gaps in PAECs, but revealed gap formation in PMVECs. Thus, both PAECs and PMVECs possess I_{SOC}, although the mechanisms underlying current activation are apparently different among these cells. Most importantly, these findings suggest that the I_{SOC} provides an important calcium source that controls endothelial cell permeability. Since TRPC4 comprises part of the channel that underlies I_{SOC}, it is not surprising that TRPC4 knockout mice exhibit a reduced permeability response to inflammatory agonists that act, in part, by stimulating SOC entry (78).

SOC Entry and Lung Permeability in Heart Failure

Heart failure increases vascular pressure across the pulmonary circulation, particularly in the venous segment. Such high pulmonary vascular pressure causes hydrostatic edema, with perivenular fluid accumulation. In severe cases,

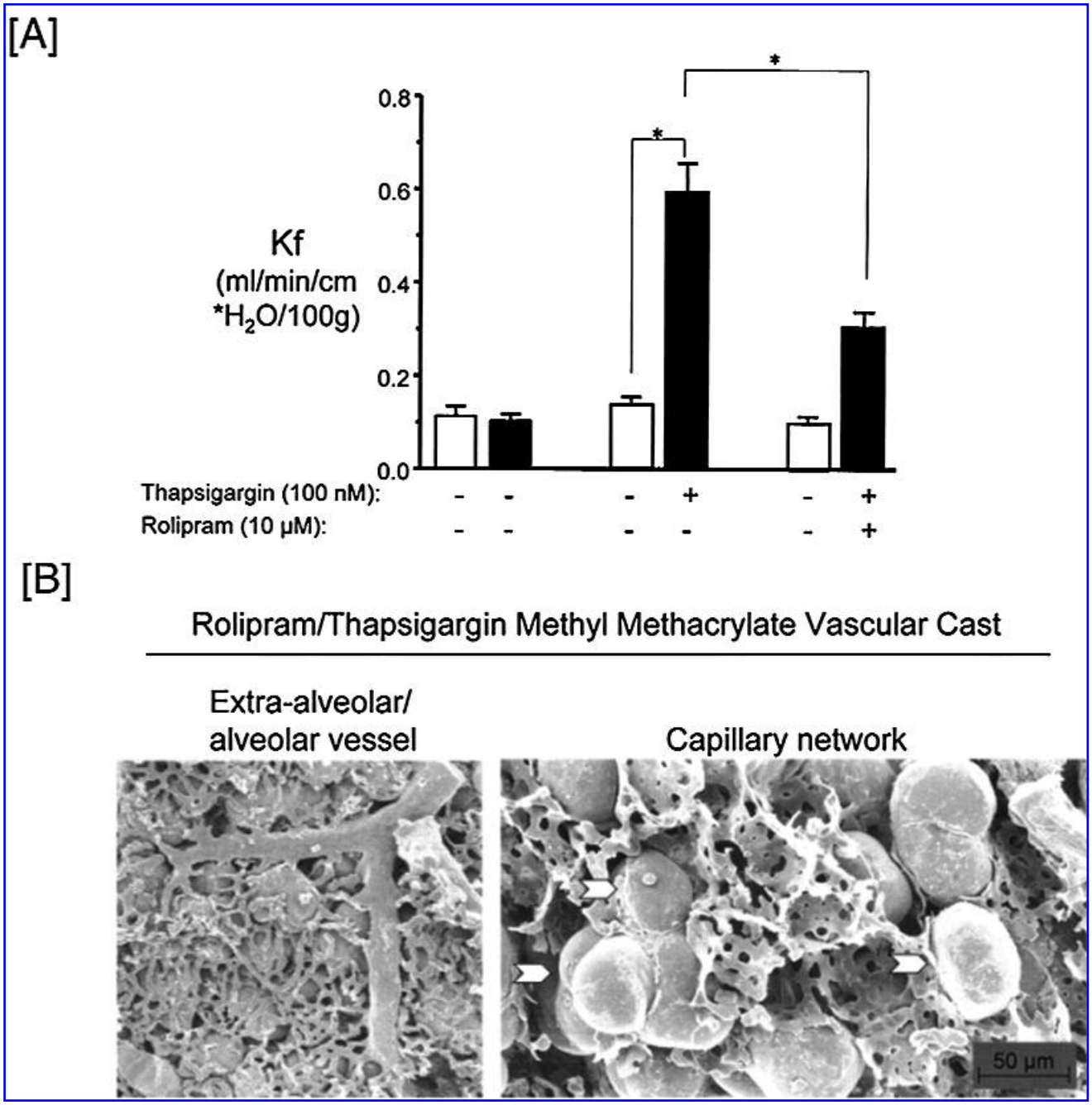

FIG. 6. Thapsigargin increases segment-specific permeability. (**A**) Thapsigargin application to isolated perfused rat lungs increases permeability, as determined by the filtration coefficient (Kf). The type 4 phosphodiesterase inhibitor, rolipram, increases endothelial cell cAMP and reduces whole lung permeability. (**B**) Whereas thapsigargin induces leak sites in extra-alveolar vessel segments (see Figure 5), rolipram pretreatment prevents thapsigargin from inducing extra-alveolar leak. Methyl methacrylate casting material was perfused through rat lungs after thapsigargin and rolipram treatment. After hardening, the cast depicts leak sites that can be resolved by scanning electron microscopy. A precapillary segment and a capillary plexus are shown on the *left panel*. Whereas leak sites are absent in the precapillary segment, leaks sites are resolved as bulges of the casting material in the capillary segment. Bulges of the casting material (*arrowheads*) are shown at higher magnification on the right panel. For experimental details, see ref. 86.

interstitial fluid activates J receptors, resulting in orthopnea. Exactly how a sustained increase in vascular pressure influences endothelial cell behavior is unclear.

Alvarez and co-workers (2) examined whether chronic heart failure alters the thapsigargin-induced permeability response, using an infrarenal aortocaval fistula model in rats. In sham-treated animals, thapsigargin increased lung permeability. However, in animals in which aortocaval fistulas were placed, with documented evidence of right ventricular thickening and increased left ventricular end diastolic pressure, the thapsigargin-induced increase in Kf was abolished (Fig. 7). This insensitivity to thapsigargin was accompanied

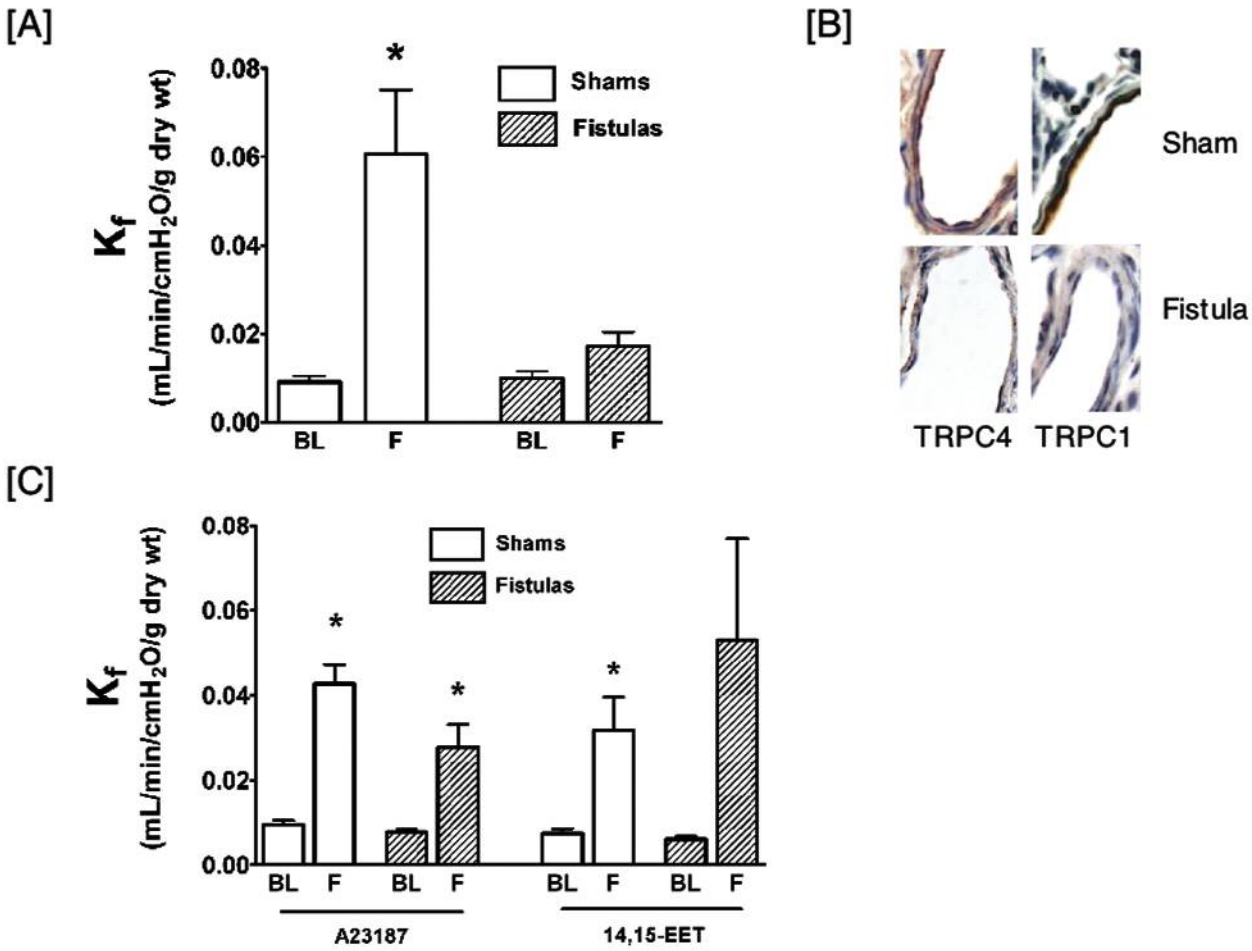

FIG. 7. Lungs from animals with chronic heart failure do not respond to thapsigargin with an increase in permeability, whereas they do respond to 14,15-EET with an increase in permeability. (**A**) Thapsigargin increases permeability in isolated lungs from sham-operated animals, but does not increase permeability in isolated lungs from animals chronically adapted to an aortocaval fistula. BL, baseline Kf; F, final Kf after thapsigargin infusion. For experimental details, see (2). (**B**) TRPC1 and TRPC4 are downregulated in endothelium from the lungs of animals with chronic heart failure. Immunostains from sham-treated animals and animals receiving aortocaval shunts show decreased immunoreactivity in the endothelium. (**C**) Calcium ionophore (A23187) and 14,15-EET increase permeability in both sham-operated and aortaocaval-containing animals. Kf was measured in lungs from control animals and animals with heart failure. In both cases, A23187 and 14,15-EET increased permeability. BL, baseline Kf; and F, final Kf after thapsigargin infusion. For experimental details, see ref. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

by the downregulation of TRPC1 and TRPC4 proteins in artery and vein endothelium, consistent with the idea that thapsigargin activates a TRPC1/TRPC4-containing channel necessary to increase permeability. Interestingly, the permeability response to 14,15-epoxyeicosatrienoic acid (EET) was retained, bringing into question how the permeability response to one agonist, thapsigargin, is abolished in heart failure, while the permeability response to another agonist, 14,15-EET, is not abolished.

14,15-EET and TRPV4 Channels

Watanabe and colleagues (84) have recently observed that 14,15-EET activates a channel belonging to the TRP protein superfamily (Fig. 3). The 14,15-EET-activated channel, TRPV4, belongs to the vanilloid subfamily. There are currently six known mammalian TRPV homologues (TRPV 1–6), each of which can be activated by a variety of stimuli. Perhaps most notable is the activation of TRPV1 by capsaicin, the compound responsible for the "hot" in hot chili peppers (14). TRPV4 is activated by physical stimuli such as mild heat (27°C–34°C), hypoosmotic conditions, and membrane deformation (82). The TRPV4 current is outwardly rectifying and moderately calcium selective with a P_{Ca}:P_{Na} ratio of 5.8 ± 0.5 (83). In addition to endogenous stimuli, TRPV4 can also be directly activated by the phorbol derivative 4α-phorbol 12,13-didecanoate (4αPDD) (83). Similar to the TRPC subfamily members, TRPV ion channels are believed to form tetrameric structures. Whereas the TRPC homologues tend to form heteromultimers, at least TRPVs 1-4 prefer to form homomeric channels (31).

Immunolocalization studies revealed that TRPV4 is abundantly expressed in lung microvascular endothelium and, to a lesser extent, in extra-alveolar endothelium (1). As in earlier studies using 14,15-EET, direct TRPV4 activation using 4αPDD increased lung permeability (1). Ultrastructural anal-

ysis of the 4αPDD-induced leak site revealed disruption of cell-matrix tethering in capillary endothelium. Thus, the 4αPDD-dependent increase in permeability starkly contrasted that seen with thapsigargin, where thapsigargin-induced I_{SOC} activation lead to extra-alveolar vessel leak and TRPV4 channel activation induced capillary leak (1). Supportive results were obtained using TRPV4-knockout mice. Whereas 4αPDD increased permeability in wild-type mice, it was without effect in TRPV4 knockout mice. Thus, TRPV4 channel expression is prominent in lung capillaries, and channel activation results in increased capillary permeability.

Perivascular Cuffs and Alveolar Flooding

In vitro and *in vivo* studies have demonstrated that calcium signaling plays an important role in lung endothelial barrier integrity. More specifically, direct I_{SOC} (TRPC1/TRPC4) activation results in perivascular fluid cuffs surrounding extra-alveolar vessels (12, 86), whereas direct TRPV4 activation causes alveolar flooding (1). The pathophysiological consequences of alveolar flooding have been studied for many years. Alveolar fluid accumulation is a cause of shunting and ventilation:perfusion mismatch, resulting in arterial hypoxemia. In addition, alveolar fluid inactivates surfactant and decreases lung compliance. Both hypoxemia and decreased lung compliance commonly occur in severe lung injury, as in the acute respiratory distress syndrome (ARDS; although decreased compliance is no longer a component of the ARDS definition).

The pathophysiological consequences of perivascular cuffs are less well understood. Perivascular cuffs have been thought to be a thoroughfare for fluid to access lymphatic drainage. Excessive cuffing was thought to be a necessary prerequisite to alveolar flooding. This appears not to be the case. Perivascular cuffs are observed as a part of pulmonary edema in multiple inflammatory and clinical settings, including ischemia/reperfusion injury, high altitude pulmonary edema, infection, and even in asthma. We examined whether the presence of perivascular cuffs impact upon lung compliance (40), and observed that thapsigargin treatment reduced dynamic and static compliance without causing hypoxemia (unpublished observations). High airway resistance could cause a decrease in dynamic compliance, although it would not affect static compliance. Thus, these observations suggest that the activation of SOC entry, and the presence of perivascular cuffs, impacts upon lung mechanics, even in the absence of frank alveolar edema and surfactant inactivation. Further studies will be needed to better understand how fluid accumulation around extra-alveolar vessels impacts upon lung elasticity and distensibility.

Summary

I_{SOC} is a calcium-selective current whose activation leads to interendothelial cell gap formation and increased permeability, in both extra-alveolar and alveolar vessels (86). I_{SOC} activation in extra-alveolar vessels results in the formation of perivascular cuffs in the absence of alveolar flooding (12, 86), which in turn leads to decreased lung compliance (40). Moreover, I_{SOC} activation within the capillary segments causes alveolar flooding (86). As pulmonary edema is an important step in the pathological sequela of acute lung injury, the I_{SOC} channel may indeed represent an important therapeutic target. However, before this goal can be realized, more work needs to be done to resolve the molecular identity of the I_{SOC} channel. To date, it is known that both TRPC1 and TRPC4 contribute to the molecular make-up of I_{SOC}, yet it is still not clear whether additional proteins (*e.g.*, TRPC3 or orai1) contribute to the channel architecture. The subunit stoichiometry (*e.g.*, how many TRPC1 subunits and how many TRPC4 subunits) also remains to be resolved.

TRPV4 forms a calcium-selective channel in endothelial cells of the capillary segment. TRPV4 activation leads to alveolar flooding (1). Thus, similar to the I_{SOC} channel, TRPV4 is an attractive therapeutic target, although the channel architecture and molecular make-up requires better resolution. For instance, although it has been proposed that TRPV4 forms a homomeric channel (31), it is important to note that this study was performed in a heterologous expression system, and it is unclear whether the homomeric channel mimics the native channel.

Calcium entry through I_{SOC} and TRPV4 channels leads to the formation of interendothelial cell gaps. To date, the downstream calcium targets leading to gap formation are incompletely understood. Whereas some calcium targets of the I_{SOC} channel are likely the same as those of the TRPV4 channel, others are likely different. For example, one target of SOC entry channels in the pulmonary endothelium is the type 6 adenylyl cyclase (AC6) (73). Adenylyl cyclases are a family of enzymes that convert ATP into cyclic AMP (cAMP). Increased cAMP is barrier protective (*e.g.,* prevent interendothelial gap formation). AC6 is calcium inhibited such that increases in cytosolic calcium inhibit function of the enzyme, leading to a decrease in cAMP and loss of barrier protection (73). SOC entry channels preferentially associate with AC6 in the cholesterol-rich membrane compartments (*i.e.*, caveolae), and it is calcium entry through these SOC entry channels that is important for AC6 inhibition (18). However, localization of TRPV4 to caveolae and association with AC6 has yet to be explored. Other potential calcium targets include the membrane skeleton. Activation of SOC entry through Gq linked agonists such as thrombin result in disruption of the cortical actin rim and formation of actin-based stress fibers. Reorganization of the actin-based cytoskeleton is an important step in interendothelial cell gap formation (50). Molecular players likely associated with reorganization of the membrane skeleton include calcium-sensitive proteins such as the actin severing protein gelsolin and other calcium-dependent proteases leading to hydrolysis of actin-binding protein (24). As actin is cross-linked by spectrin to form the membrane skeleton (8), the spectrin–actin association may also be a downstream calcium target. Protein 4.1 binds to both spectrin and actin, forming a ternary complex that is much stronger than the spectrin–actin complex alone (17, 19, 23, 58, 81). Studies *in vitro* have shown that erythrocyte β-spectrin binding to actin, mediated by protein 4.1, is inhibited by calmodulin in a calcium-dependent manner (3). It will ultimately be necessary to determine which intracellular calcium targets are effectors of the TRPC1/TRPC4 channel, and which calcium targets are effectors of the TRPV4 channel.

It is noteworthy that therapeutic interventions for severe acute lung injury, the ARDS, have universally failed in clinical trials. Indeed, the only intervention shown to effectively decrease mortality in ARDS is reduced ventilatory volume.

As calcium channel blockers, particularly of the voltage-gated calcium channel family, have been successful therapeutics for many years in other applications, it is our hope that TRP channel blockers can eventually be developed as effective anti-inflammatory drugs, with endothelium representing an important target cell. Our current appreciation that TRP-related calcium channels are differentially expressed among arterial, capillary, and venous lung vascular segments underscores the importance of defining the TRP channel molecular architecture with greater anatomical precision, in order for the therapeutic potential of putative TRP-channel blockers to be realized.

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Abbreviations

AC6, type 6 adenylyl cyclase; 4αPDD, 4 alpha-phorbol 12,13-didecanoate; ARDS, acute respiratory distress syndrome; ATP, adenosine trisphosphate; cAMP, adenosine 3′5′-cyclic monophosphate; EC_{50}, 50% effective concentration; EET, epoxyeicosatrienoic acid; ER, endoplasmic reticulum; IC_{50}, 50% inhibitory concentration; $InsP_3$, inositol 1,4,5-trisphosphate; I_{SOC}, store-operated calcium entry current; Kf, filtration coefficient; PAEC, pulmonary artery endothelial cell; PMVEC, pulmonary microvascular endothelial cells; SOC, store-operated calcium; TRP, transient receptor potential; TRPC, transient receptor potential canonical family; TRPM, transient receptor potential melastatin family; TRPV, transient receptor potential vanilloid family.

Disclosure Statement

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