Store-Operated Calcium Entry Channels in Pulmonary Endothelium: The Emerging Story of TRPCS and Orai1

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Abstract Cells of diverse origin utilize shifts in cytosolic calcium concentrations as intracellular signals to elicit physiological responses. In endothelium, inflammatory first messengers increase cytosolic calcium as a signal to disrupt cell-cell borders and produce inter-cellular gaps. Calcium influx across the plasma membrane is required to initiate barrier disruption, although the calcium entry mechanism responsible for this effect remains poorly understood. This chapter highlights recent efforts to define the molecular anatomy of the ion channel responsible for triggering endothelial cell gap formation. Resolving the identity and function of this calcium channel will pave the way for new anti-inflammatory therapeutic targets.

Keywords Acute lung injury • Vascular barrier dysfunction • Endothelial cells • transient receptor potential channel • lung microvascular endothelium

1 Introduction

Lung endothelium forms a semipermeable barrier that restricts water, solute, and macromolecular access to the interstitium, which is important in optimizing gas exchange. Inflammation disrupts this barrier function, causing accumulation of a protein-rich fluid in interstitial and alveolar compartments that compromises gas

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exchange and contributes to the genesis of acute respiratory distress syndrome. A unifying finding is that multiple different inflammatory agents promote calcium influx across the endothelial cell plasma membrane. This calcium influx triggers cytoskeletal reorganization that initiates intercellular gap formation and increases permeability. However, endothelial cells express many different ion channels, bringing into question exactly which channel provides the calcium source that reorganizes the cytoskeleton and induces gap formation necessary to increase permeability. Findings indicate that transient receptor proteins (TRPs) within the canonical family (TRPC) contribute subunits of an ion channel that provides the calcium source needed to disrupt cell-cell adhesion and induce endothelial cell gaps. Indeed, we have found that endothelial cells, both in vitro and in vivo, express an endogenous channel that possesses TRPC1, TRPC3, and TRPC4 subunits, a so-called TRPC1/3/4 channel, which importantly regulates endothelial cell barrier function. This chapter reviews the calcium channels that are expressed in lung endothelium and addresses regulation and function of the TRPC1/3/4 channel.

2 Fidelity of Calcium Signals in Endothelium

Many neurohumoral inflammatory mediators bind membrane receptors and promote calcium influx across the plasma membrane, resulting in increased endothelial cell permeability.[1–3] However, the molecular composition of the calcium channels responsible for increased endothelial cell permeability is poorly understood. It is remarkable to consider that as recently as 1990, not a single endothelial cell calcium channel was known.[4] We presently know of at least six different endothelial cell calcium channels, including the TRPC1/3/4 channel that is the focus of this chapter, an Orai1-containing channel,[5] a TRPC3/6-containing channel,[6–11] a T-type calcium channel,[12–14] a TRPV4 (TRP channel of the vanilloid subfamily)-containing channel,[6,15–21] and a cyclic nucleotide gated channel.[22–27] While we recognize that endothelial cells express a diversity of calcium channels, the molecular anatomy, regulation, and physiological function of these channels represent important areas of ongoing investigation.

Study of calcium channel diversity has led to new and unexpected insight regarding the unique behaviors of endothelium along the pulmonary vascular tree. For example, the T-type calcium channel is expressed only in lung capillary endothelium and is not found in extraalveolar pulmonary artery or vein endothelium.[14] Activation of this channel is essential for P-selectin surface expression but does not increase endothelial cell permeability (Songwei Wu, personal communication). The TRPV4 channel is also expressed predominantly in lung capillary endothelium,[17] but in contrast to the T-type calcium channel, its activation increases capillary endothelial cell permeability and does not influence P-selectin surface expression[90]. Thus, T-type and TRPV4 calcium channels are expressed in the same vascular segment, and while activation of each of these channels increases endothelial cell calcium, the T channel calcium signal translocates P-selectin from the cytosol to the plasma membrane, whereas the TRPV4 calcium signal increases endothelial cell permeability.

Like the TRPV4 channel, activation of the TRPC1/3/4 channel increases endothelial cell permeability.[28–32] However, two critical differences have been noted regarding the activation and function of these channels. First, the TRPC1/3/4 channel is expressed in pulmonary artery, capillary, and vein endothelium; when the TRPC1/3/4 channel is activated, it increases permeability in all three vascular segments,[28–32] whereas TRPV4 increases permeability primarily in the capillary segment.[17] Second, TRPC1/3/4 activation induces interendothelial cell gaps, both in vivo and in vitro, resulting in a paracellular pathway for fluid and macromolecular permeability,[31,32] as originally described by Majno and Palade in 1961.[33,34] In contrast, TRPV4 activation does not induce interendothelial cell gap formation; rather, calcium influx through TRPV4 decreases cell-matrix tethering and results in endothelial cell sluffing.[17] Thus, TRPC1/3/4 and TRPV4 channels provide calcium influx pathways that regulate quite different cell functions.

3 Activation of Store-Operated Calcium Entry Increases Endothelial Cell Permeability

Formation of interendothelial cell gaps is now a well-recognized cause of tissue edema. Pioneering studies by Majno and Palade[33,34] first revealed that neurohumoral inflammatory mediators induce interendothelial cell gaps in postcapillary venules of the systemic circulation, although the cellular basis of this observation was not known. Since the time of these original observations, many G_q-linked calcium agonists, such as bradykinin, histamine, thrombin, and platelet-activating factor, have been shown to activate membrane calcium channels and promote calcium influx that induces gap formation in both systemic and pulmonary endothelium.[1] G_q-linked agonists hydrolyze phosphatidyl inositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, both of which are important intracellular signals. $InsP_3$ releases calcium from the endoplasmic reticulum, and the transiently depleted calcium store triggers calcium entry across the cell membrane through so-called store-operated calcium entry channels, as originally described by Putney.[35] The activation of store-operated calcium entry serves two functions; it initiates physiologically important intracellular responses, and it replenishes the depleted calcium store. The endoplasmic reticulum calcium-filling state is therefore inversely related to how much calcium enters the cell from across the plasma membrane. In contrast to this mechanism of calcium entry, diacylglycerol activates another calcium entry pathway, generally referred to as receptor-operated calcium entry. Our specific focus has been to resolve the molecular anatomy of store-operated calcium entry pathways and not receptor-operated calcium entry pathways as considerable evidence demonstrated that activation of store-operated calcium entry induces interendothelial cell gaps and increases endothelial cell permeability.

Store-operated calcium entry channels can be directly activated by agents that deplete endoplasmic reticulum calcium.[35] Endoplasmic reticulum calcium can be reduced by chelating intracellular calcium, using the calcium chelators *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) and 1,2-bis(o-aminophenoxy)

ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), by photoactivation of caged $InsP_3$ or by inhibiting the sarcoplasmic, endoplasmic reticulum calcium adenosine triphosphatase (ATPase) with either thapsigargin or cyclopiazonic acid. Both in vitro and in vivo, thapsigargin (and cyclopiazonic acid) induces pulmonary endothelial cell gaps and increases lung permeability; this increase in permeability is abolished when calcium entry through store-operated calcium entry channels is inhibited.[36,37] Moreover, photoactivation of caged $InsP_3$ is sufficient to induce interendothelial cell gap formation in cultured endothelial cells. Thus, activation of store-operated calcium entry, using physiologically relevant agonists (e.g., thrombin, platelet-activating factor), thapsigargin, or photoactivation of caged $InsP_3$, induces endothelial cell gap formation and increases permeability.

Thapsigargin promotes a slowly developing and sustained increase in endothelial cell cytosolic calcium (Fig. 9.1). To better understand whether thapsigargin activates one or more calcium influx pathways, we examined the cationic permeation characteristics of store-operated calcium channels in live cells. Thapsigargin activated at least two separate pathways in endothelial cells. One store-operated calcium entry mechanism was calcium nonselective, meaning that both monovalent and divalent cations permeated the channel pore.[27] A second store-operated calcium entry mechanism was calcium selective, meaning that calcium permeated the channel pore with preference over other divalent cations.[28–32] As we examined the biophysical nature of these two distinct store-operated calcium entry pathways and their physiological function, we came to learn that it was the calcium-selective channel that provides the calcium source necessary to induce interendothelial cell gaps.[31,32]

Calcium nonselective and selective store-operated calcium entry pathways can be systematically studied using whole-cell electrophysiology approaches. Endothelial cells possess a store-operated nonselective current that is activated by thapsigargin.[27,30] This current is a large, linear current that possesses a reversal potential of 0 mV and conducts various mono- and divalent cations. Endothelial cells also possess a store-operated calcium-selective current that is activated by thapsigargin, cyclopiazonic acid, $InsP_3$, intracellular BAPTA, and TPEN; this calcium-selective current is activated when cytosolic calcium is buffered by ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), indicating it is calcium store depletion, and not a rise in cytosolic calcium, that is responsible for current activation.[28–32] This current is small (1–1.5 pA/pF) and inwardly rectifying, with a reversal potential of approximately -40 mV (Fig. 9.2). The fundamental biophysical properties of this current, called I_{SOC}, are similar in pulmonary artery and capillary endothelial cells, although mechanisms controlling channel activation differ among cell types.[31,32]

We were curious to know whether the store-operated nonselective and calcium-selective currents represent different ion channels with unique activation properties or whether they reflect the same ion channel with different permeation characteristics. As we sought experimental approaches to distinguish nonselective from calcium-selective store-operated calcium entry pathways, we became intrigued by studies in platelets incriminating the actin cytoskeleton in the activation of store-operated calcium entry.[38–40] Actin is not thought to directly interact with transmembrane proteins, such as ion channels. Rather, the spectrin membrane skeleton cross-links actin imme-

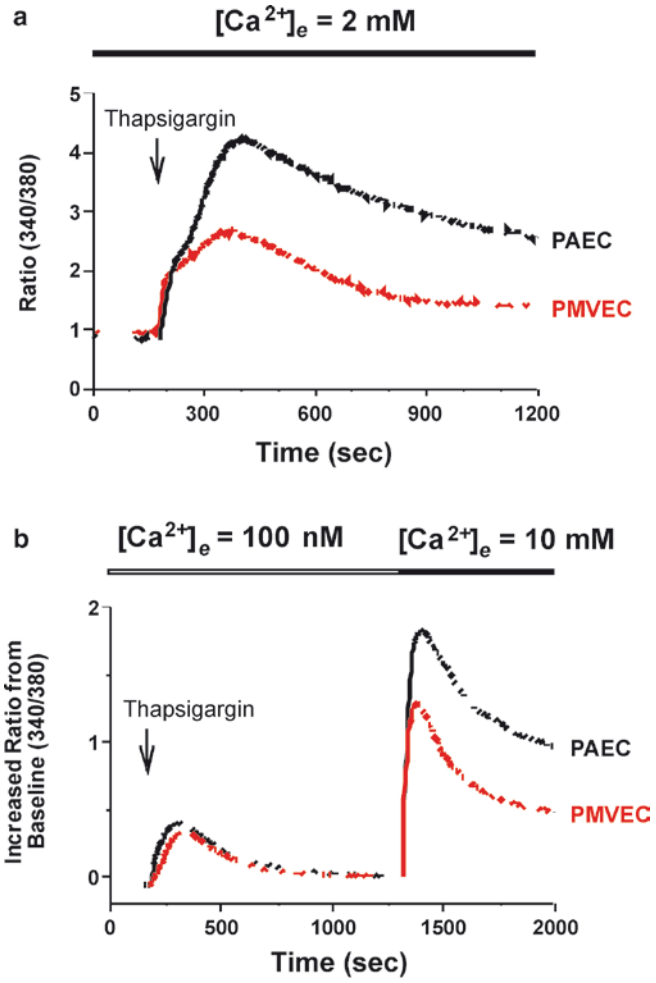

Fig. 9.1 Thapsigargin activates store-operated calcium entry channels and increases cytosolic calcium. (**a**) Confluent monolayers of pulmonary artery endothelial cells (PAEC) and pulmonary microvascular endothelial cells (PMVEC) endothelial cells were loaded with the calcium indicator fura-2. Thapsigargin (1 μ*M*) was applied at the indicated time, and cytosolic calcium concentrations were monitored. Thapsigargin induces a slowly developing and sustained rise in cytosolic calcium in endothelial cells, although the calcium rise is greater in PAECs than it is in PMVECs. (**b**) Thapsigargin inhibits the sarcoplasmic, endoplasmic calcium ATPase, causing depletion of stored calcium. Such depletion of endoplasmic reticulum calcium opens store-operated calcium entry channels on the plasma membrane, resulting in calcium influx into the cell. These separate phases can be distinguished using a recalcification protocol in which thapsigargin is first applied to fura-2-loaded cells in low extracellular calcium. A transient rise in cytosolic calcium is observed as calcium is released from the endoplasmic reticulum. As extracellular calcium is replenished, it enters the cell through open store-operated calcium entry channels. For experimental details, see Ref. [31]

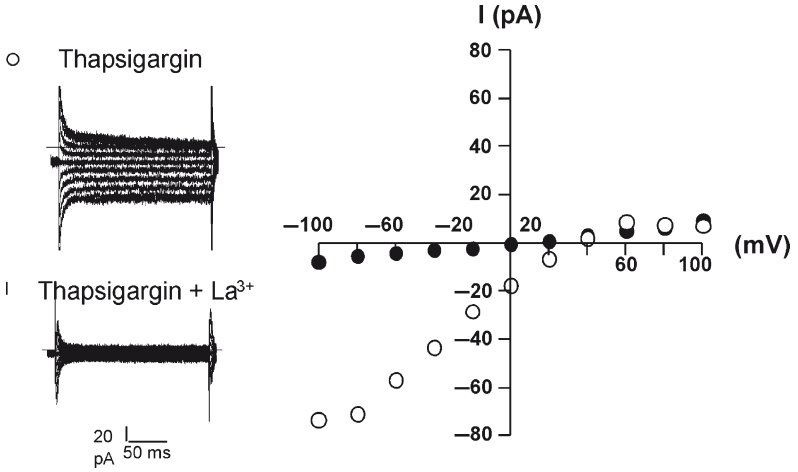

Fig. 9.2 Thapsigargin activates a calcium-selective store-operated current in endothelial cells, referred to as I_{SOC}. Macroscopic currents were resolved in single pulmonary artery endothelial cells. Thapsigargin (1 μ*M*) was applied in the patch pipet in the whole-cell configuration. The holding potential was 0 mV, and a ramp protocol was performed from –100 to +100 mV. Thapsigargin activates a small inward calcium current at negative voltages, with a reversal potential that ranges between +30 and +40 mV. This current is abolished by lanthanum perfusion. For experimental details, see Refs. [28–32]

diately adjacent to the inner leaflet of the phospholipid bilayer.[20,41,42] Spectrin binds to protein 4.1 and ankyrin, and it is these spectrin-bound proteins that directly interact with transmembrane proteins.[43] We selectively disrupted the spectrin-actin and the spectrin-protein 4.1 interactions, and observed that only disruption of spectrin from protein 4.1 inhibited I_{SOC} activation.[30] Indeed, disrupting the spectrin-protein 4.1 interaction had no effect on the thapsigargin-activated nonselective current, and disrupting the spectrin-actin association had no impact on activation of either the I_{SOC} or the nonselective current. These findings provided evidence that channels with different molecular compositions account for the calcium-selective (e.g., I_{SOC}) and nonselective currents. We were interested in resolving the molecular composition of the I_{SOC} channel as functional studies indicated that activation of this current is essential for inter-endothelial cell gap formation.

4 TRPC Proteins Form the I_{SOC} Channel

Evidence that the I_{SOC} channel, and not the nonselective channel, interacts with protein 4.1 suggested that protein 4.1 could be used as "bait" to resolve the channel's molecular identity. We therefore began to screen for channel proteins that interact with protein 4.1. There was precedence for this idea in the spectrin field as the spectrin-binding protein ankyrin was known to interact with spectrin and fast

sodium channels in nodes of Ranvier.[44–51] Discovery of TRPC proteins revealed potential candidates for store-operated calcium entry pathways.[35] However, not all TRPC proteins are store operated; in some instances, these proteins form receptor-operated channels. Store-operated channels must be activated by depletion of endoplasmic reticulum calcium, must not require increased cytosolic calcium for their activation, and must not require diacylglycerol or its analogue OAG (1-oleoyl-2-acetyl-sn-glycerol) for activation. Based on these criteria, TRPC1, TRPC4, and TRPC5 proteins can form store-operated calcium entry channels. Interestingly, sequence alignment of all TRPC isoforms revealed that TRPC4 possesses a putative protein 4.1-binding domain (Fig. 9.3). We were excited to find that the protein 4.1-binding domain on TRPC4 was adjacent to the channel pore,[28] suggesting that a protein 4.1-TRPC4 interaction may be critical for channel activation.

Work from the Flockerzi and Nilius groups indicated that TRPC4 contributes to the molecular identity of the I_{SOC} channel.[52] Indeed, TRPC4 knockdown using small-interfering RNA (siRNA) prevented thapsigargin from activating I_{SOC}, and in endothelial cells isolated from TRPC4 deficient mice, I_{SOC} similarly could not be activated. We examined whether protein 4.1 binds to TRPC4 and regulates channel activation using two related approaches (Fig. 9.3).[28] In the first approach, the protein 4.1-binding domain was deleted from TRPC4 and the chimera expressed in endothelium. The chimeric protein was appropriately expressed and targeted to the membrane. The chimeric TRPC4 protein did not interact with protein 4.1, and in cells expressing this chimera, thapsigargin was unable to activate I_{SOC}. In the second approach, a competitive peptide was generated to target the protein 4.1-binding domain on TRPC4. Introduction of the competitive peptide into endothelium inhibited protein 4.1 binding to the endogenously expressed channel and prevented thapsigargin from activating I_{SOC}. These data, taken together with those of the Flockerzi and Nilius groups,[52] indicated that the protein 4.1-TRPC4 interaction is essential for I_{SOC} activation.

Studies establishing the pivotal role for TRPC4 in store-operated calcium entry, and in I_{SOC} activation more specifically, represented an exciting advance. Yet, molecular cloning studies and hydropathy plots faithfully projected that functional channels would be comprised of four TRPC subunits.[53] Thus, it was not clear whether the I_{SOC} channel was encoded by four TRPC4 subunits or by TRPC4 and some combination of other TRPC proteins. Indeed, siRNA inhibition of TRPC1 reduced the magnitude of the I_{SOC}, suggesting it may contribute a subunit,[29] and in heterologous expression studies, TRPC1 was shown to interact with TRPC4 as an essential step in channel membrane insertion.[54] Moreover, biochemical approaches documented interaction between TRPC1 and TRPC4.[55,56] These collective findings were taken as evidence that TRPC1 and TRPC4 interact and together contribute to the molecular basis of the I_{SOC} channel.

Physiological studies further supported the assertion that TRPC1 and TRPC4 interact as each protein has been implicated in control of endothelial cell permeability. In key studies published by the Malik and Tiruppathi groups, TRPC4-deficient mice were protected from thrombin (or protease-activated receptor ligand)-induced permeability.[57,58] Moreover, inflammatory mediators increase TRPC1 expression, and the upregulated expression of TRPC1 potentiates store-operated calcium entry

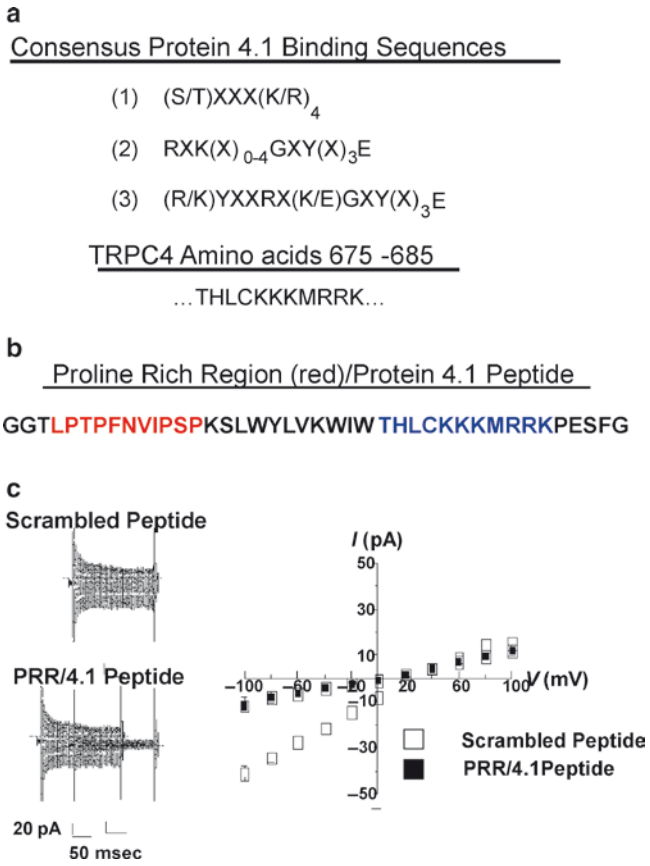

Fig. 9.3 TRPC4 possesses a conserved protein 4.1-binding sequence that is critical for I_{SOC} activation. (**a**) Consensus protein 4.1-binding sequences have been identified in MAGUKs (membrane-associated guanylate kinases), glycophorin C, and other transmembrane proteins.[88,89] A conserved protein 4.1-binding sequence was identified on TRPC4 between amino acids 675 and 685. (**b**) A peptide with sequence corresponding to the protein 4.1-binding domain was generated. (**c**) This peptide, which encompassed the proline-rich region and adjacent protein 4.1-binding domain, was introduced into endothelial cells, and the thapsigargin-induced I_{SOC} was measured. Introduction of the competitive peptide abolished I_{SOC} activation. For experimental details, see Ref. [28]

responses.[59] Perhaps most compelling for our work, however, were studies undertaken by the Townsley group, in which an aortocaval fistula was placed in rats to generate a model of congestive heart failure.[60–62] In their studies, thapsigargin was applied to wild-type, sham-operated, and heart failure animals to determine how development of heart failure has an impact on pulmonary endothelial cell barrier function. Whereas thapsigargin increased permeability in wild-type and sham-operated animals, it was without effect in heart failure animals. Immunohistochemical analysis revealed that development of heart failure was accompanied by downregulation of three TRPC proteins (TRPC1, TRPC3, and TRPC4) (Fig. 9.4).

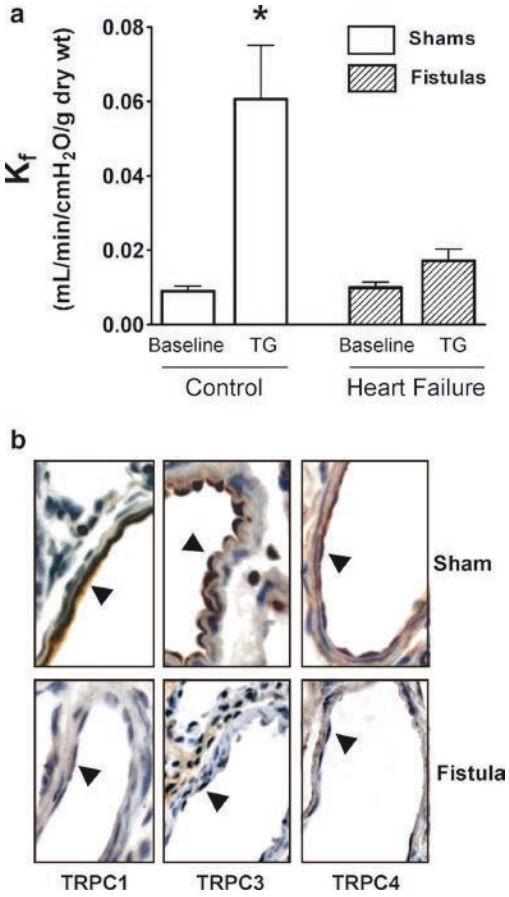

Fig. 9.4 Animals with heart failure do not respond to thapsigargin with an increase in lung endothelial cell permeability. (**a**) Aortocaval fistulas were placed in rats to induce heart failure. Heart and lungs were isolated en bloc, and permeability responses to thapsigargin were measured. Whereas thapsigargin increased permeability in sham-operated animals, animals with heart failure failed to respond to thapsigargin with an increase in permeability. (**b**) This absence of permeability response was accompanied by the selective downregulation of TRPC1, TRPC3, and TRPC4 proteins in lung endothelium. *Arrowheads* denote endothelium. For experimental details, see Ref. [62]

We were struck by the significance of this observation and wondered whether the downregulation of TRPC1, TRPC3, and TRPC4 was coincidental, representing the simultaneous downregulation of multiple channels, or whether all three proteins were a part of a common channel, perhaps the I_{SOC} channel. It was clear that determining the oligomeric state (number of subunits in an endogenous channel) and stoichiometry (how many of each subunit is in the channel) of the endogenously expressed I_{SOC} channel would be essential if we were to identify how inflammatory agonists induce endothelial cell gap formation and increase permeability.

Our group has spent considerable effort resolving both the oligomeric state and stoichiometry of the endogenous I_{SOC} channel in pulmonary endothelium. We have discovered that the observation by Townsley and colleagues that TRPC1, TRPC3, and TRPC4 are each downregulated in endothelium following heart failure is not coincidental. We utilized protein 4.1 as bait to enrich for the endogenously expressed channel and then developed a novel Förster resonance energy transfer (FRET) approach in collaboration with Drs. Claudette St. Croix and Bruce Pitt (at the Center for Biologic Imaging, University of Pittsburgh) to resolve subunit stoichiometry (D.L. Cioffi and T. Stevens, unpublished data). Our findings indicated that each of these proteins contributes subunits to the endogenous I_{SOC} channel; the I_{SOC} channel is comprised of one TRPC1, one TRPC3, and two TRPC4 subunits. Moreover, in these studies we utilized membranes derived from pulmonary artery endothelial cells, pulmonary microvascular endothelial cells, and caveolin-rich fractions isolated from the intact pulmonary circulation. In each case, both in vitro and in vivo, the channel's oligomeric state and stoichiometry were the same. These findings represent the first evidence for an endogenously expressed TRPC channel stoichiometry, the I_{SOC} channel, which is directly incriminated in endothelial cell gap formation and increased permeability.

5 Orai Proteins and Their Relationship to TRPC Channels

Our emerging data indicate that TRPC1/3/4 proteins coalesce to form a channel in which four proteins, each with six transmembrane domains, are required to generate an ion pore. This heterotetramer complex resembles the voltage-gated T-type calcium channel, with the exception that in the latter case, the four repeats of six transmembrane-spanning domains are all contained within a single gene product, the α_1 subunit.[63,64] Indeed, we were struck by the anatomic similarity between TRPC1/3/4 and T-type calcium channel topologies (Fig. 9.5). A more detailed examination of voltage-gated calcium channel organization revealed that its channel complex includes an associated subunit, the γ subunit, that interacts with the pore-forming α_1 subunit and contributes to channel activation and ionic permeation. If such an ancillary protein is critical to channel ionic permeation and if TRPC1/3/4 and voltage-gated channels possess a conserved anatomy, then it is likely that the TRPC1/3/4 channel interacts with another subunit that influences its calcium selectivity as well. Indeed, Orai1 may fulfill the role of a regulatory subunit of the TRPC1/3/4 channel.

The T-type calcium channel's γ subunit is a four-transmembrane-spanning domain protein with amino and carboxy termini that reside in the cytosol.[63,64] Functional interaction between the α_1 pore-forming subunit and the γ subunit requires a conserved sequence [(G/A/S) XXX (G/A/S)] in the γ subunit's first transmembrane-spanning domain (Fig. 9.5). This sequence is not required for α_1–γ subunit binding but is required for the γ subunit to control calcium permeation through the α_1 pore. We therefore screened for protein domains with homology to this sequence and were intrigued to find that a protein incriminated in store-operated

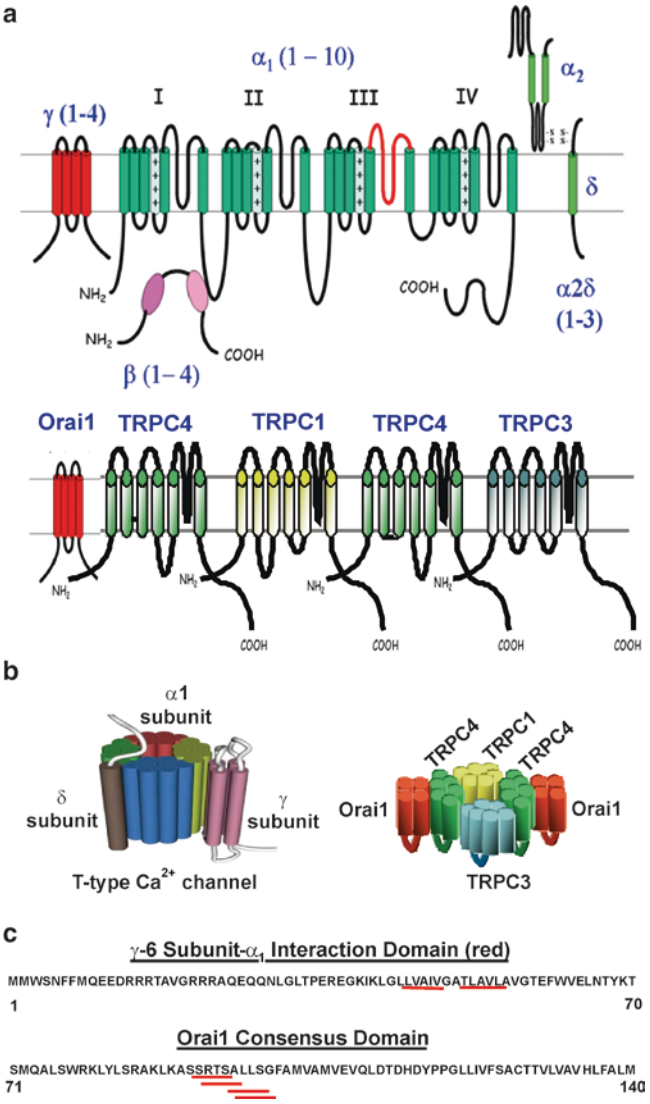

Fig. 9.5 The TRPC1/3/4 channel topology is reminiscent of the voltage-gated T-type calcium channel. (**a**) Schematic of the T-type calcium channel (*top*) and TRPC1/3/4 channel (*bottom*) illustrating a pore-forming unit formed from four cassettes of six transmembrane-spanning domains.[64] Similar to the γ subunit of the T-type calcium channel, Orai1 is a four-transmembrane-spanning domain protein that may interact with the pore-forming unit. (**b**) Schematic is shown of the three-dimensional T-type calcium channel and TRPC1/3/4 channel arrangements.[63] Channel interaction with the γ subunit (for the T-type calcium channel – *top*) and Orai1 (for the TRPC1/3/4 channel – *bottom*) is highlighted. (**c**) Functional interaction between the γ and α1 subunits occurs through a conserved sequence in the first transmembrane-spanning domain of the γ subunit. A similar interaction domain is resolved in Orai1 (see *underlined* regions)

calcium entry, orai1, was enriched with this conserved motif (Fig. 9.5). Orai1 is a four-transmembrane-spanning domain protein with amino and carboxy termini residing in the cytosol, just like the γ subunit. Thus, these findings suggest that Orai1 could interact with the TRPC1/3/4 channel and influence its calcium permeation.

The role that Orai1 plays in store-operated calcium entry is debated, with some evidence that it forms a channel pore and with other evidence that it interacts with channel proteins, such as TRPCs. Orai1 was first identified in 2006 using unbiased genetic approaches to identify putative channels that contribute to store-operated calcium entry.[65–67] In one approach, a modified linkage analysis with single-nucleotide polymorphism arrays was used to screen genes in patients with hereditary severe combined immunodeficiency (SCID) syndrome.[67] Whereas thapsigargin typically activates a highly calcium-selective current in T cells, referred to as the calcium release-activated calcium current, or I_{CRAC}, T cells from SCID patients lack the thapsigargin-activated I_{CRAC}. Genetic analysis revealed a missense mutation in exon 1 of the human ORAI1 coding sequence that resulted in mutation of a conserved arginine residue to tryptophan, a R91W mutation. Expression of wild-type Orai1 in T cells derived from SCID patients rescued the thapsigargin-activated I_{CRAC}, providing direct evidence that Orai1 fulfills an essential role in the I_{CRAC}. In a second approach, a genomewide RNA interference screen in cells from *Drosophila* was used to detect proteins that inhibit store-operated calcium influx.[65,66] Using this approach, separate groups identified Orai1 as a protein necessary for activation of the I_{CRAC}, again providing strong evidence that Orai1 is an essential component of the store-operated calcium entry channel.

From this pioneering work came a series of studies specifically addressing whether Orai1, and its related proteins Orai2 and Orai3, form a channel pore.[68,69] The I_{CRAC} is highly calcium selective, as are voltage-gated calcium channels, which utilize glutamate residues in the pore region to coordinate calcium binding. Analysis of the putative Orai1 pore revealed glutamate residues on the extracellular surface, at E106 in the first transmembrane domain, and at E190 in the third transmembrane domain. Several negatively charged aspartate residues were also found in the second transmembrane domain that may bind and sieve calcium as it passes through the pore. Replacing the conserved glutamate in the first transmembrane domain with glutamine (E106Q; substitution that cannot bind calcium) prevented thapsigargin from activating the I_{CRAC}. Replacing either the glutamate in the third transmembrane-spanning domain with glutamine (E190Q) or substituting aspartate to alanine (D110A and D112A) changed calcium selectivity and allowed for the channel to nonselectively conduct cations, including monovalent cations such as sodium and divalent cations such as barium and strontium. Thus, Orai1 may form an ion pore responsible for the I_{CRAC}.

While these studies suggested that Orai1 contributes to a calcium channel pore, channel oligomeric state and stoichiometry had not been determined. To address whether Orai1 forms a tetramer in living cells, it was expressed as a fusion protein in tandem with reporter fluorophores.[70] Once expression and membrane localization were confirmed, FRET was performed. FRET studies revealed that Orai1 organizes into tetramers in the plasma membrane. To assess whether four Orai1

subunits can generate a functional channel, four individual subunits were preassembled and expressed in cells typically deficient of the I_{CRAC}.[71] Expression of this entire tetrameric channel complex reconstituted the thapsigargin-activated I_{CRAC}, providing evidence not only that a functional channel is comprised of orai1 but also that four such subunits in tandem generate a pore-forming channel.

Despite evidence that Orai1 can form a channel, it remains unclear regarding whether it comprises the pore-forming channel accounting for the I_{CRAC} in vivo. For example, thapsigargin does not activate I_{CRAC} in all cells that express Orai1,[72–75] as discussed by Ambudkar and colleagues.[76] Moreover, the Birnbaumer[77,78] and Ambudkar[76,79] groups have independently noted that the anatomy of Orai1 is unusual for a calcium channel and, as discussed, recognized that it is more reminiscent of channel ancillary proteins, such as the γ subunit of the T channel. Both Birnbaumer[77,78,80] and Ambudkar[76,79,81] groups have proposed that Orai1 is an essential subunit of TRPC channels and is required for TRPC proteins to sense calcium store depletion and hence to be "store operated." At present, this central issue remains unresolved. It is not inconceivable that Orai1 forms the channel responsible for the I_{CRAC} and associates with TRPC channels to confer their sensitivity to store depletion. Indeed, Trebak and colleagues[5] suggested that thapsigargin activates both I_{CRAC} and I_{SOC} in endothelial cells, and that Orai1 is responsible for the I_{CRAC} in these cells, while TRPC4 contributes to I_{SOC}. Our preliminary evidence supports the idea that Orai1 interacts with the TPRC1/3/4 channel. Further work is therefore required to rigorously vet the role that Orai1 plays in calcium signaling, both as a channel and as a putative TRPC channel subunit.

6 Protein 4.1 Is an Essential Determinant of TRPC1/3/4 Activation

It is important to consider whether Orai1 contributes to activation of the TRPC1/3/4 channel. For many years, the mechanisms responsible for sensing a decrease in endoplasmic reticulum calcium were poorly understood. Identification of stromal interacting molecule-1 (STIM1) provided insight into this mechanism as STIM1 is a transmembrane protein possessing an EF hand, which is a calcium binding motif, on the luminal side of the endoplasmic reticulum.[82–85] Thus, as endoplasmic reticulum calcium decreases, it is sensed by STIM1, resulting in the punctate colocalization of STIM1 with Orai1. STIM1 and Orai1 coexpression potentiates thapsigargin activation of store-operated calcium entry.[86,87] These findings are taken to suggest that calcium store depletion results in association between the endoplasmic reticulum and calcium entry channels, necessary for channel activation.

If TRPC proteins function as store-operated calcium entry channels, and if STIM1 is necessary for activation of store-operated calcium entry, then STIM1 may interact with TRPC proteins following depletion of endoplasmic reticulum calcium. Several groups have now confirmed that this is true as STIM1 reportedly interacts with TRPC1 following thapsigargin treatment.[76–81] Interestingly, it appears that the

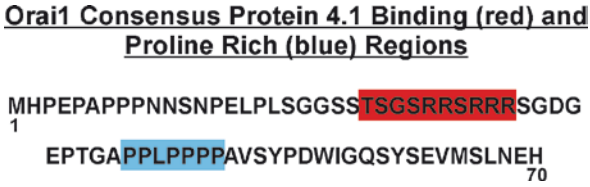

Fig. 9.6 Orai1 possesses a conserved protein 4.1-binding domain and proline-rich region in its amino-terminal domain. Similar to TRPC4, Orai1 possesses a conserved protein 4.1-binding domain and proline-rich region. At present, the function of these domains, and their relation to TRPC4-protein 4.1 binding, is unknown

interaction between STIM1 and TRPC1 is not direct but requires the coassociation of Orai1 with TRPC1. Indeed, Orai1 may be necessary for TRPC1 proteins to fulfill the criteria of a "store-operated" channel, again suggesting that Orai1 is an ancillary protein to the pore-forming TRPC channel.

While we know from preliminary work in our lab that the TRPC1/3/4 channel interacts with Orai1, we also know that TRPC1/3/4 activation requires a constitutive interaction between this channel complex and protein 4.1 and between protein 4.1 and spectrin; indeed, disruption of either of these protein-protein interactions prevents thapsigargin from activating the I_{SOC}. We began to question whether there is a relationship between the requirement for protein 4.1 to interact with the channel complex and Orai1. Remarkably, sequence alignment revealed that the Orai1 amino terminus possesses a conserved protein 4.1-binding domain immediately upstream of a proline-rich region (Fig. 9.6). It is not presently clear whether this protein 4.1-binding domain is important for the orai1-TRPC1/3/4 channel association, whether it contributes to TRPC1/3/4 channel activation, or whether it is necessary for the channel to be store operated. Thus, a principal goal of future studies must be to determine how the protein 4.1-binding domain on Orai1 contributes to TRPC1/3/4 channel activation.

7 Summary

It is an exciting time to study calcium-dependent signal transduction in endothelium. Since the mid-1990's, great strides have been made, with new protein candidates rapidly emerging as putative calcium channels. In most cases, molecular anatomy of calcium channel architecture is lacking and represents an essential goal for future work. Moreover, establishing the physiological role of calcium channels in endothelium is critically important. The TRPC1/3/4 channel provides a calcium source that disrupts cell-cell adhesion and increases endothelial cell permeability. Studies seeking to better resolve the molecular anatomy of this channel, its associated subunits, and its mode of activation will be critically important steps in refining our understanding of how the vasculature responds to inflammatory cues.

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