Chapter 2
Contribution and Regulation of Calcium Channels in Endothelial Cells

Kwong Tai Cheng, Avia Rosenhouse-Dantsker, and Asrar B. Malik

Abstract The endothelium is a highly metabolically active organ that plays a pivotal role in many physiological processes. Endothelial cells express a diversity of calcium-permeable ion channels that can be activated in response to a variety of stimuli including Ca^{2+} store depletion, oxidative stress, growth factors, and endotoxins. Emerging evidences have implicated the critical requirement of Ca^{2+} signaling in numerous vascular functions including vasomotor tone, barrier function, leukocyte homing and adhesion, inflammation, and hemostasis. The goal of this chapter is to present a comprehensive review of the expression and regulatory mechanisms of Ca^{2+} channels in endothelial cells, and discuss their contribution to vascular endothelial cell physiology and pathophysiology processes.

Keywords Endothelial cell • Endothelium • Permeability • SOCE • TRPC channels • TRPC1 • Orai1 • STIM1 • Ca^{2+} signaling • Cell function

Introduction

The endothelium is the largest organ in the body forming a highly specialized cellular network that is composed of $1–6 \times 10^{13}$ endothelial cells (ECs) [1, 2] with an estimated surface area that exceeds 1000 m^2 [3]. ECs line the inner wall of the entire vascular tree, forming a critical interface between the circulating blood and the surrounding tissues [4], thereby playing a key role in various physiological and pathological processes such as blood supply, nutrient delivery, metabolic homeostasis, immune cell trafficking and inflammation [5–7].

Under physiological conditions, ECs exert a number of functions that are important for normal homeostasis (see Fig. 2.1). The most important functions of the endothelium include regulating vasopermeability (i.e. barrier function) [8],

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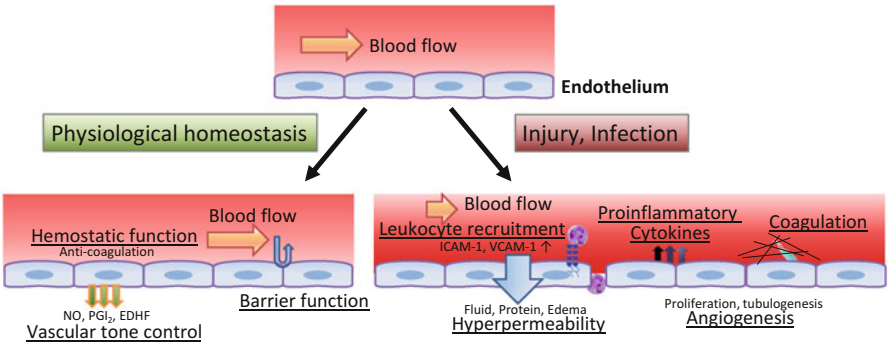


Fig. 2.1 Physiological and pathophysiology function of endothelial cells. Schematic overview of the various functions mediated by endothelial cells that impact physiological homeostasis, and their response under pathological conditions

prevention of inappropriate coagulation [9], regulation of microcirculation byproduction, and release of vasoactive mediators that can control the tones of underlying vascular smooth muscles and modulate EC functions themselves [10]. However, these functions can be significantly altered for adaptive responses under pathological conditions [7]. Specifically, ECs are the first cells exposed to invading pathogens circulating in the bloodstream, and have a prominent role in surveying the circulation of molecules of invading microbial pathogens. Furthermore, receptors of the innate immune system in EC scan be activated by microbial components and contribute to host-defense via the release of inflammatory mediators, recruit leukocytes and promote clotting as a measure to contain infections.

Ca^{2+} signaling in ECs has been shown to be critically required for the physiological and pathological processes described above [11]. In particular, it has been shown that release of vasoactive mediators is tightly governed by Ca^{2+} signaling, and that the increase in endothelial permeability depends on both Ca^{2+} release from the endoplasmic reticulum as well as Ca^{2+} entry through the plasma membrane (PM) [11]. The expression of adhesion molecules and the initiation of an inflammatory cascade have also been shown to be facilitated by Ca^{2+} signaling [12]. All these processes, which are vital for maintaining EC homeostasis, are interconnected via Ca^{2+} signaling, underpinning the importance of ion channels in the regulation of EC function. In addition, the gating and activation of ion channels are rapid processes that enable ECs to react to changes in the local environment by adjusting their function in a timely manner. Elucidating the mechanisms of Ca^{2+} signaling in ECs and the associated downstream signaling cascade promises to provide novel insights as to how these processes can be differentially regulated in a specific spatial and temporal manner as well as identify checkpoints that are critical for particular functions.

Thus, in this review, we will focus on Ca^{2+} entry pathways that have been reported in ECs. In particular, we will discuss store-operated calcium entry (SOCE) that has been shown to be critically required for multiple EC functions, and can

therefore provide insights into the role of Ca^{2+} signaling in vasculature and shed light on the potential importance of ion channels as therapeutic targets for various cardiovascular diseases.

Ca^{2+} Entry Pathway in Endothelial Cells

Store-Operated Calcium Entry

Store-operated calcium entry (SOCE) is an ubiquitous Ca^{2+} entry pathway in all excitable and non-excitable cells that was first described by Putney almost three decades ago [13]. The primary trigger for SOCE activation is the depletion of the endoplasmic reticulum (ER) Ca^{2+} store. Conversely, refilling this store leads to SOCE inactivation. Under physiological conditions, SOCE is activated in response to stimulation of G-protein coupled receptors (GPCR) in the plasma membrane that results in an increase in phospholipase C (PLC) activity, and facilitates $PI(4,5)P_2$ hydrolysis, IP_3 generation, and IP_3-mediated Ca^{2+} release from the ER (see Fig. 2.2). This decrease in ER Ca^{2+} content subsequently leads to activation of SOCE through

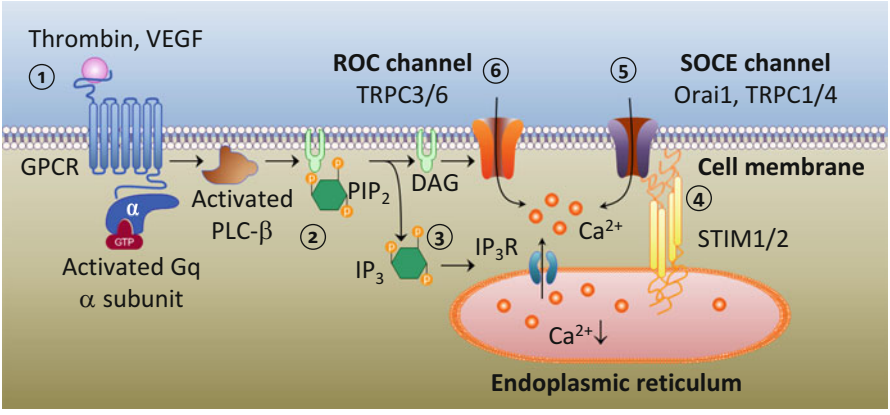


Fig. 2.2 Mechanism of Store-operated Ca^{2+} entry (SOCE) and Receptor-operated Ca^{2+} entry (ROCE) in endothelial cells. (1) An agonist (e.g. thrombin, VEGF) binds to G-protein coupled receptors or receptor tyrosine kinases. (2) Through the activation of phospholipase C (PLC), phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) is hydrolyzed into the secondary messenger inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG). (3) IP3 rapidly diffuse across the cytosol and interact with IP_3 receptors (IP_3R) located in the ER, which results in depletion of the calcium stored in the ER lumen through opening of the IP_3R. (4) Ca^{2+} dissociates from the EF-hand of STIM, facilitating STIM protein translocation and oligomerization in the plasma membrane ER junction. (5) The C-terminus of the STIM protein undergoes a conformational change to an extended structure, enabling it to directly gate plasma membrane SOCE channels (Orai1, TRPC1/4). (6) DAG directly activates receptor-operated calcium (ROC) channel(s) (TRPC3/TRPC6)

the opening of transmembrane store-operated calcium (SOC) channels. Ca^{2+} influx through these channels plays a primary role in refilling the ER Ca^{2+} store. Multiple studies have demonstrated that this Ca^{2+} influx mechanism can also regulate a broad spectrum of cellular responses such as EC migration, proliferation, T cell activation and mast cell degranulation [14]. Yet, only following the recent identification of the genes encoding the SOCE components, has it become possible to determine the structure of the fundamental units of SOCE. Furthermore, identification of the signaling mechanisms that underlie activation of SOC channels at the plasma membrane as a function of the filling status of the ER Ca^{2+} store, remain a major hurdle in SOCE research.

Molecular Components of the SOCE

Canonical Transient Receptor Potential (TRPC) Channels

The search for SOC channels led to the discovery of the mammalian transient receptor potential (TRP) channels. Among these, TRPC1 was the first mammalian TRP protein to be identified. Subsequent research revealed that mammalian TRP genes encode a superfamily of ion channels that can be grouped into seven subfamilies: TRPC, TRPV, TRPM, TRPA, TRPN, TRPP, and TRPML. All TRP channels are tetramers comprised of four subunits arranged around a central pore [15]. The transmembrane domain of each subunit consists of six membrane-spanning domains. Different TRP subunits can assemble in homomeric and/or heteromeric structures. However, little is known about the subunit stoichiometry and biophysical properties of native TRP channels expressed in different cell types. Yet, different combinations of TRP channels may confer specific cellular functions in a cell-type and tissue-specific manner. Therefore, the physiological implications of the diversity in heteromeric channel assembly, and its resultant variations in cation conductance have become a major interest in TRP channel research.

The canonical TRPC protein subfamily includes seven isoforms (TRPC1 to 7) with size range from 700 to 1000 amino acids. Members of the TRPC subfamily were proposed as candidate channels for SOCE based on their activation by stimuli that lead to $PI(4,5)P_2$ hydrolysis. Indeed, all mammalian TRPCs require PLC for activation [16]. However, there is variability in their selectivity for Ca^{2+} versus other cations, and in the mechanism coupling PLC activity to channel stimulation. Several TRPC proteins are stimulated through the SOCE mechanism including TRPC1, TRPC4, and TRPC5 in homomeric form, and TRPC3 and TRPC6 as a part of heteromultimers [17]. On the other hand, several mammalian TRPCs (TRPC3, TRPC6, and TRPC7) are coupled to PLC activation and activated in a store-independent manner by diacylglycerol (DAG). Whether these DAG-sensitive TRPC channels form homomultimers or heteromultimers remains unclear. It has also been reported that subunits from other TRP sub-families can form heteromeric channels with members of the TRPC family, and contribute to SOCE activity [18, 19]. However, this result is controversial, and additional studies are required to further investigate it.

Expression of TRPC Channels in Endothelial Cells

TRPC channels have been shown to be abundantly expressed in ECs obtained from a variety of sources. All TRPC isoforms were detected in primary ECs [10]. TRPC1 and TRPC3 to TRPC6 were identified in bovine aortic ECs [20]. In contrast, bovine pulmonary artery ECs did not express the TRPC3 isoform. Data from our group have demonstrated the expression of TRPC1, TRPC3, TRPC4 and TRPC6 in human umbilical vein ECs (HUVECs), human dermal microvascular ECs (HDMECs) and human pulmonary artery ECs (HPAECs) [21]. In addition to cultured ECs, several studies examined the expression profile of TRPC channels in ECs from intact vessels. TRPC1 and TRPC3 have been shown to be expressed in human mesenteric arteries by using single-cell RT-PCR [22] whereas in another study, in situ hybridization yielded strong labeling of TRPC1,3-6 in endothelial and smooth muscle cells of human coronary and cerebral arteries [23]. TRPC7 labeling was exclusively found in ECs but not in smooth muscle cells [23]. These studies have shown that the expression profile of TRPC channels may be different with respect to ECs derived from different vascular beds and/or from different animal species, suggesting that the function of ECs can be differentially regulated by expressing specific combinations of TRPC channels such that ECs from different vascular beds can response to environmental stimuli in a distinctive spatial and temporal manner.

STIM Proteins and Orai Channels

STIM was first identified in 2005 by an RNAi screen as a regulator of SOCE in Drosophila and HeLa cells [24, 25]. In human, there are two STIM homologs, STIM1 and STIM2. STIM1 encodes a single-pass transmembrane protein of 77 kDa, localized primarily in the ER membrane. Structural analysis of STIM1 revealed a Ca^{2+} binding EF-hand domain in the ER luminal residing portion implying that the protein functions as an ER Ca^{2+} sensor [24, 26]. The cytosolic C-terminus of STIM1 is organized in several distinct modules, the most critical of which is a region that includes roughly 100 amino acid, and is referred to as the STIM1-Orai Activation region (SOAR) [27], CRAC activation domain (CAD) [28] or CCb9 [29]. Overexpression of this region can facilitate spontaneous calcium induced activated calcium (CRAC) current (I_{CRAC}) in the absence of store depletion suggesting that this region is necessary for CRAC channel activation. The C terminal end of STIM1 contains a region with multiple lysine residues, which is referred to as the K-domain. The K-domain plays several roles. First, it interacts with $PI(4,5)P_2$ at the interface with the plasma membrane and stabilizes the STIM1 cluster at the ER-PM junction [30]. More importantly, the K-domain is required for gating TRPC channels via intermolecular electrostatic interactions [31]. Recently, the role of STIM1 in generating Ca^{2+} influx been further clarified and the underlying mechanism elucidated [32]. Specifically, when the ER Ca^{2+} store is filled, Ca^{2+} is bound to the EF-hand of STIM1. Under these conditions, STIM1 is uniformly distributed in the ER membrane. Upon agonist stimulation, generation of IP_3 will trigger IP_3R

opening and lead to depletion of the ER Ca^{2+} store. Ca^{2+} will then dissociate from the EF-hand of STIM1 and mediate a conformational change resulting in oligomerization of the STIM1 molecule. STIM1 oligomers then translocate to the ER-PM junction forming puncta. These localized STIM1 clusters interact and activate store-operated channels on the plasma membrane and generate Ca^{2+} influx.

STIM2, the second mammalian ER Ca^{2+} sensor, exhibits high structural similarity to STIM1. However, the Ca^{2+} sensitivity and activation kinetics of STIM2 have been shown to differ from those of STIM1 [33]. STIM2 also oligomerizes and translocates to the ER-PM junction in response to store depletion. However, STIM2 is a poor activator of Orai1 channel compared to STIM1. The role of STIM2 has not yet been conclusively established but recent data have shown that STIM2 can recruit STIM1 to the ER-PM junction under low stimulus intensities, thereby promoting assembly of the STIM1-Orai1 channel complex and fine tuning the agonist sensitivity of Ca^{2+} signaling. Further insights into the mechanisms that govern the function of the STIM protein will undoubtedly continue to emerge as research in this field continues.

The first store-operated Ca^{2+} current, I_{CRAC}, was measured in mast cells and T lymphocytes [34]. However, the molecular identity of this channel remains unknown after almost a decade of characterization. In 2006, Orai1 was identified as the core component of the CRAC channel through multiple studies using genome-wide RNAi screening in Drosophila [35, 36] and genetic linkage analysis in severe combined immune deficiency (SCID) patients [35]. It was demonstrated that a point mutation in the Orai1 gene (R91W) of SCID patients leads to the loss of I_{CRAC} and SOCE in SCID T-lymphocytes. Notably, expression of WT Orai1 in these cells rescued RAC channels function, thereby restoring store-operated Ca^{2+} influx [35]. Orai1 is a four-transmembrane domain protein primarily located on the plasma membrane. Early studies of the role of Orai1 in the SOCE mechanism suggested that Orai1 acts as a regulatory subunit of TRP channel [37] or that it forms a complex with STIM1 and TRPC1 [38]. However, extensive studies using site-directed mutagenesis led to the identification of a number of Orai1 mutants that were capable of altering the electrophysiological properties of I_{CRAC}, suggesting that Orai1 is the pore-forming subunit of the CRAC channel [39]. Following this observation, it is currently widely accepted that Orai1 alone constitute the I_{CRAC} channel pore and are responsible for SOCE.

Initial studies of Orai1 have primarily focused on lymphocyte and immune function. Surprisingly, Orai1 is not only expressed in haematopoietic cells, but is also widely expressed in other cell types and tissues. Earlier studies have shown that vascular endothelial growth factor (VEGF) receptor activation can facilitate Ca^{2+} release from the IP_3 receptor, and consequently induce a small Ca^{2+} current across the plasma membrane [40]. Moreover, using electrophysiology techniques, the Nilius group demonstrated that depletion of the ER calcium store can facilitate an inwardly rectifying CRAC-like current in bovine pulmonary artery ECs [41]. More recently, extensive research aimed to characterize the expression of Orai1 in the vasculature, was carried out. Our group and the groups of Trebak and Beech demonstrated the expression of Orai1 mRNA and protein in HUVEC [42–44]. Additionally, it has also been shown that Orai1 is expressed in human lung microvascular ECs [42], rat pulmonary microvessel ECs [45], and immortalized mouse lung ECs [42].

Channels Operated by Oxidative Stress

Oxidative stress can be generated in a variety of pathophysiological conditions including hypoxia, inflammatory response and injury. It has been shown that oxidative stress can modulate EC function through the activation of ion channels through the production of reactive oxygen species (ROS), subsequently leading to an increase in vascular endothelial permeability, thereby playing a crucial role in several lung diseases. The TRPM2 (melastatin) channel is among the best characterized oxidative stress sensitive channels in ECs. TRPM2 is a voltage-independent, oxidant-sensitive, nonselective cation channel that is uniquely gated by the binding of adenosine diphosphoribose (ADP-ribose) to its C-terminal domain. Recent studies demonstrated that TRPM2 may facilitate oxidative damage in the endothelium [46–50]. The proposed underlying mechanism suggested how oxidative stress could promote Ca^{2+} entry and specific Ca^{2+} dependent cellular processes. Accordingly, oxidative stress can lead to the production of hydrogen peroxide (H_2O_2) in the cytosol that would then result in nuclear and mitochondrial production of ADP-ribose. ADP-ribose would subsequently open the TRPM2 channel, resulting in increased cytosolic Ca^{2+} concentration and inducing endothelial dysfunction. In addition to the TRPM2 channel, there is emerging evidence suggesting that other TRPM channels can also be activated in response to oxidative stress and alter EC function. Knockdown of TRPM7 in HUVEC caused enhanced growth and proliferation, as well as increased expression of nitric oxide synthase and nitric oxide production [51]. In addition, knockdown of TRPM7 protects HUVEC from hyperglycemia-mediated injury [52]. Together these data suggest that TRPM7 has a prominent role in angiogenesis and smooth muscle tone control in the vasculature. In addition, a recent study has demonstrated that TRPM4 channel is critical to oxidative stress-enhanced EC migration [53]. It should be noted that the biophysical property of these various TRMP subtypes shows difference in ion selectivity, TRPM2 is a non-selective channel which show a linear current-voltage (I-V) relation that allow Na^+ and Ca^{2+} to pass through, while TRPM4 are only permeable to monovalent cations (Na^+, K^+, Cs^+) and TRPM7 carry divalent cations including Mg^{2+} as well as to a variable degree of Ca^{2+}, the difference in their ion selectivity thus may explain how these oxidative stress regulated channel can differentially affect multiple downstream EC function.

Cyclic Nucleotide-Gated Channels

The cyclic nucleotide-gated (CNG) channel is a family of Ca^{2+} permeable nonselective cation channels that open in response to elevated levels of cyclic nucleotides in the cytosol, particularly cAMP and cGMP. CNG channels were first identified in the visual and olfactory systems where they were shown to play pivotal roles in sensory detection.

Six CNG isoforms have been identified. In native cells, CNG channels usually form heterotetrameric complexes consisting of A and B subunits [54]. The A1–A3 subunits may also form functional channels on their own, whereas the B and A4 subunits serve modulatory functions. CNG channels are widely expressed in vascular tissues across species [55, 56]. CNGA1 channels have been shown to be abundantly expressed in the endothelium layer [56]. They also express in smooth muscle cells albeit at a much lower level [56]. In contrast, robust expression of CNGA2 channel was shown in both the endothelium and smooth muscle layers of human arteries [55]. Moreover, the expression of CNGA2 in endothelium can be upregulated by nitric oxide (NO), and it has been shown that this leads to elevated cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and SOCE in porcine PAEC [57].

Endothelial Cell Functions Regulated by Ca^{2+} Signaling

Permeability

The endothelium serves as a selective barrier for the exchange of fluid and macromolecules from the circulation to the tissue environment. Endothelial barrier function can be adjusted in response to tissue injury or infections. Inflammatory mediators (e.g. thrombin, TNF-α, histamine) bind to G-protein coupled receptors located on the EC plasma membrane, which then leads to an increase in $[Ca^{2+}]_i$ via the SOCE machinery. The increase in $[Ca^{2+}]_i$ facilitates cytoskeletal protein reorganization by myosin light chain-dependent EC contraction and disassembly of vascular endothelial cadherin (VE-cadherin) at adherens junctions, subsequently leading to gap formation and an increase in endothelial permeability [11]. Thrombin, the ligand of the proteinase-activated receptor (PAR), has been extensively used by us to study the mechanism of endothelial permeability *in vitro* and *in vivo* [8]. We have shown that thrombin receptor cleavage and the consequent combined elevation of intracellular Ca^{2+} and PKC activation is required for permeability increase in bovine pulmonary arterial ECs [58]. We also demonstrated that this process is mediated through activating the proteinase-activated receptor-1 (PAR-1) GPCR [59], and confirmed these observations by using a transgenic mouse model deficient in PAR-1 [60]. The question remaining to be answered is which Ca^{2+} entry pathway, and more specifically, which Ca^{2+} channel is responsible for this thrombin induced increase in endothelial permeability response.

Several reports from our group as well as others have demonstrated the involvement of Ca^{2+} entry through TRPC1, TRPC4 and TRPC6 channels in the disruption of the barrier function in pulmonary arteries [61, 62]. We have shown that TRPC1 channels make a major contribution to the increase in vascular permeability induced by thrombin and VEGF through the activation of store-operated Ca^{2+} influx [21, 63]. The expression of TRPC1 channel is upregulated by inflammatory mediators through the NF-κB pathway, and its activation can be controlled by Rho activation

[64] and PKCα phosphorylation [21, 65]. In addition, it has been reported that TRPC1 may have a role in cytoskeletal rearrangement [66], strongly supporting the role of TRPC1 in permeability regulation. We have also shown that TRPC4-deficient mice were protected from thrombin-induced permeability *in vivo* [11, 62]. ECs generated from TRPC4−/− mice did not respond to thrombin or PAR-1 agonist peptide (Figs. 2.3 and 2.4). Furthermore, *ex vivo* lung preparation from TRPC4−/− mice displayed a reduction in permeability increase in response to PAR-1 activation. In summary, TRPC4 channel deficiency led to reduced Ca^{2+} entry in ECs, suppressed thrombin-mediated actin-stress fiber formation and thus reduced permeability increase (Fig. 2.5). These observations were further confirmed by other research groups showing a direct interaction between TRPC4 and cytoskeletal proteins in ECs [67, 68].

TRPC6 is another non-selective Ca^{2+} permeable ion channel involved in vascular permeability control. In contrast to TRPC1 and TRPC4, which are regulated by the SOCE mechanism, activation of TRPC6 can also be triggered by Diacylglycerol (DAG), a second product of PLC activity, which is independent of store depletion [69]. It has been previously demonstrated that DAG or its analog 1-oleoyl-2-acetyl-sn-glycerol (OAG) can induce EC contraction and subsequently gap formation through protein kinase C-α (PKCα)-dependent activation of RhoA. TRPC6 siRNA attenuated Ca^{2+} entry induced by thrombin in concert with the decrease in permeability [61]. It was later shown that the phosphatase and tensin homolog (PTEN) exclusively interacts with TRPC6 and is necessary for OAG-induced calcium entry and the subsequent increases in monolayer permeability [70]. Consistent with this finding, ECs isolated from TRPC6−/− mice lungs displayed reduced Ca^{2+} signaling as well as reduced permeability increase in response to ischemia and OAG stimulation. Accordingly, TRPC6−/− mouse lungs are protected from ischemia/reperfusion-induced increases in lung permeability and edema [70].

Although it has been demonstrated in multiple studies that Orai1 and STIM1 form the core components of the CRAC channel, overexpression of these proteins are sufficient to generate I_{CRAC} and SOCE in T lymphocytes as well as other cell types. However, the involvement of Orai1 and STIM1 in generating SOCE in ECs as well as their role in permeability regulation yielded mixed results. The Trebak group first showed that STIM1 and Orai1 mediate CRAC currents and store-operated calcium entry in HUVECs. RNA silencing of either STIM1 or Orai1 essentially abolished SOCE and I_{CRAC} in ECs [44]. Surprisingly, in another report by the same group, they challenged the requirement for Ca^{2+} signaling in permeability control, and suggested that STIM1 alone controls endothelial barrier function independently of Orai1 and Ca^{2+} entry [71]. The authors' rationale was based on the observations that in HUVECs, STIM1 directly coupled the thrombin receptor to RhoA activation and myosin light chain phosphorylation, leading to formation of actin stress fibers, and loss of cell-cell adhesion. These data suggested that endothelial permeability regulated by STIM1 was independent of Ca^{2+} entry. In contrast to these studies, we observed that neither Orai1 knockdown nor expression of a dominant-negative Orai1 mutant could inhibit SOCE induced by either thrombin or Thapsigargin (Tg) in murine and human lung microvascular ECs. Whereas inhibition

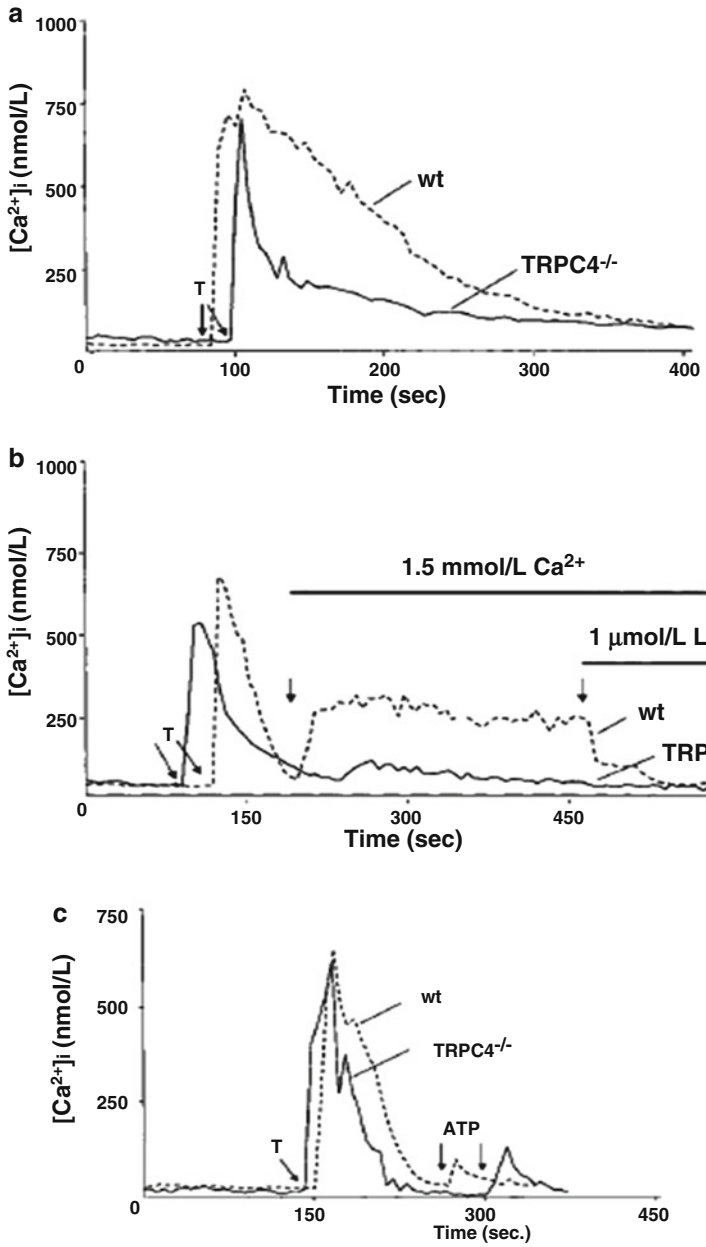


Fig. 2.3 (**a**) Effects of thrombin on $[Ca^{2+}]_i$ in LECs. Thrombin-induced increase in $[Ca^{2+}]_i$ was measured. In each experiment, 25–35 cells were selected to measure changes in $[Ca^{2+}]_i$. Extracellular Ca^{2+} concentration was 1.26 mmol/L. The *arrows* signify the time thrombin (5 U/mL) was added. Results are representative of four experiments. T indicates thrombin. (**b**) TRPC4−/− LECs fail to show thrombin-induced Ca^{2+} influx. Thrombin-induced Ca^{2+} influx was measured in wt and TRPC4−/− LECs. Fura 2-AM loaded cells were washed two times, placed in Ca^{2+}- and Mg^{2+}-free HBSS, and then stimulated with thrombin (5 U/mL). After return of $[Ca^{2+}]_i$ to baseline levels, cells were stimulated with $CaCl_2$ (1.5 mmol/L) and at the indicated time lanthanum chloride (1 µmol/L) was added to inhibit Ca^{2+} influx. The experiment was repeated three times with similar results. (**c**) Effects of thrombin on Ca^{2+} store depletion. Cells were first stimulated with thrombin (5 U/mL) in the absence of extracellular Ca^{2+}. After recovery to the base-line value, cells were challenged with ATP (5 µmol/L). Note that there was no significant increase in $[Ca^{2+}]_i$ in response to ATP. Experiment was repeated three times with similar results. [Adapted from Tiruppathi et al. [62]]

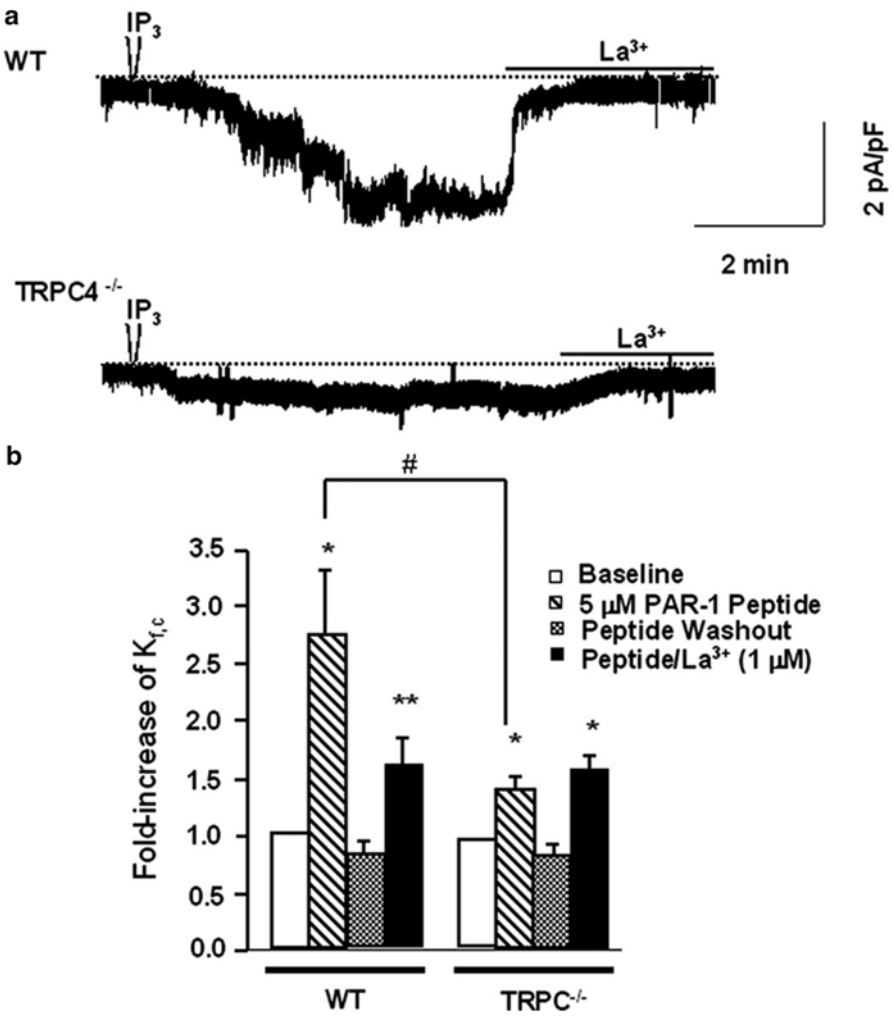


Fig. 2.4 TRPC4 activation of store-operated Ca^{2+} entry (SOCs) regulates microvessel permeability. (**a**) IP_3-induced activation of lanthanum (La^{3+})-sensitive SOCs is shown as whole cell continuous current recordings at a holding potential of −50 mV. The *traces* show that in endothelial cells isolated from wild-type (WT) mice, internal dialysis with IP_3 induced store-operated current, indicating Ca^{2+} entry. Deletion of TRPC4 in mice (TRPC4$^{-/-}$) prevented the Ca^{2+} entry in response to store depletion by IP_3. Scale represents picoampere (pA) and picofarad (pf). (**b**) In WT mice, PAR-1 peptide induced a 2.5- to 3-fold increase in lung microvessel permeability, which was reduced to 50 % by La^{3+}, the Ca^{2+} channel blocker, indicating Ca^{2+} entry contributes to the mechanism of increased lung vascular permeability. However, in TRPC4$^{-/-}$ mice, the PAR-1 peptide only induced a 1.5-fold increase in permeability, which was not affected by La^{3+}, indicating that Ca^{2+} entry via TRPC4 contributes to the regulation of microvessel permeability. *Different from baseline value. **Inhibitory effect of La^{3+}. #Significantly reduced response of TRPC4$^{-/-}$ for PAR-1 agonist peptide compared with WT. [Adapted from Tiruppathi et al. [62]]

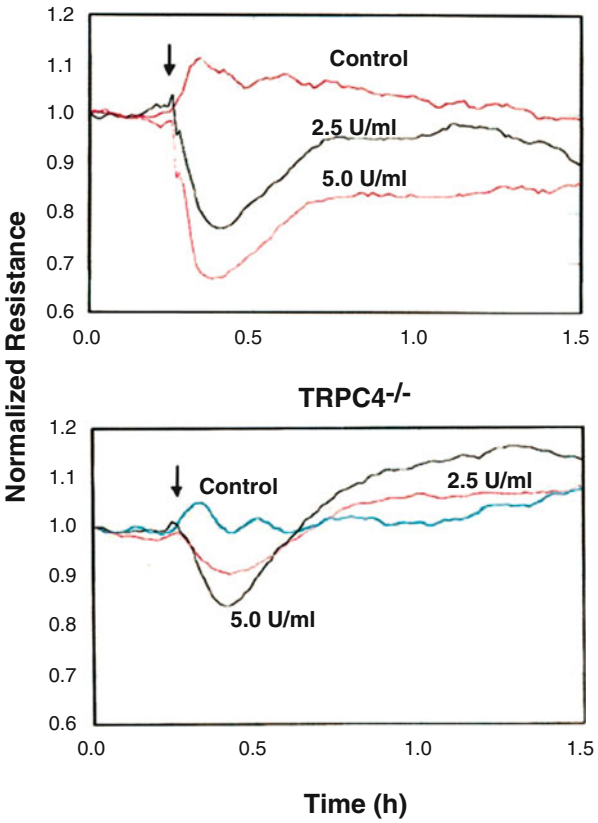


Fig. 2.5 Effects of thrombin on WT and TRPC4–/– LEC transendothelial monolayer electrical resistance values. LECs were grown to confluence on gold electrodes. Before the experiments, cells were washed twice and incubated with 1 % FBS containing growth medium for 2 h before the addition of thrombin. The *arrow* indicate the time thrombin was added. Experiment shown is representative of four trials. [Adapted from Tiruppathi et al. [62]]

of Orai1 only reduced the initial peak of SOCE, the effect was not statistically significant [42]. We further showed that knockdown of STIM1 or expression of the dominant-negative STIM1 mutant in ECs suppressed Ca^{2+} entry secondary to store depletion. Moreover, our data conclusively indicated that STIM1 could interact with TRPC1 and TRPC4 to form a functional SOC channel in ECs. Whereas both studies agreed regarding the requirement of STIM1 in permeability control, albeit through different regulatory pathways, our findings that Orai1 does not regulate SOCE in native ECs differs from the findings of Abdullaev et al. (2008) [44]. To address this difference, we also knocked-down Orai1 in HUVECs. Our data showed that in this cell type, and in agreement with the study of Abdullaev et al. [44], Orai1 plays a similar role in regulating SOCE, suggesting that the discrepancy was related to the origin of ECs being used in these studies.

Orai channels are highly Ca^{2+} selective, whereas TRPC proteins are nonselective cation channels permeable to Ca^{2+}, Na^{+}, and Cs^{+} [72]. We observed expression of Orai1, Orai2, and Orai3 proteins in HUVECs. Accordingly, Orai proteins may contribute to Ca^{2+}-selective SOC activity in HUVECs that may be required for specialized function of human umbilical veins. In contrast, exogenous expression of Orai isoforms or knockdown had no significant effect on thrombin-induced Ca^{2+} entry in both human and mouse primary ECs, indicating that TRPCs are the primary channels that conduct the nonselective cation current required for regulating native EC function.

As noted above, SOCE is activated in response to stimulation of G-protein coupled receptors (GPCRs). In addition, however, EC permeability can also increase in response to environmental stress due to, for example, ROS generation and shear stress. Specifically, ROS can directly activate plasma membrane ion channels and mobilize $[Ca^{2+}]_i$, and ECs exposed to shear stress show an increase in phospholipase activity that impacts membrane permeability [10]. Our group has also demonstrated that H_2O_2 elicited Ca^{2+} influx via TRPM2 channel activation leading to an increase in endothelial permeability [49] (see Fig. 2.6). This process could be attenuated by inhibiting TRPM2 using a pharmacological inhibitor or through the application of specific siRNA or a blocking antibody, thereby conclusively demonstrating a critical role for TRPM2 in the mechanism of endothelial barrier disruption following oxidative stress (Fig. 2.7). In addition to the full-length TRPM2 (TRPM2-L), we have also identified a short spice variant of TRPM2 (TRPM2-S) [47, 73] that could serve as a negative regulator of TRPM2 channel activity in ECs via PKCα phosphorylation upon H_2O_2 stimulation [48]. Further studies are needed to investigate the mechanism that underlies the regulation of TRPM2-mediated Ca^{2+} entry and endothelial injury by PKCα.

Permeability regulation through GPCR-dependent and GPCR-independent mechanisms merges at the calcium signaling level. An interesting question that remained to be elucidated is how ion channels can control multiple downstream processes through Ca^{2+} signaling. It has been demonstrated that the maximum increase in $[Ca^{2+}]_i$ occurs after the maximum increase in permeability [74], suggesting that regulation of EC permeability may not be determined by the magnitude of the increase in $[Ca^{2+}]_i$, but rather by the unique Ca^{2+} signature generated in distinct microdomains. For example, it has been shown that the expression of ion channels is highly compartmentalized in polarized exocrine acinar cells. Orai1 is localized in the lateral membrane towards the apical region whereas various TRPC subtypes spread from the apical region towards the lateral membrane, thereby overlapping with Orai1 in the luminal end of the lateral membrane [75, 76]. This implies that microdomains in different regions of the cell consist of specific compositions of ion channels. It is, therefore, not surprising that Ca^{2+} signals generated from these distinct microdomains display unequivocal biophysical properties (activation and regression kinetics, frequency, amplitude of the oscillations, spatial and temporal profile as well as the speed of the Ca^{2+} wave, etc.), and can differentially regulate downstream processes. In support of this notion, a recent study has shown that although Orai1, STIM1 and TRPC channels are all expressed in salivary gland

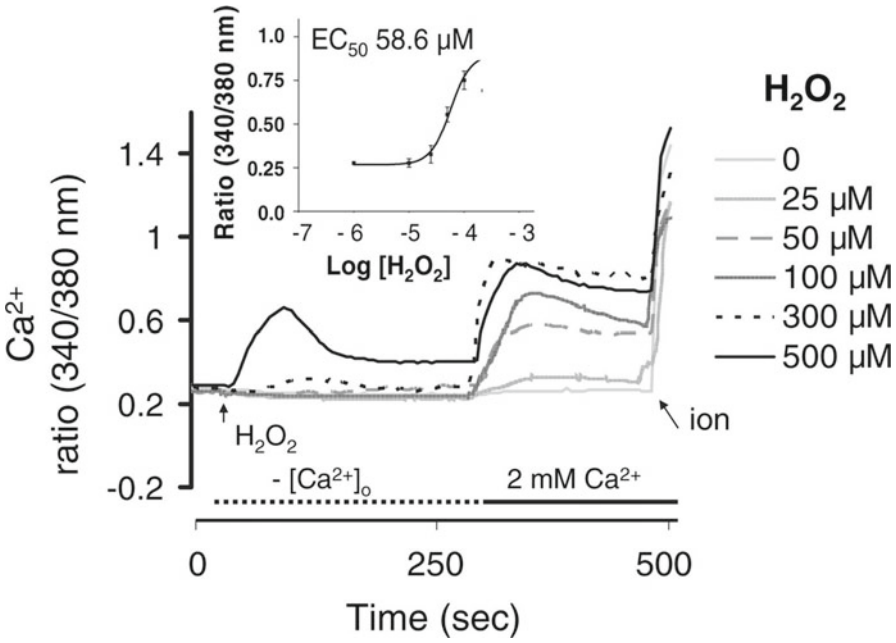


Fig. 2.6 Concentration-dependent effects of H_2O_2 on Ca^{2+} entry. HPAE cells in culture were loaded with fura-2, washed, and transferred to Ca^{2+}-free medium. H_2O_2 (0–500 μmol/L) was added at the *arrow* and $CaCl_2$ (2.0 mmol/L) was repleted at the fifth minute; the resulting Ca^{2+}-repletion transient reflects Ca^{2+} entry. The Ca^{2+} ionophore ionomycin (ion) was added at the end of the experimental recordings for calibration purposes. Each tracing is the average response of 60–99 cells in HPAE monolayers. The abscissa indicates time in seconds; the ordinate, relative $[Ca^{2+}]_i$ level. Experiments were repeated 3–5 times with similar results. The *inset* displays the dose–response curve of best fit for the calcium-repletion transient (EC_{50}, 58.6 μmol/L). The data points are mean values (n = 3 per point), and the bars indicate ± SEM. At >300 μmol/L, H_2O_2 mobilized stored intracellular Ca^{2+}. [Adapted from Hecquet et al. [48]]

acinar cells and associated with SOCE activity, the Ca^{2+} signal generated from these distinct channels can be differentially translated into diverse downstream processes [77]. Although the subcellular localization of these channels is not well characterized in ECs, the same rules are likely to apply. However, further studies are necessary to investigate the assembly and compartmentalization of these major Ca^{2+} signaling components in ECs, and determine how they control vascular endothelial permeability in a channel-specific manner.

Endothelium-Dependent Vascular Tone Control

Vascular tone, the contractile activity of vascular smooth muscle cells in the walls of small arteries and arterioles, is the major determinant of the resistance to blood flow through the circulation. Thus, vascular tone plays an important role in the

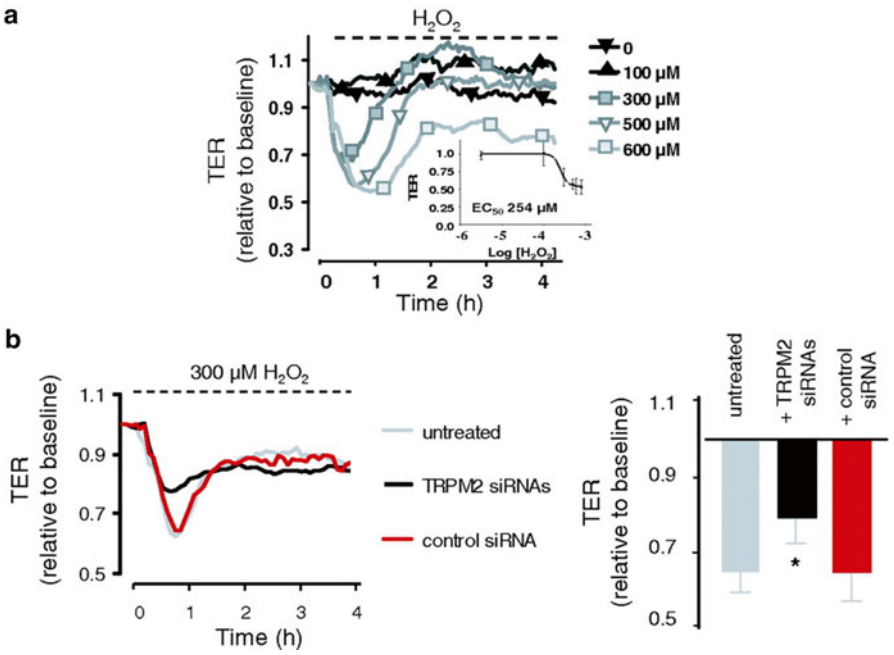


Fig. 2.7 H_2O_2-induced increase in endothelial barrier permeability depends on TRPM2 expression. (**a**) Concentration-dependent action of H_2O_2 on endothelial barrier function. HPAE cells were grown to confluence on gold microelectrodes, the cells were treated with H_2O_2 (concentration indicated), and TER was followed for 4 h. Each tracing is the average response of four wells. Experiments were repeated three times with similar results. The abscissa indicates time in hours; the ordinate, normalized resistance (relative to basal value). The *inset* shows the corresponding dose–response curve (n = 12; bars, ±SEM). H_2O_2 (0–600 µmol/L) caused a rapid, dose-dependent decrease in TER with an EC_{50} of 254 µmol/L. At <500 µmol/L, H_2O_2 effects were transitory. (**b**) TER decrease on H_2O_2 exposure (300 µmol/L). *Left*, Note that TRPM2 silencing inhibits H_2O_2 responses relative to the untreated group (no transfection) or the negative control group (scrambled siRNA; n = 4 per group). The abscissa indicates time in hours; the ordinate, normalized resistance. *Right*, Mean value (±SEM) of peak TER responses to H_2O_2 (n = 12). Experiments were repeated three times with similar results. H_2O_2-induced TER decrease was significantly attenuated in cells transfected by TRPM2-specific siRNAs compared with untreated control or negative control group transfected with a scrambled siRNA. [Adapted from Hecquet et al. [48]]

regulation of blood pressure and the distribution of blood flow between and within the tissues and organs of the body. Vascular endothelium plays a key role in controlling vascular tone by secreting a variety of endothelium-derived relaxing factors such as nitric oxide (NO), and prostacyclin (PGI2) [78–80]. Various circulating vasoactive agents (e.g. bradykinin, ATP, substance P and acetylcholine) can bind to EC surface GPCRs and lead to elevation of EC $[Ca^{2+}]_i$ and consequently activate NO synthase [81]. As noted above, TRPC channels play a critical role in agonist-induced Ca^{2+} entry in ECs. Several lines of evidence suggest that TRPC1, TRPC3 and TRPC4 channels are required for endothelium-dependent vascular tone modulation. Specifically, it has been shown that TRPC1 is responsible for

bradykinin-mediated Ca^{2+} permeable currents in human mesenteric arteries ECs [22]. It was also demonstrated that in a bovine pulmonary artery EC line, overexpression of TRPC3 could enhance Ca^{2+} entry in response to ATP and bradykinin stimulation [82]. Furthermore, in primary aortic ECs isolated from TRPC4−/− mice, acetylcholine-induced Ca^{2+} entry was significantly reduced compare to WT, leading to suppression of endothelium-dependent NO-mediated vasorelaxation of blood vessels [83]. Together, these studies indicate that there is a direct functional link between endothelial TRPC channels and vascular tone control.

However, in addition to TRPC channels, other TRP isoforms may also contribute to endothelium dependent vasorelaxation. Endocannabinoids (AEA) and 2-AG as well as the 5′,6′-epoxyeicosatrienoic acid (EET) are potent vasorelaxants [84]. The Nilius group first showed that TRPV4 channels are a molecular target for these vasorelaxants, and could be directly activated and generate Ca^{2+}-permeable channel activity in ECs, thus suggesting a potential role for TRPV4 in vascular tone modulation [85]. More recently, Sonkusare et al. demonstrated that TRPV4 channels were able to mediate local calcium sparks that activate Ca^{2+} sensitive K^+ channels (BK_{Ca}) and facilitate hyperpolarization, and induced maximal dilation of resistance arteries [86]. Furthermore, it has also been shown that TRPV4 forms heteromeric channels with TRPC1 in vascular ECs and contributes to SOCE and NO synthesis [19]. These data further support the notion that other TRP isoforms may also be involved in the regulation of vascular function. In addition to TRP channel, other Ca^{2+} entry mechanisms in ECs may also contribute to vasorelaxation. For example, endothelial cell CNG channels have been shown to play an important role in endothelium-dependent vascular dilation in response to a number of cAMP-elevating agents including adenosine, adrenaline and ATP [87–89].

Vascular Remodeling, Migration and Angiogenesis

Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) promote angiogenesis by enhancing EC proliferation and migration. These angiogenic factors can enhance the increase in intracellular Ca^{2+} concentration in ECs through the activation of plasma membrane receptors and the release of Ca^{2+} from the endoplasmic reticulum, which leads to activation of plasma membrane Ca^{2+} channels via the SOCE mechanism. In addition, receptor-operated Ca^{2+} entry (ROCE) that does not involve depletion of the ER Ca^{2+} store has also been shown to play an important role. Several studies have highlighted the importance of TRP channel-mediated Ca^{2+} signaling in the angiogenesis process [90]. TRP channels may play a major role in the initiation of vascular remodeling through two different mechanisms. First, proliferative factors may activate TRP channels and mediated TRP-dependent Ca^{2+} entry, thereby modulating downstream signal transduction pathways and leading to vascular remodeling of ECs. Second, Ca^{2+} influx through TRP channels may stimulate ECs to produce and release proliferative factors such as VEGF and PDGF, which subsequently impact

the growth, migration and differentiation of the underlying smooth muscle cells, which is critical for arterial remodeling. The interplay of these two distinct signaling pathways allows control of angiogenesis in a vascular bed specific manner. Data generated by our group as well as by others have provided a detailed description of the mechanism of VEGF-stimulated Ca^{2+} entry in human microvascular ECs (HMVECs) [63, 91, 92]. VEGF binding to the VEGF Receptor 2 (VEGFR2) facilitates tyrosine phosphorylation of the receptor, leading to the generation of IP_3 and diacylglycerol (DAG), which in turn results in the activation of TRPC3 and TRPC6 via a store-independent mechanism [93, 94]. It has been reported that TRPC6 is required for the VEGF-mediated increase in cytosolic Ca^{2+} and the subsequent downstream signaling that leads to processes associated with angiogenesis [95, 96]. Overexpression of a dominant negative TRPC6 construct in HMVECs inhibited the VEGF-mediated increases EC $[Ca^{2+}]_i$, migration, sprouting, and its proliferation in matrigel. Conversely, overexpression of a wild-type TRPC6 construct increased the proliferation and migration of HMVECs [96]. The requirement for TRPC6 in angiogenesis was further explored in another study that demonstrated that inhibition of TRPC6 in HUVECs resulted in cell cycle arrestment at the G2/M phase and suppressed VEGF-induced HUVEC proliferation and tube formation [95]. Furthermore, inhibition of TRPCs abolished VEGF-, but not FGF-induced angiogenesis in the chick embryo chorioallantoic membrane. Together, these results suggest that TRPC6 plays a critical role in VEGF-mediated angiogenesis.

Hypoxia is another major factor that promotes angiogenesis. Hypoxic conditions induce pulmonary artery smooth muscle cell (PASMC) proliferation by promoting transcriptional activation of genes encoding vasoactive agonists such as endothelin-1 (ET-1), and mitogens such as the VEGF and PDGF growth factors. Hypoxia upregulates the mRNA and protein expression of TRPC4 in ECs resulting in elevated SOCE together with increased synthesis and secretion of mitogenic and vasoactive factors. This suggests that TRPC4 may play an important role in the development of hypoxia-mediated pulmonary vascular remodeling [97].

Following the identification of Orai1 as the pore forming subunit of the I_{CRAC} channel, its role in angiogenesis has been extensively studied. As noted above, it has been shown that VEGF, the primary growth factor that drives EC migration, proliferation and angiogenesis, can evoke Ca^{2+} signaling through the SOCE mechanism. Notably, it has been shown that VEGF-mediated Ca^{2+} entry in HUVECs was attenuated by knockdown of Orai1 using specific siRNA, by its inhibition using a dominant negative Orai1 mutant, and by application of a pharmacological inhibitor. It has also been shown that Orai1 is necessary for HUVEC proliferation [44] as well as *in vitro* tube formation [43]. These results were challenged by Antigny et al. [98] who showed that in a HUVEC cell line (EA.hy926), TRPC3, TRPC4 and TRPC5 generated spontaneous Ca^{2+} oscillation upon plating on matrigel and that Ca^{2+} entry through these channels was necessary for tubulogenesis. In contrast, knockdown of Orai1 and STIM1 had no effect. Interestingly, the only phenotype associated with Orai1 knockdown in this cell type was reduced proliferation, which is similar to the effect of knockdown of other TRPC channels. Antigny et al also studied primary HUVECs using the same approach

and demonstrated the involvement of STIM1, TRPC1, and TRPC4, but not Orai1 in endothelial tube formation [98]. The requirement of STIM1 was further corroborated in another study that showed that suppression of STIM1 inhibited angiogenesis *in vivo* [99]. In a different study using endothelial progenitor cells (EPCs), it was shown that SOCE was associated with Orai1, STIM1 and TRPC1 activity. Suppression of SOCE using the SOCE inhibitor BTP-2 or by chelating intracellular Ca^{2+} using BAPTA resulted in the inhibition of EPC proliferation and tubulogenesis [100]. These data collectively demonstrated the requirement for SOCE in angiogenetic processes. The discrepancy in the involvement of Orai1 in vascular remodeling may be due to the use of HUVECs from different sources or due to differences in experimental conditions (e.g. VEGF concentration). In addition, ECs generated from various vascular beds may display different functional phenotypes as well as channel expression profiles due to heterogeneity [101]. Together, these factors may potentially contribute to the inconsistency in the findings. Other than the contributions of SOCE and TRPC channels to the angiogenic process, it has also been suggested that other TRP channels may play an important role in this process. In particular, the potential importance of TRPM6 and TRPM7 channels, and especially of TRPM7 in regulating angiogenesis and vascular remodeling has been proposed [102]. The underlying rationale in these cases is that both TRPM6 and TRPM7 are highly permeable to Mg^{2+} [103], and Mg^{2+} homeostasis has been directly linked to EC proliferation [104, 105].

ECs derived from human breast carcinomas (BTEC) exhibit enhanced angiogenic properties and are thus a useful tool for studying the mechanisms involved in vascular remodeling. Arachidonic acid (AA), a lipid second messenger released by different pro-angiogenic factors (e.g. bFGF and VEGF), is actively involved in the early stages of the angiogenic process *in vitro*, and mediates its effects by activating Ca^{2+} entry via Ca^{2+}-permeable plasma membrane channels [106]. It has been demonstrated that the expression of endogenous TRPV4 is significantly higher in BTECs compared with 'normal' ECs (HMVEC) [107]. Moreover, knockdown of TRPV4 using siRNA resulted in decreased $[Ca^{2+}]_i$ responses and a complete inhibition of AA-induced migration of BTECs. These data suggest that AA regulates angiogenesis by inducing remodeling of the cytoskeleton and stimulating TRPV4 channel activity, both of which are critical for tumor derived EC migration.

Immune Function and Inflammation

The endothelium, positioned at the interface between blood and tissue, is equipped to react quickly to local changes in biological needs caused by trauma or inflammation. Under injury or infection, circulating inflammatory mediators (e.g. endotoxins, cellular content released by damaged cells) can activate ECs and significantly modify their normal physiological functions. This may result in increased leakiness, modification of the vascular tone, enhanced procoagulant activity, and formation of

new vessels [9]. In addition, ECs can also directly contribute to the immune response by expressing cell surface-molecules that direct the trafficking of circulating blood cells. These cell-adhesion molecules determine the homing and migration of leucocytes into specific organs that are under stress or inflammatory conditions. ECs express a variety of pattern recognition receptors [108] that recognize pathogen-associated molecular patterns (PAMPs). Among these, Toll-like receptors (TLRs) are surface molecules that trigger signals resulting in proinflammatory gene expression, leukocyte chemotaxis, phagocytosis, cytotoxicity and activation of adaptive immune responses.

The immune response is tightly regulated by Ca^{2+} signaling. Indeed, the I_{CRAC} channel and Orai1 were discovered during the course of studying T lymphocyte function [35, 109]. SOCE, TRP, Orai1 and STIM1-mediated Ca^{2+} entry have been closely associated with adaptive immunity, and the molecular basis has been described in detail in numerous studies [32]. Although there are considerably less data describing the role of Ca^{2+} signaling in innate immunity, particularly the involvement of Ca^{2+} signaling in EC-mediated immune response, there is a strong indication that SOCE are involved in the pathogenesis of many inflammatory diseases [110]. In addition, a variety of inflammatory substances cause an increase in intracellular Ca^{2+}, which then leads to permeability changes in the microvascular endothelium, and hence, to tissue edema [11]. Data generated by our group as well as others support the notion that Ca^{2+} entry in ECs can modulate immunological function and the postulated involvement of ECs in contributing to the host-defense mechanism during pathological conditions.

TLR4 expression has been demonstrated in various ECs, and was found to be significantly upregulated under inflammatory conditions [111]. An increasing number of studies have indicated that TLR-mediated immune response is associated with Ca^{2+} signaling. Tauseef et al. [112] have shown that endotoxin (lipopolysaccharide; LPS) induces DAG generation in a Toll-like receptor 4 (TLR4)-dependent manner, and triggers Ca^{2+} increase through the TRPC6 channel. Importantly, the authors showed that Ca^{2+} entry through TRPC6 participates in mediating both lung vascular barrier disruption and inflammation induced by endotoxin [112].

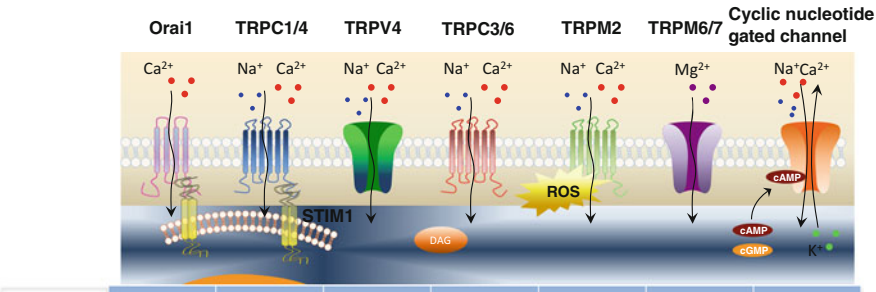
The role of STIM1 in TLR signaling was reported in another study [113]. By using EC-specific STIM1-knockout mice, the authors showed that LPS-mediated Ca^{2+} oscillations are ablated in STIM1-deficient ECs. ECs lacking STIM1 failed to trigger SOCE and NFAT nuclear translocation as well as receptor-interacting protein 3 (RIP3)-dependent cell death. Interestingly, LPS-induced vascular permeability changes were reduced in EC-specific STIM1−/− mice. Furthermore, application of a CRAC channel inhibitor in WT animals also halted LPS-induced vascular leakage and pulmonary edema. Taken together, these results demonstrate that Ca^{2+} signaling plays a critical role in promoting vascular barrier dysfunction during infection, and indicate that the SOCE machinery may provide crucial therapeutic targets to limit edema and acute lung injury (ALI) [113]. The role of STIM1-dependent signaling in EC-dependent immune response was further explored recently by using a mouse sepsis model [114]. It was shown that systemic

administration of bacterial endotoxin (LPS) enhances STIM1 expression in ECs. LPS also induced STIM1 mRNA and protein expression in human and mouse lung ECs. The induced STIM1 expression was associated with augmented SOCE as well as a permeability increase in both *in vitro* and *in vivo* models. Remarkably, inhibition of either NF-κB or p38 MAPK activation by pharmacological agents or siRNA prevented LPS-induced STIM1 expression and increased SOCE in ECs. These data suggest that TLR4-signaling mediated through cooperative signaling of NF-κB and AP1 (via p38α) amplifies STIM1 expression in ECs, and thereby contributes to the lung vascular hyperpermeability response during sepsis [114].

In addition to endotoxin, histamine and chemokines are important immunomodulators involved in allergic reactions, leukocyte homing and inflammatory responses [111]. Notably, both histamine and chemokines can bind to specific GPCRs and induce Ca^{2+} signaling through SOCE mechanism [115, 116]. It has been shown that the CXC chemokine stromal cell-derived factor 1 (SDF-1), also known as CXCL12, can bind to CXCR4 in ECs, leading to an increase in $[Ca^{2+}]_i$, thus initiating signals related to chemotaxis, cell survival, proliferation and gene transcription [115]. Histamine has also been shown to be able to facilitate SOCE in HUVEC, and is sensitive to SOCE inhibitor blockage, to knockdown of Orai1 or STIM1, and to dominant negative expression of Orai1 mutant. It was therefore suggested that histamine-mediated Ca^{2+} entry through SOCE in HUVECs can facilitate NFAT activation as well as interleukin 8 production [116]. These results highlight the central role of Ca^{2+} signaling in general, and of Ca^{2+} entry through TRPC, STIM1 and Orai1 in particular, in mediating Ca^{2+} mobilization linked to inflammatory signaling of ECs upon inflammatory mediator stimulation.

Conclusion and Future Perspective

ECs express a variety of Ca^{2+} channels, which are involved in a variety of physiological and pathophysiological processes (see Fig. 2.8). Despite remarkable progress in the understanding of the role of Ca^{2+} signaling in regulating EC function, numerous aspects of the activation of Ca^{2+} channels remain unclear. In particular, the involvement of the newly identified SOCE core components, the Orai channels and the STIM proteins, in EC function needs to be further elucidated. SOCE has been linked to multiple physiological processes in ECs. Yet, the mechanisms that underlie the differential contribution of SOCE generated through specific microdomains to distinct cellular function have only recently been identified [77]. Elucidating the roles of downstream molecules associated with specific ion channels is essential for refining our understanding of vasculature response to environmental stimuli. It is important to note that the endothelial lining of blood vessels shows remarkable heterogeneity in structure and function in time and space, and in health and disease. An interesting question is whether the



	Orai1, STIM1	TRPC1/4	TRPV4	TRPC3/6	TRPM2	TRPM6/7	CNG
Operating mechanism	SOCE	SOCE	SOCE/AA	ROCE, DAG	ROS, ADP-ribose	ROCE	cGMP, cAMP
Permeability	VEGF, Tg (34. 36, 60) LPS (104, 105)	Thrombin, VEGF, TNFα (15, 34, 45, 51-57)		Bradykinin Thrombin (50, 59) LPS (103)	H_2O_2 (65,67)		
Vascular tone		Bradykinin (16), acetylcholine (73), Tg (77)	AEA, EET , GSK, Tg (75, 76, 77)	ATP, Bradykinin (72)			ATP, Adenosine, Epinephrine, Thromboxane A2 (78-80)
Angiogenesis	VEGF (35, 36, 88, 89, 90)	Hypoxia, ECM (87,88, 90)	bFGF, VEGF, AA (96, 97)	VEGF, ECM (86, 88)		Mg^{2+} homeostasis (93-95)	
Immune response	LPS (104, 105), Histamine (108)			LPS			

Fig. 2.8 Schematic representation of Ca^{2+} channels and their functional role in endothelial cells. A variety of Ca^{2+} permeable ion channels express in endothelial cells where they regulate different physiological processes such as increase in permeability, vascular tone control, vessel growth and angiogenesis as summarized in the Table. The *numbers* in *parenthesis* indicate the respective reference. Abbreviations: *SOCE* Store-operated Ca^{2+} entry, *ROCE* receptor-operated calcium entry, *AA* Arachidonic acid, *DAG* Diacylglycerol, *ADP-ribose* adenosine diphosphoribose, *VEGF* vascular endothelial growth factor, *Tg* Thapsigargin, *LPS* lipopolysaccharide, *bFGF* basic fibroblast growth factor, *ECM* extracellular matrix, *AEA* Endocannabinoids, *EET* 5′,6′-epoxyeicosatrienoic acid

phenotypic and functional variation of ECs from different vascular beds is due to their distinct ion channel expression profile. Emerging data support the notion that Ca^{2+} signaling can be generated in a cell type and tissue dependent manner through distinctive expression patterns of Ca^{2+} channels. The majority of the studies that addressed this aspect have been carried out in cultured EC lines or in primary cultured cells *in vitro* only. Furthermore, *in situ* detection of ion channels in vessels across tissues has only been convincingly demonstrated in a few studies. It is thus of crucial importance to elucidate both the candidate channels architecture across different vascular tissues and their physiological function *in vivo*. Indeed, understanding the molecular basis for phenotypic heterogeneity is a prerequisite for developing vascular bed-specific therapies for modulating specific EC responses in a clinical setting.

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