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IP_3 Receptor Signaling and Endothelial Barrier Function

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Abstract

The endothelium, a monolayer of endothelial cells lining vessel walls, maintains tissue-fluid homeostasis by restricting the passage of the plasma proteins and blood cells into the interstitium. The ion Ca^{2+}, a ubiquitous secondary messenger, initiates signal transduction events in endothelial cells critical for control of vascular tone and endothelial permeability. The ion Ca^{2+} is stored inside the intracellular organelles and released into the cytosol in response to environmental cues. The inositol 1,4,5-trisphosphate (IP_3) messenger facilitates Ca^{2+} release through IP_3 receptors which are Ca^{2+}-selective intracellular channels located within the membrane of the endoplasmic reticulum. Binding of IP_3 to the IP_3Rs initiates assembly of IP_3Rs clusters, a key event responsible for amplification of Ca^{2+} signals in endothelial cells. This review discusses emerging concepts related to architecture and dynamics of IP_3Rs clusters, and their specific role in propagation of Ca^{2+} signals in endothelial cells.

Keywords

microtubule cytoskeleton; end-binding protein 3; endothelial permeability; signal transduction; receptor dynamics

Introduction

In the late 19th century, Wilhelm His Sr. first introduced the term “endothelium” when he described differences between the layers of cells that line the mesoderm. After the inception of the idea of an endothelium as a specific tissue type or organ, the understanding of its exact location spanned many years of debate and technological advancements to pinpoint (Reviewed in [1]. Notedly, with the discovery of its form, came the discoveries of its function. In the recent century, we have transitioned from asking where the endothelium exists to determining the precise function of the endothelium and how this function is regulated in health and disease.

The endothelium is considered as an autocrine, paracrine, and endocrine organ capable of regulating a broad range of vascular functions including tissue-fluid homeostasis, vascular tone, thrombogenesis, inflammation, and vessel growth [2], [3], [4]; Reviewed in [5], [6], [7]. The large body of data related to the functions of endothelial cells have nowadays

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centered upon an important feature: vascular permeability. Considering that the inner vascular lining is formed by mature, quiescent endothelial cells, its primary function is not to proliferate or migrate but rather to act as a semi-permeable barrier between the vascular system and surrounding tissues.

Permeability of the endothelial barrier represents one of the hallmark processes for the maintenance of proper tissue-fluid homeostasis [8], [6], [7]. Once the endothelial barrier is compromised in response to humoral or mechanical stimuli, the barrier loses its selective control, causing excessive leakage of protein-rich fluids across the barrier. Vascular leakage is not only an integral part of many diseases - including systemic capillary leak syndrome [9], dengue fever [10] and Ebola viruses [11], [12], angioedema [13], anaphylaxis [14], acute lung injury [15], [16], variety of eye [17] and central nervous system [18], [19] disorders - but also the major dose-limiting factor in many immunotherapies [20]. The basis of such an event lies in the disruption of the inter-endothelial junctions, which are finely regulated, in part, by intracellular calcium signaling.

The ion Ca^{2+} is a secondary messenger paramount to a multitude of physiological and pathological processes in human body - muscle contraction, metabolic regulation, and fertilization to name a few. Within endothelial cells, the ion Ca^{2+} also plays a fundamental role in the majority of intracellular processes including, but not limited to, cell proliferation, motility, cell-cell adhesion, and cell death. While few recent reviews focus on the role of intracellular calcium in regulation of vascular tone through control of nitric oxide production [21], [22], [23], endothelial cell migration and proliferation - a hallmark of angiogenic response of mature endothelium [24], [25], and endothelial progenitor cells [26] to environmental cues, we focus here on the role of IP_3R channels, an ion channel whose gating is important to the control of calcium homeostasis. Novel findings in IP_3R architecture and dynamics give further insight into regulation of IP_3-evoked calcium signaling and, thereby, regulation of endothelial permeability.

The role of intracellular Ca^{2+} signaling in regulating endothelial barrier function

Endothelial barrier function—Endothelial cells form an inner monolayer, termed the endothelium, lining the vessels of the blood, and lymphatic systems, and the endocardium. The endothelium acts as a semi-permeable barrier that regulates passage of fluids, solutes, gases, nutrients, as well as transmigration of blood cells from circulation into surrounding tissues. A passage of macromolecules such as albumin across the endothelial barrier is essential to the maintenance of tissue-fluid homeostasis. Two main pathways, paracellular and transcellular, are involved in this process. The transcellular pathway, also known as transcytosis, uses receptor-mediated vesicular transport to actively distribute macromolecules across the endothelium. In contrast, the paracellular pathway utilizes interendothelial cell-cell junctions that connect adjacent endothelial cells and restrict permeability to macromolecules larger than 3 nm in diameter [27], [28], [29]. Hence, albumin, a molecule of 3.8 nm in diameter and 15 nm in length [30], [31], which represents ~60% of all plasma proteins [32], [33], is mainly retained in circulation. This higher concentration of albumin in the circulation compared to the interstitial space creates transendothelial oncotic pressure, a driving force of fluid reabsorption from the interstitium

[34], [35]. Loss of interendothelial junctions in disease settings causes proteinaceous tissue edema, a pathological condition associated with leakage of protein-rich fluids in the interstitum [13], [15, 16].

Interendothelial junctions are comprised of adherens junctions (AJs), tight junctions (TJs), and gap junction (GJs) [36], [37, 38], [39]. AJs, composed of Vascular Endothelial (VE)-cadherin adhesion complexes, are the primary cell-cell adhesions in the endothelium except for the brain and retinal blood barriers [40], [41], [42], also reviewed in [7]. Numerous signaling pathways - including those induced by free cytosolic calcium (Ca^{2+}) - regulate the integrity of AJs [43, 44], [45], [46, 47]. The ion Ca^{2+} is a versatile and ubiquitous secondary messenger implicated in destabilization of endothelial barrier function [48], [49], [50], [51], [52], [53]. It participates in signal transduction by binding to intracellular proteins, most of which contain the EF-hand motif [54, 55], and causing conformational changes in their tertiary structure [56], [57]. In most cases, these conformational changes result in activation of protein functions.

The role of calcium-dependent kinases—In endothelial cells, the ion Ca^{2+} orchestrates a set of signal transduction events through activation of calcium-dependent kinases and phosphatases (Figure 1). These signaling molecules alter endothelial barrier function by multiple convergent mechanisms [6], [7]. The ion Ca^{2+} activates serine/threonine kinases including protein kinase Cα (PKCα), myosin light chain kinase (MLCK)-210 (endothelial-specific isoform), and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII). In turn, these kinases induce disassembly of AJs, thereby increasing permeability of endothelial barrier to plasma proteins [58], [59], [60], [61], [62, 63], [47], [64]. For example, PKCα phosphorylates p120-catenin [65], [66], [47], a protein of the VE-cadherin adhesion complex that binds to the juxtamembrane region of the VE-cadherin [67]. Phosphorylation of p120-catenin at the S879 residue decreases binding affinity of p120-catenin to VE-cadherin, resulting in dissociation of p120-catenin from the VE-cadherin adhesion complex [47]. This event enables binding of an adaptor protein (AP)-2 complex to VE-cadherin [68], [69] and subsequent initiation of internalization [47], [67] and proteolytic processing of VE-cadherin [70], [71].

PKCα is also responsible for the activation of RhoA, a small GTPase that reorganizes the actin cytoskeleton through activation of the downstream effectors, Rho-associated coiled-coil forming protein kinase (ROCK) and Diaphanous-related formin-1 (mDia1) [72], [73], [74]. PKCα induces activation of RhoA signaling by phosphorylating both a Guanine Nucleotide Dissociation Inhibitor (GDI) [44, 75] and a Guanine Nucleotide Exchange Factor (GEF) p115RhoGEF [76], [77], [78]. Phosphorylation enables GTP exchange mediate by p115RhoGEF by inhibiting GDI binding to RhoA [75, 76]. RhoA activates ROCK, which in turn phosphorylates myosin light chain phosphatase (MLCP) at Thr-695, Ser-894, and Thr-850 [79, 80], [81], inhibiting MLCP activity [79]. This process enables phosphorylation of myosin-II, an actin motor that assembles the acto-myosin contractile apparatus, at Ser19/Thr18 by MLCK-210 [82], [83], [84]. RhoA also mediates translocation of mDia1 to AJs, allowing polymerization of F-actin filaments [85]. mDia1-dependent assembly of actin filaments is reinforced by CaMKII-dependent activation of Mitogen-activated protein kinase

3/1 (MAPK3/1 or ERK1/2), which phosphorylates actin-binding protein caldesmon at Ser789 to prompt re-organization of the actin cytoskeleton [58], [60], [63].

Another mechanism of endothelial barrier destabilization involves proline-rich tyrosine kinase 2 (Pyk2) that is responsible for phosphorylation of Vascular Endothelial Tyrosine-Protein Phosphatase (VE-PTP), a constituent of AJs [86], [87]. VE-PTP, also known as PTPβ, provides a constitutive mechanism for VE-cadherin dephosphorylation at critical tyrosine residues Y658 and Y685 and, hence, prevents VE-cadherin internalization [88], [89]. Phosphorylation of VE-PTP at Y1981 residue causes a dissociation of VE-PTP from VE-cadherin thereby increasing endothelial permeability [89]. In addition, Pyk-2 positively regulates Ca^{2+} entry in endothelial cells by inducing phosphorylation at Y361 of stromal interaction molecule 1 (STIM1), a calcium sensor localized in the endoplasmic reticulum [90, 91], [92]. Pyk-2-dependent phosphorylation of STIM1 is required for its interaction with calcium release-activated calcium channel (Orai1) and Ca^{2+} entry from extracellular stores [92]. Importantly, phosphorylation of STIM1 at Y361 is required for development of pulmonary edema in inflammatory lung [92]. Although, studies in dermal microvascular endothelial cells isolated from neonatal foreskin challenge the role of Orai1 in agonist-induced barrier disruption [93], further understanding of Orai1 function with respect to intracellular signaling might explain tissue-specific role of Orai1 in microvascular endothelial cells.

The role of calcium-dependent phosphatases—The ion Ca^{2+} also enables the activation of calcineurin (CaN), a serine/threonine phosphatase [94, 95]. CaN dephosphorylates the microtubule end binding protein 3 (EB3) at S162 allowing reorganization of the microtubule cytoskeleton [46]. It also dephosphorylates STIM1 and prolongs calcium entry from extracellular stores [96]. In addition, activation of CaN rapidly dephosphorylates the transcription factor nuclear factor of activated T-cells (NFAT) protein to initiate NFAT nuclear import [94], [97], [98]. NFAT signaling in endothelial cells is linked to upregulation of transcripts associated with cytokine-cytokine receptor interaction (C-X-C motif chemokines [CXCL] 10 and 11) and the cell adhesion (Vascular cell adhesion protein 1 [VCAM1]) pathways [52]. These factors facilitate entry of neutrophils into pulmonary circulation, augmenting vascular injury. Therefore, calcium signals also contribute to the inflammatory response by inducing expression of specific proteins in activated endothelium (Figure 1).

Intracellular calcium-release channels in endothelium

Calcium homeostasis is finely regulated in endothelial cells through gating of selective and non-selective cation channels that enable cells to uptake, store, and release Ca^{2+} ions in order to manipulate their cytosolic concentration in a spatio-temporal manner [99]. The Endoplasmic Reticulum (ER) retains about 75% of total stored Ca^{2+} ions [100], [101] while other stores such as mitochondria, lysosomes, and endosomes hold about 25% [100], [102, 103], recently reviewed in [104]. Movement of Ca^{2+} ions in and out the cytosol is commonly regulated by channels and pumps [105], [106], [107], also reviewed in [108], [109], [110], [111]. The channels, when open, enable rapid diffusion of the ion Ca^{2+} from intracellular or extracellular stores into the cytoplasm downhill concentration gradients. In contrast, the

pumps move the ion Ca^{2+} in an energy-dependent manner against the concentration gradient [reviewed in [112], [104, 113]]. The plasma membrane Ca^{2+} ATPase (PMCA) and the sodium calcium exchanger (NCX) both continuously remove the ion Ca^{2+} outside of the cells [114–116] whereas the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) moves the ion Ca^{2+} for storage into ER and SR lumina [117]. These stores release the ion Ca^{2+} in response to extracellular stimuli in order to increase the ion Ca^{2+} concentration and induce calcium signaling in endothelial cells.

There two main types of Ca^{2+}-selective intracellular channels, the inositol 1,4,5-trisphosphate receptors (IP_3Rs) and the ryanodine receptors (RyRs). IP_3Rs release Ca^{2+} ions from the ER whereas RyRs release Ca^{2+} ions from the sarcoplasmic reticulum (SR) [118], [119], [120], [121], [122, 123], [124], [125], [126], reviewed in [127], [128]. IP_3Rs are ubiquitously expressed IP_3-gated channels localized on the membrane of the ER [129]. These channels consist of four ~260kDa subunits [130], [131], [132], [133] that appear to be mostly mobile structures randomly organized into small clusters across the surface of the ER membrane [134, 135], [136], [137], [138], [139], [140]. Some evidence from patch clamp experiments using isolated nuclei suggest that IP_3R clusters of about four channels are spontaneously induced by IP_3 [141, 142]. Although the underlying mechanism of IP_3 -evoked cluster formation remains unknown, the current view is that binding of IP_3 to the IP_3Rs may trigger conformational changes within the receptor that favor assembly of the clusters. Interestingly, at basal cytosolic Ca^{2+} concentrations, clustered IP_3Rs have a ~50% reduction in mean open time than lone IP_3Rs suggesting that lone receptors are more active than clustered ones. However, the presence of free Ca^{2+} ions in cytosol reverses this inhibition [141]. The cytosolic portion of IP_3Rs contains a putative Ca^{2+}-sensor region (residues 1933–2271), which may modulate properties of the channels. In the presence of Ca^{2+} ions, the clustered IP_3Rs demonstrate a higher open probability while showing reduced close time as compared to lone receptors; in addition, the receptors within the clusters exhibit simultaneous openings and closing [141], indicative of cooperative behavior. Therefore, the current model proposes that both secondary messengers, IP_3 and Ca^{2+} ion, bind to the IP_3Rs and attribute a positive regulation of Ca^{2+} release. Whereas IP_3 rapidly promotes assembly of IP_3Rs into the clusters, Ca^{2+} ions allow recruitment of Ca^{2+} release events through cooperative gating behavior of the receptors within the cluster.

IP_3 is generated by a class of membrane-associated enzymes, known as phospholipase C (PLCs), that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) [143], [144]. These enzymes are classified into six distinct families: PLC-β, γ, δ, ε, ζ and η [145]. Eight out of thirteen known PLC isoforms have been identified in freshly isolated murine endothelial cells [146] from mesenteric arteries (PLCβ1, β3 and β4; PLCγ1 and γ2; PLCδ1, δ3; and PLCε) whereas two additional isoforms have been detected in pulmonary (PLCη1 and η2) and middle cerebral arteries (PLCδ4 and PLCη1) indicating tissue-specific diversity of the phosphoinositide signaling pathways across the arterial beds. Whereas function of PLCδ, PLCε, and PLCη isoforms are mainly unknown in endothelial cells, our previous work indicates that PLCγ1 is a major isoform responsible for maintenance of basal level of IP_3 in human lung microvascular endothelial cells [46]. This is consistent with the phenotype of PLCγ1 knock-out mice which dies *in utero* due to loss of both erythroid and endothelial progenitor cells [147], [148]. In contrast, PLCγ2 is responsible for IP_3 generation

downstream of VE-cadherin adhesion disruption suggesting a potential role of PLCγ2 in endothelial cell proliferation and migration downstream of inflammatory and pro-angiogenic stimuli [46]. PLCβ isoforms act downstream of G-protein-coupled receptors (GPCRs) coupled to the G-proteins G_q and G_{11} [149], [150], [151], [152] or VEGFR2 signaling [153].

Given the fact that sustained elevation of cytosolic Ca^{2+} is toxic to cells, several negative feedback mechanisms regulating the channel activity have evolved [154], [155]. As the intracellular ion Ca^{2+} concentration exceeds a threshold value of 0.92 millimolar, release of the Ca^{2+} ions is inhibited due to the Ca^{2+} binding to the inhibitory sites of IP_3Rs [156], [157], [158], [159]. To date, seven Ca^{2+}- binding sites have been discovered in the cytosolic portion of IP_3R1 [160, 161], [162], [163], yet their significance in IP_3R gating remains unclear. Mutagenesis analysis of residues overlapping these Ca^{2+}- binding sites have no significant effect on IP_3R gating [164]. Another model proposes that luminal concentration of Ca^{2+} is directly linked to sensitivity of IP_3Rs to the Ca^{2+} ions [165]. As the luminal concentration of Ca^{2+} falls, IP_3Rs loses its sensitivity to IP_3 even in the presence of cytosolic IP_3 and Ca^{2+}. This model, however, needs to be supported with further experiments.

It has also been proposed that several proteins including Ca^{2+} -binding proteins containing the tetra EF- hand, calmodulin (CaM), and the calmodulin (CaM)-like neuronal Ca^{2+}-binding proteins (CaBPs), attribute to negative regulation of the IP_3Rs [166], [167], [168], [169, 170], recently reviewed in [171]. These proteins bind IP_3R1 within the first 128 amino acid sequence [170, 172] and inhibit binding of IP_3 to the receptor at 0.15 millimolar free Ca^{2+} ions in cytosol leading to blocked Ca^{2+} release [166], [169]. Hence, cytosolic Ca^{2+} ions attribute to both positive and negative feedback regulation of Ca^{2+} release through IP_3Rs.

Another type of Ca^{2+}-selective intracellular channel, the ryanodine receptors (RyRs) form Ca^{2+} channels located on the membrane of the sarcoplasmic reticulum (SR). The tetrameric RyR channel consist of subunits ~5000 residues in size [173], [174], [175], [176]. There exist three isoforms of RyRs in mammals with RyR1 and RyR2 being preferentially expressed in skeletal muscles and cardiomyocytes [174], [177], [178]. Endothelial cells predominantly express RyR3 isoform [179], [180]. RyRs activity is regulated by ryanodine, a plant alkaloid from *Ryania speciose* [181], [182], which induces channel pore opening at nanomolar concentrations and promotes pore closing at micromolar concentrations [183], [184], [154], [185]. Furthermore, Ca^{2+} ions and ryanodine demonstrate cooperative behavior [186, 187]. An increase in free cytosolic Ca^{2+} facilitates stronger binding of ryanodine to the RyRs as shown in endothelial cells [188]. In this respect, RyRs and IP_3Rs share analogous regulation of Ca^{2+} release by cytosolic Ca^{2+}. Since our review is mainly focused on the function of IP_3Rs in endothelial cells, we refer to recent publications for additional information on structure and function of RyRs [189], [190], [191].

Recent studies have also revealed the presence of intracellular two-pore channels (TPCs) in endothelial cells that belong to a family of the voltage-gated ion channels [192], [193], [194]. The family is presented by three distantly related proteins (TPC1–3) with TPC2 specifically localized to the lysosomal, and TPC1 to the endolysosomal systems [192], [195]. TPC3 is also targeted to acidic organelles as well as the plasma membrane [196],

[197] but is lost in human and rodent species [198]. The crystal structure of a two-pore channel from *Arabidopsis thaliana* suggests that Ca^{2+} and membrane potential activate two distinct six transmembrane (6-TM) domains [199] indicating potential mechanism for Ca^{2+} and voltage sensing. It also proposes that luminal Ca^{2+} might stabilize the second voltage-sensing domain in the resting state and thereby shift voltage activation towards more positive potentials.

In endothelial cells, TPCs are mainly involved in regulation of cell proliferation [193], [194]. TPC2, which promotes Ca^{2+} release from lysosomal stores, potentiates pro-angiogenic effect of Vascular Endothelial Growth Factor (VEGF) on endothelial cells, suggesting a specific role of TPC2 in VEGFR2-mediated angiogenesis [193]. In contrast, TPC1 is involved in intracellular calcium release from endolysosomal store in response to arachidonic acid and mediates specific calcium signaling associated with proliferation of circulating endothelial colony forming cells [194].

Function of IP_3R channels in endothelium

IP_3Rs isoforms—Through cDNA cloning on mammalian cells, three isoforms of IP_3Rs (IP_3R1, IP_3R2, IP_3R3) have been discovered and characterized in various cell culture models [118], [200], [201], [135]. Even though all three isoforms demonstrate about 65–85% sequence similarity, they each produce a different magnitude of Ca^{2+} release due to their distinct affinities for IP_3 [202], [131], [203], [204], [205]. The *in-vitro* studies have shown that IP_3R2 is the most sensitive to IP_3 [135], [206] and produces long-lasting and regular Ca^{2+} oscillations [207], [203], [208]. IP_3R3, the least sensitive for both secondary messengers, instigates monophasic Ca^{2+} transients [203], [209], [206]. IP_3R1 demonstrates intermediate affinity for IP_3 and generates Ca^{2+} oscillations, which appear more irregular when compared with IP_3R2-mediated oscillations [207, 209], [206]. These differences in binding affinities for IP_3 among receptor types can be further modulated through the interaction of the N-terminus ligand-binding domain (LBD; see *'Organization and structure of IP_3Rs'*) with adenosine triphosphate (ATP), the regulatory proteins - such as CaM, CaMBP, CaN, STIM 1 - and homer protein homolog (HOMER) proteins, as well as through the receptor phosphorylation [210], [166], [167], [168], [169, 170], [211], [212], [213], [214, 215], [216], [209], [217], [218]; recently reviewed in [128].

Expression of endothelial IP_3R isoforms vary among the different vascular systems [219], [220], [221], [53]. Whereas all three isoforms are expressed in the endothelium of the aorta and arteries of systemic circulation in rats and domestic cattle [220], [222], [223], [224], [225], [226], IP_3R1 is not present in the pulmonary endothelium of either human or mouse [53]. Interestingly, some striking difference in the expression pattern of IP_3Rs is also noted among species. This difference might be attributed to specialized function of the receptor isoform in human since in IP_3R1 is predominant in endothelial cells of human brain microvasculature and mesenteric artery [219], [220] while IP_3R3 and IP_3R2 are preferentially expressed in pulmonary microvascular, and aorta endothelial cells [53], [221] as well as in circulating endothelial colony forming cells [227].

The role of IP_3R1 in regulating myogenic vascular tone—Interestingly enough, endothelial cell-specific knockout of IP_3R1 causes high blood pressure in mice due to disruption of NFAT/endothelial nitric oxide synthase (eNOS) signaling [228]. Consistent with this finding, other studies demonstrate that expression of IP_3R1 in aortic endothelial cell is downregulated in spontaneously hypertensive rats [222]. Both IP_3R1s and IP_3R2s are localized at myo-endothelial projections between endothelial and smooth muscle cells and selectively regulate inter-cellular communications. They are also involved in control vascular tone by activating intermediate conductance of calcium-activated potassium channels, thereby hyperpolarizing adjacent smooth muscle cells [224], [229], [230]. It is well-known that fluid shear stress induces intracellular Ca^{2+} oscillations through activation of heterotrimeric G_q/G_{11} proteins, which is coupled to purinergic receptor [231], [232] or bound to the plasma membrane in endothelial cells [233], [234]. Deficiency in IP_3R1 seems to disrupt these adaptive responses to increased blood flow in arteries of systemic circulation and causes hypertension [228, 235, 236]. Cumulatively, these findings suggest, that IP_3R1-dependent Ca^{2+} handling might play a critical role in regulating myogenic vascular tone in arteries. It is plausible that IP_3R1 is involved in physiological adaptive responses to hemodynamic changes in endothelium of systemic circulation whereas it is dispensable in the low-pressure system such as pulmonary circulation.

The role of IP_3R2 and IP_3R3 in regulating endothelial permeability in lung—The other IP_3R isoforms IP_3R2 and IP_3R3 are expressed in endothelial cells and have implications in both increased endothelial permeability and cytokine production during inflammation in the lung [52], [53]. Pro-inflammatory mediators such as serine protease α-thrombin and histamine induce Ca^{2+} ion release from ER stores. These mediators bind to and activate respective G-protein-coupled receptors (GPCRs), protease-activated receptor 1 (PAR-1) [237], [238] or histamine receptor 1 (H1) [239], [240], at the surface of endothelial cells (Figure 2).

IP_3 binds to IP_3Rs and initiates Ca^{2+} release from ER stores [120], [241], [242]. Depletion of intracellular stores, in turn, activates a set of intracellular events that mediate capacitive or store-operated Ca^{2+} entry (CCE or SOCE) and thus replenishing Ca^{2+} in ER (Figure 2) [243], [244]. The level of Ca^{2+} ions within ER is sensed by transmembrane proteins localized on ER membrane, STIM1 [90], [245], [246]. STIM1 binds to the Ca^{2+} ions through EF-hand motif at the N-terminus, a portion of the protein located in the ER lumen [247], [248]. Depletion of Ca^{2+} inside the ER leads to oligomerization as well as interaction of STIM1 with both the calcium release-activated calcium channel proteins (Orai) and transient receptor potential channel 1 (TRPC1) located at the plasma membrane of endothelial cells [249–255].

As mentioned above, distinct stimuli can engage specific IP_3R isoforms in activated endothelium. IP_3R3 triggers monophasic Ca^{2+} transients in response to pro-inflammatory mediators such as α-thrombin, and promotes increased permeability of endothelial barrier [53]. IP_3R2 induces Ca^{2+} oscillations in response to endotoxin lipopolysaccharide (LPS), which is found in the outer membrane of Gram-negative bacteria [52]. This isoform is responsible for sustained activation of NFAT-mediated transcripts encoding the proteins associated with inflammation [52], [256]. In addition, DAG promotes Ca^{2+} influx through

receptor-operated Ca^{2+} channels (ROC), the pathway unique from SOCE (Figure 2) [257], [258], [259], [260]. DAG interacts with TRPC6 at the plasma membrane [259] and permits Ca^{2+} entry through this non-selective cation channel in endothelial cells [49], [261], [51]. DAG can also contribute to inflammatory responses in endothelial cells through direct activation of protein kinase C (PKC) [262], [263], [44], [59], [264], [47] or generation of lipid secondary messengers such as leukotrienes [265], [266], [267]. Thus, calcium signaling emanated at the level of ER channels is amplified by Ca^{2+} flux from extracellular stores through SOCE and ROCE mechanisms.

Organization and structure of IP_3Rs

A potential mechanism of channel gating has been suggested based on the recently solved quaternary structure of the IP_3R1 channel in non-conducting state [133]. The IP_3R1 tetrameric structure consists of four subunits organized around a central axis [133]. Each subunit consists of two β-trefoil domains (β-TF1 and β-TF2), three armadillo solenoid folds (ARM1-ARM3), α-helical domain (HD), intervening lateral domain (ILD), six α-helices (TM1–TM6 or TMD), C-terminal domain (CTD), and a helical linker domain (LNK), which connects the transmembrane and the cytosolic bundles of each subunit (Figure 3a–b).

Organization of the permeation pathway—The proposed model suggests that the TMD region is essential for Ca^{2+} ion conduction [133]. The Ca^{2+} permeation path is lined by four TM6 α-helices, which are oriented at 37° with respect to the ER membrane (Figure 3c). A luminal loop between TMD5 and TMD6 α-helices assembles a luminal vestibule. This loop contains both a short P-helix and a selectivity filter comprised of highly conserved residues [268], [133]. An important feature of a luminal vestibule is the presence of a positively charged ring, which is formed by the His2541 residues located within the P-helix of a tetrameric channel (Figure 3c). This ring is predicted to repel the positively charged Ca^{2+} ions in the non-conducting channel. In conductive state, the P-helices are anticipated to undergo a structural rearrangement allowing passage of Ca^{2+} ions beyond a luminal vestibule [133]. The gateway for ion-permeation is located at the constriction spot between four of the TM6 helices that each contain several hydrophobic residues oriented towards the permeation pathway (Figure 3c). In the non-conducting channel, these hydrophobic residues form a pore of 5Å in diameter that restricts passage of the hydrated Ca^{2+} ion of 8–10 Å in diameter [133]. In contrast to a luminal vestibule, a cytosolic vestibule formed above the pore is comprised of negatively charged residues, which are anticipated to promote a translocation of the Ca^{2+} ions into the cytosol [133].

Function of the cytosolic region—The cytosolic part of IP_3R1 contains two β-trefoil domains, three ARMs, and an α-helical domain (Figure 3a–b). The modular organization of armadillo folds predicts their role in assembling different interfaces for binding of IP_3, Ca^{2+} ion, and the regulatory proteins [171]. The β-TF1 domain at the N-terminus (residues 5–225) constitutes a suppressor domain whereas β-TF2 and two α-helices armadillo repeats of ARM1 assemble the IP_3-binding core (IBC) region. These three domains form a triangular structure, also known as ligand-binding domain (LBD) [269], [270], [126], above the CTD bundle. The α-helical, ARM2, and ARM3 domains connect the LBD to the channel-forming region. Even though the exact mechanism underlying IP_3-evoked conformational changes

within the pore is not fully understood, it is proposed that gating of IP_3Rs occurs through a long-range communication between the LBD region and the pore [133].

The current model suggests that the binding of IP_3 to the IBC causes a closure of IP_3 -binding pocket and, sequentially, a twist-like movement of the suppressor domain [133]. The model indicates that the interactions between the suppressor and ARM3 domains carry the signal from the LBD to the pore. Interestingly, the suppressor domain also establishes interaction with β-TF2 and ARM2 domains of adjacent subunit suggesting that movement of the suppressor domain might transmit the signals to adjacent subunits. Consistent with this model, deletion of the suppressor domain or mutation of the Y167, site involved in the interaction with β-TF2, prevents IP_3-evoked gating of the channel [271], [272], indicating an importance of the suppressor domain in the transmission of long-range allosteric changes.

It also has been proposed that CTD domain plays an important role in gating of the IP_3R channel. The studies using IP_3R mutant lacking the 43 residues from the CTD domain have demonstrated that it is required for channel gating [273]. The CTD domain is comprised of the long helical bundle spanning the cytosolic portion of the receptor [133]. Both biochemical and structural studies indicate that the N-terminal and the C-terminal parts of the receptor are closely associated with respect to each other [274]. The quaternary structure of the receptor reveals the presence of the electrostatic interactions between the CTD and β-TF2 domains from the adjacent subunits. The CTD also interacts with LNK, the bride between the cytosolic and transmembrane parts of the receptor, and possibly transmits the signal to the TMD6 [133].

Therefore, ligand-evoked gating of the channel relies on both extensive inter- and intra-subunit interactions within the receptor comprised of four subunits. The first near-atomic resolution structure of the IP_3R1 [133] reveals conservation of the ion-conduction pore among tetrameric channels [275]. The specific features are carried through additional domains attached to the pore. A unique architecture in the CTD domain suggests a distinctive mechanism of IP_3R gating that requires direct coupling between the CTD and LBD domains of adjacent subunits [133].

IP_3-evoked Ca^{2+} release events

The fundamental question is “How does the ion Ca^{2+} elicit an array of signaling events alternating from proliferation, survival, and death?” Because diffusion of the ion Ca^{2+} is altered due to binding to numerous proteins and lipids in the cytosol, in many instances, it is only effective in initiating signal transduction events locally, in the proximity of the ion Ca^{2+} release or entry. The ion Ca^{2+} is released from intracellular stores through IP_3Rs in three major events called blips, puffs, and waves (Figure 4). A blip is the elementary release event, a result of a passive diffusion of the Ca^{2+} ions from the ER through the lone channel (Figure 4), [276]. This event allows movement of the ion Ca^{2+} along the gradient in a stochastic and autonomous process occurring at intermittent IP_3R sites across the ER [277], [276]. Blips typically last for 10 ms and are responsible for basal cytosolic Ca^{2+} concentration of about 40 – 80 nanomolar in resting endothelial cells [53].

A greater release of Ca^{2+} ions lead to puffs, a result of simultaneous openings of 5–6 channels grouped into the clusters lasting for about 100 milliseconds, [278], [279], [280], [281], [282], [283]. As opposed to blip events, puffs originate within the clusters suggesting that engagement of lone IP_3R channels into multichannel assembly plays a critical role in transition from blip to puff (Figure 4) [284, 285]. The clusters igniting puffs are ~50 nm wide and distributed at ~3 μm from each other [286]. Multiple studies have experimentally established a direct correlation between frequency of puffs ignition and the number of IP_3Rs present within the cluster [285], [287], [276], [288]. This relationship suggests that larger IP_3R clusters have a disproportional influence on intracellular Ca^{2+} signaling and may act as an important ignition switch for regenerative Ca^{2+} waves (Figure 4). However, it should be considered that blips and puffs do not solely serve as components of a global Ca^{2+} release, rather, they are signaling events in themselves.

As IP_3 concentration increases, puffs can trigger regenerative Ca^{2+} waves that spread across cell [286], [289] due to coordinated opening of a numerous channels of IP_3Rs within the ER (Figure 4). It has been proposed that Ca^{2+} ions trigger higher frequency puffs through activation of neighboring channels. The current model suggests that puff frequency might set up a threshold for regenerative Ca^{2+} waves where greater oscillation frequency attributes to higher excitability of nearest IP_3Rs and thus the transition from local (puffs) to global (wave) calcium release event [290–293], [294]. In this respect, organization and distribution of IP_3R clusters become critical features. Below, we discuss the mechanisms regulating the receptor clustering in light of our recent work [53].

Regulation of IP_3R clustering in endothelial cells

Dynamics of IP_3Rs clusters in endothelial cells—Regenerative ion Ca^{2+} release relies on recruitment of neighboring IP_3Rs via a process known as Ca^{2+}-induced Ca^{2+} release (CICR). Therefore, propagation of Ca^{2+} signals is critically dependent on organization of the IP_3Rs as well as optimal distribution of the receptors within the ER membranes. IP_3Rs assemble the clusters in unstimulated pulmonary endothelial cells [53] as well as other cell types [139], [134, 135]. IP_3R3s form less abundant clusters than IP_3R2s in endothelial monolayers, which can be explained by their different binding affinities to IP_3. This suggests that local spikes in basal IP_3 production that are mediated by PLCγ1 [46], [295] are sufficient to promote clustering of both IP_3Rs in unstimulated endothelial cells [53].

Consistent with IP_3Rs distribution in other cell types [135], [134], [138], [139], GFP-IP_3R3 form highly mobile structures in resting endothelial monolayers [53]. The IP_3R3 clusters appear to be very infrequent structures within the ER membrane that undergo spontaneous assembly and disassembly. Furthermore, consistent with hypothesis of “hot spots” for calcium release [277, 296, 297], some clusters demonstrate sporadic assembly and disassembly at the same location, perhaps in response to changes in local concentrations of IP_3. Furthermore, stimulation of the endothelial cells with α-thrombin promptly induces *de novo* formation of IP_3R3 clusters, which demonstrates exponential growth in size [53] Our recent work identified the microtubule-associated protein EB3 as a key regulator of formation of IP_3R3 clusters in endothelial cells [53].

The role of microtubule cytoskeleton in IP_3Rs clustering—The role of the microtubule cytoskeleton in organization of IP_3-evoked Ca^{2+} signals has been established in various cell types [298], [299], [300], [301], [302], [303], [225], [53]. IP_3Rs are shown to establish the transient associations with microtubules [304], [53], which in turn, actively participate in the redistribution of IP_3Rs within the ER membrane [301], [305], [300]. The microtubule cytoskeleton also contributes to initiation and propagation of Ca^{2+} waves in endothelial cells [225, 299], that may occur through spatial organization and temporal dynamics of IP_3R3s within ER membranes [306], [53], [307]. Furthermore, few reports indicate that the microtubule cytoskeleton facilitates delivery of IP_3 to IP_3Rs [308, 309], possibly contributing to initiation of Ca^{2+} spikes in secretory cells [310]. More recent studies in endothelial cells indicate that microtubules establish transient interactions with IP_3R3 localized at the ER membrane and play a critical role in assembling agonist-evoked IP_3R3 clusters [53].

While it is possible that the microtubule cytoskeleton promotes calcium signaling through multiple independent mechanisms, it has become apparent that it contributes to spatio-temporal organization of calcium release from ER stores. Microtubule dynamic is critical for this function because accumulation of specific proteins at the growing microtubule plus ends might provide the mechanism for tethering and stabilizing protein complexes such as IP_3Rs clusters at ER membrane.

EB3 is a major regulator of IP_3Rs clusters in stimulated endothelial cells—The EB family consists of three members: EB1, EB2, and EB3 [311]. The EB proteins have demonstrated a high affinity for the outer part of growing microtubules, so-called the '+tips' [312], [313], [314]. EBs are involved in establishing a protein network at the '+tips' through recruitment of other proteins. Specifically, EB proteins interact with the CAP-Gly domain proteins (such as cytoplasmic linker and p150-Glued proteins) and proteins containing the short EB-binding motif S/TxIP [315], [316], [317], [318], [319], [320].

IP_3Rs contain a EB-binding motif within a short unstructured region of the α-helical domain (HD), which connects the LBD to the channel-forming region [133]. The S/TxIP - containing motif conserved among mammalian IP_3Rs enables the interaction of IP_3Rs with EB3 and, albeit, a much weaker interaction with EB1 [53]. Intriguingly, EB3, but not EB1, is required for α-thrombin-evoked clustering and gating of IP_3R3 in endothelial cells. Depletion of EB3 or disruption of the IP_3R3 binding interface through a single-point mutation within EB-binding motif reduces number of the IP_3R3 clusters in response to α-thrombin challenge [53]. The formed clusters are short-lived [53], suggesting that tethering of IP_3R3s to microtubule +tips by EB3 might play an essential role in stabilization of IP_3R3s intremolecular associations within the cluster.

Fascinatingly, IP_3R3 mutant bearing a single-point mutation (T804 to A) - this mutation disrupts the interaction with EB3 - reveals a striking difference in cluster dynamics of the mutant as compared to native protein [53]. Stimulation of endothelial cells with α-thrombin causes *de novo* formation of IP_3R3(T804A) clusters; however, the number of clusters is significantly less than those formed by native receptor [53] indicating that the interaction of the receptor with microtubule tips might stabilize receptor clusters. Disruption of this

interaction, either through depletion of EB3 or mutation of IP_3R3, alters organization of IP_3R3 clusters and thereby inhibits propagation of calcium signals in endothelial cells.

Our proposed model also suggests that formation of IP_3R3 clusters is essential for recruitment of neighboring IP_3Rs via Ca^{2+}-induced Ca^{2+} release since IP_3-evoked Ca^{2+} signals are massively attenuated in EB3 deficient cells. The importance of EB3 in calcium signaling is clear from analyses of isolated lungs where knockout of EB3 in endothelial cells protects lungs from increased vascular leakage and pulmonary edema [53]. Thus, the microtubule cytoskeleton provides a critical mechanism for assembling stable IP_3Rs clusters and effective Ca^{2+} signals in endothelium in the context of inflammation. Targeting this interaction with specific inhibitors might provide an attractive strategy for treatment of vascular leakage and pulmonary edema.

Conclusion remarks and future directions

Intracellular Ca^{2+} signaling is one of the major mechanisms involved in the communication between endothelial cells and the environment. Changes in mechanical (pressure and shear stress) and chemical (growth factors, hormones, and cytokines) stimuli are processed by endothelial cells through receptor-mediated outside-in signaling. The ion Ca^{2+} is a second massager that is released from ER stores and ignites a myriad of signal transduction pathways upon the activation of endothelial cell surface receptors. The hierarchy of IP_3-evoked Ca^{2+} release events is predicted to differentially regulate the cellular responses. While elementary Ca^{2+} signals in form of blips and puffs might be critical for regulation of vascular tone, globalization of Ca^{2+} signals through regenerative Ca^{2+} waves might contribute to vascular inflammation and leakage. Understanding a spatial-temporal organization of Ca^{2+} release in endothelial cells remains a major challenge in the field of endothelial biology.

Dynamic reorganization of IP_3Rs within ER membrane is crucial in igniting neighboring channels and transitioning from local to global calcium release events. The microtubule cytoskeleton contributes to assembly of stable IP_3Rs cluster and sets up a positive-feedback loop for the amplification of Ca^{2+} signals associated with the progression of several pathologies including vascular leakage and inflammation. Increasing our understanding of IP_3R regulation at the molecular level will lay a ground work for the design of therapeutic interventions that target specific function of the receptor associated with pathology while leaving intact physiologically important function.

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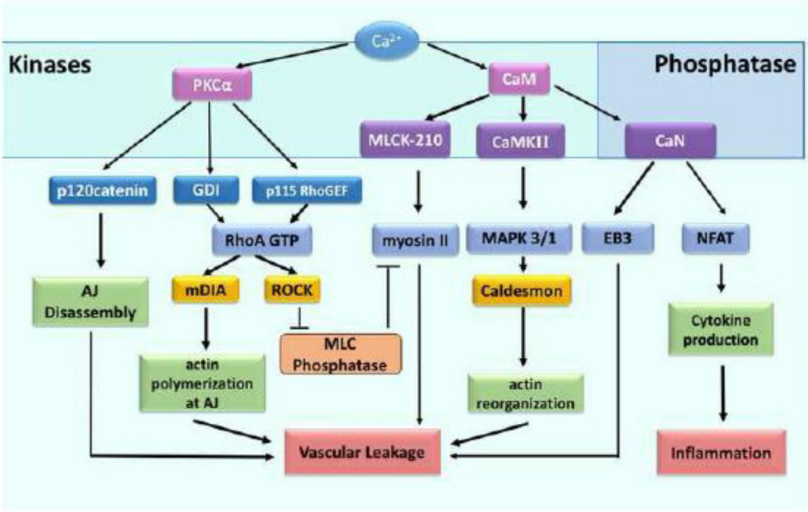

Figure 1. Intracellular Ca^{2+} signaling in endothelial cells associated with vascular leakage and inflammation

Increase in cytosolic Ca^{2+} concentration activates specific kinases and phosphatases that, in turn, promote vascular leakage and inflammation. The serine/threonine kinase PKCα induces phosphorylation of p120-catenin and, through this mechanism, contributes to disassembly of AJs. PKCα also promotes reorganization of actin cytoskeleton and acto-myosin contractility by activating RhoA signaling. CaM-dependent activation of both MLCK-210 and CaMKII facilitates reorganization of actin cytoskeleton through the phosphorylation of actin motor myosin-II and caldesmon. In addition, CaM activates the phosphatase CaN, which dephosphorylates the microtubule accessory factor EB3, thereby coordinating reorganization of the actin and microtubule cytoskeleton. CaN also mediates activation of NFAT and promotes cytokine production by endothelium.

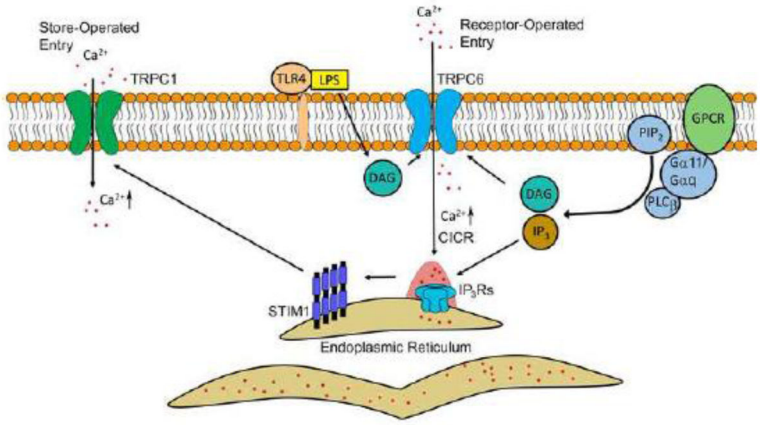

Figure 2. Model of receptor-mediated signaling in activating Ca^{2+} release and entry in endothelial cells during inflammation

The signal transduction begins with the binding of the ligand (hormone, cytokine, and etc.) to specific G-protein coupled receptor (GPCR) at the surface of endothelial cells. Initial $G\alpha_{11}/G\alpha_q$-coupled activation of phospholipase C (PLC) β directs cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into the two secondary messengers, diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 induces clustering and activation of IP_3Rs on the ER membrane whereas DAG instigates Ca^{2+} entry through Transient Receptor Potential Cation channels, member 6 (TRPC6) located at the plasma membrane. The latter might contribute to increase in cytosolic Ca^{2+} concentration igniting global Ca^{2+} release through IP_3Rs. Depletion of the ER Ca^{2+} stores triggers oligomerization of Stromal interaction molecule 1 (STIM1) that is required for activation of TRPC1 channel and Ca^{2+} entry from extracellular space. Lipopolysaccharide (LPS) also can activate Ca^{2+} entry through the TRPC6 channel by binding to toll-like receptor 4 (TLR4).

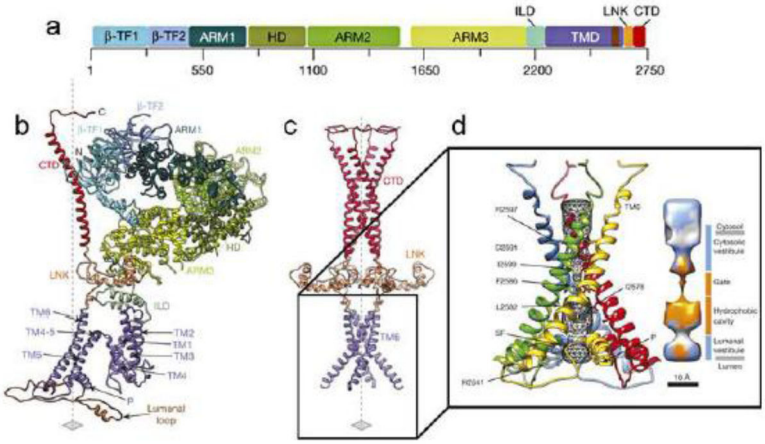

Figure 3. Domain structure of IP_3R1

(a) Schematic representation of IP_3R1 domains with corresponding amino acids. **(b)** Organization of individual IP_3R1 molecule within tetrameric channel; different domains are indicated by color as in (a). **(c)** Structure of the central core of IP_3R1 channel (tetramer); axis is indicated by the dashed line. **(d)** The bundle of TM6 helices forming the conduction pathway of the IP_3R1 channel; four individual TM6 domains are highlighted by color. The P-helices (P) lining the luminal vestibule and selectivity filter (SF) loops are indicated. The pore is formed by chain of hydrophobic residues as indicated by color on the right. β-TF1 and β-TF2, β-trefoil domains; ARM1-ARM3, armadillo solenoid folds; HD, α-helical domain; ILD, intervening lateral domain; TM1–TM6 or TMD, α-helices; CTD, C-terminal domain; LNK, a helical linker domain. Modified from Fan et al [133] with permission of the publisher. Copyright ©2015, Nature Publishing Group.

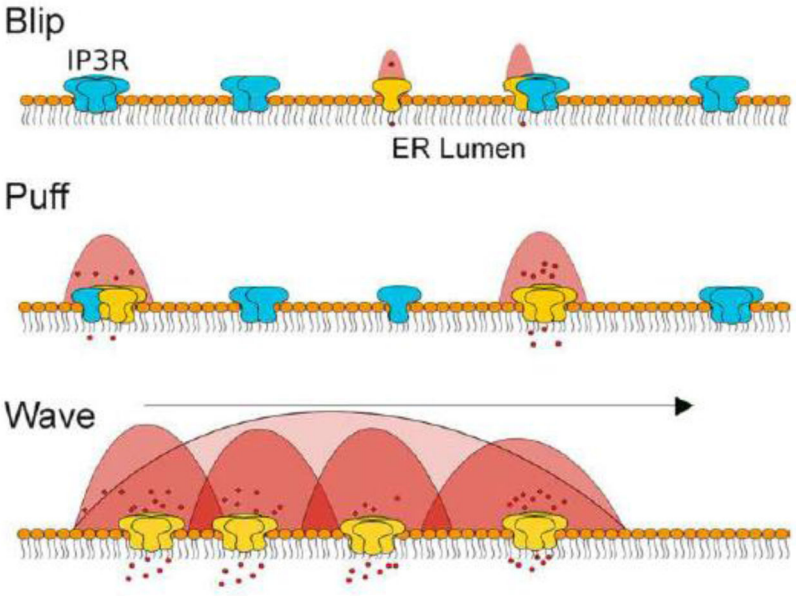

Figure 4. Elementary Ca^{2+} release events evoked by IP_3

Ca^{2+} release occurs in 3 major events: blip, puff and wave. Blips represent release of Ca^{2+} through a single IP_3R channel, which can be lone or part of the cluster. Puffs are characterized by coordinated opening of multiple channels organized into the cluster. Regenerative Ca^{2+} waves are attributed to activation of IP_3R across the entire cell. Both IP_3 and Ca^{2+} ignite Ca^{2+} release form neighboring IP_3Rs clusters.