

Update on vascular endothelial Ca²⁺ signalling: A tale of ion channels, pumps and transporters

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Received: May 17, 2012 Revised: July 4, 2012
Accepted: July 11, 2012
Published online: July 26, 2012

Abstract

A monolayer of endothelial cells (ECs) lines the lumen of blood vessels and forms a multifunctional transducing organ that mediates a plethora of cardiovascular processes. The activation of ECs from a state of quiescence is, therefore, regarded among the early events leading to the onset and progression of potentially lethal diseases, such as hypertension, myocardial infarction, brain stroke, and tumor. Intracellular Ca²⁺ signals have long been known to play a central role in the complex network of signaling pathways regulating the endothelial functions. Notably, recent work has outlined how any change in the pattern of expression of endothelial channels, transporters and pumps involved in the modulation of intracellular Ca²⁺ levels may dramatically affect whole body homeostasis. Vascular ECs may react to both mechanical and chemical stimuli by generating a variety of intracellular Ca²⁺ signals, ranging from brief, localized Ca²⁺ pulses to prolonged Ca²⁺ oscillations engulfing the whole cytoplasm. The well-defined spatio-temporal profile of the subcellular Ca²⁺ signals elicited in ECs by specific extracellular inputs depends on the interaction between Ca²⁺ releasing channels, which are

located both on the plasma membrane and in a number of intracellular organelles, and Ca²⁺ removing systems. The present article aims to summarize both the past and recent literature in the field to provide a clear-cut picture of our current knowledge on the molecular nature and the role played by the components of the Ca²⁺ machinery in vascular ECs under both physiological and pathological conditions.

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Key words: Endothelial cells; Ca²⁺ signalling; Plasma membrane; Endoplasmic reticulum; Intracellular Ca²⁺ release; Ca²⁺ entry; Ca²⁺ removal; Ca²⁺ oscillations

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Moccia F, Berra-Romani R, Tanzi F. Update on vascular endothelial Ca²⁺ signalling: A tale of ion channels, pumps and transporters. *World J Biol Chem* 2012; 3(7): 127-158 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v3/i7/127.htm>
DOI: <http://dx.doi.org/10.4331/wjbc.v3.i7.127>

INTRODUCTION

It has long been known that an increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i) plays a key role in the intricate network of signal transduction pathways exploited by endothelial cells (ECs) to maintain cardiovascular homeostasis^[1]. Due to its strategic location at the interface between the vascular wall and bloodstream, the endothelium is exposed to a myriad of transmitters (released by autonomic and sensory nerves or platelets), circulating hormones, autacoids, cytokines, growth factors, and drugs, as well as to mechanical stimuli, such as pulsatile

stretch, shear stress, and changes in the local osmotic pressure^[2,3]. Moreover, vascular ECs might serve as thermosensors and modulate peripheral vasoconstriction or vasodilation depending on the environmental temperature^[4]. A remarkable blend of membrane receptors, transporters, and ion channels, which are located both on the plasma membrane and within the intracellular Ca²⁺ reservoirs (namely, endoplasmic reticulum, lysosomes, and Golgi), may be utilized by ECs to detect and react selectively to the incoming stimulus with the most suitable Ca²⁺ signal^[1,4-8]. A number of excellent reviews have recently outlined features and activation mechanisms of Ca²⁺-permeable channels in vascular endothelium^[1,7,8]. The present article will survey the Ca²⁺ signaling toolkit whereby ECs shape their Ca²⁺ responses to chemical and mechanical stimuli by summarizing the most recent developments in the field. Recent work has shown that endothelial progenitor cells (EPCs), a subpopulation of mononuclear cells that are mobilized from bone marrow upon an ischemic insult, impinge on Ca²⁺ signals to proliferate, traffic to the injured tissue, and form capillary-like structures. However, we refer to a recent review from our laboratory for an exhaustive description of our current knowledge about the functional role and the molecular underpinnings of Ca²⁺ signalling in human EPCs^[9].

Ca²⁺ SIGNALLING IN VASCULAR ECs

In resting, i.e., non-stimulated cells, [Ca²⁺]_i is very low (around 100 nmol/L) due to the activity of various active mechanisms that remove Ca²⁺ out of the cytosol^[10]. More specifically, the plasma membrane Ca²⁺-ATPase (PMCA) and the sarco-endoplasmic reticulum Ca²⁺-ATPase (SERCA) extrude Ca²⁺ by direct ATP hydrolysis, while the Na⁺/Ca²⁺ exchanger (NCX) clears cytosolic Ca²⁺ by exploiting the Na⁺ gradient across the plasma membrane^[10]. An increase in [Ca²⁺]_i up to 1 μmol/L is the key signal to activate vascular ECs following recruitment of either tyrosine-kinase linked receptors (TRKs) or G-protein coupled receptors (GPCRs) by growth factor and vasoactive agents, respectively^[1,6,7,9,11,12]. The endothelial Ca²⁺ response may comprise an initial Ca²⁺ spike, shaped by Ca²⁺ mobilization from the intracellular Ca²⁺ reservoir, followed by an intermediate plateau level due to Ca²⁺ entry across the plasma membrane^[6,7,9]. However, at low-dose agonist stimulation, the Ca²⁺ signal may adopt an oscillatory pattern driven by the interplay between intracellular Ca²⁺ release and Ca²⁺ influx from the extracellular space^[1,6,13]. Responding cells with an intermediate behavior between the extremes described above can also be found. Accordingly, in most microvascular ECs, chemical stimulation does not elicit a remarkable Ca²⁺ inflow and results in either a transient Ca²⁺ spike or localized, high frequency Ca²⁺ oscillations, which are attributable to Ca²⁺ mobilization from the intracellular store^[14,16]. Conversely, when the Ca²⁺ signal is uniquely modelled by Ca²⁺ entry, a sustained and monotonic increase in [Ca²⁺]_i occurs^[17,18]. Mechanical stimulation may also affect

vascular endothelium by causing an elevation in [Ca²⁺]_i. Indeed, exposure of ECs to fluid shear stress, pulsatile stretch, and cell swelling may evoke both Ca²⁺ discharge from intracellular Ca²⁺ pools and Ca²⁺ inflow through mechanosensitive channels on the plasmalemma^[4,7,19]. Recent studies have argued that ion channels serve as direct sensor of mechanical stimuli, but rather as a relay that is engaged by a yet to identify upstream receptor^[19]. Unlike the Ca²⁺ response to extracellular ligands, mechanosensitive Ca²⁺ signals normally lack the biphasic kinetics and exhibit either a transient^[20,21] or an oscillatory pattern^[21-25]. In a few cases, a dose-dependent stepwise elevation in [Ca²⁺]_i was observed in cultured ECs exposed to laminar shear stress^[24,26,27]. The contact between air microbubbles and cultured ECs may also result in an intracellular Ca²⁺ wave^[28]. Finally, an increase in [Ca²⁺]_i arises in vascular ECs upon contact with circulating leukocytes^[29] or metastasizing tumor cells^[30,31].

Ca²⁺ RELEASE FROM INTRACELLULAR STORES

The endoplasmic reticulum (ER) houses the largest intracellular Ca²⁺ reservoir in ECs, amounting to approximately 75% of the total cellular storage capacity^[32]. A recent ultrastructural analysis has revealed that the endothelial ER consists of tubulovesicular structures that are interconnected at various sites to form a widespread network within the ECs. In the periphery of the cells, they lie immediately adjacent to the plasma membrane and the caveolae at the apical, lateral, and basal surfaces of the cells and occasionally run closely parallel to the plasma membrane itself^[33-35]. Intriguingly, the ER-plasmalemma coupling is tighter (around 8 nm) in macrovascular than in microvascular (around 87 nm) ECs^[33]. As Ca²⁺ entry may be governed by the physical interaction between an ER Ca²⁺-sensor and the plasmalemmal Ca²⁺-permeable channels (see below), this feature might help explain why microvascular ECs normally lack the plateau phase^[33]. In the middle of the cells, the tubulovesicular structures may be in close contact with the mitochondria, sometimes enveloping them^[34,36], and are in continuity with the nuclear membrane^[34]. Ca²⁺ is sequestered into the ER lumen, where it attains a concentration of about 100-500 μmol/L, by high-affinity (K_m around 1 μmol/L) SERCA pumps, which can be inhibited by pharmacological agents, such as the marine toxin thapsigargin and cyclopiazonic acid (CPA)^[37,38]. Inhibition of the SERCA pump with either drug leads to Ca²⁺ store depletion, which indicates that ER membrane is constitutively leaky to Ca²⁺. Candidates to mediate the leak channel in a variety of cell types include polycystin-2, presenilins, and the ribosome-translocon complex^[37]. No study has, however, hitherto addressed the molecular underpinnings of passive Ca²⁺ leak from ER in ECs^[1]. Calreticulin represents the major Ca²⁺ buffer within the ER lumen in all non-muscle cells^[38], as well as in vascular endothelium^[34,35]. Maintenance of a constant luminal level of Ca²⁺ in ECs

is also fulfilled by a number of additional Ca²⁺-binding proteins, including calnexin, glucose-regulated protein (GRP)-78, and GRP-94^[38]. All these Ca²⁺ buffers also serve as molecular chaperones and their protein folding activity is regulated by ER Ca²⁺ concentration ([Ca²⁺]_{ER})^[38]. Monitoring of intraluminal Ca²⁺ levels in ECs transiently transfected with a cameleon-based Ca²⁺ probe targeted to the ER, D1_{ER}, has shown that, in the presence of extracellular Ca²⁺, extracellular autacoids do not induce a massive decline in [Ca²⁺]_{ER}^[39-42]. Consequently, Ca²⁺-dependent protein folding is largely maintained during physiological cell stimulation and does not activate any stress signaling pathway^[38,42]. Conversely, a rapid drop in ER Ca²⁺ content occurs when ECs are stimulated in the absence of extracellular Ca²⁺ entry^[43]. Owing to the large electrochemical gradient existing between ER lumen and the cytosol, rapid release of Ca²⁺ from the intracellular organelle occurs upon the opening of two largely homologous receptor types on its membrane, which are named for their affinity for either the cell metabolite inositol-1,4,5-trisphosphate (InsP₃) or the plant alkaloid ryanodine^[1,6,11]. Ryanodine receptors (RyRs), in particular, may be engaged by either a local increase in intracellular Ca²⁺ levels through a process called Ca²⁺-induced Ca²⁺ release (CICR) or the second messenger, cyclic ADPr (cADPr)^[44-48]. The remaining 25% of the total stored Ca²⁺ in ECs is loaded in non-ER intracellular pools, mainly mitochondria, which contribute to agonists-induced intracellular Ca²⁺ signaling^[32,36,40,41]. Ca²⁺ enters the organelles through a uniporter, present in the inner membrane, and can be exported *via* the stimulation of a mitochondrial NCX (NCX_{mito})^[36,41], thus creating a Ca²⁺ cycling system for the control of mitochondrial Ca²⁺ concentration that, under resting conditions, is equal to around 200 nmol/L in ECs^[49]. Mitochondria give rise to an impressive network in ECs, which is in tight apposition with either Ca²⁺ release channels on the ER or Ca²⁺-permeable pathways, mainly store-operated Ca²⁺ channels (SOCs), on the plasma membrane^[34-36]. Acidic, lysosome-like vesicles may also serve as Ca²⁺ storage organelles in vascular endothelium^[50,51]. Unlike the large network that is the ER, lysosomes constitute relatively small, discrete and mobile vesicles with a limited Ca²⁺ content, which can be discharged by NAADP^[50,51]. An attempt to quantify the relative lysosomal Ca²⁺ content suggested the latter to amount to around 79% of the ER Ca²⁺ pool^[52]. Such a value, which is at odds with a previous analysis of the relative capacities of intracellular Ca²⁺ storage organelles^[32], does not truly reflect lysosomal Ca²⁺ levels. Indeed, unlike the large network that is the ER, lysosomes generally constitute relatively small, discrete and mobile vesicles with a limited Ca²⁺ content^[53]. However, such acidic stores may be situated very close to the Ca²⁺-releasing channels on ER. As a consequence, NAADP induces scattered, local Ca²⁺ signals that are capable of generating a global Ca²⁺ wave only by triggering further Ca²⁺ mobilization from InsP₃Rs and/or RyRs *via* the CICR mechanism^[53] (see below). Along with InsP₃, cADPr, and NAADP, ECs

may liberate intracellular Ca²⁺ in response to other second messengers, such as arachidonic acid (AA)^[54], whose intracellular target remains to be elucidated, and reactive oxygen species (ROS), such as H₂O₂^[55,56] and superoxide anion (O₂⁻), which directly evoke Ca²⁺ release from InsP₃Rs^[57]. As to the sphingolipid-derived messenger, sphingosine-1-phosphate (S1P), that may elicit intracellular Ca²⁺ release independently from InsP₃Rs and RyRs^[10], it has been shown that, when endogenously synthesized in autacoids-stimulated ECs, it modulates SOCs, but does not stimulate Ca²⁺ mobilization^[58]. In blood vessels, however, plasma S1P may be produced and released by erythrocytes and platelets and induce Ca²⁺ mobilization in ECs upon binding to the G_i protein-coupled S1P1 receptor^[59]. The latter, in turn, recruits phospholipase C (PLC) to generate InsP₃ and, thus, stimulates InsP₃Rs and activates SOCs^[60,61].

Phospholipase C, InsP₃, and InsP₃-receptors

The intracellular second messenger InsP₃ liberates Ca²⁺ ions stored within the ER and, in ECs, is synthesized following activation of either GPCRs or TRKs^[1,6,11,62]. InsP₃ is a highly diffusible hydrophilic messenger that is produced by the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP₂). This reaction is catalyzed by a family of PLC isozymes, which, depending on the subtype, can be activated by G-proteins (G_{q/11}), phosphorylation or Ca²⁺ itself^[63,64]. The other product of PIP₂ hydrolysis is diacylglycerol (DAG), which stays within the plasma membrane where it either activates protein kinase C (PKC) or gates members of the transient receptor potential (TRP) canonical (TRPC) subfamily of Ca²⁺-permeable channels^[10]. The 13 mammalian PLC isozymes (excluding alternatively spliced forms) identified to date are all single polypeptides and can be divided into five types: PLCβ (β1-4), PLCδ (δ1-4), PLCγ (γ1 and γ2), PLCε, PLCζ, and PLCη (η1 and η2)^[63,64]. The amino acid sequences of PLC isozymes are relatively nonconserved with the exception of two regions, known as the X and Y domains, that form the catalytic core and are associated to various combinations of regulatory domains that are common to many other signalling proteins. In more detail, in γ-type isozymes, this region contains two additional Src homology 2 (SH2) domains and one SH3 domain, which bind phosphorylated tyrosine residues and proline-rich sequences, respectively^[63,64]. All PLC isoforms, with the exception of PLCε, are endowed with at least one potential polyphosphoinositides binding site, the pleckstrin homology (PH) domain, which is located in the NH₂-terminal region preceding the X domain^[63,64]. Moreover, the COOH-terminal region possesses a C2 domain which confers a strong Ca²⁺ sensitivity to the enzymatic activity of PLC. The C2 domain is, however, absent in the γ-type^[63,64]. In this view, four EF domains are located between the PH and X domains of all PLC subclasses but their functional significance is yet to be fully elucidated^[63,64]. PLCε, finally, exhibits a NH₂-terminal Ras guanine nucleotide exchange factor (RasGEF)-like domain and at least one COOH-

terminal Ras binding (RA) domains that are involved in its activation^[63,64]. ECs express a variety of PLC isoforms. PLC β 1 is enlisted following GPCR binding to their specific agonists, which include a plethora of circulating autacoids: acetylcholine, ATP, ADP, thrombin, bradykinin, serotonin, angiotensin II, and anandamide^[1,7,65]. Receptor occupancy results in the activation of the pertussis toxin (PTX)-insensitive heterotrimeric G $_{q/11}$ protein family. As a consequence, the GTP-bound G $_{\alpha q/11}$ subunit dissociates from the G $_{\beta\gamma}$ dimer and activates the enzyme^[1,7,63,64]. A remarkable exception is provided by the proteinase-activated receptor 1, which utilizes both G $_{\alpha q}$ ^[66,67] and G $_{\alpha o}$ ^[68] to trigger Ca²⁺ signals in thrombin-stimulated ECs^[69], albeit only the former leads to InsP₃ production^[70]. The G $_{\alpha o}$ -dependent activation is mediated by the G $_{\beta\gamma}$ dimer^[68], which may recruit all PLC- β subtypes with the exception of the β 4 isoform^[63,64]. This mechanism is also involved in PLC β engagement by the PTX-sensitive family of G $_i$ proteins^[63,64], as observed in ECs exposed to S1P^[59,61]. PLC γ 1 is the main γ -type expressed in ECs^[9], with PLC γ 2 being primarily restricted to cells of the hematopoietic lineage^[71]. PLC γ 1 is the isozyme mediating the increase in [Ca²⁺]_i elicited in vascular endothelium by growth factors, such as epidermal growth factor (EGF)^[72], vascular endothelial growth factor (VEGF)^[73,75], platelet-derived growth factor (PDGF)^[76], and basic fibroblast growth factor (bFGF)^[73,77]. Upon growth factor binding to its cognate receptor, PLC- γ 1 is recruited to the activated receptors *via* SH2-domain-mediated phosphotyrosine interaction and then subjected to phosphorylation by receptor tyrosine kinase^[63,64]. It has, however, been reported that PLC- γ 1 may be also stimulated by GPCR in ECs. For instance, it may associate with the COOH-terminal intracellular domain of the bradykinin BR receptor (B2), which results in a transient phosphorylation and InsP₃ production^[78,79]. On the other hand, VEGF has been recently shown to elicit PLC β 3 lipase activity upon binding to VEGFR-2 (KDR), which in turn physically interacts with G $_{q/11}$ proteins to induce G $_{\beta\gamma}$ dissociation from G $_{\alpha}$ subunits^[80]. G $_{\beta\gamma}$ may thus stimulate PLC β 3 to generate InsP₃ and trigger an intracellular Ca²⁺ wave^[81]. PLC δ 1 has been so far detected in a limited number of vascular beds, although its ability to evoke InsP₃-dependent Ca²⁺ mobilization has been probed only in human umbilical vein ECs (HUVECs) transfected with PLC δ 1-containing plasmids^[82]. While there is no report of PLC η expression in ECs and PLC ζ is present almost exclusively in mammal sperms^[6], PLC ϵ may promote angiogenesis in a mouse model of adenocarcinoma, but its role in Ca²⁺ signaling has not been explored^[83]. It is, however, remarkable that β 2-adrenergic excitation results to InsP₃-dependent Ca²⁺ discharge upon recruitment of the exchange protein activated by cAMP-1 (Epac-1)^[84], which has long been known to stimulate PLC ϵ ^[64]. In agreement with PLC activation by both GPCRs and TKRs, InsP₃ production has been evaluated in a variety of stimulated ECs^[11]. In [³H]myoinositol-labeled cultured cells, both thrombin and histamine-induced increases in InsP₃ occurred in less

than 15 s, and were temporally correlated with the Ca²⁺ signals^[70,85-87]. Bradykinin and ATP also evoked an elevation in InsP₃ concentration, which attained a peak value at 15 s after agonist application and then decayed to a plateau level with kinetics similar to those of the ensuing Ca²⁺ signals^[70,88,89]. The same methodology revealed that platelet activating factor (PAF)-induced InsP₃ formation is maximal after 1 min of stimulation^[90]. Similarly, a radioreceptor assay of InsP₃ accumulation in ECs revealed that VEGF induced a rapid increase in InsP₃ levels, which achieved a peak after 4 min of exposure^[91]. Due to the objective difficulties entailed by these pioneering measurements, it is unlikely that they truly reflect the dynamics of agonists-induced InsP₃ accumulation in vascular ECs. A more recent investigation, based on an innovative analytical method, revealed that InsP₃ levels peak within < 1 s from ATP stimulation and then rapidly subside due to degradation to lower order inositol phosphates^[92]. Besides agonist stimulation, InsP₃ production in vascular ECs may be stimulated by reactive oxygen species (ROS), such as O₂^{·-}^[93] and H₂O₂^[94].

In addition to extracellular agonists, mechanical stimuli may induce InsP₃ formation in vascular endothelium. Cyclic strain was found to elicit a transient spike of immunoreactive InsP₃ in bovine aortic ECs (BAECs): peak levels occurred 10 s after the initiation of strain and decayed to control values 25 s later^[95]. Moreover, BAEC exposure to shear stress induced a biphasic increase in intracellular InsP₃ concentration, whereas a transient InsP₃ peak arising within the first 15 s was followed by a major peak observed at 15 min after the onset of the stimulus. InsP₃ levels returned to pre-shear stress level within the following 15 min^[96]. Recent studies have demonstrated that the vascular system is endowed with mechanosensitive GPCRs translating mechanical stimuli into an InsP₃-dependent Ca²⁺ mobilization^[97]. Consistently, shear stress induces bradykinin B2 receptor to undergo a conformational transition that triggers the downstream signaling cascades, i.e., InsP₃ and DAG production, even in the absence of the agonist^[97,98]. The kinetics of InsP₃ synthesis under these conditions, however, has not been determined.

Once produced by PLC isozymes, InsP₃ diffuses into the cytosol and evokes a short-lasting Ca²⁺ efflux from ER^[99]. InsP₃-induced Ca²⁺ mobilization from ER has been characterized both in saponin-permeabilized ECs loaded with Ca²⁺ and intact ECs perfused with caged InsP₃ *via* a patch pipette^[45,99-101]. The release of Ca²⁺ by this second messenger displayed an EC₅₀ around 1 μ mol/L and was independent of InsP₃ metabolism^[99,101]. InsP₃ acts on a tetrameric receptor characterized by a very long cytosolic NH₂-terminal tail that contains the InsP₃-binding site, which has been crystallized recently^[102]. Three InsP₃ receptor subtypes have been identified that may assemble to form either homo- or hetero-tetrameric Ca²⁺-releasing channels. InsP₃ binds to the very long cytosolic NH₂-terminal tail, while the proper Ca²⁺ channel is located in the COOH-terminal portion of the molecule, which contains

Table 1 Pattern of expression of InsP₃R_s, RyR_s and TPC_s in vascular endothelial cells as detected by reverse transcription/polymerase chain reaction, immunostaining for cultured cells, immunohistochemistry, Western blotting, and immunoprecipitation

	InsP ₃ R 1	InsP ₃ R 2	InsP ₃ R 3	RyR1	RyR2	RyR3	TPCs1-2
Human brain microvascular ECs ^[113]	+(RT-PCR, WB, IC)						
Human umbilical vein ECs ^[106]	+(RT-PCR)						
Human microvascular dermal ECs ^[213]	+(RT-PCR)						
Human mesenteric artery ECs ^[127]	+(RT-PCR)						
EA.hy926 cells ^[51,127]	+(RT-PCR)						
Mouse cremasteric ECs ^[108]	+(WB, IC)						
Rat hippocampus ECs ^[413]	-(IHC)						
Rat aortic ECs ^[106,107]	+(RT-PCR, IC, IHC)						
Rat mesenteric artery ECs ^[107]	+(RT-PCR, IC, IHC)						
Rat basilar artery ECs ^[107]	+(RT-PCR, IC, IHC)						
Rat adrenal medulla microvascular ECs ^[106]	+(RT-PCR)						
Rat splenic sinus ECs ^[35]	+(WB, IC)						
Bovine aortic ECs ^[104,109]	+(WB, IC)						
Ovine uterine artery ECs ^[414]	+(WB, IC)						

RT-PCR: Reverse transcription/polymerase chain reaction; WB: Western blotting; IC: Immunostaining for cultured cells; IHC: Immunohistochemistry; ISH: *In situ* hybridization; IP: Immunoprecipitation; ECs: Endothelial cells.

the sixth transmembraneTM domain motif and the loop connecting helices 5 and 6 folding back into the membrane^[102]. The molecular mass of each InsP₃R isoform is around 260 kDa^[103,104], which indicates that the native protein is around 1 MDa^[102]. The Hill coefficient for InsP₃-elicited Ca²⁺ release is around 3.8 and suggests that InsP₃ binding to all subunits is required to induce channel opening^[99]. All InsP₃R subtypes have been detected in ECs (Table 1), both under culture conditions^[104-106] and in intact vessels^[107,108]. The expression pattern of InsP₃R isoforms in both freshly isolated and cultured ECs is: InsP₃R-3 > InsP₃R-2 > InsP₃R-1^[106]. This feature becomes even more remarkable in proliferating cells, which suggests that distinct isoforms may impact on different aspects of EC physiology^[106]. In this view, recent work has revealed that InsP₃R forms mostly, albeit not exclusively, homotetramers in vascular ECs^[109]. The subcellular localization of InsP₃R subtypes may vary with cell isolation and culture. In cultured ECs, InsP₃R-1 is mainly localized to the ER, InsP₃R-2 resides within the nucleus, and InsP₃R-3 is detected at the perinuclear region^[104], while, in the intact vascular wall, all isoforms are tightly associated to F-actin fibers and InsP₃R-1 and InsP₃R-2 lie along with InsP₃R-3 near the edge of the nucleus^[107]. None of the three subtypes has been detected in close association with the plasma membrane^[104,107], although some studies have reported a protein with high homology to the InsP₃R-1 in endothelial caveolae *via* binding to F-actin^[34,110]. Such plasmalemmal InsP₃ might mediate the InsP₃-gated Ca²⁺ entry originally described by Vaca and Kunze^[111]. Moreover, InsP₃R-1 may be associated to the EC side of myoendothelial junctions *in vivo* and thus plays a key role in transmitting intracellular Ca²⁺ waves from vascular endothelium to the adjoining smooth muscle cell layer^[108]. InsP₃R expression may be altered under pathological conditions, such as hypertension, whereas ECs isolated from spontaneously hypertensive rats display an increase in InsP₃R-2 transcripts and a decrease in InsP₃R-1 mRNA^[112]. Moreover, InsP₃R levels augment as a consequence of

ethanol metabolism and oxidative stress^[113]. InsP₃R gating is regulated by both InsP₃ and Ca²⁺. InsP₃ is able to activate InsP₃R_s when the surrounding Ca²⁺ is between 50 nmol/L and 200 nmol/L, whereas higher Ca²⁺ levels inhibit Ca²⁺ release even at saturating InsP₃ concentrations^[102]. Such a biphasic dependence on ambient Ca²⁺ is due to two independent Ca²⁺-binding sites that mediate channel activation or closure, respectively. More specifically, InsP₃ tunes InsP₃R sensitivity to cytosolic Ca²⁺: it favors Ca²⁺ binding to the stimulatory site, which is the trigger for pore opening, and inhibits Ca²⁺ binding to the inhibitory one^[102,114]. Consistent with this model, InsP₃-induced Ca²⁺ mobilization is enhanced at lower (200 nmol/L) than at higher (2 μmol/L) Ca²⁺ concentrations in saponin-permeabilized BAECs^[99,115]. The large modulatory domain between the InsP₃-binding site and the channel-forming region presents consensus sequences for numerous protein kinases, including PKC, protein kinase A (PKA), cGMP-dependent protein kinase (PKG), Akt kinase, calmodulin-dependent protein kinase II (CaMKII), tyrosine kinases, and cyclin-dependent kinase 1/cyclin B (cdc2/CyB) complex^[102]. Additionally, InsP₃R channel activity is sensitive to a variety of modulators, including ATP, redox balance, and luminal Ca²⁺, and interacting proteins, such as calmodulin, Ca²⁺-binding proteins (CaBPs), chromogranins, InsP₃R binding protein released with inositol-1,4,5-trisphosphate (IRBIT), RACK1 and G_{βγ}, ERp44, FKBP12, cytochrome C, Bcl-2 proteins, huntingtin, adapter proteins (ankyrin, homer, protein 4.1N), and proteases (caspase-3 and calpain)^[102]. As to vascular ECs, PKC and CaMKII block InsP₃-dependent Ca²⁺ release *via* ATP-dependent phosphorylation of InsP₃R_s^[116-118]. Conversely, PKA and mammalian target of rapamycin (mTOR) phosphorylate InsP₃R_s to render them more sensitive to ligand binding and, thereby, increase the velocity of InsP₃-dependent Ca²⁺ waves^[118]. In addition, an InsP₃R-ankyrin complex has been detected in cells exposed to hyaluronic acid (HA). Such stimulation favors the ankyrin-mediated recruitment of ER In-

sP₃Rs into plasmalemmal caveolae; an event that triggers Ca²⁺ release independently on agonist binding^[119]. InsP₃R modulation in ECs may be further accomplished by the gasotransmitters, NO and H₂S. While the former induces Ca²⁺ release likely *via* receptor S-nitrosylation^[120,121], the latter inhibits InsP₃-induced Ca²⁺ discharge probably through receptor S-sulfhydration^[122,123]. An additional post-translational modification of endothelial InsP₃Rs is provided by S-glutathionylation, which might sensitize the receptor to the basal level of InsP₃^[124]. In this view, the InsP₃R may be directly regulated by ROS, which may oxidize the two highly conserved thiol groups located in the COOH-tail of the protein^[102]. It has, indeed, been shown that Ca²⁺ release from endothelial InsP₃Rs may be evoked by H₂O₂^[55-57], which may increase the sensitivity of the intracellular Ca²⁺ pool to InsP₃^[56].

RyRs and cADPr

Although InsP₃R is the predominant ER Ca²⁺-releasing channels in ECs, RyR-dependent signalling has also been reported in vascular endothelium. Three RyR isoforms have been identified (RyR-1, RyR-2, RyR-3) which assemble as homo-tetrameric channels consisting of four 560-kDa polypeptides^[125]. They possess a very large cytosolic NH₂-domain comprising around 90% of the receptor amino acid sequence. This region is part of a macromolecular complex and serves as a scaffold for numerous regulatory or modulatory proteins, such as calmodulin, calstabin, sorcin, and presents binding sites for cytosolic ions and modulators, including Ca²⁺, Mg²⁺, and adenine nucleotides. RyRs may also be modulated PKA- and CamKII-mediated phosphorylation, may undergo oxidative/nitrosative modifications, and are subject to pH modulation^[126]. In addition, RyRs are modulated by SR luminal Ca²⁺ *via* a signaling pathway that includes calsequestrin (CSQ), triadin and junction^[126]. Comparative studies carried out by either single-cell reverse transcription/polymerase chain reaction (RT-PCR) or immunocytochemistry have revealed that ECs mainly express RyR-3^[34,127], but not RyR-1 and RyR-2 (Table 1). Immunogold labeling with anti-RyR indicates that RyR-3 is located in the nuclear membranes, the ER tubulovesicular system, and the cristae membrane of mitochondria^[34]. In the light of the ability of RyRs to modulate sub-plasmalemmal Ca²⁺ signaling in ECs (see below), it is remarkable that RyR-3 is largely expressed in the ER structures facing the plasma membrane^[34]. Ca²⁺ release from RyRs in ECs, as well as in both excitable and non-excitable cells^[126], is triggered by CICR^[44,45], that occurs when 1-10 μmol/L Ca²⁺ nearby the receptor and is prevented when Ca²⁺ is 1-10 mM^[126]. RyRs may be pharmacologically activated by the methylxanthine derivative, caffeine, which sensitizes the receptor to resting Ca²⁺ levels, and by ryanodine, which binds to and locks the receptor in an open state^[128]. In a number of ECs from different vascular districts, caffeine/ryanodine may evoke either fast Ca²⁺ transients^[46,129,130] or slow Ca²⁺ release from ER^[131,132]. Other studies, however, failed to report any increase in

bulk [Ca²⁺]_i in ECs exposed to either caffeine^[72,130] or ryanodine^[132,133]. Similarly, RyR contribution to agonists-induced Ca²⁺ signals has been detected in some^[44,47,132,134,135], but not all ECs^[72,136,137]. RyR-dependent signaling might be masked by SERCA activity, so that Ca²⁺ ions liberated into the cytosol by RyR opening are immediately sequestered into the InsP₃-sensitive region of ER lumen by its Ca²⁺-ATPase^[130]. In addition, monitoring of subplasmalemmal and perinuclear Ca²⁺ levels revealed that RyR-sensitive Ca²⁺ mobilization may selectively occur beneath the plasma membrane, but not in the bulk cytosol^[133]. Indeed, RyRs may be part of a subplasmalemmal Ca²⁺ control unit (SCCU), as confirmed by their localization in the ER membrane facing the cytosolic leaflet of the plasma membrane (see above). The SCCU enables a functional coupling between RyRs expressed on superficial ER and plasmalemmal NCX, so that Ca²⁺ released from RyRs is vectorially pumped out by NCX without affecting [Ca²⁺]_i. Under these conditions, RyR contribution to endothelial Ca²⁺ signaling may be appreciated only upon NCX inhibition^[133,138,139] or by monitoring the local activation of large conductance Ca²⁺-dependent K⁺ channels^[140,141]. Besides CICR, RyRs may be stimulated by cADPr^[10], a second messenger that can be generated in response to either agonist stimulation^[142] or oxidative stress through the synthesis of H₂O₂^[143,144]. The ADP ribosyl cyclase, CD38, which is located both on the cell surface and on intracellular membranes, catalyzes nicotinamide adenine dinucleotide (NAD) conversion into cADPr by a cyclization reaction occurring at neutral pH^[142]. cADPr, in turn, acts by displacing the FK506 binding protein 12 (FKBP12 or calstabin1) or its related isoform FKBP12.6 (FKBP12.6 or calstabin2) from RyRs^[48], which increases their open probability and leads to Ca²⁺ release^[125]. A recent study has demonstrated that bradykinin induces Ca²⁺ release by stimulating cADPr synthesis and RyR activation in human coronary artery ECs^[47]. Moreover, genetic ablation of FKBP12/12.6 from endothelial RyRs caused an intracellular Ca²⁺ leak associated with the impairment of NO production and endothelial dysfunction^[145,146]. It is, however, yet to demonstrate that cADPr triggers Ca²⁺ release by dissociating FKBP12/12.6 from RyRs in ECs. It should also be noticed that a number of studies have failed to find direct activation of RyRs by cADPr, as discussed previously^[48]. For instance, cADPr has been reported to promote the activity of the SERCA pump, thereby increasing the rate of ER refilling and Ca²⁺ release *via* the luminal excitation of RyRs^[142]. Mechanical stimulation may also lead to RyR activation in vascular ECs. More specifically, shear stress may induce oscillations in [Ca²⁺]_i by stimulating the interplay between mechano-sensitive Ca²⁺ influx and RyRs through the CICR mechanism^[25].

NAADP and two-pore channels

NAADP is the newest and most efficacious member of the family of Ca²⁺-mobilizing messengers, being active already at nanomolar concentrations^[53]. NAADP is synthe-

sized from NADP by CD38 in a base-exchange reaction occurring at acidic pH and targets acidic organelles, such as the endolysosomal Ca²⁺ storage compartment^[53,142], although other signaling pathways may control NAADP production^[53]. Ca²⁺ is sequestered into lysosomal lumen by a putative Ca²⁺/H⁺ exchanger, which is driven by a vacuolar-H⁺ ATPase^[53]. NAADP-gated Ca²⁺ release is mediated by TPC2, whose primary sequence places them in the superfamily of voltage-gated cation channels. Hydrophathy analysis has revealed that the full-length protein contains 12 putative TM (TM) α helices that are organized into two homologous domains of six TM segments, each with a membrane re-entrant pore loop between the fifth and sixth segments. Although three TPCs have been identified (TRPC1-3), only TPC2 is endowed with both high (5 nmol/L) and low (10 μ mol/L) affinity binding sites for NAADP and is selectively targeted to lysosomes^[53]. Due to the limited amount of Ca²⁺ in the endolysosomal compartment, which consists of small vesicles (0.2-1 μ m in diameter) moving throughout the cytosol, NAADP-induced Ca²⁺ signals *per se* appear as scattered, discrete events^[53]. The relatively small quantity of Ca²⁺ mobilized by endolysosomes may, however, be amplified into a global Ca²⁺ wave by recruitment of InsP₃Rs and RyRs *via* CICR^[53,147]. This feature requires the physical proximity between the NAADP-sensitive Ca²⁺ pool and the Ca²⁺-releasing channels on ER, that has been documented in a number of cell types^[53]. NAADP has recently been involved in endothelial Ca²⁺ signaling (Table 1). A cell-permeable NAADP analog induced an increase in [Ca²⁺]_i in human aortic ECs, which was associated with membrane hyperpolarization and NO production. Importantly, NAADP-dependent Ca²⁺ release triggered the Ca²⁺ response to acetylcholine and thrombin, but not ATP^[50]. Importantly, NAADP-dependent Ca²⁺ release triggered the Ca²⁺ response to acetylcholine and thrombin, but not ATP^[50]. Consistent with these data, it was subsequently shown that histamine binding to H1 receptors caused an increase in intracellular NAADP levels in EA.hy926 cells, which evoked a TPC2-mediated Ca²⁺ signal and secretion of von Willebrand factor^[51]. Unlike human aortic ECs, however, the Ca²⁺ response to thrombin was insensitive to the inhibition of NAADP-dependent signalling^[51].

Ca²⁺ ENTRY FROM THE EXTRACELLULAR SPACE

Ca²⁺ influx across the plasma membrane sustains the Ca²⁺ response to both chemical and mechanical stimuli in ECs, as well as most other nonexcitable cells. In addition to refilling the intracellular Ca²⁺ stores, Ca²⁺ inflow is responsible for the activation of Ca²⁺-sensitive effectors which are strategically located near the inner mouth of endothelial Ca²⁺ channels^[6,9,11,62]. Ca²⁺ entry in vascular endothelium may be mediated by voltage-operated Ca²⁺ channels (VOCs), agonist-operated Ca²⁺ channels (AOCs), and mechanosensitive channels (MSCs)^[7,11,13,62].

AOCs, in turn, comprise at least three types of Ca²⁺-permeable membrane pathways, which may be gated by distinct signaling mechanisms: (1) receptor-operated channels (ROCs), which are ionotropic receptors activated by direct binding of the ligand; (2) second-messenger-operated channels (SMOCs), which are stimulated by intracellularly generated mediators, such as DAG, AA and its derivatives (eicosanoids and anandamide), ADPr, and cyclic nucleotides; and (3) SOCs, which open in response to a depletion of the ER Ca²⁺ pool. Most, but not all, endothelial SMOCs, as well as MSCs, belong to the TRP family of nonselective cation channels^[4,8,19,148-150], whereas the molecular nature of SOCs is still debated^[151,152]. The inrush of Ca²⁺ ions consequent to the opening of the Ca²⁺-permeable membrane route is governed by the following equation:

$$J_{Ca} = 2 \times F \times N \times p \times \gamma_{Ca} \times (V_M - E_{Ca})$$

where J_{Ca} is Ca²⁺ influx, V_M is the membrane potential, E_{Ca} is the equilibrium potential for Ca²⁺, F is the Faraday constant, N is the number of channels, p and γ_{Ca} , respectively, are their open probability and conductance^[7]. Ca²⁺ entry may, therefore, be modulated by any change in V_M according to the following general rule: hyperpolarization increases the driving-force pushing Ca²⁺ into the cytosol, while depolarization reduces Ca²⁺ influx across the plasmalemma^[7,153]. This rule, however, may not apply to microvascular ECs, which may be endowed with VOCs, as depicted below. We refer the reader interested in the ion channels controlling endothelial V_M to a number of exhaustive and relatively recent reviews^[7,153]. The description of the Ca²⁺-permeable membrane pathways in ECs will be addressed by focussing on the following topics: (1) VOCs; (2) ROCs; (3) cyclic-nucleotide-gated channels, which belong to SMOCs; (4) TRP channels, which comprise SMOCs, SOCs, and SACs; and (5) Orai1 and Stim1, which mediate SOCs.

Voltage-gated Ca²⁺ influx in ECs

Although classically considered as non-excitable cells, ECs from capillaries, but not from large vessels, may express both L- and T-type VOCs^[154-159]. VOCs have been detected in microvascular ECs from bovine adrenal glands (BAMCECs)^[154-156], rat and pig brain^[160,161], rat and mice lungs^[157,162-165]. The following features suggest that the voltage-gated currents measured in BAMCECs belong to the L- and T-type described in endocrine secretory cells: their kinetic and gating properties, current-voltage relationships, sensitivity to BAY K 8644, nifedipine, and amiloride, and their different selectivity for Ba²⁺ and Ca²⁺^[156]. The T-type transient current displayed a conductance of 8 pS and may contribute to depolarisation-induced Ca²⁺ influx^[155,166], while the L-type large current manifests a single-channel conductance of 20 pS and is affected by dihydropyridines^[154]. Besides T- and L-type VOC, BAMCECs express a voltage-dependent Ca²⁺ channel (termed SB) that shows a single-channel conductance of 2.8 pS in 100 mmol/L Ba²⁺ and is sensitive to BAY K 8644, but not nifedipine^[154]. Such a path-

way is operative at negative resting potentials and might contribute to low-threshold Ca²⁺ inflow^[154]. Pulmonary microvascular vascular ECs (PMVECs) exhibit a Ca_v3.1 (α_{1G}) voltage-gated T-type Ca²⁺ current that is transiently activated at ≥ -60 mV and peaks at around -10 mV^[158,159]. Although α_{1G} may mediate T-type voltage-dependent Ca²⁺ currents without any interaction with its ancillary proteins (β -, γ -, and $\alpha\delta$ subunits), $\alpha\delta$ -, β_1 - and β_3 -, but not β_2 - and β_4 -subunits are expressed in PMVECs^[158]. The window current of T-type Ca_v3.1 channels occurs at membrane potentials ranging from -60 mV to -30 mV, a voltage interval that may be achieved in ECs challenged with extracellular agonists when their V_M is close to the K⁺ equilibrium potential, i.e., -70/-80 mV^[7]. Thrombin, in particular, utilize SOCs to depolarize PMVECs and exploit T-type Ca_v3.1-mediated Ca²⁺ entry to drive the exocytosis of von Willebrand factor^[162]. Exposure of PMVECs to shear stress may upregulate Ca_v3.1 (α_{1G}) expression, so that flow cessation results in an abrupt Ca²⁺ entry through T-type Ca²⁺ channels^[157]. Ca²⁺ entry through T-type Ca_v3.1 channels has also been observed in mouse pulmonary capillary endothelium, where it selectively drives P-selectin expression^[163]. An R-type VOC has been, finally, described in canine aortic ECs, that may contribute to PAF-induced Ca²⁺ entry^[167]. This channel does not inactivate during long-lasting depolarizations, therefore, it is well suited to mediate prolonged Ca²⁺ inflow^[7]. It is, finally, noticeable that angiotensin II causes α_{1G} through AT1 receptors, Ras and MEK^[168]. This study, however, did not assess the appearance of voltage-gated Ca²⁺ signals, although it showed that mibefradil, a T-type Ca²⁺ channel inhibitor, prevented the regeneration of an injured monolayer^[168].

ROCs in ECs

Vascular ECs may express at least two distinct ROCs, namely the nicotinic acetylcholine (ACh) receptor (nAChR) and the purinergic P_{2X} receptor, which are gated by ACh and ATP, respectively. These pathways are also permeable to K⁺ and Na⁺ and gate an inward current at physiological V_M . Therefore, in capillary ECs endowed with VOCs, ROCs may also affect Ca²⁺ signaling by depolarizing the cells and inducing a voltage-dependent Ca²⁺ inrush.

Neuronal nAChRs: nAChRs are ionotropic receptors that mediate fast cationic currents in response to their selective agonist, ACh. They are named after nicotine, a psychoactive ingredient of tobacco that can mimic the action of ACh in opening these channels. The neuronal nAChRs are pentameric channels comprising either combinations of two different types of subunit (α and β) or five copies of the same α subunit symmetrically arranged in a barrel-like configuration^[169]. Each subunit consists of four α -helical spanning domains (TM1-TM4), with TM2 of each subunit lining the receptor pore^[169,170]. Ten α isoforms (α_1 - α_{10}) and four β subtypes [β_1 - β_4], which may assemble in either homomeric or heteromeric

configurations, have been identified^[170,171]. The diverse stoichiometric combinations may result in ion channels displaying different pharmacological [ligand affinity and α -bungarotoxin (BTX) sensitivity] and biophysical (mean open time and Ca²⁺ permeability) features^[172]. The neuronal α -BTX-sensitive nAChRs are formed by α_7 - α_9 homopentamers, are inhibited by α -BTX, and display the highest Ca²⁺:Na⁺ permeability ratio (P_{Ca}/P_{Na}), while the non- α -BTX nAChRs are heteropentamers containing at least one α (out of α_2 - α_6) and one β (out of β_2 - β_4) subunits and exhibit a much lower P_{Ca}/P_{Na} ^[172]. As reviewed by Arias *et al.*^[173] and Gahring and Rogers^[170], ECs from both macro- and microvascular origin present a pattern of α [α_2 - α_5 , α_7] and β [β_2 and β_4] isoforms consistent with the expression of both nAChR7 and nAChR3. Activation of the latter does not elicit any detectable Ca²⁺ influx in vascular endothelium^[174], while the former triggers a proangiogenic Ca²⁺ entry that might be exploited to enhance tissue revascularization^[173,175]. Moreover, nAChRs-mediated membrane depolarization induces VOCs-dependent Ca²⁺ inrush in BAMCECs^[166].

P_{2X} receptors: The ionotropic P_{2X} receptors are channels that respond to extracellular ATP to induce membrane depolarization and an influx of Ca²⁺. Seven P_{2X} receptor subtypes (P_{2X1-7}) have been identified: each of them possesses two TM domains (TM1 and TM2) connected by a large extracellular loop and with the NH₂- and COOH-terminal tails extending into the cytoplasm^[176]. The extracellular loop contains the ATP binding site, while TM1 and TM2 are thought to line the channel pore^[176]. The P_{2X} receptors assemble as homo- or heterotrimers with a range of biophysical features and functions, including different P_{Ca}/P_{Na} values (which may range from 1 to 71) and rates of inactivation^[177]. For instance, P_{2X3}-mediated currents rapidly decay during prolonged exposition to ATP, whereas P_{2X7} remains open for several minutes^[176]. Vascular ECs express all P_{2X} receptor isoforms in both arteries and veins^[178,179], although P_{2X4} levels are higher in the latter^[179]. P_{2X4} expression in the arterial endothelial layer undergoes a dramatic increase following balloon injury, which hints at this subunit as a key player in EC regeneration^[179]. The electrophysiological analysis showed that channels with the electrophysiological and pharmacological properties of both P_{2X4} (fast inactivation, inward rectification, conductance of around 36 pS, insensitivity to reactive blue) and P_{2X7} (slow inactivation, conductance of 9 pS, linear current-to-voltage relationship, sensitivity to reactive blue) are functionally present on EC plasma membranes^[180]. P_{2X5} subunits may assemble with P_{2X4} subtypes and form a heterotrimer associated to a current displaying the characteristics of a P_{2X4} current^[180]. Ca²⁺ imaging recordings demonstrated that P_{2X4} is the main mediator of ATP- and flow-induced Ca²⁺ inflow in vascular endothelium^[181,182]. Shear stress induces ATP release from ECs through different mechanisms, including exocytosis or ATP binding of cassette transporters^[183,184], or it may stimulate a cell surface ATP-synthase^[185]. ATP, in turn, causes a step-like elevation in [Ca²⁺]_i^[181,182], which

leads to NO production and vasorelaxation *in vivo*^[186]. ATP-dependent Ca²⁺ entry is linearly correlated with the amplitude of the mechanical stimulation^[187].

Cyclic nucleotide-gated Ca²⁺ channels

Cyclic nucleotide-gated channels (CNGs) are nonselective cation channels gated by direct binding of the cyclic nucleotides, cAMP and cGMP^[188]. There are six members of the CNGC family: four α subunits (CNGA1-4) and two β subunits (CNGB1 and CNGB3). They all share structural homology with voltage-gated channels and have six TM domains with both the NH₂- and COOH-termini facing the cytoplasm. A pore region lies between TM5 and TM6. The CNGA1-3 subunits form channels whereas CNGA4 and the two CNGB isoforms have a modulatory function. The cyclic nucleotide binding site is located at the COOH-terminal domain of each subunit^[188]. All CNGs conduct Ca²⁺ better than Na⁺, a feature which enables these conductances to affect significantly intracellular Ca²⁺ signaling^[189]. Both CNGA1, which is selectively gated by cGMP, and CNGA2, which opens in response to cAMP, are widely expressed by ECs from several vascular beds, although cultured cells undergo downregulation of both subunits^[190,191]. Both cAMP and cGMP levels may increase in ECs challenged with β -receptor agonists, such as epinephrine, noradrenaline and isoprenaline^[192,193]. Moreover, intracellular levels of both cyclic nucleotides may be raised when ECs are stimulated with adenosine and ATP^[192-194], which are coupled, respectively, to A_{2B} and P_{2Y1} receptors^[194-196], and VEGF, which acts through VEGFR-2^[196]. Finally, SOCs-mediated Ca²⁺ entry may lead to the intracellular accumulation of cGMP, which in turn leads to CNG opening and membrane depolarization, thus establishing a negative feedback responsible for SOC closure^[197]. CNGA2 is the main contributor to cyclic-nucleotide-induced Ca²⁺ inflow in vascular endothelium stimulated with epinephrine^[198], ATP^[195] or adenosine^[194]. CNGA2-mediated Ca²⁺ influx, in turn, results in NO production and vasorelaxation^[194]. Moreover, NO may upregulate CNGA2 levels in ECs^[199].

TRP channels

The super-family of TRP channels comprises 28 members, which have been subdivided in six categories based on the homology of their amino-acid sequence: the TRPC (canonical; TRPC1-TRPC7), TRPV (vanilloid; TRPV1-TRPV6), TRPM (melastatin; TRPM1-TRPM8), TRPP (polycystin; TRPP1-TRPP3), TRPML (mucolipin; TRPML1-TRPML3), and the TRPA (ankyrin; TRPA1) families^[200]. They all are nonselective cation channels, with the exception of TRPM4 and TRPM5, which are only permeable to Na⁺ and K⁺ ($P_{Ca}/P_{Na} < 0.05$), and TRPV5 and TRPV6, which are Ca²⁺ selective ($P_{Ca}/P_{Na} > 100$)^[200]. All the family members have the same basic structure consisting of six TM domains (TM1-TM6) with the NH₂- and COOH-termini located in the cytoplasm. Between TM5 and TM6, which are highly conserved among all the homologs, there is a re-entrant pore loop constituted by a short hydrophobic stretch lining the channel pore. This

molecular architecture resembles that of the archetypal 6-TM voltage-gated channels, although TM4 lacks the conserved block of arginine residues forming the voltage sensor of transmembrane electrical potential^[201]. Most of the differences between the three families are found within the NH₂- and COOH-terminal tails. The TRPC and TRPV family members contain three or four ankyrin repeats at their NH₂ terminus, while TRPA1 contains 14 ankyrin repeats. All mammalian TRP families present a homologous sequence of around 25 amino acids immediately COOH-terminal to TM6, which has been termed TRP domain and consists of a highly conserved six-amino-acid TRP box (EWKFAR) and a proline-rich motif. The TRP domain is, however, lacking in TRPA and TRPP^[201]. TRPC proteins contain a number of calmodulin/InsP₃R binding domains (CIRB) at both their NH₂- and COOH-tails, while a predicted coil:coil region may be present in both locations of TRPC, TRPM, and TRPP^[19]. The TRPM family is characterized by having very long N and C termini. The latter contain enzymatic activity in some of the channels, e.g., TRPM2 has an ADP-ribose pyrophosphatase, whereas TRPM7 contains an atypical protein kinase^[200]. TRP subunits within one subfamily may assemble in either homo- or heterotetramers and this gives rise to large variability in their biophysical properties and activating mechanisms^[19,200,201]. Vascular ECs have been shown to express TRP channels from all subfamilies^[8,150]. More specifically, all the TRPC, all TRPV, all TRPM except TRPM5, TRPP1, TRPP2 and TRPA1 are present in ECs (Tables 2-4). Endothelial TRP channels serve as polymodal cell sensors, as each TRP subunit may be engaged by more than one signaling pathway. The selective coupling to distinct downstream Ca²⁺-sensitive decoders enables TRP-mediated Ca²⁺ influx to govern a plethora of processes in ECs^[4,19,148,149]. For sake of clarity, we will separately address the activating mechanisms and the main functions fulfilled by each TRP isoform in vascular endothelium.

TRPC channels

The TRPC family may be subdivided in four subfamilies according to their amino acid homology: TRPC1, TRPC2, TRPC3/6/7 (around 80% homology), and TRPC4/5 (around 60% homology). TRPC2 is a pseudogene in humans and does not fulfil any functional role in vascular ECs. TRPC channels are nonselective cation channels whose P_{Ca}/P_{Na} may vary from 0.9 to 9^[202]. The TRPC subunits may assemble as homo- and heterotetramers. In the latter case, they may combine with members of their own subfamily or TRPC1 may form heteromers with TRPC1, TRPC4/5 and TRPC3^[200]. Endothelial TRPC channels are activated by GPCRs and TKRs coupled to PIP₂ hydrolysis by PLC activation and, depending on their stimulating mechanism, may be part of either SMOCs or SOCs.

TRPC1: TRPC1 is widespread in both cultured and native ECs (Table 2), where it mainly serves as SOC and controls transendothelial permeability^[203,204], although

Table 2 Pattern of expression of transient receptor potential canonical channels in vascular endothelial cells as detected by reverse transcription/polymerase chain reaction, immunostaining for cultured cells, immunohistochemistry, Western blotting, northern blots, and in situ hybridization

	TRPC1	TRPC2	TRPC3	TRPC4	TRPC5	TRPC6	TRPC7
PCa/PNa and conductance (pS) ^[202]	Non-selective, 16	2.7, 42	1.6, 60-66	1.1, 30-42	9, 47-66	5, 28-37	0.5-5.4, 25-50
Human pulmonary artery ECs ^[210,211,219,222,249,261,415,416]	+(RT-PCR, NB, WB, IC)		+/- (RT-PCR)	+(RT-PCR, WB, IC)	-(RT-PCR)	+/- (RT-PCR) +(WB)	
Human coronary artery ECs ^[234,237]	+(RT-PCR, WB, ISH, IHC)		+(RT-PCR, WB, ISH, IHC)	+(RT-PCR, WB, ISH, IHC)	+(RT-PCR, WB, ISH, IHC)	+(RT-PCR, WB, ISH, IHC)	+(RT-PCR, WB, ISH)
Human cerebral artery ECs	+(RT-PCR, ISH, IHC)		+(RT-PCR, ISH, IHC)	+(RT-PCR, ISH, IHC)	+(RT-PCR, ISH, IHC)	+(RT-PCR, ISH, IHC)	+(RT-PCR, ISH)
Human intact mesenteric artery ^[127]	+(RT-PCR)		+(RT-PCR)	-(RT-PCR)	-(RT-PCR)		
Human glomerular ECs						+(WB, IC)	
Human preglomerular ECs ^[244,245]			+(RT-PCR, WB, IHC)			+(IHC)	
Human dermal microvascular ECs ^[118,212,221,222,264]	+(RT-PCR, WB)	-(RT-PCR)	+(RT-PCR)	+(RT-PCR, WB)	-(RT-PCR)	+/- (RT-PCR) +(WB)	+/- (RT-PCR)
HUVECs ^[127,205,212,215,218,220-223,227,231]	+(RT-PCR, WB)	-(RT-PCR)	+/- (RT-PCR)	+/- (RT-PCR)	+/- (RT-PCR)	+/- (RT-PCR) +(WB)	+(RT-PCR)
EA.hy926 cells ^[127,243,269]	+(RT-PCR)		+/- (RT-PCR) +(WB)	+/- (RT-PCR)	+(RT-PCR, WB)	+/- (RT-PCR)	
Mouse aortic ECs ^[216,228,248,258]	+(RT-PCR, WB, IC)		+(WB)	+(RT-PCR, WB)	+(WB)	+(WB)	
Mouse pulmonary artery ECs ^[218,251,259,262]	+(RT-PCR, WB)	-(RT-PCR)	+/- (RT-PCR)	+(RT-PCR, WB)	-(RT-PCR)	+(RT-PCR, WB)	-(RT-PCR)
Mouse brain capillary ECs	+(RT-PCR)	+(RT-PCR)	+(RT-PCR)	+(RT-PCR)	+(RT-PCR)	+(RT-PCR)	+(RT-PCR)
Bovine pulmonary artery ECs ^[232]	+(RT-PCR)		-(RT-PCR)	+(RT-PCR)			
Bovine aortic ECs ^[207-209,248,267,268]	+(RT-PCR, WB, IC)		+/- (RT-PCR) +(WB)	+(RT-PCR, WB)	+(RT-PCR, WB)	+(RT-PCR, WB)	
Bovine brain capillary ECs	+(RT-PCR)	-(RT-PCR)	+(RT-PCR)	-(RT-PCR)	+(RT-PCR)	-(RT-PCR)	-(RT-PCR)
Bovine adrenal capillary ECs ^[292]							
Rat aortic ECs ^[233,239]	-(WB)		+(WB)	-(WB)	+(WB)	+(WB)	
Rat pulmonary artery ECs ^[260]	+(RT-PCR)	+(RT-PCR)	+/- (RT-PCR)	+(WB, IC) -(RT-PCR)		-(RT-PCR)	-(RT-PCR)
Rat coronary arterioles ECs	+(RT-PCR, IHC)						
Rat cerebral artery ECs ^[311]	+(RT-PCR)						
Rat mesenteric artery ECs ^[227,312]	+(RT-PCR, WB, IHC)		+(WB, IHC)	+(WB, IHC)	+(WB, IHC)		
Rat descending vasa recta ECs ^[254]				+(RT-PCR, WB, IC)	-(RT-PCR)		
Rat splenic sinus ECs ^[35]	+(IHC)						
Porcine aortic ECs ^[241,242]			+(RT-PCR, WB)	+(WB)			
Porcine coronary artery ECs ^[235]			+(RT-PCR, WB)				
Zebrafish aortic ECs ^[225]	+(IHC, ISH)					+(ISH)	
Ovine uterine artery ECs ^[414]	+(WB)		+(WB)	+(WB)	+(WB)	+(WB)	

RT-PCR: Reverse transcription/polymerase chain reaction; WB: Western blotting; IC: Immunostaining for cultured cells; IHC: Immunohistochemistry; ISH: *In situ* hybridization; NB: Northern blotting; ECs: Endothelial cells; HUVECs: Human umbilical vein ECs.

recent studies have argued against this conclusion^[205,206]. TRPC1 is engaged by a number of inflammatory agonists, such as ATP, thrombin, tumor necrosis factor (TNF)- α , and angiotensin II (AngII), and by growth factors, such as VEGF and bFGF^[5,12,148,207-209]. TRPC1 may act as a SOC due to its ability to interact with InsP₃Rs through several signaling cascades. The first model implies RhoA-dependent actin rearrangement, which leads InsP₃Rs to couple to and gate TRPC1^[210]. RhoA activation, in turn, is induced by TRPC6 *via* PKC α -mediated phosphorylation^[211]. At the same time, PKC α also phos-

phorylates TRPC1 to enhance RhoA-dependent stimulation^[212]. Alternatively, thrombin utilizes caveolin-1 (Cav-1) to trigger TRPC1-mediated Ca²⁺ entry: Cav-1 scaffolding domain (CSD) enables the physical and functional interaction of TRPC1 and of InsP₃R3 with Cav-1 protein within the caveolae^[213]. In this view, a recent work has demonstrated that polyunsaturated fatty acids, such as docosahexaenoic acid, may reduce store-operated Ca²⁺ entry (SOCE) by displacing TRPC1 from membrane caveolae lipid rafts^[214]. Finally, VEGF recruits TRPC1 *via* direct coupling to InsP₃R2 in an angiotensin-1-regulated

Table 3 Pattern of expression of transient receptor potential vanilloid channels in vascular endothelial cells as detected by reverse transcription/polymerase chain reaction, immunostaining for cultured cells, immunohistochemistry, Western blotting, and *in situ* hybridization

	TRPV1	TRPV2	TRPV3	TRPV4	TRPV5	TRPV6
PCa/PNa and conductance (pS) ^[202]	10, 35-80	1-3, NM	12, 172	6, 90	> 100, 75	> 100, 40-70
Human coronary artery ECs ^[300]				(+RT-PCR, WB, IHC)		
Human pulmonary artery ECs ^[415]	(+RT-PCR)	(+RT-PCR)		(+RT-PCR)		
Human pulmonary microvascular ECs	(+RT-PCR)					
Human cerebral microvascular ECs ^[272]	(+RT-PCR, IC)					
Human cerebral arterioles ECs ^[305]			(+RT-PCR, IHC)			
Human dermal microvascular ECs ^[319]				(+RT-PCR, WB)		
Breast cancer derived microvascular ECs ^[319]				(+RT-PCR, WB)		
Human umbilical vein ECs ^[227,277,296,301]	(+RT-PCR)			(+WB, IHC)		
Mouse aortic ECs ^[280,285,299,306]	(+WB)			(+RT-PCR, WB, NM, IHC)		
Mouse pulmonary artery ECs ^[313]				(+WB, IHC)		
Mouse mesenteric artery ECs ^[27,280,302]	(+RT-PCR, WB, IC)			(+RT-PCR, IC)		
Mouse cerebral microvascular ECs		(+RT-PCR)		(+RT-PCR, WB)		(+RT-PCR)
Mouse dermal microvascular ECs ^[307]				(+RT-PCR, WB)		
Mouse carotid artery ECs ^[290,296]				(+IHC)		
Rat mesenteric artery ECs ^[226,275,302]	(+WB)			(+WB, IC, IHC)		
Rat femoral artery ECs ^[312]				(+RT-PCR, IC)		
Rat pulmonary artery ECs ^[303]				(+WB, IHC)		
Rat renal artery ECs ^[303]				(+IHC)		
Rat cardiac microvascular ECs ^[303,316]				(+IHC)		
Rat cerebral artery ECs ^[287,297,311]		(+RT-PCR)	(+RT-PCR, IC)	(+RT-PCR, IC)		
Rat carotid artery ECs ^[291]				(+RT-PCR)		
Porcine aortic ECs ^[312,317]				(+RT-PCR, IC, WB)		
Porcine coronary artery ECs ^[273]	(+IHC)					
Bovine aortic ECs ^[271]	(+WB)					
Bovine adrenal capillary ECs ^[292]				(+RT-PCR, WB)		
Zebrafish ventricular ECs				(+ISH)		

RT-PCR: Reverse transcription/polymerase chain reaction; WB:Western blotting; IC: Immunostaining for cultured cells; IHC: Immunohistochemistry; ISH: *In situ* hybridization; NM: Not measured; ECs: Endothelial cells.

fashion^[215]. The circulating protein, klotho, may bind to both VEGFR-2 and TRPC1 to promote their co-internalization, reduce Ca²⁺ influx and maintain endothelial integrity^[216]. A number of studies have, however, reported that TRPC1 must combine with TRPC4 to be operated by intracellular Ca²⁺ stores^[217,218]. This issue will be addressed below. TRPC1-mediated Ca²⁺ entry enlists calmodulin to activate the myosin light chain kinase (MLCK) and induce EC retraction, thus disassembling intercellular junctions and increasing endothelial permeability^[204]. Moreover, TRPC1-gated Ca²⁺ entry is capable of stimulating nuclear factor (NF)- κ B *via* AMP-activated protein kinase and PKC δ , so that NF- κ B may initiate the transcriptional programme involved in the host defense to inflammatory stimuli and in EC resistance to apoptosis^[219,220]. NF- κ B, in turn, increases TRPC1 expression in TNF- α -stimulated cells, thus establishing a feed-forward loop that amplifies agonist-elicited SOCE and the increase in transendothelial permeability^[221,222]. Similarly, AngII upregulates TRPC1 levels in an NF- κ B-dependent manner in ECs^[223]. Finally, TRPC1 may be involved in endothelial tube formation both *in vitro*^[224] and *in vivo*^[225]. The expression of TRPC1 in vascular ECs may be increased by high glucose levels, with a consequent increase in the amplitude of agonist-induced Ca²⁺ influx and a dramatic impact on endothelial function in diabetic patients^[209]. TRPC1 not only acts as an SOC in vascular endothelium, but may also

serve as an MSC by assembling with TRPV4 to mediate flow-induced Ca²⁺ entry in vascular endothelium^[226]. A drop in luminal Ca²⁺ favors the translocation of the TRPC1-TRPV4 heteromeric channel from the ER to plasmalemmal caveolae^[227], where the complex amplifies shear-stress-induced Ca²⁺ influx and augments the ensuing vascular dilation^[227]. In addition, such an enhanced vesicular trafficking of TRPC1-TRPV4 channels may contribute to endothelial SOCs^[228]. Similarly, TRPC1 may mediate stretch-induced Ca²⁺ entry after traumatic injury in microvessel endothelial cells, thereby resulting in NO synthesis and actin stress fiber formation^[229].

TRPC3/6/7: TRPC3, TRPC6 and TRPC7 are Ca²⁺-permeable channels that may be activated by DAG in a PKC-independent manner and may thus be regarded as SMOCs^[200,201]. TRPC3 and TRPC6 are widely expressed both in cultured ECs and *in situ* endothelium, while TRPC7 is far less abundant (Table 2). DAG stimulates TRPC3- and TRPC6-gated Ca²⁺ entry into ECs exposed to a number of agonists, including VEGF^[118,230,231], ATP^[232-234], and bradykinin^[232,235]. TRPC3- and TRPC6-induced Ca²⁺ inflow underpin the stimulatory effect of VEGF on endothelial proliferation, migration and permeability^[118,231,236], while ATP exploits TRPC3 to activate NF- κ B and increase vascular cell adhesion molecule-1 expression^[237]. TRPC3-driven NO synthesis and vasore-

Table 4 Pattern of expression of transient receptor potential melastatin channels in vascular endothelial cells as detected by reverse transcription/polymerase chain reaction, Western blotting, and in situ hybridization

	TRPM1	TRPM2	TRPM3	TRPM4	TRPM5	TRPM6	TRPM7	TRPM8
PCa/PNa and conductance (pS) ^[202]	NM, NM	0.5-1.6, 52-80	0.1-10, 65 (Ca ²⁺)-130	Not permeable, 25	Not permeable, 16-25	PCa/PNa~6, NM	3, 40-105	1-3, 83
Human Pulmonary artery ECs ^[220]	+(RT-PCR)	+/(RT-PCR) +(WB)	+(RT-PCR)	+(RT-PCR)	-(RT-PCR)	+(RT-PCR)	+(RT-PCR)	+(RT-PCR)
Human umbilical vein ECs ^[325,327,328]				+(RT-PCR, WB)		-(RT-PCR)	+(RT-PCR, WB)	
Mouse aortic ECs ^[329]				+(RT-PCR, WB)			+(RT-PCR)	
Human dermal microvascular ECs ^[327]							+(RT-PCR, WB)	
Mouse cerebral microvascular ECs	-(RT-PCR)	+(RT-PCR)	+(RT-PCR)	+(RT-PCR)	-(RT-PCR)	-(RT-PCR)	+(RT-PCR)	-(RT-PCR)
Mouse cardiac microvascular ECs ^[8]		+(RT-PCR, WB)					+(RT-PCR)	
Rat cerebral artery ECs ^[311]				+(RT-PCR)		+(RT-PCR)	+(RT-PCR)	
Rat spinal cord capillary ECs ^[324]				+(ISH)				
Rat mesenteric artery ECs ^[312]							+(RT-PCR)	

RT-PCR: Reverse transcription/polymerase chain reaction; WB: Western blotting; IHC: Immunohistochemistry; ISH: *In situ* hybridization; NM: Not measured; ECs: Endothelial cells.

laxation may be attenuated by the hypoxia-reoxygenation injury of vascular ECs, which prevents TRPC3 trafficking to the plasma membrane^[235]. Moreover, TRPC3 supports both spontaneous and histamine-evoked Ca²⁺ oscillations arising, respectively, during and after EC tubule formation^[224]. Finally, TRPC3-mediated Ca²⁺ inflow may stimulate flow-dependent endothelium-dependent vasodilation^[238]. Endothelial TRPC3 and TRPC6 may be inhibited by NO in a protein-kinase-G-dependent fashion^[239,240], while TRPC3 may also be inhibited by a PKC-mediated phosphorylation on Ser-712^[239]. Both TRPC3 and TRPC6 may be recruited by signaling mechanisms other than DAG in vascular ECs. TRPC3 may provide a leakage Ca²⁺ entry pathway in unstimulated ECs^[234] or behave as an SOC owing to its ability to physically interact with InsP₃R2 in uterine artery ECs^[105]. Connexin-43-mediated intercellular communication between adjacent ECs favors TRPC3/InsP₃R2 association, although *via* a yet to be identified mechanism^[105]. Moreover, endothelial TRPC3 may assemble with its distant relative, TRPC4, and form a heteromeric channel sensitive to oxidative stress and with biophysical properties distinct from both TRPC3 and TRPC4 homomers^[241,242]. Finally, TRPC3 may be activated by thapsigargin-induced ER Ca²⁺ store depletion following PLC-dependent DAG synthesis and PKC η activation. PKC η , in turn, engages Src tyrosine kinase to phosphorylate and gate TRPC3^[243]. TRPC3 levels in vascular ECs may be downregulated by VEGF in a PI3K-sensitive manner, and upregulated by an increase in blood pressure^[244,245]. TRPC6, on the other hand, may be translocated from intracellular sites to the caveolae compartment of the plasma membrane by cytochrome P450 (CYP)-derived epoxyeicosanotrienoic acids (EETs). This process enhances bradykinin-induced Ca²⁺ entry and thus prolongs the activation of small and intermediate Ca²⁺-activated K⁺ channels responsible for endothelial hyperpolarization^[246]. The functional consequence of TRPC6 insertion into the plasmalemma is related to the

ability of ECs to transmit their negative shift in V_M to the adjacent smooth muscle cells and trigger vasorelaxation (the so-called endothelial-dependent hyperpolarizing factor, EDHF)^[247]. TRPC6 externalization may also be induced by lysophosphatidylcholine (LPC): the following Ca²⁺ influx induces TRPC5 translocation to the plasma membrane, thus allowing sustained Ca²⁺ entry into ECs^[248]. TRPC5-mediated Ca²⁺ inflow, in turn, is essential to restrict endothelial movement and might be relevant in impairing the healing process of the intimal layer in atherosclerotic arteries, where LPC is abundant^[248]. TRPC6 externalization requires the phosphatase and tensine homolog (PTEN), whose tail 394-403 residues associate with the channel and favour its plasmalemmal localization^[249]. It has yet to be elucidated whether EETs and LPC utilize PTEN to induce the intracellular movements of TRPC6 in ECs. Recent studies have shown that TRPC6 plays a key role in the onset of the lung ischemia-reperfusion edema (LIRE) as a consequence of endothelial NOX2-dependent ROS, which, in turns, leads to PLC γ activation, DAG kinase inhibition and an increase in submembranal DAG levels^[250]. Consistently, PAF-induced lung edema depends upon the activation of acid sphingomyelinase (ASM), which engages TRPC6 to caveolae, thereby resulting in Ca²⁺ influx and following increase in endothelial permeability^[251]. This process is inhibited by NO donors^[251].

TRPC4/5: TRPC4 and TRPC5 form a subfamily with the closest homology with TRPC1^[201]. Both channels open in response to the Gq/11 family GPCRs and TKRs, although neither InsP₃ nor DAG alone are able to activate them^[200,201,252]. It has, however, been demonstrated that TRPC4 opening is favored by PIP₂ hydrolysis, although it also requires G β recruitment and intracellular Ca²⁺^[253]. The scenario is further complicated by the necessity for TRPC4 to interact with the adaptor NHERF, ezrin/radixin/moesin ERM proteins, and cortical actin

via its COOH-terminal PDZ-binding domain to undergo PIP₂ modulation^[253,254]. Similarly, TRPC5 activation may be potentiated by intracellular Ca²⁺ release and is modulated by the scaffolding ERM-binding phosphoprotein-50 (EBP50)^[255,256]. TRPC4 and TRPC5 may assemble either as homo- or heterotetramers *via* their first ankyrin repeat in the NH₂-terminal^[257] and are abundantly expressed in both cultured cells and naive tissues. TRPC4 has long been considered as the key component of SOC in vascular endothelium^[203], because TRPC4-deficient mice lack SOCE and exhibit impaired NO production and increased vascular permeability^[258,259]. According to this model, the extent of store depletion is conveyed to PM by protein 4.1N, which links the cytoskeletal protein, spectrin, to TRPC4^[260]. The latter, in turn, contributes pore-forming subunits to the store-dependent channel along with TRPC1 according to the following stoichiometry, 1 TRPC1 and 3 TRPC4^[203,217,260,261]. The association between the two subunits is favoured by Cav-1, which also recruits InsP₃R1 into the membranal signalplex^[262]. InsP₃R1, in turn, may interact with protein 4.1N, a feature which would explain the dependence of the TRPC1-TRPC4 complex on intracellular Ca²⁺ stores^[263]. An alternative model proposes that this heterotetramer is activated by a protein termed stromal interaction molecule-1 (Stim1)^[218], which senses intraluminal ER Ca²⁺ levels, as more widely illustrated below. A recent study, however, questioned TRPC1 and TRPC4 involvement in endothelial SOCE^[205,206]. The controversy was explained by the finding that TRPC4 is externalized to the plasma membrane upon binding to β -catenin only in subconfluent, migrating ECs, but not in quiescent, barrier-forming cells, which is the typical condition for Ca²⁺ signaling studies^[264]. Therefore, TRPC4 might serve as an agonist-recruited SOC during endothelial cell-cycle transitions from a proliferating to a quiescent phenotype^[265]. Whether TRPC4 contributes to the repetitive Ca²⁺ spikes arising in HUVECs both before (spontaneous) and after (histamine-evoked) tubule formation in a store-dependent or independent manner has yet to be elucidated^[224]. A single nucleotide polymorphism in the TRPC4 gene has been associated with protection from myocardial infarction and results in a larger current density when the mis-sense channel is expressed in heterologous cell systems^[266]. This feature is likely due to a less bulky Val-957, which favors TRPC4 insertion into the plasma membrane and its interaction with the catalytic site of the tyrosine kinase that phosphorylates the channel at Tyr-959^[266]. Less information is available on the role played by TRPC5 in ECs, where it may be constitutively open and also participate to ATP-induced Ca²⁺ entry^[267]. TRPCs have been suggested to be activated also by NO *via* direct S-nitrosylation^[186], but this mode of regulation is absent in vascular endothelium, where TRPC5 is actually inhibited by NO^[267]. Conversely, the endothelial TRPC5 channel may be potentiated by the plant-derived isoflavone, genistein, independently on tyrosine kinases^[268] and is upregulated by erythropoietin^[269].

TRPV channels

The TRPV family consists of four subfamilies on the basis of their structure and function: TRPV1/2, TRPV3, TRPV4, and TRPV5/6^[201]. The latter are highly Ca²⁺-selective pathways, while TRPV1-4 are nonselective cation channels whose P_{Ca}/P_{Na} ranges between 1 and 15 pS^[202]. All TRPV channels are endowed with 3-6 NH₂-terminal ankyrin repeats, which enable the protein to traffic to the plasma membrane, are required for tetramerization, and control protein-protein interactions^[270]. TRPV channels may assemble either as homo- or heterotetramers, although their exact stoichiometry *in situ* is still unknown^[270]. TRPV1-4, which are the only isoforms expressed in vascular ECs, may be gated by a number of stimuli, including G_q signaling, endocannabinoids, dietary agonists, changes in temperature and osmolality, pulsatile stretch and shear stress^[270]. TRPV channels are, therefore, a remarkable example of polymodal channels and may serve as both MSCs and SMOCs.

TRPV1: TRPV1 is a nonselective cation channel with a preference for calcium that can be expressed in both cultured and native ECs (Table 3). Endothelial TRPV1 channels may be activated by the dietary agonist, capsaicin, and the endogenous cannabinoids, anandamide and 2-arachidonoyl-glycerol (2-AG)^[149,270-272]. TRPV1 may, therefore, serve as an SMOC, although the physiological ligands that are capable of recruiting this channel in ECs are yet to be elucidated. Pharmacological stimulation of TRPV1, either by capsaicin or anandamide, causes vascular relaxation upon release of several vasodilators, including NO^[273-275], calcitonin gene-related peptide (CGRP)^[276,277], and EDHF^[273]. CGRP may also afford a protective effect against LPC- and lipopolysaccharide (LPS)-dependent injury^[276,277]. Furthermore, TRPV1 mediates the vasorelaxing effects of the synthetic, cannabinoid-like compound, VSN16, albeit it is still unknown whether TRPV1 is engaged by VSN16 directly or *via* the "abnormal-cannabinoid receptor"^[278]. The signaling cascade responsible for endothelial NO synthase (eNOS) activation by TRPV1 has been unravelled. TRPV1 may trigger Ca²⁺-dependent PI3K/Akt/CaMKII signaling, which leads to TRPV1 phosphorylation, increases TRPV1-eNOS complex formation, eNOS stimulation, and NO synthesis^[271]. This signaling pathway might involve the AMP-activated protein kinase (AMPK), a multifunctional regulator of energy homeostasis that is phosphorylated upon TRPV1-mediated Ca²⁺ influx^[279]. TRPV1-induced vasodilation is impaired in obese Ossabaw swine with the metabolic syndrome and thus could be a mechanism involved in endothelial dysfunction and development of coronary disease^[273]. Chronic stimulation of TRPV1 by capsaicin enhances PKA and eNOS phosphorylation, which, in turn, results in vasorelaxation, reduces blood pressure and delays the onset of brain stroke in spontaneously hypertensive rats^[280]. Moreover, dietary capsaicin reduces the high-salt-diet-induced endothelial dysfunction and nocturnal hypertension by pre-

venting the generation of O₂⁻ anions and NO reduction through vascular TRPV1 activation^[281]. Baseline mean arterial pressures are, however, not statistically different in wild-type and TRPV1^{-/-} mice^[282,283]. Besides chemical agonists, TRPV1 may be gated by temperature higher than 40 °C in ECs^[277,284]. This feature gives support to the hypothesis that TRPV1 contribute to peripheral vascular dilatation and high temperatures^[8]. Conversely, a recent report described TRPV1-induced endothelin production by cultured ECs, which might cause the activation of vascular ET_A receptors and increase the mean arterial pressure *in vivo*^[285]. Finally, capsaicin opposes angiogenesis by causing G1 arrest of ECs *via* downregulation of cyclin D1 and inhibited VEGF-triggered signaling pathways^[286]. Whether this effect depends on TRPV1 stimulation is yet to be demonstrated.

TRPV2: TRPV2 has been mainly located in vascular smooth muscle cells (VSMCs), where it may be activated by cell swelling and act as an MSC^[270]. Only a few studies have reported TRPV2 expression in ECs (Table 3) and, therefore, its function in vascular endothelium is still unclear. Due to its ability to mediate Ca²⁺ inflow in ECs exposed to moderate heat, however, it might aid TRPV1 to stimulate NO synthesis and peripheral vasorelaxation at > 43 °C^[8,284].

TRPV3: TRPV3 is a polymodal channel that may be gated by either natural compounds, such as carvacrol, eugenol, and camphor, or innocuous warm temperatures (> 30 °C)^[200]. TRPV3 expression has recently been shown in both *in situ* and cultured ECs (Table 3). In intact tissue, TRPV3 stimulation by carvacrol induces VSMC hyperpolarization *via* the Ca²⁺-dependent opening of intermediate- (IK_{Ca}) and small-conductance (SK_{Ca}) K⁺ channels. The consequent K⁺ efflux activates smooth muscle inwardly rectifying potassium channels (K_{IR}) and amplifies the negative shift in VSMC V_M, thereby promoting arterial relaxation^[287]. In human corneal ECs, TRPV3-induced Ca²⁺ entry is evoked by raising bath temperature from 25 to > 44 °C^[284]. If present also in vascular endothelium, this feature would render the channel capable of sensing high temperatures in peripheral circulation along with TRPV1 and TRPV2.

TRPV4: TRPV4 is the most polymodal TRP channel, being a molecular integrator of both chemical and physical stimuli^[200,270], and may be expressed in both native and cultured ECs (Table 3). More specifically, endothelial TRPV4 may be activated by non-noxious temperatures (> 24 °C)^[288], hypo-osmotic cell swelling^[289], shear stress^[290,291], cyclic strain^[292,293], and pharmacologically, by the non-PKC-activating phorbol ester, 4 α -phorbol-12,13-didecanoate (4 α PDD)^[294] and by the plant-derived flavone, apigenin^[295]. Cell swelling and shear stress do not gate TRPV4 directly, but *via* the PLA₂-dependent synthesis of AA, and its subsequent metabolization to 5',6'-epoxyeicosatrienoic acid (EET) through a CYP

epoxygenase-dependent pathway^[290,291,296-299]. A unique mechanism has been described in human coronary arterioles isolated from patients suffering from coronary artery disease, where the endothelial TRPV4 stimulate mitochondria to release ROS, which in turn cause VSMCs to relax^[300]. Conversely, TRPV4 signaling induced by mechanical strain is mediated by direct force transfer from β 1 integrins to the integrin-associated transmembrane CD98 protein within focal adhesions^[293]. This feature renders TRPV4 a pure MSC that can be activated within only 4 ms by external force application^[293]. TRPV4-mediated Ca²⁺ responses to heat and 4 α PDD require an aromatic amino acid at the NH₂-terminal of the third transmembrane domain^[289]. Recent work proposed that Cav-1 governs TRPV4 activity and location to the plasma membrane in ECs^[301]. NO, PGI₂, and IK_{Ca}/SK_{Ca}/MaxiK-mediated EDHF are vasodilatory pathways that are activated following by pharmacological activation of TRPV4^[27,291,302-304]. An alternative mechanism has been unravelled in human coronary arterioles from patients with coronary artery disease, where flow-induced TRPV4 activation causes ECs to release mitochondrial ROS, thereby relaxing the adjoining VSMCs^[300]. Under physiological conditions, TRPV4-mediated vasodilation, mainly *via* NO and EDHF production, is induced by both shear stress^[27,290,291,296] and extracellular autacoids, such as Ach and UTP^[297,301,305,306]. Although the cellular mechanisms linking membrane receptors to TRPV4 opening are still unclear, UTP utilizes PLA₂ to gate the channel^[297], while PKC α mediates Ach-dependent activation^[307]. Despite the evidence for TRPV4-evoked vasodilation, the baseline blood pressure is not affected in TRPV4^{-/-} mice^[305,306]. Nevertheless, systemic activation of the channel may induce vasorelaxation *in vivo*^[302,303,308,309] and, eventually, lead to circulatory collapse^[303]. The vasorelaxing effect of TRPV4 is more evident in rodent models of N^o-nitro-L-arginine (L-NNA)- and salt-induced hypertension^[305,308,309]: this feature suggests that its main function might be to adverse excessive vasoconstricting stimuli and protect the vasculature during times of extreme stress^[270]. It is, therefore, intriguing that TRPV4 is downregulated in a genetic model of salt-susceptible hypertensive rats exposed to a salt-enriched diet, whereas its function and expression are enhanced in salt-resistant rat strains^[308]. In this view, apigenin might afford a protective effect on the development of cardiovascular diseases by stimulating TRPV4 to release EDHF^[295]. An additional role for TRPV4-dependent Ca²⁺ signals consists in driving the shear stress-induced growth of collateral vessels (arteriogenesis) which occurs as a consequence of arterial occlusion^[310-312]. Ca²⁺ entry may, indeed, recruit several Ca²⁺-sensitive transcription factors, including nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), Kv channel interacting protein 3, calsenilin (KCNP3/CSEN/DREAM), and myocyte enhancer factor 2C (MEF2C)^[310]. On the other hand, TRPV4-mediated Ca²⁺ influx elicited by cyclic strain triggers cytoskeletal remodeling and movement necessary for cell

realignment in the vasculature^[292]. TRPV4 channels are extensively expressed in alveolar septal capillaries^[163,313], where they control lung endothelial permeability and barrier integrity in response to intravascular and airway pressures^[314-316]. More specifically, TRPV4-gated Ca²⁺ entry increases vascular permeability to such an extent that barrier disruption and alveolar flooding may occur^[313]. Finally, TRPV4 may enable ECs to sense body temperature, because the threshold for the heat-dependent activation for this channel is set at 25-27 °C and it is activated by moderate heat in ECs^[4,288,317,318]. The results provided by knocked out mice, however, suggest that the steady state release of NO from endothelium is not governed by such a mechanism. A recent study implicated TRPV4 in tumor angiogenesis, as Florio Pla and coworkers reported an increase in TRPV4 levels in breast tumor ECs (b-TECs) as compared to control cells belonging to a human dermal microvascular endothelial cell line (HMVEC). In particular, AA-dependent TRPV4-mediated Ca²⁺ influx selectively drives cell migration *via* remodeling of the actin cytoskeleton in b-TECs, but not in HMVECs^[319].

TRPM channels

The melastatin-related TRP subfamily was named based on the first discovered member, melastatin 1 (TRPM1), the gene of which was identified from melanomas. Members of the TRPM family are divided into four groups: TRPM1/3, TRPM2/8, TRPM4/5, and TRPM6/7^[200,201]. TRPM channels exhibit highly varying cation permeability, from Ca²⁺ impermeable (TRPM4/5) to highly Ca²⁺ and Mg²⁺ permeable (TRPM6 and TRPM7)^[202]. All the TRPM isoforms except TRPM5 are expressed in ECs (Table 4), although only the role of TRPM2 and TRPM7 has been extensively elucidated in these cells^[270]. These channels are regarded as chanzymes, as they result from the fusion of an ion-channel pore-forming domain with an enzymatic domain^[200].

TRPM2: TRPM2 is a non-elective cation channel whose predominant feature is the so-called Nudix box, a consensus region for pyrophosphatases that is localized in the cytoplasmic COOH-terminal tail of the channel protein and confers a unique activation mechanism, i.e., gating by ADPr, on the channel^[144]. ADPr arises from breakdown of β-NAD, CD38, or other enzymes acting on cADPr and hydrolysis of ADP polymers by poly-ADP ribose glycohydrolase (PARP). TRPM2 is also activated by oxidative or nitrosative stress (e.g., H₂O₂)^[4,144], perhaps mediated by mitochondrial ADPr. Therefore, TRPM2 serves as a sensor for the intracellular redox status by mediating oxidative stress-induced Ca²⁺ entry and triggering the subsequent Ca²⁺-dependent elevation in endothelial permeability^[4,200,320]. This chain of reactions is suppressed by a truncated TRPM2 isoform (TRPM2-S), generated by alternative splicing of the full-length protein (TRPM2-L)^[144]. Moreover, TRPM2 may underlie neutrophil-elicited increase in endothelial [Ca²⁺]_i, which is a prerequisite for transendothelial migration and,

consequently, for sepsis-induced increase in lung vascular permeability^[321]. The NH₂-terminal domain possesses a calmodulin-binding site which renders the channel sensitive to changes in sub-plasmalemmal Ca²⁺, which shifts the dose-response curve to ADPr to the left^[144]. In addition, the NH₂-terminal cytosolic tail of TRPM2 also contains a high affinity binding site for PKCα, which rapidly colocalizes with TRPM2-S in ECs challenged with H₂O₂^[4,321]. PKCα might, thus, increase TRPM2-mediated Ca²⁺ influx and increase in endothelial permeability by phosphorylating TRPM2-S^[4,321].

TRPM4: TRPM4, which is expressed as two splice variants, TRPM4a (non functional) and TRPM4b (functional, traditionally referred to as TRPM4), which is selective for monovalent cations, with an order of permeability Na⁺ > K⁺ > Cs⁺ > Li⁺, but not for Ca²⁺^[200,202]. TRPM4 is present in both intact vessels and cultured ECs (Table 4) and is gated by cytosolic Ca²⁺ with a K_D ranging from 0.4 to 9.8 μmol/L^[522]. Therefore, TRPM4 represents the long-sought Ca²⁺-activated nonselective cation channels^[200]. A short stretch of six acidic amino acids in the pore loop determines its monovalent selectivity^[202]. We refer the reader to other recent and comprehensive reviews for the molecular details of TRPM4 modulation in cell types other than ECs^[322,323]. Briefly, the Ca²⁺-dependent activation is regulated by ATP, Ca²⁺-calmodulin, and PKC. The current-voltage exhibits a large outward rectification, indicative of a voltage-dependent behavior which is further enhanced by increasing the temperature between 15 and 35 °C. PIP₂ also modulates channel activity by influencing its Ca²⁺ and voltage sensitivity^[322]. The main function attributed to TRPM4 in ECs is that, when activated, it protects against Ca²⁺ overload by depolarizing the cell, thereby decreasing the driving force for Ca²⁺ entry. It has, however, recently been shown that TRPM4 plays a crucial role in promoting EC death. TRPM4 is upregulated in capillary ECs of injured spinal cord, which leads to microvessel fragmentation, formation of satellite (petecchial) hemorrhagic lesions, and secondary hemorrhages^[324]. In addition, TRPM4 mediates LPS-induced EC death by a yet to be unveiled mechanism^[325].

TRPM7: TRPM7 is a nonselective cation channel that exhibits moderate permeability to Mg²⁺ (albeit at very low conductances) and is, therefore, involved in intracellular Mg²⁺ homeostasis^[200]. In addition, TRPM7 is permeable to Zn²⁺, Co²⁺, and Mn²⁺, thereby providing a membrane pathway for metal ions^[323]. TRPM7 might form either homo- or heteromultimers with its close homolog TRPM6, and has been found both in *in situ* and *in vitro* ECs (Table 4). Similar to TRPM2, TRPM7 serves as a chanzyme, its primary structure resulting from the fusion of a cation-selective ion channel pore with a COOH-terminal region possessing homology to a unique family of serine/threonine kinase domains, named the α-kinase family^[200,326]. The enzymatic activity of the α-kinase domain is not required for pore opening, although evidence

exists that it may have an alternative means of influencing channel gating^[323,326]. The mechanism underlying the constitutive activation of the channel is, therefore, still unresolved^[323]. The phosphotransferase activity of the kinase domain is, nevertheless, able to respond to local changes in free Mg²⁺ occurring as the result of Mg²⁺ flux through the channel. TRPM7 might, therefore, act as “Mg²⁺-sensor”^[323,326]. Consistently, silencing TRPM7 mimics the effect of lowering extracellular Mg²⁺ in human microvascular ECs by impairing cell migration and arresting the cells in G0/G1 and G2/M phases^[327]. Similarly, TRPM7 may inhibit EC proliferation *via* an extracellular signal-regulated kinase (ERK)-dependent reduction in eNOS levels^[328]. This finding has been supported by *in vivo* observations performed in mice with inherited hypomagnesemia, TRPM7 upregulation is associated with endothelial dysfunction consequent to a decrease in eNOS levels and an increase in systolic blood pressure, to an augmented expression of vascular cell adhesion molecule-1 and plasminogen activator inhibitor-1, and to the downregulation of annexin-1, a downstream target of TRPM7^[329]. Unlike VSMCs, the endothelial TRPM7 is insensitive to laminar shear stress^[330].

TRPP channels or polycystins

The TRPP family consists of eight members, only two of which, i.e., polycystic kidney disease 2 (PKD2 or TRPP2) and PKD2-like (or TRPP3), form Ca²⁺-permeable channels^[200,323]. TRPP2 may, however, assemble with the non-channel protein, TRPP1 (or PKD1), in order to form a heterocomplex able to transduce laminar shear stress in intracellular [Ca²⁺]_i signals^[200,323]. Both TRPP1 and TRPP2 have been found in cultured and *in situ* vascular ECs (Table 5). TRPP1 is an integral membrane glycoprotein with 11 transmembrane domains, an extensive NH₂-terminal extracellular tail involved in cell-cell, cell-matrix interaction and signaling pathways, and a cytosolic COOH-terminal endowed with a coiled-coil domain^[331]. TRPP1 interacts with TRPP2, a six-transmembrane domain protein of the TRP ion channel family^[200,323]. Polycystins also interact with multiple partners, including the TRP channel subunits TRPC1 and TRPV4, as well as several elements of the cytoskeleton^[331]. By the coiled-coil COOH-terminal domain, TRPP2 and TRPP1 can form a functional polycystin complex in the apical cilia of both cultured and native ECs^[332,333]. Due to its large extracellular NH₂-terminal tail, TRPP1 has been suggested to be a mechanical sensor regulating the opening of the associated calcium-permeable channel TRPP2^[325]. Shear-stress-induced and TRPP1/TRPP2-dependent Ca²⁺ entry, in turn, triggers NO production by recruiting PKC, Ca²⁺-calmodulin and the PI3K/Akt pathway^[332,333]. Moreover, TRPP2 may aid TRPC1 in mediating Ca²⁺ influx, NO production, and actin reorganization in bEnd3 cells exposed to mechanical stress^[229], as aforementioned.

TRPA1 channels

TRPA1 is the sole member of the TRPA family, whose

structural hallmark is represented by the 14 ankyrin repeats at its NH₂ terminus^[200,323]. These repeats might be required for the channel to interact with cytoskeletal components or to bind to specific agonists^[323]. In addition, the NH₂ terminus possesses a Ca²⁺-binding EF-domain^[323]. TRPA1 homotetramers assemble to form a nonselective cation channel displaying a relatively high Ca²⁺ permeability when compared with other TRP channels, including TRPV1 (Table 5). TRPA1 may be activated by electrophilic compounds such as acrolein (an active component of tear gas), allicin (found in garlic), and allyl isothiocyanate (AITC) (derived from mustard oil). α , β -Unsaturated aldehydes produced endogenously in response to oxidative stress, such as 4-hydroxy-2-nonenal, 4-oxo-nonenal, and 4-hydroxyhexenal, also activate the channel^[323]. Most identified TRPA1 agonists act *via* covalent modification of cysteines in the NH₂ termini, with specific binding residues identified^[323]. In addition, TRPA1 might open in response to noxious cold (< 17 °C), but the thermosensitivity of the channel is still debated^[323]. TRPA1 has been detected in native ECs, localized to endothelial cell membrane projections through the internal elastic lamina in the direction of VSMCs. TRPA1-mediated Ca²⁺ influx causes endothelium-dependent SMC hyperpolarization and relaxation of cerebral arteries by activating IK_{Ca} and SK_{Ca}^[304]. IK_{Ca}- and SK_{Ca}-gated K⁺ efflux, in turn, activates K_{IR} in SMCs, thus amplifying myocyte relaxation and dilation^[304].

Stim1 and Orai1

The physical interaction between the ER Ca²⁺-sensor, Stim1, and the plasmalemmal, Ca²⁺-permeable channel, Orai1, has recently been shown to mediate SOCE in a number of vascular ECs^[205,206,243,334]. Stim1 is an ER-resident type I single transmembrane protein with an NH₂ terminus located either in the ER lumen or facing the extracellular space. It contains two NH₂-terminal EF hand domains (a canonical and a hidden one) followed by a sterile α -motif (SAM) domain, the transmembrane region, three predicted coiled-coil domains comprising an ezrin-radixin-moesin (ERM) motif and at the COOH terminus, a proline-rich and a lysine-rich domain^[335,336]. The negatively charged NH₂-terminal EF hand domain is the Ca²⁺ sensing subunit of the protein. In the resting state, Stim1 seems to be present as a dimer in the ER membrane. The dimerization happens through the COOH-terminal coiled-coil domains. In case of store release, and subsequent Ca²⁺ dissociation from the canonical EF hand domain, further oligomerization of Stim1 through the SAM domain occurs. These Stim1 oligomers migrate towards sites of the ER that are in close vicinity to the plasma membrane, where they assemble into punctuate clusters and activate SOCE by directly binding to Orai1^[335,336]. Accordingly, the latter has been found to be an essential component of SOCE by forming the pore unit of the channel in a variety of cell types^[335,337], including vascular ECs^[205,206,338]. Orai1 is a four-transmembrane protein with intracellular NH₂ and COOH termini. In the resting state, it is present as a dimer in the plasma mem-

Table 5 Pattern of expression of transient receptor potential ankyrin and polycystic channels in vascular endothelial cells as detected by reverse transcription/polymerase chain reaction, immunohistochemistry, and Western blotting

	TRPA1	TRPP1	TRPP2
PCa/PNa and conductance (pS) ^[202]	0.8-1.4, NM	1-5, 40-177	4, 137
Human renal artery ECs ^[332]			+(RT-PCR, WB, IC)
Human umbilical vein ECs ^[332]			+(RT-PCR, WB)
Mouse aortic ECs ^[332,333]		+(RT-PCR, WB, IC)	+(RT-PCR, WB)
Mouse femoral artery ECs ^[332]			+(RT-PCR, WB)
Rat cerebral arteries ^[287,304,311]	+(RT-PCR, IC)		+(RT-PCR)

RT-PCR: Reverse transcription/polymerase chain reaction; WB: Western blotting; IC: Immunostaining for cultured cells; NM: Not measured; ECs: Endothelial cells.

brane, however, the functional SOC channel seems to be formed by Orai1 tetramers. The interaction between Stim1 and Orai1 is mediated by the CRAC activation domain (CAD) located at the COOH terminus of Stim1 and the cytosolic NH₂ and COOH tails of Orai1^[335,336]. Accordingly, the genetic suppression of either Orai1 or Stim1 suppresses SOCE induced by either pharmacological (CPA and thapsigargin) and physiological (VEGF, thrombin) stimulation in HUVECs, EA.hy296 cells, and porcine aortic ECs^[205,206,218,243,334]. This, in turn, leads to the blockade of several key steps of the angiogenic process, such as proliferation and tubulogenesis^[205,206,338]. These data are supported by the finding that, under resting conditions, the ER Ca²⁺ sensor is homogeneously distributed throughout the cytosol, whereas emptying of the InsP₃-sensitive Ca²⁺ reservoir in the absence of extracellular Ca²⁺ induces Stim1 homo-oligomerization and redistribution into sub-plasmalemmal puncta^[206,218,339]. Both processes are recovered upon Ca²⁺ restoration to the bathing solution, as Ca²⁺ entry recharges the intracellular Ca²⁺ pool and inactivates Stim1^[340]. Stim1 puncta in vascular EC reassembly at the same loci during repetitive cycles of ER Ca²⁺ depletion and refilling suggest that this process is driven by accessory proteins, such as the microtubule-plus-end tracking protein, EB1^[335,336,340]. The engagement of Stim1 is, however, limited when ECs are stimulated in the presence of extracellular Ca²⁺, which is consistent with modest reduction in the ER Ca²⁺ load observed under these conditions^[40,41,340]. An important aspect of Stim1 dynamics in ECs regards its modulation by cytosolic Ca²⁺: an increase in submembranal Ca²⁺ levels prevents Stim1 clustering, thereby impeding prolonged SOC activity and intracellular Ca²⁺ overload^[340]. This feature might contribute to explain the long-known inhibition of SOCE by Ca²⁺ microdomains arising at the mouth of the channel^[341]. SOCE mediated by Stim1 and Orai1 is gated by an inwardly rectifying, Ca²⁺-selective current^[205], whose amplitude may fall below the resolution limit of a whole-

cell patch-clamp recording system^[206]. Orai1 has two homologs in mammals, namely Orai2 and Orai3, which do not contribute to SOCE in ECs^[205,218,338]. In contrast to Stim1, Stim2 has no major effect on SOCE, but might be involved in the regulation and stabilization of basal cytosolic and ER Ca²⁺ levels in ECs^[342]. The involvement of Stim1 and Orai1 to endothelial SOCE is, however, still controversial and might depend on both the vascular bed and the cell type. For instance, recent work ruled out any detectable role for Orai1 in thrombin- and thapsigargin-induced Ca²⁺ inflow in mouse lung ECs^[218], where SOCE activity requires functional TRPC1 and TRPC4^[218]. The same study reported that, in these cells, Stim1 is mobilized into sub-plasmalemmal puncta in a TRPC4-dependent manner and, in turn, it signals TRPC1 and TRPC4 about the ER Ca²⁺ content.

Ca²⁺ EXTRUSION FROM THE CYTOSOL

A variety of pumps and exchangers fulfil the function to remove Ca²⁺ from the cytosol following EC stimulation, namely SERCA, NCX, PMCA, and the mitochondrial uniporter^[36,40,41,139,343-349]. SERCA, NCX, PMCA, mitochondria may be aligned either in series or in parallel in ECs in order to remove Ca²⁺ from the cytosol properly, to achieve successful ER Ca²⁺ refilling, and facilitate SOCE by buffering sub-plasmalemmal Ca²⁺. These functions are facilitated by their optimal coupling to either InsP₃Rs or RyRs.

Expression of SERCA, NCX and PMCA in ECs

SERCA is encoded by three different genes (ATP2A1, ATP2A2, and ATP2A3) with numerous alternative splice isoforms at the 3' ends of the mRNA. More specifically, ATP2A1 encodes for SERCA1a-1b, ATP2A2 for SERCA2a-2c, and ATP2A3 for SERCA3a-3f^[350]. The SERCA isoforms differ mainly by their affinity for Ca²⁺ (2b > 2a = 1 >> 3) and their Ca²⁺ transport turnover rates, SERCA2b having the lowest transport capacity of all SERCAs^[350]. Both SERCA2a and SERCA3 have been found in isolated and *in situ* ECs^[106,351-356], albeit SERCA3 transcript levels decrease during EC proliferation in culture and in hypertension^[106,112]. SERCA3 expression is regulated by Ca²⁺ itself in a calcineurin/NFAT-dependent manner^[357] and its genetic deletion leads to a decrease in agonist-induced elevation in [Ca²⁺]_i and NO synthesis^[358]. The Ca²⁺-ATPase activity in vascular endothelium may be enhanced by NO, which considerably increases Ca²⁺ loading into ER lumen^[359]. Moreover, ECs may be endowed with phospholamban (PLB), a 24-27-kDa phosphoprotein that is closely associated with the cardiac sarcoplasmic reticulum, where it modulates SERCA function^[360]. More specifically, PLB inhibits Ca²⁺ uptake by SERCA in Ach-stimulated cells and dampens the endothelial-dependent vasorelaxation^[360]. In addition, SERCA activity is more resistant to peroxynitrite and peroxide in ECs as compared to VSMCs^[361,362]; a feature that may confer a protective effect during the massive production of free

radicals observed under pathological conditions, such as ischemia-reperfusion. Conversely, selective targeting of thiol groups (i.e., Cys residues) by myeloperoxidase-derived oxidants may inhibit the Ca²⁺-ATPase activity and lead to an unbalanced increase in [Ca²⁺]_i, which might contribute to the endothelial dysfunction occurring during several inflammatory diseases^[363]. In this view, oxidized and glycated low-density lipoprotein causes SERCA oxidation, aberrant ER stress and atherosclerosis *in vivo*, although this detrimental chain of events is suppressed by AMPK^[364,365].

The Na⁺/Ca²⁺ exchanger family comprises three members, namely NCX1, NCX2, and NCX3, which act by expelling one Ca²⁺ ion for the uptake of three Na⁺ ions (the so-called “forward-mode” of NCX). The electrochemical gradient for Na⁺ provides the energy necessary to drive Ca²⁺ out of the cell against its large gradient and with a stoichiometric ratio of 3 Na⁺ in: 1 Ca²⁺ out^[366]. Cultured ECs may express all of the three NCX subtypes^[355,367,368], while the only evidence about NCX presence *in situ* has been reported in rat brain^[369]. NCX expression may be upregulated by an increase in [Ca²⁺]_i^[370]. In addition to extruding Ca²⁺ out of the cytosol, NCX may contribute to Ca²⁺ entry when operating in the so-called “reverse-mode” (3 Na⁺ out: 1 Ca²⁺ in). More specifically, the close proximity between NCX and the TRPC channels, which may either physically interact^[371] or be clustered in limited membrane nanodomains^[372], causes NCX to switch into the reverse-mode upon Na⁺ accumulation beneath the plasma membrane^[371,372]. Intriguingly, NCX-mediated Ca²⁺ entry in ECs may be stimulated by Ach^[373], histamine^[43], and VEGF^[374], but not by ATP^[375]. In particular, NCX-driven Ca²⁺ influx underpins PKC α translocation to the plasma membrane, where it stimulates ERK1/2 to trigger a number of steps (proliferation, migration, and tubulogenesis) involved in VEGF-dependent angiogenesis^[374]. It is, therefore, conceivable that NCX role in the regulation of Ca²⁺-dependent processes in vascular endothelium may be governed by the incoming stimuli.

PMCA is encoded by four genes, termed PMCA1-4. Alternative splicing of their primary transcripts yields a large variety of PMCA proteins that differ in their kinetics and regulation^[350]. The pump operates with high Ca²⁺ affinity and low transport capacity, with a 1:1 Ca²⁺/ATP stoichiometry^[350]. PMCA1 is the main isoform found in vascular endothelium, although lower levels of PMCA2 and PMCA4 may also be present^[355,376]. PMCA-dependent Ca²⁺ clearance is triggered by local elevation in intracellular Ca²⁺, which acts by promoting the association between CaM and a specific CaM-binding domain located at the COOH terminus of the pump^[348,377]. PMCA activity and expression is negatively regulated by endothelin-1, which may thus lead to an increase in [Ca²⁺]_i by reducing Ca²⁺ clearance from the cytosol^[378]. PMCA is preferentially located at caveolae on the luminal surface of ECs, and its activity is reduced by phosphatidylserine externalization^[379,380]. In addition to extruding Ca²⁺ across

the plasma membrane, PMCA suppresses both eNOS activity and the subsequent NO production by promoting Thr-495 phosphorylation in resting and activated ECs^[381]. PMCA activity in vascular ECs is, in turn, inhibited by S-glutathionylation, which leads to a progressive raise in [Ca²⁺]_i^[124]. The elucidation of the role served by PMCA in endothelial signaling might benefit from the recent discovery of caloxin 1b3, a specific PMCA1 inhibitor^[382].

Mitochondrial Ca²⁺ uptake

Mitochondria contribute to the dynamics of Ca²⁺ signaling by aiding SERCA, NCX, and PMCA in removing Ca²⁺ from the cytoplasm during the recovery phase. Mitochondrial Ca²⁺ release mechanisms, then, return Ca²⁺ back into the cytosol, where it can be sequestered by SERCA. Indeed, there is a close functional relationship between mitochondria and ER^[10]. This interaction is reinforced by the chaperon glucose-regulated protein 75 (grp75), which tethers ER InsP₃Rs to the voltage-dependent anion channels (VDACs) in the outer mitochondrial membrane. VDACs enable the transfer of cytosolic Ca²⁺ into the mitochondrial intermembrane space, from where the electrochemical gradient (the inner mitochondrial membrane potential is equal to about -180 mV) drives Ca²⁺ into the mitochondrial matrix mainly by two transport mechanisms^[383]. The mitochondrial Ca²⁺ uniporter (MCU), which is located in the inner mitochondrial membrane, operates at the micromolar Ca²⁺ concentrations that are only transiently reached in cells near Ca²⁺ release channels, and might be contributed by uncoupling proteins 2 and 3 (UCP2 and UCP3) in ECs^[384]. However, UCP2/3 buffer intracellularly mobilized Ca²⁺, but not that entering the cells *via* SOCs^[385]. Mitochondrial Ca²⁺ uptake from SOCE in ECs is accomplished by a second type of MCU, which mediates Ca²⁺ uptake into mitochondria at nanomolar cytosolic Ca²⁺ concentrations and has been termed Letm1^[383]. This is a leucine-zipper-EF hand-containing a TM region which catalyzes the 1:1 electrogenic exchange of Ca²⁺ for H⁺^[386]. Conversely, the contribution of the so-called mitochondrial calcium uptake 1 (MICU1), which has recently been discovered by working on HeLa and HEK-293 cells^[387], to endothelial Ca²⁺ signaling has not been ascertained yet. The mitochondrial NCX (mNCX) is the main mechanism responsible for the outward transport of Ca²⁺ towards the cytosol with a stoichiometry of 3Na⁺:1Ca²⁺ (as for the plasmalemmal NCX)^[36,41]: the Na⁺ that enters down the electrochemical gradient is exchanged for Ca²⁺^[383].

Microarchitecture of the Ca²⁺ transporting systems in ECs

The microstructural physical localization of Ca²⁺ pumps, transporters and channels in the ER and plasma membrane relative to each other determines the pattern of Ca²⁺ clearance from the endothelial cytosol^[36,40,41,138,139,345,388]. This organization of the Ca²⁺ transporting systems creates Ca²⁺ gradients between the sub-plasmalemmal region and bulk cytoplasm, which, in turn, enables the same second

messenger, i.e., Ca²⁺, to regulate diverse cellular functions depending on its proximity to specific Ca²⁺-sensitive effectors^[10]. The sequence of events leading to removal of cytosolic Ca²⁺ may be different in ECs from different vascular beds and three main Ca²⁺-buffering pathways have hitherto been identified. (1) In-series arrangement of RyRs and NCX and of SERCA and RyRs^[139,345]: according to this model, that has been described in rabbit aortic ECs, cytosolic Ca²⁺ is captured by SERCA, released from the ER *via* RyRs, and extruded across the plasma membrane *via* the NCX. PMCA acts in parallel to this pathway to bind to and deliver Ca²⁺ to the extracellular space; (2) Vectorial Ca²⁺ release from sub-plasmalemmal InsP₃Rs to the extracellular space *via* NCX^[589]: this system operates following either a moderate or a slow increase in InsP₃ levels in porcine coronary artery ECs, prevents sub-plasmalemmal Ca²⁺ from reaching the deeper cytosol by triggering CICR on RyRs, and has, therefore, been termed SCCU. The SCCU has also been found in EA.hy926 cells, where it subserves a signaling role by activating the endothelial Ca²⁺-dependent large conductance K⁺ channels (BK_{Ca}) after cell stimulation by low concentrations of histamine^[140,141]. An increase in the agonist dose overcomes the moderate RyRs-dependent subplasmalemmal Ca²⁺ release due to the higher InsP₃-dependent Ca²⁺ mobilization; and (3) Mitochondrion-mediated ER refilling: this pathway redirects entering Ca²⁺ through SOCs to the ER in the presence of an InsP₃-generating agonist^[36,41,388]. SOCE is buffered by sub-plasmalemmal mitochondria and vectorially transferred *via* mNCX to SERCA. When SOCE occurs in the absence of InsP₃, the superficial domains of the ER mimic mitochondrial Ca²⁺ buffering by immediately capturing entering Ca²⁺. This Ca²⁺-buffering mode has been mainly characterized in EA.hy926 cells, where it coexists with the SCCU system described above. The former is responsible for the extrusion of Ca²⁺ entering into the cells *via* SOCE, whereas the latter removes from the cytosol the intracellularly-released calcium ions. The mitochondria-mediated ER recharging might also be present in other vascular beds, but this mechanism has not been thoroughly investigated in mature endothelium.

The endothelial Ca²⁺-buffering system may, however, operate in parallel, as depicted by a number of studies conducted on microvascular endothelial cells. In rat brain capillary ECs, for instance, the SERCA provides the major pathway responsible for clearing cytosolic Ca²⁺, whereas NCX extrudes across the PM a minor percentage (around 30%) of the calcium ions mobilized from ER^[343]. Conversely, in rat cardiac microvascular cells, PMCA and SERCA have been shown to restore endothelial Ca²⁺ to prestimulation levels during exposure to the agonist. However, upon interruption of the stimulation, SERCA quickly recaptures Ca²⁺ back into the ER lumen^[349].

SPATIOTEMPORAL ORGANIZATION OF INTRACELLULAR Ca²⁺ SIGNALS IN ECS

The remarkable versatility of Ca²⁺ is due to the multitude

of Ca²⁺ signals that can be generated by extracellular stimuli within a single cell. Such heterogeneity depends on the recruitment of specific components of the Ca²⁺ toolkit which lead to the generation of intracellular signals varying in amplitude, spatial dimension and frequency^[10,390,391]. The opening of Ca²⁺-permeable pathways either on the ER or plasma membrane may give rise to local or elementary Ca²⁺ signals, which are spatially restricted within hundreds of nanometers to micrometers from the mouth of the channel pore^[392]. The local nature of these events is attributable to the concerted action of Ca²⁺ buffering and reuptake mechanisms that limit the diffusion of the Ca²⁺ signal^[37]. The most simple Ca²⁺ release events are the Ca²⁺ blips and the Ca²⁺ quarks, which arise from the opening of single InsP₃Rs and RyRs, respectively^[10,392]. Endothelial Ca²⁺ blips have average amplitude of 23 nmol/L, are 1- to 3- μ m wide, and last < 100 ms^[393]. Conversely, there is no report of endothelial Ca²⁺ quarks. The concerted opening of a fixed cluster of InsP₃Rs and RyRs give rise, respectively, to Ca²⁺ puffs and Ca²⁺ sparks^[10,392]. Spontaneous Ca²⁺ puffs have been reported in native ECs^[394-396], which can be excited by adjoining SMCs through gap junctional communication^[396-398]. Isolated ECs display only agonist-induced Ca²⁺ puffs, which reach a peak of about 50 nmol/L and spread for no longer than 30 μ m^[393]. Elementary Ca²⁺ release events mediated by InsP₃Rs, but different from the classic Ca²⁺ puffs, may occur in restricted spaces of myoendothelial projections and have been termed Ca²⁺ pulsars^[16,399]. The endothelial Ca²⁺ pulsars colocalize with the intermediate conductance, Ca²⁺-sensitive K⁺ channels (K_{Ca}3.1) so that local Ca²⁺ signals may be translated into a negative shift in V_M which is rapidly transmitted to the adjacent VSMCs in terms of EDHF^[399]. Consistently, sympathetic nerve stimulation activates α -adrenergic receptors on SMCs to recruit new pulsar sites in the adjoining ECs in order to oppose vasoconstriction^[400]. The spatiotemporal summation of adjacent Ca²⁺ puffs leads to a regenerative intracellular Ca²⁺ wave that can spread throughout the cytosol at an average speed of 5-60 μ m/s^[118,393,401]. The regenerative propagation of the Ca²⁺ elevation is, indeed, faster in the perinuclear region and at the initiation site^[118]. The endothelial Ca²⁺ sweep tends to originate from multiple peripheral foci localized in the caveolae compartments of the plasma membrane^[402]. These triggering sites remain constant during repetitive cell stimulation^[393,401,402]. Indeed, when the information embedded within the [Ca²⁺]_i increase must be conveyed over a long time, both the elementary events and the global Ca²⁺ sweeps can adopt an oscillatory pattern^[16,72,339,396]. However, the physiological outcome of local Ca²⁺ spikes depends on the Ca²⁺-sensitive decoders located within a few nanometers from the channel pore^[395], and the frequency of repetitive Ca²⁺ waves encodes for the selective engagement of specific downstream targets^[403-405]. The interspike interval of the Ca²⁺ transients is inversely correlated to the strength of the stimulus until, at least at high agonist doses, they apparently fuse to generate a plateau level^[16,72,406,407]. Endothelial Ca²⁺ oscillations may,

indeed, be elicited by mechanical forces, extracellular autacoids or oxidative stress. Shear stress and pressure may induce ECs to undergo repetitive Ca²⁺ transients by triggering Ca²⁺ entry through Gd³⁺-sensitive MSCs followed by CICR from RyRs^[22,24,25]. The opening of the MSC generates a transient local Ca²⁺ event that has been termed Ca²⁺ hotspot^[24]. Unlike the oscillatory Ca²⁺ response to extracellular ligands^[72,406,407], the amplitude of the Ca²⁺ spikes increases with the intensity of the mechanical stimulation^[22-25]. Agonist-evoked Ca²⁺ oscillations require PLC activation by either GPCRs or TKRs^[72,339,404,405,408]. The following InsP₃-dependent Ca²⁺ mobilization may be amplified by adjacent RyRs *via* CICR^[44,134] and requires Ca²⁺ inflow to supply releasable Ca²⁺ over time and maintain InsP₃R sensitivity to the ligand^[72,407,409,410]. Ca²⁺ entering through either SOCE or the reverse-mode of NCX is sequestered by SERCA into ER lumen before being released again into the cytosol through the open InsP₃Rs^[72,134,339,404,405]. Some^[16], but not all^[72], microvascular ECs may be independent on Ca²⁺ inflow. In addition to bringing about the downstroke of each Ca²⁺ spike, SERCA shapes both the rate of the initial Ca²⁺ release and the amplitude of the regenerative upstroke, whereas it does not affect the Ca²⁺ wave speed^[339,411]. As a consequence of the rhythmic interplay between InsP₃-induced Ca²⁺ release and SERCA-mediated Ca²⁺ reuptake, SOCE is not constant during the Ca²⁺ train. Accordingly, store-sensitive Ca²⁺ inflow is activated by the partial depletion of the Ca²⁺ pool occurring during each Ca²⁺ spike and is then turned off by store replenishment^[410,412]. Finally, ECs may display repetitive Ca²⁺ oscillations in response to the NAD(P)H oxidase-derived H₂O₂ that is endogenously produced during the ischemia/reperfusion injury^[56,413]. H₂O₂-elicited Ca²⁺ spikes are supported by the periodic opening of InsP₃Rs, but rapidly run down in absence of extracellular Ca²⁺^[56,413]. It would, therefore, be interesting to assess whether the H₂O₂-sensitive plasmalemmal channel, TRPM2, is involved in the signal transduction pathways recruited under these conditions^[320].

CONCLUSION

Once regarded as an inert barrier between circulation and vascular tissue, ECs constitute the largest signal transduction platform of the organism, which is essential for the proper functioning of the cardiovascular system. Any alteration in the endothelial capability to detect and decode both physical and humoral stimuli into the most suitable vessel wall phenotype may lead to an astonishing variety of severe diseases, including myocardial infarction, brain stroke, atherosclerosis, peripheral artery disease, and tumor. As recently outlined by Munaron and Florio Pla^[11], it is imperative to understand the molecular mechanisms that enable vascular ECs to fulfil their multifaceted role in order to either prevent or reduce the adverse consequences of so-called endothelial dysfunction. The Ca²⁺ signalling toolkit plays a key role in the control of a wide array of endothelial processes and, therefore, any change in the pattern of expression of each of its components is

somehow involved in the pathogenesis of an increasing number of pathologies. Despite the most recent advances in the comprehension of the molecular underpinnings of the Ca²⁺ responses to both physical and chemical stimulation, a huge amount of work is standing in front of us. First, the blend of endothelial ion channels, transporters and receptors may vary with species, age, sex, hormonal status, vascular bed, and blood vessel diameter. This issue has been further stressed by the recent finding that Stim1 and Orai1 control *in vitro* tubulogenesis in HUVECs^[206], but not in the HUV-derived EC line, EA.hy926^[224]. As a consequence, the information on the Ca²⁺ machinery acquired on animal or cell line models cannot undergo a straightforward clinical translation, but must be confirmed in humans and in the vascular region implicated in the disease under investigation. This task may not be easy due to the difficulties of obtaining human samples from either healthy or pathological vessels. Second, the controversy on the molecular nature of SOCs, which are regarded as the most important pathway for Ca²⁺ influx into vascular endothelium, is far from being fully resolved. As described above, a recent study suggested that Orai1 and Stim1, whose physical interaction mediates SOCE in the immune system, underlie this Ca²⁺ entry route only in HUVECs, whereas TRPC1 and TRPC4 are activated by the emptying of the Ca²⁺ pool in other vascular beds. The same study, however, contributed to boost such controversy by demonstrating that the genetic suppression of TRPC1 dampens SOCE in HUVECs, a finding which is not consistent with those previously reported on the same preparation. This conflict is not surprising when considering the abundance of discrepant data about TRPC1 modulation by Stim1. The possibility that the molecular structure of SOCE (i.e., either the ER Ca²⁺ sensor and the Ca²⁺-permeable pore) might differ in distinct species and vascular beds, as put forward by Sundivakkam *et al.*^[218], should be also considered. Moreover, the possibility that the components of SOCE may be different in distinct phases of the cell cycle should be taken in account. Yet, it is evident that further studies will have to be devoted to clarify this issue in vascular ECs. A recent study attempted to solve this controversy by investigating the association between Orai1 and TRPC1, which has been described in a growing number of nonexcitable cells, in rat pulmonary artery ECs. In these cells, SOCE has long been associated with TRPC1 and TRPC4, as widely described above^[217]. Surprisingly, Cioffi *et al.*^[414] have found that Orai1 constitutively interacts with TRPC4 and drives the activation of the TRPC1-TRPC complex upon intracellular Ca²⁺ release. It is not clear, however, whether and how protein 4.1 and Stim1 independently confer store-sensitivity to the ternary complex on the plasma membrane. Third, whereas the intracellular target of InsP₃ has long been recognized, the selective receptor of the two newly discovered second messengers, cADPr and NAADP, has recently been questioned. Indeed, a couple of recent investigations demonstrated that the genetic suppression of TPC1-2 does not prevent cell labeling by NAADP^[415,416]. Conversely, the same studies suggested

that a 22- and 23-kDa pair of accessory proteins might serve as NAADP receptors and regulate the activity of its target channels, including TPC1-2 in ECs and RyRs in the immune system. The same controversy holds true also for the molecular effector of cADPr-dependent signalling in mature ECs. As mentioned already, the mechanistic link between cADPr and RyRs-mediated Ca²⁺ is yet to be elucidated. Fourth, ECs establish either transient or permanent intimate contacts with a variety of cell types, such as circulating blood cells or tumoral cells, fibroblasts, and SMCs. Only a few studies have hitherto addressed whether and how these tight relationships may affect endothelial Ca²⁺ homeostasis. In addition, ECs may undergo a rearrangement of their Ca²⁺ signaling proteins when detached from the vessel walls and cultured *in vitro*^[7,153]. It follows that future studies should be conducted both *in vitro* and *in vivo*, by exploiting the genetic Ca²⁺-sensors that have been described in the literature, to explore this fascinating aspect of Ca²⁺ signaling. Finally, intracellular Ca²⁺ waves have recently been shown to drive EPC proliferation, homing, and tubulogenesis. Only a few investigations have so far been devoted to unmask the players of the Ca²⁺ machinery involved in these tasks. In the light of the promising therapeutic potential of the Ca²⁺-permeable channels in these cells, either as a target or facilitators of clinical interventions, we are confident that this field will gain the interest of researchers worldwide. Therefore, it appears that the way towards full comprehension of the intricate network of membrane receptors, ion channels, transporters, pumps, and cytosolic decoders that convert an extracellular stimulus into a meaningful Ca²⁺ signal still has a long way to go and overcome many obstacles, but a multidisciplinary approach will certainly be helpful to reach this goal.

ACKNOWLEDGMENTS

The authors are grateful to all their collaborators for the ongoing and fruitful discussions on Ca²⁺ signalling in endothelial cells and endothelial progenitor cells. They are also delighted to acknowledge Mrs Simona Laudadia for her excellent bibliographic assistance.

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