Calcium signalling in endothelial cells

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Abstract

Vascular endothelial cells are ubiquitous for their presence in each and every vessel and unique for their multifunctional nature. A large number of endothelial functions depend to various extents on changes in intracellular Ca\textsuperscript{2+} concentration. Reviewed are endothelial Ca\textsuperscript{2+} stores, Ca\textsuperscript{2+} channels, and in-out-in Ca\textsuperscript{2+} signalling events, from ligand-binding on the plasma membrane into depletion of intracellular Ca\textsuperscript{2+} stores and therefrom out to transplasmalemmal Ca\textsuperscript{2+} entry that is of prime importance for many endothelial functions. Special emphasis is placed on mechanisms regulating store-operated Ca\textsuperscript{2+} entry including a Ca\textsuperscript{2+} influx factor, the vesicle secretion-like model, the conformational coupling model, the membrane potential, cytochrome P450, protein tyrosine kinase, myosin light chain kinase and nitric oxide.

Keywords: Endothelial factors; Calcium (cellular); Ca-channel; Signal transduction; Protein kinases

1. Introduction

Endothelial cells (ECs) are unique for their multifunctional nature. They regulate immune responses, control blood coagulation states, serve as an interface adjusting blood–tissue permeability, contribute to angiogenesis and vessel repair, and modulate vascular tone. These diverse functions confer on ECs indispensable roles in the body's normal homeostasis and in many pathological conditions. In the cardiovascular system, ECs play key regulatory roles by producing several potent vasoactive agents and regulating coagulation states. Endothelial dysfunction has been implicated in many cardiovascular diseases, which renders modulation of endothelial functions a promising therapeutic approach. Most endothelial functions depend to various extents on changes in intracellular calcium concentration ([Ca\textsuperscript{2+}]). In particular, store-operated Ca\textsuperscript{2+} entry has been shown to regulate many of endothelial functions. This review is intended to briefly go over the mechanisms that regulate the various Ca\textsuperscript{2+} signalling events in this cell type, with an emphasis on recent findings concerning store-operated Ca\textsuperscript{2+} entry.

2. Ligand–receptor interaction and the production of inositol triphosphate (IP\textsubscript{3})

Many hormones, neurotransmitters, and secretagogues act by increasing [Ca\textsuperscript{2+}], in their target cells. In ECs, agonists such as bradykinin, angiotensin II, serotonin and acetylcholine, when occupying their specific receptors on the cell membrane, couple with and activate a guanosine nucleotide-binding protein, which subsequently activates phospholipase C-\textdelta1 in a Ca\textsuperscript{2+}-independent manner. Phospholipase C-\textbeta1 then hydrolyses the lipid precursor phosphatidylinositol-4,5-biphosphate (PIP\textsubscript{2}) to yield IP\textsubscript{3} and diacylglycerol. On the other hand, growth factors such as platelet-derived growth factor, epidermal growth factor or antigens bind to tyrosine kinase-linked receptors, which activate phospholipase C-\textgamma1. Apart from hydrolysing PIP\textsubscript{2} to yield IP\textsubscript{3} and diacylglycerol like PLC-\textbeta1, phospholipase C-\textgamma1 also activates phosphatidyl inositol 3-OH kinase (PI\textsubscript{3}K)}
kinase), which generates phosphatidylinositol 3,4,5-triphosphate (PIP$_3$) and GTPase-activating protein that regulates ras and activates mitogen-activated protein kinases [1,2]. Mechanical stimuli such as high shear stress also stimulate biphasic and self-subsiding IP$_3$ production in ECs, such that IP$_3$ concentration returns to basal levels despite continuous stimulation [3]. IP$_3$ is thus formed by two pathways: a G protein (GTP-binding protein)-coupled pathway and a tyrosine kinase-linked one. Once formed, IP$_3$ binds to its specific receptors (IP$_3$R) on the surface of the endoplasmic reticulum (ER) and activates IP$_3$R Ca$^{2+}$ release channels, which depletes internal Ca$^{2+}$ stores and results in a transient increase in [Ca$^{2+}$]$_i$. Synthetic analogues of IP$_3$ have been developed with slightly lower biological activity than that of IP$_3$ to stimulate Ca$^{2+}$ mobilization [4]. Diacylglycerol, on the other hand, together with Ca$^{2+}$ released from the ER, activates protein kinase C, whose maintained activation, possibly following a sustained formation of diacylglycerol from phosphatidylcholine, allows persistence of many Ca$^{2+}$-dependent physiological responses despite short-lived initiating Ca$^{2+}$ triggers [5–7].

IP$_3$ is metabolized by two known routes. Dephosphorylation by IP$_3$-5-phosphatase results in the formation of 1,4-IP$_2$ that is subsequently degraded to free inositol by other phosphatase activities [8]. Alternatively, phosphorylation of IP$_3$ on the 3-hydroxy group by an ATP-dependent kinase produces 1,3,4,5-inositol tetrakisphosphate (IP$_5$) [9]. IP$_4$ has been implicated in intracellular Ca$^{2+}$ signalling. Injection of IP$_4$ together with IP$_3$ into sea urchin eggs causes a Ca$^{2+}$ influx [10]; combination of IP$_3$ and IP$_4$ in lacrimal cells increases [Ca$^{2+}$]$_i$ and Ca$^{2+}$-activated K$^+$ channels [11]; and it was hypothesized that IP$_3$ and IP$_4$ act in conjunction to deplete internal Ca$^{2+}$ stores [12]. In ECs, IP$_4$ and increasing [Ca$^{2+}$]$_i$, may cooperatively activate a plasmalemmal Ca$^{2+}$-permeable channel [13]. The role of IP$_4$ in Ca$^{2+}$ signalling, however, needs further investigation [14,15].

Down the signaling cascade from ligand-binding receptors to IP$_3$Rs, inhibitors of phospholipase C such as U-71223 and U-73122 [16] or of the binding of IP$_3$ to its receptors such as heparin, all block to different extents downstream Ca$^{2+}$ signalling events. Recently, antimalarials such as chloroquine, quinidine and quinine were also shown to block binding of IP$_3$ to IP$_3$R [17].

3. Intracellular Ca$^{2+}$ stores

3.1. ER as the main intracellular Ca$^{2+}$ stores

The ER is the main Ca$^{2+}$ stores in nonmuscle cells [18,19] and controls many cellular functions [20,21]. It contains many Ca$^{2+}$ binding proteins such as GRP 94, BiP (GRP 78), RP 60 and calreticulin [22,23]. With each of their molecules being able to sequester as many as 30 Ca$^{2+}$ ions and with these proteins existing in large amounts, Ca$^{2+}$ concentration in the ER can be as high as 3 mM [24]. In ECs the ER accounts for approximately 75% of the total intracellular Ca$^{2+}$ reserve. Extending like a net over the entire cytoplasm, the ER is in virtually immediate exposure to any intracellular Ca$^{2+}$ signals or Ca$^{2+}$ releasing factors. The ER determines in great part the generation of important Ca$^{2+}$ signals that are involved in most other vital functions of the cell. A question yet to be answered is whether the high Ca$^{2+}$ concentration in the ER can affect its other functions like synthesis and modification of proteins and synthesis of lipids or whether the ER as such just functions as a storehouse for Ca$^{2+}$. A Ca$^{2+}$-ATPase that pumps Ca$^{2+}$ into the ER lumen is the major membrane protein of the ER.

3.2. Mitochondria

Mitochondria are the other important containers of intracellular Ca$^{2+}$ in ECs, accounting for the remaining 25% of the cell’s Ca$^{2+}$ reserve. The relative quantification of the Ca$^{2+}$ storage capacity of the ER and mitochondria in ECs was made by comparing the total Ca$^{2+}$ uptake into permeabilised ECs in the presence of inhibitors of mitochondria or of the ER Ca$^{2+}$-ATPase inhibitor thapsigargin [25]. Mechanisms mobilizing mitochondrial Ca$^{2+}$ in ECs have not been so fully investigated as in other cells. Mitochondrial Ca$^{2+}$ uptake, 10–100 times kinetically slower than mitochondrial Ca$^{2+}$ efflux [26], is believed to be a unipporter that facilitates the diffusion of Ca$^{2+}$ down the electrochemical gradient across mitochondrial membrane. Previously, mitochondria were simply considered as high-capacity, low-affinity Ca$^{2+}$ storage pools that serve in states of Ca$^{2+}$ overload as a life-rescuing mechanism by taking up the amount of Ca$^{2+}$ that would otherwise overburden the ER [26]. Recent work, however, has shown that these organelles themselves are excitable, capable of generating and conveying electrical and Ca$^{2+}$ signals [27]. Release of Ca$^{2+}$ from mitochondria requires Ca$^{2+}$ to be triggered and in turn plays a critical role in forming Ca$^{2+}$ oscillation patterns [28]. Thus, mitochondrial Ca$^{2+}$ is released following the release from the ER. Mitochondrial Ca$^{2+}$-induced Ca$^{2+}$ release (mCICR) in ECs is triggered during IP$_3$-induced Ca$^{2+}$ mobilization and amplifies the Ca$^{2+}$ signals primarily emitted from the ER. Mitochondria appear in close association with regions of the ER enriched in IP$_3$R and are particularly responsive to IP$_3$-induced increases in Ca$^{2+}$. Each mitochondrial Ca$^{2+}$ uptake site faces multiple IP$_3$Rs, a concurrent activation of which is required for optimal activation of mitochondrial Ca$^{2+}$ uptake and there seems to be a synaptic way of transmission of Ca$^{2+}$ signals between mitochondria and the ER [29]. Ca$^{2+}$ uptake by the mitochondria was recently shown to suppress the local positive feedback effects of Ca$^{2+}$ on the IP$_3$Rs, giving rise to subcellular heterogeneity in IP$_3$. 

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sensitivity and IP$_3$R excitability [30]. Cross talks between mitochondrial and ER Ca$^{2+}$ signals appear to be important in controlling the Ca$^{2+}$ homeostasis of the cell in basal as well as in stimulated conditions.

Recently a volume-sensitive, IP$_3$-insensitive Ca$^{2+}$ store was described in ECs. This store is released in hypotonic conditions and appears to possess Ca$^{2+}$-ATPase as a Ca$^{2+}$ pump, since loading of Ca$^{2+}$ into this pool was prevented by thapsigargin [31]. It remains to be determined whether this store represents an IP$_3$-insensitive portion of the ER or yet another store for intracellular Ca$^{2+}$ in ECs.

4. Intracellular Ca$^{2+}$ channels

4.1. Ca$^{2+}$ release channels on the ER

These include IP$_3$Rs, predominant in non-excitable cells including ECs, and ryanodine receptors (RyRs), most extensively characterized in smooth muscle and cardiac muscle.

4.1.1. IP$_3$Rs

IP$_3$Rs located on the ER surface constitute the most clearly identified Ca$^{2+}$ channels that pump Ca$^{2+}$ from this intracellular store. Three forms of IP$_3$R (types 1, 2 and 3) have been characterized by cDNA cloning in both animal and human cells [32–34]. Most cells have at least one form of IP$_3$R, and many express all three. The IP$_3$R channels are tetramers composed of four subunits. IP$_3$R1 function is regulated by at least two major cellular signalling pathways: the second messenger IP$_3$ activates the channel and phosphorylation by non-receptor protein kinases increases its opening probability. In ECs, the identified form of IP$_3$R is both structurally and functionally analogous to that detected in neuronal tissues, is preferentially located at the perinuclear region, and is approximately 260 kDa in molecular weight [35]. IP$_3$Rs function as Ca$^{2+}$ pumps to release Ca$^{2+}$ when occupied by IP$_3$. This Ca$^{2+}$ release, however, terminates, even in the continued presence of IP$_3$. Explanations for this include rapid hydrolysis of IP$_3$ and hence deactivation of IP$_3$Rs, feedback effects of cytoplasmic and/or luminal Ca$^{2+}$ concentration on specific Ca$^{2+}$ binding sites on the IP$_3$Rs/channel complex, and intrinsic deactivation properties of IP$_3$Rs [36]. Recent work suggests that Ca$^{2+}$ release in ECs is partly dependent on a movement of K$^+$ through K$^+$ channels in the store membranes and that there possibly is a counter-ion system that controls Ca$^{2+}$ release. Using saponin-permeabilised bovine aortic ECs, the ability of different ions to allow IP$_3$-induced Ca$^{2+}$ release was found to be K$^+>$Na$^+>$Cs$^+$>$Rb$^+>$Co$^{2+}$ [37]. Most recently, it was shown in smooth muscle cells that an IP$_3$R-associated cGMP kinase substrate (IRAG), a protein of relative molecular mass of 125 kDa, serves as an essential regulator of IP$_3$-induced Ca$^{2+}$ release from intracellular stores [38]. It remains to be determined, however, if such a molecule exists in ECs.

Factors controlling the rate of Ca$^{2+}$ discharge from intracellular stores contribute to the regulation of cytosolic Ca$^{2+}$ oscillations. IP$_3$Rs, however, are not necessarily the only mechanism responsible for Ca$^{2+}$ release from intracellular stores. In porcine aortic ECs, it was shown that bradykinin, an agonist that stimulates IP$_3$ production, and thapsigargin, a strong inhibitor of the ER Ca$^{2+}$-ATPase, released Ca$^{2+}$ from the same source [39]. Recent technology has allowed Ca$^{2+}$ release from intracellular stores to be regulated at will in terms of both amplitude and frequency, which has shown that the frequency of Ca$^{2+}$ release can control gene expression [40].

4.1.2. RyRs

RyRs constitute another family of proteins responsible for Ca$^{2+}$ releasing channels. Ryanodine binds to RyRs on the sarcoplasmic reticulum [41,42] and thus changes their Ca$^{2+}$ release properties; at low concentrations, ryanodine keeps the Ca$^{2+}$ release channels in an open state, causing Ca$^{2+}$ release [43]. Recently, RyRs were reported to exist in ECs of porcine endocardium and thoracic aorta [44]. The RyR isofrom detected is more homologous to the cardiac isofrom than to the skeletal isofrom. RyRs had actually been suggested to participate in Ca$^{2+}$ oscillations in ECs [45–47]. Soon after detection of RyRs in ECs, evidence was provided that the channels represented by these receptors are functional. Prestimulation of rat aortic, human aortic, human umbilical vein and bovine pulmonary ECs with ryanodine significantly reduced bradykinin-induced Ca$^{2+}$ release. Increasing Ca$^{2+}$ concentrations enhance binding of ryanodine to its receptors in human umbilical vein ECs [48,49]. Molecular characteristics of endothelial RyR channels are not determined.

4.1.3. Ca$^{2+}$ leak

Apart from IP$_3$R and RyR channels, which require binding of a second messenger for their activation, Ca$^{2+}$ from inside the endoplasmic reticulum can also be spontaneously released via other mechanisms. ER luminal Ca$^{2+}$, constantly in high concentration, leaks out into the cytosol. This leak is normally compensated for by Ca$^{2+}$ uptake mechanisms, to be mentioned in Section 4.2. In coronary ECs, 10-min incubation in Ca$^{2+}$-free medium could deplete bradykinin-sensitive store by 59%, and bradykinin- and ryanodine-sensitive stores are suggested to differ in functions [50].

4.2. ER Ca$^{2+}$ uptake channels

The major ER surface protein is the Ca$^{2+}$ ATPase, an ATP-dependent Ca$^{2+}$ pump that continuously takes up Ca$^{2+}$ from the cytosol. This pump can take up Ca$^{2+}$ at much greater rates than Ca$^{2+}$ spontaneously leaks out of the ER lumen. Under treatment with inhibitors of this
pump such as thapsigargin, dibenzohydroquinone (BHQ), or cyclopiazonic acid, the ER is depleted. This mode of store depletion is not related to the production of IP₃ and these agents have served as very useful tools to investigate intracellular Ca²⁺ signalling. Antagonists of the ubiquitous Ca²⁺-binding protein calmodulin were recently suggested to trigger Ca²⁺ responses in porcine aortic ECs the same way as does thapsigargin. This suggests that calmodulin, generally thought to play a passive role of binding to Ca²⁺, might play an active role in endothelial Ca²⁺ signalling [51].

5. Transplasmalemmal Ca²⁺ entry channels

Transplasmalemmal Ca²⁺ entry pathways are of particular importance because Ca²⁺ entry from the extracellular space modulates many cellular functions. This Ca²⁺ entry is a necessary intermediate for long-term cellular responses [52]. Enormous efforts have been made to elucidate the Ca²⁺ entry pathways and a variety of entry pathways have been described.

5.1. Voltage-dependent Ca²⁺ channels

These channels have been described in ECs [53–56], but the lack of potent and specific blockers makes it difficult to assess their influence on endothelial functions. Furthermore, most of the channels described were of small conductance, and are generally considered to be of little functional importance [57]. Indeed, agonist-induced Ca²⁺ entry, known to regulate many endothelial functions, is largely diminished by depolarization of the plasma membrane with high potassium solutions [58,59]. This strongly argues against a significant contribution of voltage-dependent Ca²⁺ channels to agonist-induced Ca²⁺ entry in ECs, for depolarization of the plasma membrane would enhance a Ca²⁺ entry that is due to activation of voltage-dependent Ca²⁺ channels, not inhibit it. Moreover, classical inhibitors of voltage-dependent Ca²⁺ channels like diltiazem and verapamil did not affect agonist-induced Ca²⁺ entry in ECs [59,60]. This makes voltage-dependent Ca²⁺ channels not important in ECs as they are in smooth muscle cells.

5.2. Non-selective cation channels

Evidence has accumulated that in ECs non-selective cation channels may account for agonist-induced Ca²⁺ entry — also called capacitative Ca²⁺ entry — that will be dealt with in a separate section. Agonists such as thrombin, bradykinin, serotonin, ATP and endothelin-1 activate non-selective cation channels in ECs [61–65]. EC swelling also activates non-selective cation channels with conductance of 13$p$ for calcium. ER Ca²⁺ ATPase inhibitors as well as IP₃ applied intracellularly, all of which deplete intracellular Ca²⁺ stores, also activate non-selective cation channels [66–68]. Evidence also exists that in ECs, agonist-induced Ca²⁺ entry channels share some behavioral similarities with type-3 IP₃R. In Xenopus oocytes, expression of this IP₃R specifically enhances Ca²⁺ entry without affecting Ca²⁺ release, suggesting that in some cases, type-3 IP₃R might also function as a plasmalemmal Ca²⁺ channel [69].

5.3. Molecular structure of agonist-induced Ca²⁺ channels

Molecular characterization of agonist-induced Ca²⁺ entry channels has been intensive lately. Much work has been made on mammalian homologues of the Drosophila trp (transient receptor potential) protein as potential candidates for agonist-induced Ca²⁺ entry channels. Trp is a Drosophila photoreceptor mutant incapable of maintaining a sustained potential in response to photostimulation [70]. The fact that these receptors use a phospholipase C signalling pathway gave the first hint that trp might encode a component of the Ca²⁺ entry pathway [71]. Light-induced phosphoinositide hydrolysis in Drosophila activates two classes of channels, one selective for Ca²⁺ and absent in the transient receptor potential mutant trp, the other a non-selective cation channel that requires Ca²⁺ for activation. As well as being a major charge carrier for the light-induced current, Ca²⁺ entry via the trp-dependent channels appear to be required for refilling IP₃-sensitive Ca²⁺ stores and for feedback regulation (light adaptation) of the transduction cascade. The trp-dependent Ca²⁺ channels are stimulated by depletion of internal Ca²⁺ stores, being activated by the endoplasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin [72]. Six mammalian trp genes homologous to the Drosophila trp mutant have been identified. In pulmonary ECs, expression of Trp1 gene was reported as a likely candidate for Ca²⁺ entry, whereas Trp3 and Trp6 are not likely expressed in this cell type [73]. For a review see Ref. [74].

6. Store-operated Ca²⁺ entry

6.1. Capacitative model

In non-excitable cells, agonists trigger a biphasic rise in $[\text{Ca}^{2+}]_i$, which consists of an initial transient component followed by a large and sustained one. Under Ca²⁺-free conditions, the response is only a small and transient rise in $[\text{Ca}^{2+}]_i$, which should reflect the release of intracellular Ca²⁺ stores. When extracellular Ca²⁺ is reintroduced in the absence of the agonist, there is a large rise in $[\text{Ca}^{2+}]_i$. Since the depletion of intracellular Ca²⁺ stores is apparently the single mechanism at work under such a circumstance, the Ca²⁺ entry has been hypothesized to be activated by the depletion of these stores and this Ca²⁺
entry is thus called store-operated Ca$^{2+}$ entry (SOC), or capacitative Ca$^{2+}$ entry (CCE) [75]. It was originally proposed that the biphasic nature of agonist-activated Ca$^{2+}$ mobilization could be seen as an initial emptying of the intracellular Ca$^{2+}$ stores by IP$_3$, followed by rapid entry of Ca$^{2+}$ into the stores and, in the continued presence of IP$_3$, into the cytosol. Upon withdrawal of agonist, IP$_3$ would be rapidly degraded, the pathway from the stores to the cytosol closed, and rapid entry from the outside would continue until the stores’ Ca$^{2+}$ content could reach a level that would inactivate Ca$^{2+}$ entry. The model was later adjusted that Ca$^{2+}$ entry would first enter the cytosol and then the store refilling process would take place [76]. Non-selective cation channels, as discussed above, are believed to be responsible for this entry. Although it might not be a universal mechanism [77], CCE probably is the main mechanism of agonist-induced Ca$^{2+}$ entry in vascular ECs [78–81]. Shear stress also activates CCE in ECs by several mechanisms. First, flow transfers blood-borne agonists to the cell surface; flow has been shown to activate phospholipase C and increase IP$_3$. Second, the permeability of the cell membrane to extracellular Ca$^{2+}$ increases upon exposure to flow. Shear stress also activates heterotrimeric G-proteins and small G proteins, which participate in Ca$^{2+}$ signalling; for a review see Ref. [81].

Most recently, it was reported that a mechano-sensitive nonselective cation channel might account for shear-stimulated Ca$^{2+}$ entry in rat aortic endothelial cells [82]. This channel has relative permeability ratios of $P_{Ca}:P_{Na}:P_K = 5:1:1$, and is inhibited by 8-Br-cGMP, suggesting that a protein kinase G-dependent mechanism is involved. The channel, however, might not be capacitative in nature, as $[\text{Ca}^{2+}]_i$ did not increase under flow when extracellular Ca$^{2+}$ was absent.

6.2. Signalling mechanisms of CCE

The events from ligand–receptor binding to activation of intracellular Ca$^{2+}$ channels and Ca$^{2+}$ store depletion in non-excitable cells are fairly well understood. Nevertheless, it remains unclear how Ca$^{2+}$ entry into the cell is gated by store depletion. Factors that regulate CCE have been intensively investigated over the years and several mechanisms have been proposed.

6.2.1. Calcium influx factor

The diffusible messenger theory states that when intracellular Ca$^{2+}$ stores are depleted, some diffusible messengers are produced that would transmit the signal from the stores to the plasma membrane to activate CCE [83]. A putative calcium influx factor (CIF) was proposed when an extract of Jurkat lymphocytes collected following store depletion under extracellular Ca$^{2+}$-free condition was able to activate Ca$^{2+}$ entry in macrophages [84]. Several molecules were excluded as CIF, including ATP, ADP, GTP, IP$_3$, IP$_4$, cAMP, cGMP, cis-unsaturated fatty acids, platelet activating factor, eicosanoids, arachidonic acid metabolites and cytochrome P450. CIF was suggested to possess several characteristics: stable and non-protein-like, containing hydroxyl groups on adjacent carbons, a phosphate, some hydrophobic portions, and a negative charge, more likely to reside on a phosphate than a carboxylate group.

6.2.2. Vesicle secretion-like model

CIF’s role was recently negated in SOC activation in Xenopus oocytes, such that the SOC in this cell type required SNAP-25 to be activated, not a diffusible messenger [85]. This finding is in support of the vesicle secretion-like signalling model, which states that the transmission of information from depleted intracellular stores resembles the secretion of vesicles to the extracellular matrix. This hypothesis receives further support from recent findings on smooth muscle cells that coupling between store depletion and Ca$^{2+}$ entry is probably the work of a modified cytoskeleton [86]. Although these findings do not rule out a possible role of a CIF in other cell types, it is clear that further identification is required to establish the role of such a factor in CCE.

6.2.3. Conformational coupling model

This model proposes that information transfer from store depletion to the plasma membrane is mediated by the IP$_R$, functioning as the go-between of the two membrane systems [14,87]. Specifically, it was proposed that the IP$_R$ would integrate in its cytoplasmic head information that signals the capacitative Ca$^{2+}$ entry and then transmit this information to the Ca$^{2+}$ release-activated Ca$^{2+}$ channels in the plasma membrane via direct protein–protein interaction. The IP$_R$s are hypothesized to have two possible distinct functions: they can either release Ca$^{2+}$ to the cytosol or use their large cytoplasmic heads to transmit information to the surface membrane. These two functions can be performed by two different isoforms; for a review see Ref. [87].

6.2.4. Membrane potential

As noted above, membrane potential of ECs is strongly implicated in the regulation of CCE [58]. When EC membrane was depolarized, either by high K$^+$ concentration or by the K$^+$ channel blocker tetraethylammonium, Ca$^{2+}$ entry in response to ATP and bradykinin was significantly diminished while release of Ca$^{2+}$ from intracellular stores remained unaffected. Membrane potential is determined, among other things, by potassium and chloride concentrations. Ca$^{2+}$ influx into ECs in response to histamine and ATP was also shown to be sensitive to chloride concentration [88] and there is recent report that both bradykinin and thapsigargin provoke chloride influxes that partly regulate CCE [89]. Although it is not clear whether a “permissive” membrane potential is required for Ca$^{2+}$ entry through non-selective cation channels, these
findings suggest that membrane potential could control CCE in ECs.

6.2.5. Cytochrome P450

Cytochrome P450 (CYP450) has also been suggested to participate in the regulation of SOC [90]. Unsaturated fatty acids that antagonize the dealkylation of benzyl-resorufin, a CYP450-mediated activity, were found to inhibit $\text{Ca}^{2+}$ entry in rat thymocytes and human neutrophils. In agreement with these findings, Mn$^{2+}$ influx in human platelets promoted by thapsigargin and tBuBHQ, or by preincubation in $\text{Ca}^{2+}$-free medium, was inhibited by the imidazole antimycotics, econazole and miconazole, which inhibit CYP450 activity [91]. It has been hypothesized that when $\text{Ca}^{2+}$ is released from intracellular stores, calmodulin, normally bound to $\text{Ca}^{2+}$ inside the stores and now free, restores an inhibited microsomal CYP450 function, which will open a $\text{Ca}^{2+}$ entry pathway. When $\text{Ca}^{2+}$ subsequently enters the cell, intracellular stores are refilled, $\text{Ca}^{2+}$ then binds to calmodulin in the stores, which inhibits CYP450 activity and thus reduces membrane permeability to $\text{Ca}^{2+}$. In ECs, inhibitor of CYP450 mono-oxygenase inhibited acetylcholine (Ach)-induced changes in $[\text{Ca}^{2+}]$, as well as Ach-induced hyperpolarization [92] and it was hypothesized that the endothelium-derived hyperpolarizing factor released from coronary ECs in response to bradykinin could be a CYP450 metabolite of arachidonic acid. CYP450 was suggested to play an important role in the regulation of endothelial SOC when CYP450 MO was shown to be activated by $\text{Ca}^{2+}$ store depletion and CYP450 MO produced compounds to hyperpolarize ECs [93].

6.2.6. Protein tyrosine kinase

Information from the depleted internal stores perhaps can also be transferred to the plasma membrane through protein–protein interactions. It has been postulated that some protein kinases or phosphatases in the cytosol might be capable of changing the phosphorylation state of $\text{Ca}^{2+}$ entry channels. The bulk of evidence for involvement of protein kinases in the regulation of CCE has come from biochemical experiments using kinase inhibitors. Protein kinase C activation was shown to have an inhibitory effect on CCE in different cell types [94,95]. In porcine aortic ECs, however, neither strong inhibitors of protein kinase C nor PKA inhibitor peptide had any effect on agonist-induced $\text{Ca}^{2+}$ entry [39]. Many studies have implicated tyrosine phosphorylation in the store depletion–membrane entry coupling process [96–98]. In ECs, the role of tyrosine phosphorylation in the regulation of agonist-induced $\text{Ca}^{2+}$ entry has also been well-documented [99]. Thus, bradykinin, histamine and thapsigargin induced tyrosine phosphorylation of a 42/44 kDa mitogen-activated kinase, which was dependent on extracellular $\text{Ca}^{2+}$ concentration and was inhibited by pretreatment with genistein [100]. The inhibition of the thapsigargin-induced $\text{Ca}^{2+}$ entry and tyrosine phosphorylation lends good proof to the role of tyrosine kinase in agonist-induced CCE in ECs, since some other stimuli may use tyrosine kinase-linked receptors to activate phospholipase C early in the signalling cascade and thus would make it difficult to narrow the inhibitory effects seen with tyrosine kinase inhibitors on the $\text{Ca}^{2+}$ responses down to inhibition of CCE alone. Cytoskeleton remodeling and cell shape change following capacitative $\text{Ca}^{2+}$ entry stimulated by fluid shear stress is also regulated by tyrosine kinase [101].

6.2.7. Myosin light chain kinase

In porcine aortic ECs, CCE provoked by both IP$_1$-dependent and -independent mechanisms was completely inhibited by strong inhibitors of myosin light-chain kinase (MLCK) (Fig. 1) [39]. In human platelets, wortmannin also inhibited significantly thrombin-induced $\text{Ca}^{2+}$ entry and MLC phosphorylation without affecting intracellular store release [102]. More recently, in porcine aortic ECs, MLCK inhibitors were demonstrated to inhibit agonist-induced $\text{Ca}^{2+}$ entry more strongly than did tyrosine kinase inhibitors, whereas the opposite was true for agonist-induced Mn$^{2+}$ influx [103]. Further implication of MLCK in capacitative $\text{Ca}^{2+}$ entry in ECs came as various specific MLCK inhibitors were shown to inhibit agonist-induced $\text{Ca}^{2+}$ entry according to their potencies to inhibit the enzyme: agonist-induced $\text{Ca}^{2+}$ entry was correlated with diphosphorylation of myosin light chain by MLCK; and shear stress-induced $\text{Ca}^{2+}$ entry was also completely prevented by MLCK inhibition (Fig. 2) [104]. These findings indicate that MLCK is probably a strong candidate for the control of CCE in vascular ECs regardless of the store depletion mechanism. Further substantiation of its role with methods other than use of kinase inhibitors is awaited.

6.2.8. Nitric oxide

Nitric oxide (NO) produced by ECs is a potent vasodilator that relaxes smooth muscle cells (SMCs) by increasing cytosolic cGMP. NO is known to inhibit $\text{Ca}^{2+}$ entry through L-type $\text{Ca}^{2+}$ channels in SMCs via cGMP-dependent mechanisms or via membrane hyperpolarization due to cGMP-dependent activation of $\text{Ca}^{2+}$-dependent K$^+$ channels. Interestingly, NO was recently shown to reduce agonist-induced $\text{Ca}^{2+}$ entry in SMCs by sequestering cytoplasmic $\text{Ca}^{2+}$ into intracellular stores via SERCA, the refilling of which takes away the trigger for CCE [105]. Thus, in SMCs, NO exerts dual effects of inhibiting both voltage-dependent and store-operated $\text{Ca}^{2+}$ entry pathways. The effects of NO on $\text{Ca}^{2+}$ signalling in other non-excitable cells are contradictory. In human platelets, NO was recently demonstrated to inhibit SOC by promoting refilling of $\text{Ca}^{2+}$ stores as in SMCs [106]. If such an action of NO also applied to ECs, there would be a logical
negative feedback mechanism since NO itself is produced from ECs under conditions that trigger SOC. Paradoxically enough, in porcine aortic ECs, exogenous NO was most recently shown to trigger Ca$^{2+}$ entry [107]. It is not yet known, however, if this entry is gated by store depletion and thus it is necessary to further delineate NO’s effect on intracellular Ca$^{2+}$ stores in ECs. Inhibitors of NO production were also shown to inhibit shear stress-stimulated Ca$^{2+}$ response in human aortic ECs [108]. These diverse effects of NO are interesting, as factors that regulate CCE in ECs have been shown to regulate endothelial NO production. Tyrosine kinase has been shown to regulate endothelial NO production through regulation of eNOS. Most recently, MLCK was shown to strongly control production of both NO and EDHF in porcine aortic ECs [109]. Further investigation is certainly required to clarify the role of NO in signalling in ECs.
7. Concluding remarks

Factors that regulate Ca\(^{2+}\) mobilization in ECs regulate many important EC functions, the production of NO mentioned above being a case in point. While the signalling events from ligand–receptor binding to activation of intracellular Ca\(^{2+}\) channels and store depletion are fairly well understood, the quest is still underway for a clear identification of the CCE channels as well as their gating mechanisms. So far most reports have described the actions of such regulating mechanisms (Fig. 3) on the product of the activation of CCE channels — the increase in \([\text{Ca}\(^{2+}\)]\). What is critical, now, that molecular structures of CCE channels are being brought to light, is to see how (for example, enzyme substrates) and where (binding sites) inositol-1,4,5-trisphosphate activates an endothelial Ca\(^{2+}\)-permeable channel. Nature 1992;355:356–358.

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