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Emerging Roles for Native ORAI Ca^{2+} Channels in Cardiovascular Disease

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Abstract

Orai proteins form highly calcium (Ca^{2+})-selective channels located in the plasma membrane of both non-excitable and excitable cells, where they make important contributions to many cellular processes. The well-characterized Ca^{2+} release-activated Ca^{2+} (CRAC) current is mediated by Orai1 multimers and is activated, upon depletion of inositol 1,4,5-trisphosphate (IP_3)-sensitive stores, by direct interaction of Orai1 with the endoplasmic reticulum (ER) Ca^{2+} sensor, stromal interaction molecule 1 (STIM1). This pathway is known as capacitative Ca^{2+} entry or store-operated Ca^{2+} entry (SOCE). While most investigations have focused on STIM1 and Orai1 in their store-dependent mode, emerging evidence suggest that Orai1 and Orai3 heteromultimeric channels can form store-independent Ca^{2+} selective channels. The role of store-dependent and store-independent channels in excitation-transcription coupling and the pathological remodeling of the cardiovascular system are beginning to come forth. Recent evidence suggests that STIM/Orai-generated Ca^{2+} signaling couples to gene transcription and subsequent phenotypic changes associated with the processes of cardiac and vascular remodeling. This short review will explore the contributions of native Orai channels to heart and vessel physiology and their role in cardiovascular diseases.

Introduction

Ion channels are membrane proteins with essential functions in a variety of physiological and pathophysiological situations. Defective ion channel function produces clinical syndromes typically termed channelopathies. Mammalian Orai1 proteins (mammals have three Orai genes, Orai1 through 3) are evolutionarily conserved calcium (Ca^{2+}) channels displaying a remarkably high selectivity for Ca^{2+} [1-4] The channelopathy resulting from Orai1 deficiency is primarily characterized by a severe combined immunodeficiency (SCID)-like phenotype attributed to an impairment in T-cell activation; other notable findings include congenital myopathy and anhidrotic ectodermal dysplasia with defective dental enamel calcification [5]. Mice Homozygous for Orai1 deficiency ($Orai1^{-/-}$) generated in a mixed ICR genetic background have a phenotype analogous to CRAC-deficient patients bearing the Orai1 homozygous mutation R91W. $Orai1^{-/-}$ mice in a pure inbred C57BL/6 background are characterized by perinatal lethality, presumably from defective skeletal

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muscle development [6-8]. Despite Orai1 channelopathy being mainly associated with defects of the immune system and skeletal muscle, there is emerging evidence that Orai1 channels (and their homologues) are important to homeostatic cell function as well as disease in almost every cell type, including cardiac and vascular tissues. Thus, the lack of obvious cardiovascular defects in Orai1 channelopathy would suggest that Orai channels likely become more relevant under stressful or pathophysiological conditions. As argued earlier, compensatory mechanisms by Orai2 or Orai3 in Orai1$^{-/-}$ mice are unlikely and all three Orai isoforms probably serve non-redundant functions [9, 10].

Orai channels

Store-operated Ca^{2+} (SOC) channels

A variety of growth factors, neurohormonal stimuli and inflammatory mediators induce store-operated Ca^{2+} entry (SOCE) activity. Ligation of agonists to G-protein coupled receptors (GPCR) or receptor tyrosine kinases activates isoforms of phospholipase C (PLC) enzymes, which convert phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 diffuses into the cytosol and depletes endoplasmic reticulum (ER) Ca^{2+} stores by opening IP_3 receptor (IP_3R) Ca^{2+} release channels. The drop in Ca^{2+} concentration within the ER lumen activates the single transmembrane ER-resident Ca^{2+}-sensor protein STIM1. When STIM1 loses Ca^{2+} from its Ca^{2+}-binding EF hand, it undergoes a conformational change that prompts STIM1 oligomerization in areas adjacent to the plasma membrane where its C-terminus achieves an extended conformation to directly gate CRAC channels formed by multimers of Orai1 [1, 3, 4]; this gating involves the binding of a minimal 100 amino acid C-terminal region of STIM1 (called SOAR for STIM/Orai activating region) to both the C- and N-terminus of Orai1 [11, 12]. A homozygous missense mutation in human Orai1, changing amino acid 91 from arginine to tryptophan (R91W) is responsible for defective SOCE observed in a subpopulation of patients with hereditary SCID [1]. Restoration of SOCE in immune cells isolated from these patients was achieved by ectopically expressing wild-type Orai1 [1]. Experiments involving mutagenesis of conserved acidic residues lining the putative pore region further strengthened the notion of Orai1 as an essential pore subunit of CRAC/SOCE [2, 13]. It was also realized that patients with deficient STIM1 function displayed a clinical phenotype similar to that seen in Orai1-deficient patients, including immunodeficiency, nonprogressive myopathy, and malformation of dental enamel [14]. CRAC channels deliver sustained Ca^{2+} signals essential for a multitude of cellular processes; Ca^{2+} delivered intracellularly through CRAC channels likely serves to replenish depleted internal stores, thus maintaining a relatively stable concentration of Ca^{2+} in the lumen of organelles, such as ER and mitochondria, essential for cell homeostasis. A more important function of CRAC is likely the generation of spatial and temporal Ca^{2+} gradients crucial for activation of specific cell signaling pathways and subsequent gene transcription to coordinate complex long-term cell functions such as cell secretion, migration, growth and proliferation [15-17]. Nuclear factor for activated T cells (NFAT) has been uniformly shown to depend on CRAC channel activity for its nuclear translocation and activity in a number of cell types [18].

There are two human homologues of *Drosophila* STIM (STIM1 and STIM2). Compared to STIM1, the function of STIM2 remains relatively less understood. STIM2 is a more sensitive Ca^{2+} sensor and appears to be a weaker activator of CRAC channels compared to STIM1 [19]. STIM2 was proposed to play a role in maintaining ER Ca^{2+} homeostasis [20]. However, STIM2 is likely endowed with broader signaling functions that remain to be elucidated. Members of mammalian Orai proteins (Orai1, Orai2, Orai3) have been identified based on their homology to the single *Drosophila* Orai gene [1]. While Orai1 deficiency gives rise to the SCID phenotype described above, channelopathies stemming from defects in either Orai2 or Orai3 have yet to be described. Using ectopic expression in HEK293 cells, all three Orai proteins were found capable of mediating SOCE when co-expressed with STIM1 [21, 22]. However, as argued recently [9], Orai1 consistently and exclusively encodes native CRAC channels in a number of different mammalian cell types despite co-expression of other Orai isoforms by these cells. Ectopic expression of Orai3 in lymphocytes and fibroblasts from SCID patients only weakly reconstituted SOCE while expression of Orai2 had no appreciable effect, suggesting lack of redundancy of Orai proteins, at least in these cell types[18]. There is however at least one reported exception where Orai3 mediates SOCE in a subset of breast cancer cells [23].

Given that Orai proteins lack any obvious homology to other families of ion channels, the structural and functional properties of CRAC channels are inherently unique and many efforts have been directed at understanding such features. CRAC channels are characterized by an extraordinarily high selectivity for Ca^{2+}, paralleled only by voltage-gated Ca^{2+} channels, and demonstrate poor permeability to Cs^{+} ions [24]. Further, CRAC channels exhibit an unusually long and narrow pore lined exclusively by residues on the predicted transmembrane domain 1 [25, 26]. These properties are noticeably absent when STIM1 gating of Orai1 channels is circumvented by mutations in Orai1 yielding a constitutive CRAC channel, indicating that STIM1 regulates ion selectivity and the pore architecture of native CRAC channels [27].

Another hallmark of CRAC channels is their rapid Ca^{2+}-dependent inactivation, whereby local elevations of cytosolic Ca^{2+} quickly diminish the amplitude of CRAC currents [28]. Interestingly, this phenomenon has also been attributed to a direct and specific interaction of Ca^{2+} and calmodulin with STIM1 [29, 30], further suggesting that STIM1 becomes an intimate component of Orai1 channel during CRAC activity. Lastly, CRAC channels are pharmacologically distinguished by a high sensitivity to blockade by lanthanides mediated by residues glutamine108, aspartate110, and aspartate112 in human Orai1 located in the extracellular TM1-TM2 loop [13, 25]. Previous studies have suggested that functional CRAC channels exist in the plasma membrane as tetrameric complexes [11, 31, 32], with each Orai subunit containing four transmembrane segments and arranged such that both amino and carboxy termini are in the cytosol [1, 3]. Penna *et al* proposed that in the resting state Orai1 exists as a dimer, and upon channel activation assembles into functional tetramers [32]. All these previous studies are difficult to reconcile with the crystal structure of *Drosophila* Orai that was recently resolved at 3.35 angstroms, suggesting that drosophila Orai proteins form hexameric structures [33].

Store-independent Ca^{2+} channels

With native CRAC channels being preferentially composed of Orai1 subunits, it is not surprising that Orai3 (and perhaps Orai2) would contribute to Ca^{2+} selective currents, either as homomultimeric or heteromutlimeric channels that are activated by mechanisms distinct from store depletion [34, 35]. One particularly well-characterized store-independent Ca^{2+} channel is the arachidonic acid activated Ca^{2+} (ARC) channel that is formed by Orai1 and Orai3 [35]. Like CRAC channels, ARC channels are similarly Ca^{2+} selective and share many of the biophysical properties of CRAC, including dependence on STIM1 for their activation [36, 37]; however, the pool of STIM1 involved was suggested to be the one residing at plasma membrane [37]. Based on experiments using concatenated multimers of Orai, ARC channels were proposed to involve a pentameric assembly of two Orai3 and three Orai1 molecules [34]. We have recently identified a native ARC-like channel in vascular smooth muscle cells (VSMC; discussed below) that is encoded by both Orai1 and Orai3 subunits. This channel is store independent, requires STIM1 for activation and is gated by cytosolic leukotrieneC_4 (LTC_4) [38]. We have termed this channel LRC (pronounced *Lark*; for LTC_4-regulated Ca^{2+} channel). To date, the existence of native store-operated or store-independent Ca^{2+} ionic currents contributed by Orai2 in any cell type remains unknown.

Orai channels in the heart

The mechanical function of the heart is dependent on a continuous alternation between contraction and relaxation of cardiac muscle fibers. Myocardial inotropy is governed by temporally and spatially controlled cytosolic Ca^{2+} sparks that can be finely tuned to regulate cardiac output and blood pressure [39]. In excitation-contraction coupling, membrane depolarization opens voltage-gated Ca^{2+} (CaV) channels clustered on plasma membrane invaginations. Due to their spatial proximity with the sarcoplasmic reticulum (SR), these channels direct Ca^{2+} ions onto nearby ryanodine receptor (RyR) Ca^{2+}-release channels and induce Ca^{2+} release from the SR into the cytosol to turn on actin-myosin cross bridge cycling [39, 40].

Human patients with Orai1 deficiency do not exhibit any obvious defects in cardiac function, but as mentioned above skeletal myopathy is a prominent clinical feature [41, 42]. SOCE has been shown to be an important regulator of skeletal muscle contractility, although the contribution of SOCE to either cytosolic Ca^{2+} or Ca^{2+} store refilling during contractility of skeletal muscle is a contentious issue [43]. CRAC channels can be rapidly activated upon store depletion of skeletal muscle, yet SOCE does not become relevant until Ca^{2+} stores are substantially reduced [44, 45]. Under physiological conditions, such a state is infrequently achieved, as skeletal muscle is equipped with proteins that minimize store depletion including Ca^{2+}-ATPases, allowing for rapid re-uptake of released Ca^{2+}, as well as highly dynamic Ca^{2+} buffering proteins within the SR lumen (i.e. calsequestrin) [46]. This would suggest that Ca^{2+} entry via CRAC channels is likely involved in adaptive signaling required for maintaining muscle fiber homeostasis during development, growth or prolonged muscle work [42]. Supporting this idea, isolated skeletal muscle from STIM1-deficient mice fatigued earlier and generated less force upon high frequency stimulation [45]. Very little is known about how CRAC/SOCE contributes to contraction in other excitable cells including

cardiomyocytes. Recently, SOCE was demonstrated in HL-1 cells, a useful *in vitro* model for investigating Ca^{2+} handling in unstressed cardiomyocytes [47]. Orai1 knockdown in this cell type not only prevented SOCE, but also lowered baseline Ca^{2+} levels. These authors proposed that CRAC channels might function in healthy cardiomyocytes to replenish Ca^{2+} stores and maintain intracellular Ca^{2+} levels over time [47]. While functional CRAC channels are found in both neonatal and adult hearts, it is unlikely that SOCE is significantly involved in cardiac excitation-contraction coupling. The magnitude of Ca^{2+} entry in postnatal mouse cardiomyocytes is reportedly half of what has been observed in embryonic cells, and the detection of SOCE in adult mice has been variable [48, 49]. Likewise, the expression of Orai1 dramatically decreases after birth [50]. CaV channels are coincidentally upregulated [51], and it was recently proposed that activated STIM1 impedes CaV channel conductance in VSMC [52]. Indeed, STIM1 knockdown in neonatal rat ventricle cardiomyocytes (NRCM) lowered diastolic Ca^{2+} levels and also diminished SR Ca^{2+} content, while silencing Orai1 expression did not alter these parameters [17]. However, as will be discussed below, several studies have proposed a role for STIM1/Orai1 signaling complexes in altered Ca^{2+} signaling underlying the development of cardiac hypertrophy.

As terminally differentiated cells, cardiomyocytes are largely incapable of cell proliferation. Hypertrophic growth is the primary mechanism by which the heart is able to preserve pump function and maintain adequate cardiovascular support when faced with an increased workload; by increasing wall thickness such growth necessarily decreases stress on the ventricular walls [53]. Cardiac growth during pregnancy or endurance training is transient and occurs without impact on myocardial structure or function [53]. On the other hand, hypertrophy from different pathologic stimuli, including hypertension, coronary insufficiency or valvular defects, is associated with transcription programs that alter myocardial architecture and invariably lead to cardiac dysfunction [54]. The molecular mechanisms underlying the development of physiological and pathological cardiac hypertrophy may be overlapping but are clearly distinct and remain incompletely understood.

Several agonists that converge on common intracellular signaling pathways have been implicated in the activation of hypertrophic gene programs. For example, endothlin-1, angiotensin II and catecholamines were proposed to activate the calcineurin-NFAT and Ca^{2+}/calmodulin-dependent Kinase II (CaMKII) pathways to induce the molecular stress fetal gene program linked to pathologic growth and remodeling. Calcineurin is a Ca^{2+}-dependent serine/threonine phosphatase activated by calmodulin as well as structural proteins localized in the z-disc [55]. Prolonged increases in cystosolic Ca^{2+} levels maintain calcineurin activation, and in turn keep NFAT transcription factors localized to the nucleus where they induce pro-hypertrophic gene expression [56]. Similarly, CaMKII contributes to hypertrophic gene expression downstream of spatial Ca^{2+} signals by phosphorylating type II histone deacetylases (HDAC), prompting HDAC exportation from the nucleus and relieving MEF-2-mediated gene expression [57] (Figure 1). However, the sources of Ca^{2+} responsible for the activation of these Ca^{2+}-dependent signaling circuits are not clear, remain the subject of intensive investigations, with multiple channels being implicated in this process (Figure 1). Ca^{2+} elevations required for transcriptional activation may be mediated by Ca^{2+} release from internal stores or influx across the plasma membrane, with evidence supporting

involvement of Ca^{2+} release through cardiac RyR [58] and IP_3R [57, 59], and Ca^{2+} influx through CRAC channels [17], TRPC channels [60] and CaV channels [61].

In NRCM, SOCE activity has been detected upon treating cells with either the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin or IP_3-generating agonists such as angiotensin II and endothelin-1 [62]. Further, these hypertrophic stimuli were shown to promote NFAT nuclear translocation [62]. Agonist-induced Ca^{2+} elevations and hypertrophic gene expression were more strongly inhibited when pretreating cells with either glucosamine or SKF96365, both nonselective inhibitors of SOCE, than with the CaV channel antagonist verapamil [62]. Orai1 and STIM1 have since been demonstrated as essential elements of SOCE in cardiac cells [17, 63]. In an *in vitro* model of cardiac hypertrophy, silencing either STIM1 or Orai1 in cultured NRCM attenuated the phenylephrine-induced hypertrophic response [17, 63]. While protein expression of either Orai2 or Orai3 was not observed in NRCM, Orai2 mRNA level was significantly increased in Orai1 siRNA-treated cells [17]. Hulot *et al* showed enhanced SOCE in hypertrophic cardiomyocytes [64]. STIM1 expression is elevated in pressure-overloaded hearts, and silencing STIM1 *in vivo* using cardiotropic viruses prevented hypertrophic responses following abdominal aortic banding [64]. Consistently, overexpressing STIM1 in NRCM enhanced NFAT activity and cell size. However, in the presence of SKF96365, STIM1 overexpression did not induce cellular hypertrophy [64], compatible with the idea that CRAC channel function mediates pathologic cardiac growth. Not surprising, pressure overload in mice induces upregulation of Orai1 in cardiomyocytes [50]. Recently, Volkers *et al* examined Orai1 in zebrafish development [50]. Injection of zebrafish zygotes with Orai1 antisense oligonucleotides resulted in severe impairments in both skeletal and cardiac muscle function. The cardiac phenotype in this model was characterized by severe heart failure, though no effect on initial myocyte differentiation was reported. The histological findings of cardiomyocytes from Orai1-deficient zebrafish included dramatic alterations in sarcomeric structure, with fewer myofilaments and variable z-disks, suggesting that Orai1 channels are necessary for proper sarcomeric growth and function in cardiac cells [50]. The absence of a comparable cardiac deficit in human patients and mice lacking Orai1 may be attributed to the presence of Orai3 in mammals [65].

Orai channels in vessels

The principle cellular constituents of blood vessel walls are VSMC and endothelial cells (EC). VSMC are most abundant in the tunica media, particularly in the case of muscular arteries and arterioles. While VSMC are maintained in a partially constricted state, moment by moment regulation of intracellular Ca^{2+} concentrations enables rapid adjustments to vascular tone essential for hemodynamic stability [66]. Although VSMC infrequently divide, they lose their homeostatic quiescence with arterial injury or inflammatory activation [67-69]. Proliferation and migration of VSMC is a key step in the pathogenesis of vascular occlusive diseases such as arterial stenosis in atherosclerosis, neointimal hyperplasia following angioplasty or stent placement, and arteriolar remodeling in hypertension. Similarly, VSMC in the pulmonary circulation adopt this pathologic or “synthetic” phenotype to contribute to vascular remodeling in pulmonary hypertension [70]. EC are the main constituents of the intimal layer of blood vessels, and participate in a wide variety of

functions. In addition to forming a selective barrier for cellular and nutrient trafficking, the endothelium also modulates local blood flow and vascular tone, and offers resistance to oxidative stress, inflammation, thrombosis, hemostasis, and VSMC proliferation [71]. Importantly, the EC phenotype is remarkably diverse. Not only do EC display significant structural and functional heterogeneity throughout the cardiovascular system [72], but like VSMC, with the right environmental cues, EC will adopt a pathological phenotype distinguished by the ability to migrate and proliferate [73]. Understanding the regulation of signaling pathways that govern phenotype switching in VSMC and EC is important to the development of targeted therapies against cardiovascular disease.

Endothelial cells

The growth of new vessels or angiogenesis occurs either from preexisting blood vessels or form endothelial precursor cells (EPC) originating in the bone marrow, and is necessary for replacing damaged tissues resulting, for instance, from tissue hypoxia [73]. However, angiogenesis also contributes to the development of vascular networks that support tumor growth and metastasis [73]. EC proliferation and migration is induced by activation of signaling cascades in response to various growth factors, most notably vascular endothelial growth factor (VEGF) [73]. Early studies showed that activation of VEGF receptor triggers Ca^{2+} release from IP_3-sensitive stores and induces a low Ca^{2+} conductance across the plasma membrane necessary for EC proliferation [74, 75]. Electrophysiological characterization of this conductance was initially problematic given its relatively low and often variable amplitude in endothelial cells. Fasolato and Nilius were first to provide evidence that EC activate a small inward rectifying CRAC-like current in response to store depletion by either IP_3, thapsigargin or Ca^{2+} ionophores [76]. Subsequently, Abdullaev *et al* showed a requirement for Orai1 and STIM1 in mediating CRAC currents in human umbilical vein EC (HUVEC) after passive store depletion, and that CRAC channels contributed to EC proliferation [77]. Inhibition of Orai1 function by either siRNA, expression of a dominant negative Orai1 mutant or the CRAC channel blocker S66 prevented endothelial tube formation *in vitro* [78]. However, these results have been challenged by experiments using primary HUVEC, which showed involvement of STIM1 and transient receptor potential canonical channel isoforms, TRPC1 and TRPC4, but not Orai1, in endothelial tube formation [79], suggesting a dissociation between STIM1 and Orai1 in tubulogenesis. Furthermore, knockdown of either STIM1 or STIM2 had only a modest inhibition of EC proliferation relative to that observed with Orai1 knockdown [77]. Furthermore, normal endothelial migration and vasculogenesis was reported in EC-specific STIM1-knockout mice [80].

EPC are present in postnatal circulation where they can proliferate, migrate, and adopt a mature endothelial phenotype [81]. They incorporate into vascular networks associated with tumors, help in the formation of *de novo* blood vessels and mediate improvements in blood flow to ischemic regions [82, 83]. Sanchez-Hernandez *et al* provided the first evidence of SOCE expression in EPC [84]. Further characterization of SOCE in this cell type revealed that stimulation with hepatocyte growth factor enhanced STIM1-dependent SOCE activity [85], with STIM1 being required for EPC migration and proliferation [86]. VEGF-treated EPC displayed IP_3-dependent Ca^{2+} oscillations that were mediated through SOCE, and these

signals were necessary for the recruitment of NF-κB [87]. These data suggest CRAC channel involvement in EPC Ca^{2+} signaling pathways governing cell proliferation and tubulogenesis. Importantly, Orai1 channel function has recently been linked to EPC-mediated angiogenesis in patients with renal cell carcinoma [88]. The majority of these tumors arise from functional inactivation of von Hippel-Lindau tumor suppressor, which leads to elevated levels of pro-angiogenic hypoxia-inducible factors (HIF) [89]. EPC isolated form the peripheral blood of patients with renal cell carcinoma were found to have increased expression in both Orai1 and STIM1 in comparison to control EPC, with concordant enhancement of SOCE activity [88]. Pharmacological inhibition of CRAC channel activation using either lanthanides or BTP2 impaired proliferation and *in vitro* tubulogenesis [88]. It is important to note that in EPC from renal cell carcinoma, VEGF failed to elicit Ca^{2+} oscillations despite normal expression levels of VEGF receptor; the authors proposed CRAC channel activation may be dependent on alternative physiological agonists in this cell type [88].

EC activation is an adaptive response employed in a variety of physiological and pathological situations. However, when the activation of EC is exaggerated and generalized, as is the case in sepsis and the systemic inflammatory response syndrome (SIRS), endothelial dysfunction follows leading to organ failure and potentially death [90]. An important stimulus of endothelial dysfunction is the bacterial endotoxin, lipopolysaccharide (LPS), which modifies EC phenotype in an organ-specific manner [90]. LPS binding to EC surface receptors, including toll-like receptor 4, activates signaling cascades that can lead to enhanced coagulation, increased vessel permeability, recruitment of inflammatory cells, secretion of vasoactive substances and apoptosis [90]. Ca^{2+} influx through CRAC channels is suggested to be an important step in the LPS-signaling cascade. The mechanism proposed involves production of reactive oxygen species (ROS) by EC exposed to LPS; ROS would then activate PLC to trigger release of IP_3-sensitive Ca^{2+} stores causing subsequent activation of SOCE [80, 91]. SOCE would mediate its effects by providing sustained Ca^{2+} entry necessary for sustaining Ca^{2+}-dependent transcriptional activation, namely NFAT transcriptional programs [80]. Additionally, ROS can engage STIM1 independently of Ca^{2+} store-depletion [92]. Through this pathway, ROS was proposed to cause S-glutathionylation of an evolutionary conserved cysteine residue (cysteine 56) on STIM1, resulting in constitutive STIM1 and CRAC channel activation. While the significance of this redundancy remains unclear, these studies highlight a critical role for STIM1 in EC activation. Indeed, LPS do not elicit a significant inflammatory response in EC-specific STIM1-knockout mice in comparison to wild-type mice. EC isolated from STIM1-knockout mice failed to trigger Ca^{2+} oscillations or activate NFAT-mediated transcription in response to LPS [80].

Vascular smooth muscle cells

The contribution of SOCE to VSMC contractility is a contentious issue. A number of studies have proposed a role for SOCE in VSMC contractility based on the use of non-specific SOCE inhibitors such as 2-Aminoethoxydiphenyl borate (2-APB) and lanthanides [93-95]. We and others have reported minimal SOCE/CRAC activity and STIM1/Orai1 protein expression in contractile freshly isolated VSMC by comparison to cultured proliferative synthetic VSMC, consistent with a minimal role for SOCE in VSMC contractility [16, 38,

96, 97]. However, the contribution of CRAC channels to vascular tone may be accomplished through their role in EC-mediated vasorelaxation by delivery of Ca^{2+} signals important for nitric oxide (NO) production in vascular EC [98]. Vasoactive agonists initiate NO-dependent vasodilation by elevating cytosolic endothelial Ca^{2+} levels and causing activation of endothelial NO synthase [99]. In fact, STIM1 suppression in porcine aortic EC inhibited SOCE, and was associated with a partial reduction in NO production induced by thrombin [100]. Additionally, a reduction in SOCE activity in mouse aortic EC was associated with impaired acetylcholine-induced vessel relaxation [101]. In diabetic mice, hyperglycemia leads to down regulation of STIM1 in EC with attenuation in coronary vascular relaxation, and restoration of STIM1 levels can recover endothelium-dependent relaxation [102].

Dysfunction of the endothelial monolayer is a common inciting event in the development of vascular occlusive diseases, activating inflammatory cells and platelets that result in the local release of a plethora of chemokines, growth factors, and vasoactive molecules [103]. Importantly, these agonists contribute to the phenotypic modulation of VSMC by activating signaling cascades that decrease expression of contractile proteins while upregulating extracellular matrix macromolecule secretion characteristic of VSMC in stenosed vessels [103]. A similar phenotypic modulation is observed when VSMC are cultured *in vitro* in the presence of serum [104], providing a useful model for the study of proliferating mitogenic synthetic VSMC. In culture, VSMC display enhanced SOCE in comparison to freshly isolated cells [16, 38, 96, 97]. Synthetic VSMC increase expression of STIM1 and Orai1, and knockdown experiments confirmed a requirement for STIM1/Orai1 in mediating SOCE in these cells [96, 97]. Higher expression of Orai1 was demonstrated in aortas of stroke prone hypertensive rats [105] and in neointimal VSMC from either balloon-injured rat carotids or ligation-injured mouse carotids [16]. Inhibition of either STIM1 or Orai1 expression *in vivo* using specific shRNA encoded by viral vectors lead to impaired CRAC channel function and significantly hindered VSMC proliferation and neointima formation [16, 106-108], implicating Orai1 as a modulator of VSMC phenotype. Orai1 channels appear to be important in sustaining Ca^{2+} entry required for NFAT nuclear translocation and its continued activation in VSMC proliferation and migration [16].

Interestingly, while Orai1 was detectable in cultured human aortic VSMC, it was relatively low in comparison to abundantly expressed Orai2 and Orai3 when normalized to expression levels in lymphocytes [97]. Orai2 and Orai3 expression was also increased in aortic synthetic VSMC in culture compared with contractile cells [38, 95]. However, silencing either Orai2 or Orai3 *in vitro* in synthetic VSMC did not affect the amplitude of SOCE, suggesting that CRAC channels contain only Orai1 subunits in these cells [96]. The expression of Orai3 was enhanced in VSMC following carotid injury and *in vivo* knockdown of Orai3 significantly inhibited neointima formation [38], indicating that Orai3 contributes to additional channels (discussed in detail below) or signaling pathways that promote VSMC synthetic phenotypes.

As noted above, multiple agonists have been implicated in driving VSMC proliferation and migration during vascular remodeling. Paracrine release of platelet-derived growth factor (PDGF) by EC, macrophages and VSMC induces VSMC migration, whereas promotion of intimal proliferation appears to be a secondary phenomenon [109]. Indeed, multiple reports have shown that treatment with PDGF receptor inhibitors significantly attenuates neointima

formation in animal models of restenosis [110-112], though the clinical utility of such therapeutics has yet to be achieved. Ligation of PDGF to its receptor induces cytosolic Ca^{2+} transients [113]; Bisaillon *et al* showed PDGF-induced Ca^{2+} entry is specifically contributed by SOCE through STIM1 and Orai1 [106] (Figure 2A). STIM1 and Orai1 are essential for migration of VSMC and airway smooth muscle in response to *in vitro* stimulation with PDGF [106, 114].

Thrombin serves as a physiological agonist for proteinase-activated receptors (PAR) on both EC and VSMC. Although the expression of these receptors on VSMC in healthy arteries is limited, in vascular lesions there is an upregulation of PAR [115]. Thrombin simulation is important in mediating VSMC contraction, migration, proliferation, hypertrophy and production of extracellular matrix [116]. As such, thrombin signaling in VSMC is considered an important contributor to the pathogenesis of vascular occlusive disease. *In vitro* stimulation of synthetic VSMC with thrombin activated a novel Ca^{2+} entry pathway distinct from SOCE/CRAC. Surprisingly, thrombin-activated Ca^{2+} channels were highly Ca^{2+} selective and required STIM1, Orai1 and Orai3 [38] in manner similar to the arachidonic acid (AA)-regulated Ca^{2+} (ARC) channels [35, 37]. Indeed, despite the requirement for STIM1, this novel thrombin-activated channel was store-independent and was instead, gated by leukotrieneC_4 (LTC_4) produced downstream of receptor activation and acting from the cytosolic side (Figure 2B) [38]. Whether these channels functionally couple to STIM1 residing on the ER or STIM1 on plasma membrane remains to be determined. Thus, thrombin-activated LTC_4-regulated Ca^{2+} (LRC) channels are the first example of a native store-independent Orai channel in the vasculature activated by a physiological agonist. As previously mentioned, in EC thrombin has been shown to activate store-dependent CRAC channels [77]. Taken together, these findings highlight the diversity of Ca^{2+} signaling and suggest that not only, distinct Ca^{2+} signals can be generated by distinct agonists in the same cell type but that, the same agonist can generate distinct Ca^{2+} entry routes depending on the cell type in question.

In contrast the systemic circulation, pulmonary vessels are not predisposed to becoming hypertensive. However, pulmonary hypertension can occur in the setting of cardiac or pulmonary diseases. Less commonly, pulmonary hypertension arises from intrinsic defects in the pulmonary bed, a rare disease termed idiopathic pulmonary arterial hypertension (IPAH). Proliferation of pulmonary arterial smooth muscle cells (PASMC) is a key step the pathogenesis of IPAH, contributing to the development of pulmonary vascular remodeling, thrombosis, sustained vasoconstriction and subsequent occlusion of pulmonary flow [117]. Studies have shown that SOCE is elevated in PASMC from IPAH patients, and serves as an essential mediator PASMC proliferation [118]. Characterization of SOCE in cultured mouse PASMC revealed that Orai1 channels were responsible for this STIM1-dependent Ca^{2+} conductance [119], and treatment of PASMC with PDGF enhanced SOCE by upregulating STIM1 and Orai1 through the Akt/mTOR signaling pathway [120]. However, Song *et al* found that protein expression of STIM2 was enhanced in PASMC from IPAH patients, whereas STIM1 expression was not significantly changed, suggesting that STIM2 may be important in enhancing SOCE in these cells [121]. STIM2 was shown to be necessary for mediating SOCE and proliferation in IPAH PASMC. Interestingly, Orai2 expression was

also enhanced in PASMC from IPAH patients, though the exact significance of Orai2 upregulation is unknown [121].

Conclusion and Perspective

STIM and Orai proteins are important regulators of intracellular Ca^{2+} homeostasis, making pivotal contributions to the Ca^{2+} signaling mechanisms that support the development of cardiac and vascular disease. As such, there is a heightened interest in targeting Orai channels to treat diseases such as cardiac hypertrophy, atherosclerosis and restenosis. One obstacle with CRAC channel-directed therapies is the absence of specific channel blockers; understanding the molecular details of STIM/Orai structures and interactions and CRAC channel gating will hopefully help in the development of improved small molecule inhibitors. Additionally, the ubiquity of CRAC channels and its prominence in the immune system naturally impedes cell type-specific targeting of these channels for treatment of cardiovascular disease. The recognition of cell type-specific differences in CRAC channel molecular organization and modulation may help in the specific targeting of Orai-mediated Ca^{2+} signaling in a given system or organ. Likewise, the identification of important cardiovascular processes that depend on Ca^{2+} currents activated by alternative mechanisms other than store depletion and utilizing other Orai isoforms, whether as homomultimers or in heteromultimeric assemblies of two or more Orais, might hopefully provide newer and perhaps better targets for treatment of cardiovascular disease.

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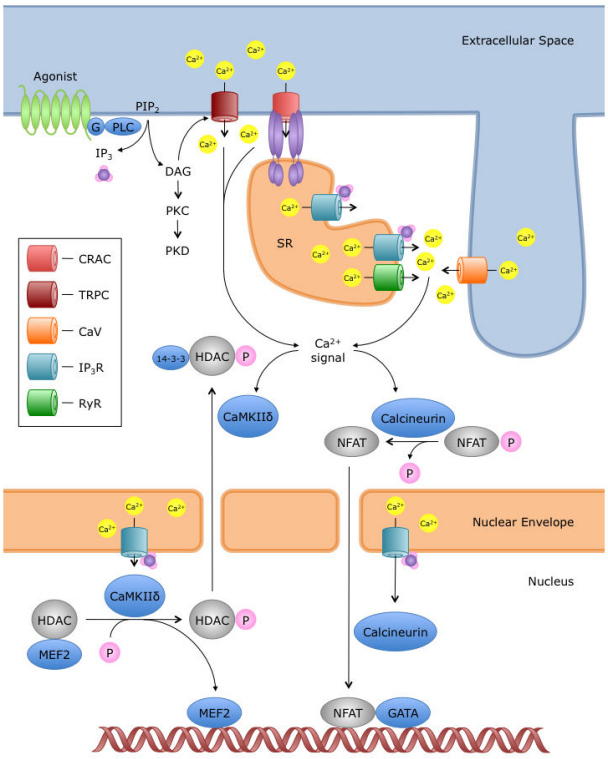

Figure 1. Ca^{2+}-dependent induction of pathologic cardiac gene expression

In cardiomyocytes, Ca^{2+} elevations are achieved by a variety of channels to cause Ca^{2+}-dependent transcriptional activation. On the plasma membrane, agonists binding to G-protein coupled receptors cause IP_3 and DAG production. DAG is a direct activator of TRPC3/6/7 channels. IP_3, on the other hand, diffuses within the cell and binds to calcium release channels, the IP_3 receptors; Ca^{2+} from SR stores can activate Ca^{2+} entry via CRAC channels by causing STIM1 clustering, as well as alter Ca^{2+} flux through RyR and CaV channels in the dyadic junctions [59]. These cytoplasmic Ca^{2+} signaling events may initiate calcineurin- and/or CaMKII-dependent transcription. Similarly, IP_3 has been shown to bind IP_3 receptors on the nuclear envelope, inducing Ca^{2+} transients that activate nearby calcineurin and CaMKII [57]. The dual compartmentalization of Ca^{2+} signals serves to initiate and maintain activation of prohypertrophic transcription regulators, including nuclear factor of activated T cells (NFAT) and histone deacetylase (HDAC). Abbreviations of Ca^{2+} channels: CRAC, Ca^{2+} release-activated Ca^{2+} channel; TRPC, Canonical transient receptor potential channel; CaV, voltage-activated L-type Ca^{2+} channel; IP_3R, Inositol 1,4,5-trisphosphate receptor; RyR, Ryanodine Receptor.

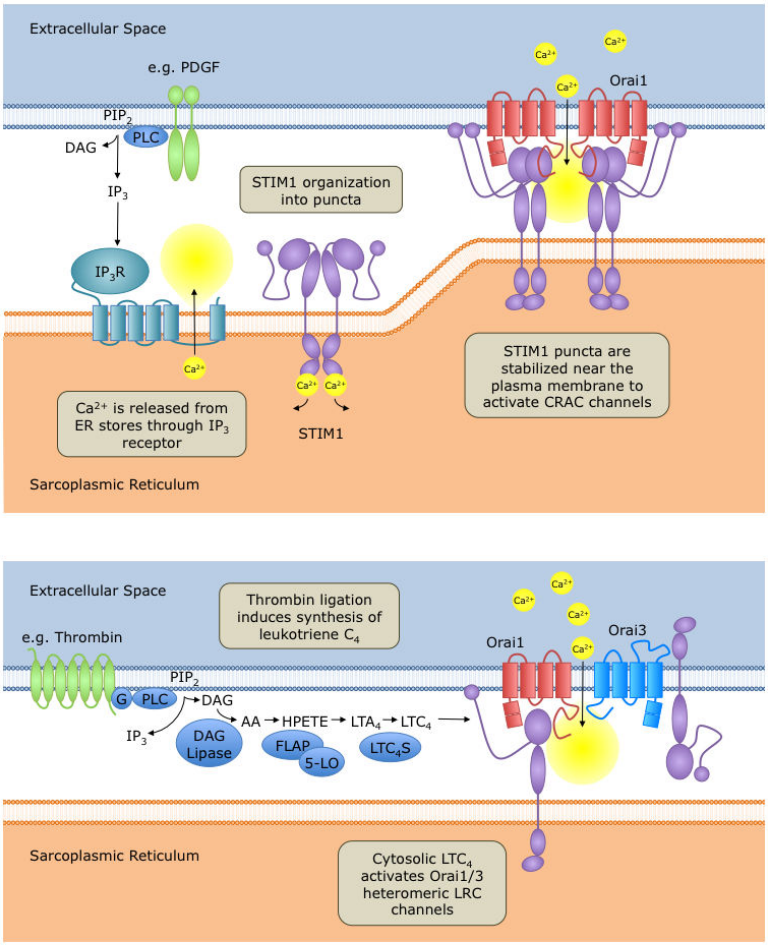

Figure 2. Orai channel activation in VSMC

The promigratory and mitogenic agonists (**A**) PDGF and (**B**) thrombin activate Orai-dependent store-operated (PDGF) [106] or store-independent (thrombin) [38] Ca^{2+} entry in VSMC. In **B**, the involvement of either ER or plasma membrane STIM1 pools, the oligomeric state of functional STIM1 proteins and the nature of the interactions between STIM1 and Orai1/Orai3 LRC channels are unknown. Abbreviations of enzymes: DAG lipase, Diacylgercerol lipase; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; LTC_4S, LeukotrieneC_4 synthase