Orai channel-mediated Ca\(^{2+}\) signals in vascular and airway smooth muscle

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Orai Proteins in Smooth Muscle

Mammalian Orai proteins are a class of Ca\(^{2+}\)-selective plasma membrane channel proteins made up of three homologs, Orai1, Orai2, and Orai3, encoded by three independent genes (77). Invertebrates have only one single Orai gene; all vertebrates possess two Orai genes, Orai1 and Orai2; the Orai3 gene is exclusive to mammals. Orai1 is expressed in human embryonic kidney (HEK-293) cells as two different isoforms: a long form, Orai1\(\alpha\), containing an additional NH\(_2\)-terminal 63 amino acids, and a short form, Orai1\(\beta\) (Fig. 1). These Orai1 isoforms are the result of alternative translation-initiation sites within the Orai1 message; the long-form, Orai1\(\alpha\), is exclusive to mammals (26).

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mRNA of all three Orai homologs is ubiquitously expressed, including expression in vascular and airway SMCs from different species, including humans. Protein expression levels in contractile vascular SMCs obtained from human or rat aorta are negligible (5, 67). However, in cultured aortic SMCs, which are reminiscent of synthetic dedifferentiated SMCs found in disease states, expression levels of STIM1, Orai1, Orai2, and Orai3 are dramatically increased (5, 6, 30, 67). Expression levels of Orai1α and Orai1β have not been characterized in SMCs, but such studies are warranted, as these isoforms have been shown to have distinct signaling and regulatory properties (17).

Orai1 contains four transmembrane domains (23), with its NH2- and COOH-terminal domains residing in the cytosol (69) (Fig. 1). The use of myc-tagged Orai1 at the NH2- or COOH-terminal end confirmed Orai1 plasma membrane (PM) localization. In vascular SMCs, Orai1 colocalizes with the well-known PM marker wheat germ agglutinin (6). Similarly, when Orai2 and Orai3 proteins are ectopically expressed, they locate at the PM (32) through their four highly conserved transmembrane domains, TMD1, TMD2, TMD3, and TMD4 (gray) with extracellular loops between TMD1 and TMD2 and between TMD3 and TMD4. TMD1 and the first extracellular loop contain a highly conserved pore region with a conserved "E" residue (red) representing the selectivity filter. The intracellular loop contains residues 153–157, proposed to mediate fast Ca2+-dependent inhibition (blue line) (82). The COOH-terminal region contains a coiled-coil domain, required for stromal-interacting molecule 1 (STIM1) binding and gating of Orai channels (pink). Orai1 contains a distinct proline-rich NH2-terminal domain (peach) from M1 to P17. The conserved COOH-terminal domain (blue line) spans E86–R91. Orai1 contains a conserved COOH-binding domain (blue line) spanning E86–R91. Orai1 also has 4 conserved transmembrane domains (TMD1, TMD2, TMD3, and TMD4), a conserved pore residue at E106 (red), and a distinct glycosylation site within the second extracellular loop at N223 (blue). The shorter form of Orai1 (Orai1β) originates from an alternative initiation-translation of the Orai1 mRNA at methionine 63 (M63; blue) (17, 26). Orai1 contains a region shown to mediate interaction with the Ca2+-sensitive adenylate cyclase 8 (AC8; green line) (98), which contains 2 serine residues (S27 and S30; green), which are targets for phosphorylation by protein kinase C (34), and a putative caveolin-binding domain (purple line). Orai2, the smallest of the 3 isoforms, contains all conserved functional domains, with its Ca2+-binding domain spanning H42–R65, its pore-selectivity filter residue at E80, and its COOH-terminal coiled-coil domain spanning residues 221–Q252. The Orai3 Ca2+-binding domain spans residues Q43–R66; the homologous residue to E106 in Orai1 is E81. Orai3 contains a distinctly long extracellular loop (olive) between TMD3 and TMD4 and its COOH-terminal coiled-coil domain spanning residues S269–V295. Conserved residues among all 3 Orai homologs; conserved residues between 2 Orai homologs.
brane domains, which are highly conserved among all Orai homologs. Between these transmembrane domains are two extracellular loops: the first is shorter, with a large number of acidic residues (69) made up of nine conserved amino acids, including a glutamate residue [E106 (Orai1), E80 (Orai2), and E81 (Orai3)], a glutamine residue [Q108 (Orai1), Q81 (Orai2), and Q83 (Orai3)], and within Orai1 three aspartate residues (D110, D112, and D114) (96). Orai1:E106, in particular, and its homologs in Orai2 and Orai3 are critical for Ca\(^{2+}\) selectivity of Orai channels (69, 94, 101). Orai1 constructs where E106 was substituted for glutamine (E106Q) or alanine (E106A) yield a dead channel, and when E106 was mutated to a similar amino acid, aspartate (E106D), Orai1 channels lose their Ca\(^{2+}\) selectivity and mediate monovalent and divalent cation currents equally (69, 101), highlighting the importance of the size and charge of pore-forming domain structures in selectivity of this ion channel. The second extracellular loop, located between transmembrane domains 3 and 4, is much longer in Orai1. The cytosolic domains are also conserved among all Orai homologs. Residues within the NH\(_2\) terminus contain a Ca\(^{2+}\)/calmodulin (CaM)-binding domain first shown in Orai1 to mediate Ca\(^{2+}\)-dependent inactivation (CDI) of the channel (45, 56). Srikanth et al. (82) showed that five residues spanning 153–157 (NVHNL) within the intracellular loop of Orai1 also participate in the fast Ca\(^{2+}\)-dependent inactivation. Within STIM1, a string of acidic residues (475–483) that are COOH-terminal to the STIM-Orai-activating region (SOAR; residues 344–442) are required for mediation of CDI of Orai1 channels (16, 41, 56). Furthermore, recent data from our laboratory showed much more pronounced CDI in Orai1α than Orai1β channels (17); however, the molecular basis of this difference and the 63 NH\(_2\)-terminal residues within Orai1α that contribute to enhanced CDI remain unknown. Orai1 contains a distinct proline- and arginine-rich region in its NH\(_2\) terminus (8) that binds much more weakly to STIM1 than does the COOH-terminal region but was, nonetheless, proposed to regulate channel gating by STIM1 (102). The NH\(_2\) terminus contains a region involved in interaction with the Ca\(^{2+}\)-sensitive adenylate cyclase 8 (98) and contains two protein kinase C phosphorylation sites (34). A putative caveolin-binding domain is also located in the NH\(_2\) terminus (Fig. 1). The COOH-terminal domain of all three Orai homologs contains a conserved STIM-binding coiled-coil domain. The importance of this coiled-coil domain in gating Orai channels was first identified in Orai1 (25). This COOH-terminal coiled-coil domain is essential for Orai1 channel activation by virtue of its role in mediating binding to STIM1. Upon store depletion, a 100-amin acid region in STIM1, SOAR, interacts with the COOH-terminal coiled-coil domain of Orai1 to activate the Orai1 channel (54, 55, 62, 102). Within this COOH-terminal coiled-coil domain of Orai channels, Lee et al. (41) showed that three conserved glutamates in the COOH terminus of Orai2 and Orai3 (E233, E235, and E236 in Orai2; Fig. 1) mediate fast Ca\(^{2+}\)-dependent inactivation, which is more prominent in Orai2 and Orai3 than in Orai1. Interestingly, Frischauf et al. (25) reported that COOH-terminal coiled-coil domain probability varies between Orai homologs, with the lowest probability observed for Orai1 and a five- to six-times-greater probability for Orai2 and Orai3 COOH-terminal coiled-coil regions; this suggests a correlation between this coiled-coil probability and the interactions between different Orai isoforms and STIM1. A single-residue mutation in the Orai1 coiled-coil region (L273S) disrupted the interaction of Orai1 with the STIM1 COOH-terminal domain, while equivalent mutations in Orai2 or Orai3 supported moderate interaction with STIM1 and channel activation. Reciprocally, decreasing coiled-coil probability of the second coiled-coil region in the COOH terminus of STIM1 by mutation of one residue (L373S) abrogated activation of Orai1 while allowing partial activation of Orai2 and Orai3 (25).

SOCE in Smooth Muscle

Vascular smooth muscle. SOCE, a widespread means of regulated Ca\(^{2+}\) entry into cells from the extracellular space, was first introduced by Putney (71). PM receptors that couple to isoforms of phospholipase C (PLC) induce production of inositol 1,4,5-triphosphate (IP\(_3\)) (83). IP\(_3\) causes Ca\(^{2+}\) release from the endoplasmic reticulum (ER) through the IP\(_3\) receptor, and this ER Ca\(^{2+}\) store depletion is the signal that triggers activation of PM store-operated Ca\(^{2+}\) channels. SOCE can be activated pharmacologically by specific inhibitors of the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), such as thapsigargin and cyclopiazonic acid (CPA), which passively deplete internal Ca\(^{2+}\) stores without producing second messengers (86). The Ca\(^{2+}\) current mediating SOCE, Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (\(I_{\text{CRAC}}\)), was first identified in mast cells by whole cell patch-clamp recordings. \(I_{\text{CRAC}}\) is activated when store depletion is achieved by inclusion of Ca\(^{2+}\) chelators (alone or with IP\(_3\)) in the patch pipette; \(I_{\text{CRAC}}\) is an inwardly rectifying Ca\(^{2+}\)-selective current that has a reversal potential around +60 mV and is voltage-insensitive (35). After nearly two decades, STIM and Orai molecules were finally identified as the genuine molecular components of SOCE and \(I_{\text{CRAC}}\) (70). STIM and Orai proteins are also expressed in cells of the vasculature and mediate SOCE in endothelial cells and SMCs (1, 67, 75). STIM proteins are single-pass transmembrane proteins located in the ER membrane with an NH\(_2\)-terminal luminal low-affinity EF-hand. STIM proteins sense Ca\(^{2+}\) store depletion within the lumen of the ER, while Orai proteins are the store-operated Ca\(^{2+}\) channels at the PM. ER Ca\(^{2+}\) store depletion causes STIM molecules to accumulate at junctional ER-PM spaces, where they trap and gate Orai channels through physical interactions between the cytosolic COOH-terminal SOAR domain of STIM and the COOH-terminal coiled-coil domain of Orai; to interact with STIM, Orai channels diffuse to these regions of the PM closely associated with the ER.

The molecular identity of the PM SOCE channels in SMCs and in other cell types was a matter of intense debate over the past decades (for review see Ref. 68). With the discovery of STIM and Orai proteins as the bona fide molecular components of SOCE in Drosophila Schneider 2 cells and in mammalian lymphocytes (70), Peel et al. (64, 65) used siRNA knockdown to document roles for STIM1 and Orai1 in mediating SOCE in cultured human airway SMCs. Our laboratory provided evidence for the existence of the highly Ca\(^{2+}\)-selective store depletion-activated current \(I_{\text{CRAC}}\) in cultured rat vascular cells as well as airway SMCs. We established a role for STIM1 and Orai1 in mediating SOCE and \(I_{\text{CRAC}}\) in these cells in response to passive store depletion or agonist stimulation, namely, by
the SMC proproliferative promigratory agonist platelet-derived growth factor (PDGF) (6, 67, 81).

Studies from our laboratory used cultured proliferative migratory vascular SMCs (also referred to as synthetic cells), which represent a good in vitro model for dedifferentiated SMCs under pathophysiological conditions such as restenosis and atherosclerosis (60). Passive store depletion in synthetic vascular SMCs activated Ca\(^{2+}\) entry, which bears pharmacological features of the classical SOCE pathway, characterized in lymphocytes and HEK-293 cells (61), including inhibition by 30–50 μM 2-aminoethoxydiphenyl borate (2-APB), 50 μM ML-9, and relatively low concentrations (5 μM) of lanthanides (93). In the same study we showed that store depletion activated \(I_{\text{CRAC}}\) in cultured synthetic vascular SMCs (67). Knockdown of STIM1 or Orai1 inhibited SOCE and \(I_{\text{CRAC}}\) in these cells, while knockdown of Orai2, Orai3, and the transient receptor potential (TRP)-canonical (TRPC) channels TRPC1, TRPC4, and TRPC6 had no effect on SOCE or \(I_{\text{CRAC}}\). These findings are supported by other studies in a number of mammalian cells where native SOCE is mediated by STIM1 and Orai1, despite ubiquitous protein expression of Orai2 and Orai3 in all these cells (90). The only exception is a subset of estrogen receptor-positive breast cancer cells, where Orai3, along with STIM1, mediates SOCE (52, 53). TRP channels, especially isoforms belonging to the TRPC subfamily, have long been proposed to mediate SOCE in many cell types, including those of the vasculature (SMCs and endothelial cells) (29, 91). However, while there is evidence that certain isoforms of TRPC channels work closely and in concert with Orai channels, are regulated by STIM1 proteins, and fulfill nonoverlapping physiological roles (11, 13), the general consensus is that TRPC channels are activated secondarily to store depletion by receptor-mediated mechanisms, including Orai1-generated Ca\(^{2+}\) signals and lipid second messengers generated downstream from the PLC pathway (19).

SOCE measured by fura 2 imaging is upregulated in synthetic vascular SMCs compared with quiescent contractile SMCs, which were acutely isolated from vessels and studied within 6 h of isolation (67). This enhanced SOCE in synthetic SMCs correlated with increased protein expression of STIM1 and Orai1 compared with levels of these proteins in freshly isolated quiescent vascular SMCs. Knockdown of STIM1 and Orai1 using siRNA attenuated proliferation and migration in synthetic vascular SMCs (67). Inhibition of Orai1 was associated with a compensatory downregulation of Na\(^+\)/Ca\(^{2+}\) exchanger type 1 and PM Ca\(^{2+}\) pump isoform 1 that would, presumably, reduce Ca\(^{2+}\) extrusion. In this same study, immunohistochemistry revealed co-clustering of Orai1 and Na\(^+\)/Ca\(^{2+}\) exchanger type 1 in regions of the PM (4).

The pathophysiological SMC agonist PDGF activates Ca\(^{2+}\) entry in synthetic vascular SMCs through STIM1/Orai1-mediated SOCE, as well as proliferation and migration of these cells. Knockdown of STIM1 or Orai1 in synthetic vascular SMCs attenuated PDGF-mediated Ca\(^{2+}\) entry and SMC migration, while silencing of Orai2, Orai3, TRPC1, TRPC4, and TRPC6 did not affect the magnitude of PDGF-mediated Ca\(^{2+}\) entry; knockdown of Orai2, Orai3, and STIM2 had a marginal effect on PDGF-induced vascular SMC migration (6). The PDGF receptor is upregulated in vascular occlusive disease and promotes vascular remodeling through initiation of SMC migration, proliferation, and extracellular matrix deposition, subse-
for pharmacological compounds and could be selectively targeted without affecting other cell types, including leukocytes, where SOCE plays a prominent role in immune function and competence.

The characteristic sustained Ca\(^{2+}\) signal that is mediated by SOCE regulates various Ca\(^{2+}\)/CaM-dependent enzymes, including the serine/threonine phosphatase calcineurin (24). In an agonist-dependent manner, calcineurin regulates transcriptional programs in vascular SMCs through activation of transcription factors, most notably isoforms of nuclear factor of activated T cells (NFAT). NFAT resides in the cytosol under basal conditions in a phosphorylated state; upon SOCE activation, NFAT is dephosphorylated by calcineurin, promoting its nuclear translocation, DNA binding, and transcriptional regulation of genes. NFAT-regulated genes are involved in various processes, including inflammation, proliferation, and migration (33). In synthetic vascular SMCs, passive store depletion by thapsigargin or receptor stimulation by PDGF leads to the nuclear translocation of NFAT (47, 104, 106). Knockdown of STIM1 or Orai1 expression prevents nuclear translocation of NFAT in vascular SMCs, with a corresponding decrease in promoter activity, as assessed by luciferase reporter assays (104). Synthetic vascular SMCs downregulate expression of SERCA2a, which is abundantly expressed in contractile vascular SMCs (44). Bobe et al. (7) reported that restoration of SERCA2a expression in synthetic human coronary artery SMCs by gene transfer altered the nature of agonist-induced Ca\(^{2+}\) signaling from sustained to oscillatory, abrogated SOCE by inhibiting STIM1-Orai1 interactions, and inhibited NFAT nuclear translocation and SMC proliferation and migration, providing further evidence that sustained, robust Ca\(^{2+}\) entry mediated by SOCE couples to NFAT and vascular SMC growth and remodeling.

Damage endured by the vessel in response to removal of the atherosclerotic plaque using a procedure called balloon angioplasty and stent insertion often leads to restenosis. Restenosis involves vessel remodeling, whereby vascular SMCs proliferate and migrate to the luminal side of the injured vessel, depositing excess matrix proteins and forming the neointimal layer, leading to recidivous occlusion of the vessel (60, 105). The use of this mechanical vascular injury procedure in rat carotid arteries provides an in vivo model to study vascular SMC dedifferentiation during disease. STIM1 and Orai1 protein expression were upregulated in the medial and neointimal layers of balloon-injured rat carotid arteries (74, 104). These injured vessels also showed an increase in vascular muscle proliferation compared with controls as assessed by increased expression of the proliferative markers Ki-67 and proliferating cell nuclear antigen in the SMCs. Infection of injured vessels with a lentivirus containing shRNA against STIM1 or Orai1 prevented Orai1 and STIM1 upregulation in the media and neointima, inhibited cell proliferation, and attenuated neointima formation (2, 104). Subsequent studies confirming these earlier findings showed that knockdown of STIM1 and Orai1 inhibited angiotensin II-activated Ca\(^{2+}\) entry and proliferation of synthetic vascular SMCs. Knockdown of STIM1 and Orai1 using siRNA inhibited neointimal growth induced by angiotensin II after vascular injury, suggesting a potential role for SOCE in angiotensin II-induced SMC proliferation during vascular disease (31).

STIM1 and Orai1 were implicated in the pathogenesis of arterial hypertension, whereby protein expression of Orai1 and STIM1 was increased in aortas derived from stroke-prone spontaneously hypertensive rats compared with wild-type Wistar-Kyoto rats. Store depletion caused a greater STIM1- and Orai1-dependent SOCE in arteries from hypertensive than normotensive rats. Giachini et al. (28) stimulated vessels with thapsigargin to activate SOCE and relied on nonspecific inhibitors such as 2-APB and Gd\(^{3+}\) at high concentrations (100 \(\mu\)M) to link SOCE function to enhanced vessel contraction in hypertensive rats. Nevertheless, enhanced SOCE in hypertensive rats might indeed account for enhanced receptor signaling through contractile mediators (e.g., phenylephrine and angiotensin II) (38, 47). Notwithstanding an effect on contractility, STIM1/Orai1 and SOCE upregulation in vessels from hypertensive animals might suggest the establishment of SMC remodeling, which is a major contributor to the chronic phase of hypertension.

Mancarella et al. (47) generated SMC-specific STIM1 knockout (sm-STIM1-KO), STIM2 knockout (sm-STIM2-KO), and STIM1/STIM2 double-knockout mice. The double-knockout mice were perinatally lethal, while sm-STIM1-KO mice exhibited high mortality and reduced body weight; sm-STIM2-KO mice did not show an obvious phenotype. The use of external potassium chloride to assess depolarization-induced contraction on aortas of sm-STIM1-KO mice showed normal contractility in these mice. However, \(\alpha_1\)-adrenergic-mediated contraction of sm-STIM1-KO mice was inhibited by \(\sim 26\%\), suggesting a defect in contractile \(\alpha_1\)-adrenergic signaling (47). Consistent with previous results with rat balloon injury, neointima formation caused by carotid artery ligation was reduced by 54% in sm-STIM1-KO mice compared with littermate controls. Furthermore, in vascular SMCs isolated from sm-STIM1-KO mice and stimulated with PDGF, in vitro proliferation was strongly inhibited, while Ca\(^{2+}\) entry and NFAT nuclear translocation were essentially abrogated (47). A subsequent study from our laboratory generated sm-STIM1-KO mice, as well as endothelial-specific STIM1 knockout (ec-STIM1-KO) mice, and showed that body weight was reduced in sm-STIM1-KO and ec-STIM1-KO mice. Consistent with the results of Mancarella et al., we also showed that artery vessel contraction to \(\alpha_1\)-adrenergic stimulation with phenylephrine was significantly reduced only in sm-STIM1-KO mice, while contraction to thromboxane \(A_2\) agonist and potassium chloride was normal. Mancarella et al. showed that sm-STIM1-KO mice show abnormally developed vascular and intestinal smooth muscle tissues with distended and thinned morphology. Therefore, using siRNA, we acutely downregulated STIM1 in arteries ex vivo and showed that the contractile response to phenylephrine was inhibited, with no effect on contractility in response to thromboxane, suggesting that defects in contractile \(\alpha_1\)-adrenergic signaling are not developmentally related (38). An Ossabaw miniature swine model was used in an animal study to address the issue of accelerated coronary artery disease in areas adjacent to stenting (peri-stent) in metabolic disease. Animals fed an excess-calorie atherogenic diet (metabolic syndrome) showed increased SOCE and coronary artery disease in non- and peri-stent segments of coronary arteries. Exercise reverses coronary artery disease and increases in SOCE, as well as STIM1, Orai1, and TRPC1 upregulation (20).
In an early study that predates the discovery of STIM and Orai proteins, Lin et al. (43) were the first to show upregulation of SOCE and diacylglycerol (DAG)-activated Ca\(^{2+}\) entry in pulmonary artery SMCs during hypoxic pulmonary hypertension and correlated these enhanced Ca\(^{2+}\) entry routes to increased expression of the TRPC isoforms TRPC1, TRPC3, and TRPC6 (TRPC3 and TRPC6 are receptor-activated channels gated by DAG). Ng et al. (58) subsequently proposed that interactions between TRPC1, Orai1, and STIM1 underlie SOCE induced by acute hypoxia in pulmonary artery SMCs. Wang et al. (97) proposed that peroxisome proliferator-activated receptor-γ (PPAR\(\gamma\)) plays a protective role in pulmonary hypertension induced by chronic hypoxia. They proposed that PPAR\(\gamma\) decreases proliferation and migration of pulmonary artery SMCs through inhibition of SOCE, which is upregulated by exposure to hypoxia. In the same study, they correlated their findings with expression of TRPC1 and TRPC6 proteins; however, STIM/Orai isoform expression was not addressed (97). In a subsequent study, Yang et al. (100) suggested that enhanced SOCE in hypoxia-exposed pulmonary artery SMCs is mediated through the upregulation of caveolin-1, arguing that SOCE channels are organized within caveolin-rich regions. Molecular knockdown of caveolin-1 inhibited hypoxia-mediated increases in SOCE, and the PPAR\(\gamma\) agonist GW1929 reduced SOCE and caveolin-1 expression under hypoxic conditions (100).

Zhang et al. (103) reported that SOCE was increased in pulmonary artery SMCs from patients with idiopathic pulmonary hypertension (IPAH) compared with control normotensive patients. In a subsequent study, Song et al. (80) showed that STIM2 expression level was increased in pulmonary artery SMCs from IPAH patients, while STIM1 levels were decreased and knockdown of STIM2 in these cells inhibited SOCE and proliferation, while STIM2 knockdown in pulmonary artery SMCs from controls had no effect on SOCE or cell proliferation. Overexpression of STIM2 in control cells did not cause an increase in SOCE or proliferation, leading the authors to conclude that STIM2 plays a necessary, but not sufficient, role in upregulation of SOCE and enhanced pulmonary artery SMC proliferation during IPAH (80). The failure of STIM2 overexpression to cause an increase in SOCE and proliferation in control cells is likely due to the fact that native Orai channel expression is limiting in control cells and that concurrent upregulation of different isoforms of Orai channels in IPAH is required for enhanced SOCE. Indeed, follow-up studies showed that STIM2 and Orai2 are upregulated in proliferating pulmonary artery SMCs compared with quiescent contractile cells, suggesting a role for STIM2/Orai2-mediated SOCE in phenotypic modulation and proliferation of pulmonary artery SMCs (22).

A recent study by Daskoulidou et al. (15) described the upregulation of all STIM and Orai proteins by chronic treatment with high glucose (25 mM), which correlated with enhanced SOCE in response to store depletion in vascular endothelial cells. This upregulation could be prevented by cyclosporin A and knockdown of NFATc3, leading the authors to conclude that upregulation of STIM/Orai proteins by hyperglycemia is mediated by the calcineurin/NFAT pathway, which enhances SOCE and causes endothelial dysfunction (15). The enhanced expression of STIM/Orai isoforms was also observed in aortas from diabetic patients and from two different animal models of diabetes (15). However, whether STIM/Orai upregulation in response to high glucose or during diabetes also occurs in vascular SMCs and whether upregulation of STIM/Orai isoforms causes enhancement of modes of regulated Ca\(^{2+}\) entry other than SOCE have not been studied.

**Airway smooth muscle.** Initial studies characterizing SOCE in acutely dispersed porcine airway SMCs showed store depletion by the use of the SERCA inhibitor CPA or caffeine-activated Ca\(^{2+}\) entry from the extracellular space. This Ca\(^{2+}\) entry pathway was unaffected by the L-type Ca\(^{2+}\) channel blocker nifedipine but was inhibited by 1 \(\mu\)M La\(^{3+}\) and 10 \(\mu\)M SKF-96365, a nonspecific SOCE inhibitor (3). SOCE has also been characterized in cultured human airway SMCs (65), in which all three Orai homologs are expressed. Molecular knockdown of Orai1 in cultured human airway SMCs inhibited thapsigargin- and CPA-induced Ca\(^{2+}\) entry. Knockdown of Orai3 also inhibited SOCE in these cells, although to a smaller degree, while knockdown of Orai2 expression had no inhibitory effects on SOCE. The same study showed that knockdown of Orai1 in cultured human airway SMCs inhibited CPA-activated \(I_{CRAC}\)-like currents. Interestingly, Orai3 knockdown also attenuated Ca\(^{2+}\) release, leading Peel et al. (65) to propose an additional role for Orai3 in regulating basal levels of ER Ca\(^{2+}\)\(^{2+}\) or in mediating Ca\(^{2+}\) release from internal stores.

The allergen-induced mucosal injury characteristic of asthmatic airways is believed to initiate a repair cascade that involves secretion of growth factors, including PDGF, which stimulate airway SMCs (95). In whole cell patch-clamp recordings, PDGF activated a small inwardly rectifying Ca\(^{2+}\) current characteristic of \(I_{CRAC}\) that could be further amplified by the use of divalent free bath solutions in rat airway SMCs (81). Knockdown of Orai1 expression in synthetic rat airway SMCs inhibited PDGF-mediated activation of SOCE and \(I_{CRAC}\) and attenuated airway SMC proliferation and migration (81). Using chemotaxis chamber assays, Suganuma et al. (84) investigated the role of STIM1 and Orai1 in PDGF-mediated human airway SMC migration. They showed that PDGF-activated Ca\(^{2+}\) entry and migration in these cells were significantly reduced by siRNA against STIM1 or Orai1, confirming the potential role for SOCE in human airway SMC remodeling; in this study, knockdown of STIM2 with siRNA had no effect on SOCE or cell migration (84). Zhou et al. (109) showed that, similar to synthetic vascular SMCs, SOCE and STIM1/Orai1 expression were enhanced during airway SMC proliferation. Pharmacological blockade of SOCE using the inhibitors SKF-96365 (10 \(\mu\)M) and BTP2 (100 nM) or molecular knockdown of STIM1 or Orai1 inhibited SOCE and reduced airway SMC proliferation in response to serum (109). Gao et al. (27) suggested that TGF\(\beta1\) (10 ng/ml) induced an increase in expression of STIM1 and Orai1 in airway SMCs with enhancement of basal cytotoxic Ca\(^{2+}\)\(^{2+}\) levels and SOCE in response to thapsigargin. They reported that rat airway SMC proliferation in response to serum and TGF\(\beta1\) was partly reduced by 10 \(\mu\)M SKF-96365 (27). Jia et al. (37) showed that IL-13 and TNF\(\alpha\) enhance puncta formation of ectopically expressed fluorescently tagged STIM1 and increased SOCE in airway SMCs, suggesting that inflammatory cytokines might contribute to airway hyperresponsiveness through increased SOCE in human airway SMCs during disease.

While airway SMC hyperresponsiveness to contractile agonists is a hallmark of asthmatic airways, airway SMC pheno-
typic modulation involving proliferation and hypertrophy is also a major contributor to the pathophysiology of asthma and other airway obstructive diseases (14, 66). Patients with severe asthma tend to have an increase in airway smooth muscle mass and enhanced distance between airway smooth muscle and luminal epithelium, suggesting increased airway SMC proliferation, hypertrophy, and rate of migration (66). STIM1 and Orai proteins are upregulated in airway SMCs from asthmatic mice, suggesting a potential role for SOCE in the pathogenesis of atopic asthma (81). However, the specific role of STIM and Orai proteins in asthma development and airway SMC remodeling has not been examined. In addition to this role in promoting the airway SMC synthetic proliferative phenotype, SOCE signaling has been proposed to play a role in regulating Ca²⁺ signals that mediate airway SMC contraction. Based on the use of 3-fluoropyridine-4-carboxylic acid, a nonspecific pharmacological blocker of SOCE in guinea pig airway SMCs, Sutovska et al. (85) proposed that G protein-coupled receptor agonists such as histamine regulate airway SMC contractility through activation of the SOCE pathway. However, extensive studies are required to clarify the role of SOCE in airway SMC contractility. Wylam et al. (99) incubated human airway SMCs with cigarette smoke extract and showed enhanced SOCE and increased expression of Orai1 and STIM1. Knockdown of Orai1 inhibited the effects of cigarette smoke extract on SOCE. These authors also reported that cigarette smoke extract enhanced proliferation of airway SMCs and, thus, concluded that enhanced expression of Ca²⁺ signaling proteins such as Orai1 and STIM1 and subsequent increase in SOCE activity could play a role in the pathogenesis of airway disease in smokers (99).

**Store-Independent Ca²⁺ Entry in Smooth Muscle**

STIM and Orai proteins also encode a different receptor-mediated Ca²⁺ entry pathway that is store-independent (74, 79). This store-independent pathway is activated by receptor-mediated production of arachidonic acid (AA) or its metabolite leukotriene C₄ (LTC₄) (30, 49, 76), and the conductance it mediates was originally characterized in HEK-293 cells and termed AA-regulated Ca²⁺ current (I_{ARC}) (48). I_{ARC} and I_{CRAC} are not additive in the same cell, suggesting that these two conductances are mediated by physically distinct channels (48). I_{ARC} is biophysically similar to I_{CRAC}, and both pathways often co-exist in the same cells, suggesting that they control distinct cellular and physiological functions. I_{ARC} has some unique features: it has a less pronounced Ca²⁺-dependent fast inactivation under a voltage-step protocol, and it is not inhibited by high concentrations (50 μM) of 2-APB or by reductions in external pH (78).

Production of AA or its downstream metabolite LTC₄ after receptor ligation can occur through a range of enzymatic pathways, including 1) direct generation of AA from membrane glycerophospholipids by the action of the phospholipase A₂ family of enzymes or 2) from sequential catalytic activities of PLC on phosphatidylinositol 4,5-bisphosphate (which generates DAG) and DAG lipase action on DAG, which will yield AA, and 3) through the activity of phosphatidic acid phosphohydrolase on phosphatidic acid produced through phospholipase D (78). Nevertheless, I_{ARC} was predominantly studied by addition of relatively low concentrations (8 μM) of exogenous AA to the bath solution, which causes activation of Ca²⁺ entry and membrane currents without measurable depletion of internal Ca²⁺ stores. Using HEK-293 cells, Shuttleworth and colleagues (50, 51, 87) showed that I_{ARC} required STIM1, Orai1, and Orai3 and proposed that the minor pool of STIM1 expressed at the plasma membrane is involved in the activation of I_{ARC} while the NH₂-terminal domain of Orai3 determines the selectivity for activation by AA over store depletion.

**Vascular smooth muscle.** Studies from our laboratory showed that the mitogenic and inflammatory agonist thrombin activates store-independent Ca²⁺ entry and membrane currents in cultured rat aortic SMCs that were additive to SOCE and I_{CRAC} activated by PDGF (30). Maximal concentrations of thrombin do not cause sustained store depletion, and blockade of IP₃ receptor-mediated Ca²⁺ release through dialysis of heparin in the patch pipette failed to inhibit thrombin-activated currents (30). Thrombin-activated currents were highly Ca²⁺-selective currents, similar to I_{CRAC}. However, they showed some unique features that were distinct from I_{CRAC} as they did not show the typical phenomenon of depotentiation in divergent free solutions (whereby large Na⁺ currents through I_{CRAC} are activated but immediately inactivate) and were not inhibited by 50 μM 2-APB. Using molecular knockdown, we showed that this store-independent thrombin-activated Ca²⁺ entry pathway requires Orai1, Orai3, and STIM1, with the pool of STIM1 that is restricted to the ER being necessary and sufficient (107). Activation of this Ca²⁺ entry route requires receptor-mediated production of AA through the actions of PLC and DAG lipase and AA metabolism into LTC₄ by the action of LTC₄ synthase (LTC₄S) (30, 106). Interestingly, exogenous AA added to the bath solution is metabolized by cells into LTC₄ for optimal channel activation; dialysis of LTC₄ through the patch pipette activated similar currents that were not additive to thrombin- or AA-activated currents (30). We showed that the STIM1 COOH-terminal coiled-coil domain is constitutively interacting with store-independent Orai1/Orai3 heteromultimeric channels through the Orai3 COOH terminus and that this interaction is required for channel activation by LTC₄ in vascular SMCs (107). We thus named this conductance LTC₄-regulated Ca²⁺ current (I_{LRC}) (30). In a follow-up study, we set out to establish whether I_{ARC} in HEK-293 cells and I_{LRC} in vascular SMCs are encoded by the same or different pools of STIM1, Orai1, and Orai3 proteins. We undertook comparative whole cell patch-clamp recordings of I_{ARC} (activated by exogenous application of 8 μM AA) and I_{LRC} (activated by dialysis of 100 nM LTC₄ through the patch pipette) in HEK-293 cells and vascular SMCs and discovered that, regardless of cell type, I_{ARC} and I_{LRC} are not additive and require STIM1, Orai1, and Orai3, strongly arguing that these two currents are mediated by the same channel. Regardless of cell type (HEK-293 cells or vascular SMCs), a nonmetabolizable form of AA, 5,8,11,14-eicosatetraenoic acid, activated ~50% of the current activated by AA. Similarly, when exogenous AA was added to cells treated with a 5-lipoxygenase inhibitor to prevent AA downstream metabolism, only ~50% of the current densities were obtained compared with currents activated by dialysis of LTC₄ into the cytosol of cells treated with the 5-lipoxygenase inhibitor (108). These data strongly argue that while AA is capable of activating these store-independent channels, AA metabolism into LTC₄ is required for full or optimal activation. We also addressed the discrepancy between data on HEK-293 cells from Thompson and Shuttleworth (88) and our own data on...
vascular SMCs (30) regarding the requirement of PM-STIM1 for I_{ARC} vs. the requirement of ER-STIM1 for I_{LRC} (108). We found that when whole cell patch-clamp recordings were used in both cell types, a construct expressing STIM1 at the ER and PM was required for current activation by LTC_{4} or AA. However, exclusive expression of STIM1 at the ER was sufficient to mediate current activation by LTC_{4} or AA when the perforated patch-clamp technique or fura 2 Ca^{2+}/H11001 imaging was used. We concluded in this study that a soluble factor might be involved in interacting with the STIM1-Orai1-Orai3 complex and required for store-independent Orai1/Orai3 channel activation by LTC_{4}; this factor would be mostly dialyzed out when whole cell patch-clamp recordings are considered, but PM-STIM1 would help maintain its interaction with the channel complex. However, in the case of an intact cytosol, the soluble factor would be retained in the cytosol and the PM-STIM1 requirement would be bypassed (89).

Orai channel heteromultimerization and store-independent Orai channel activation likely represent means to enhance the diversity of receptor-activated Ca^{2+} entry routes, whereby different PLC-coupled agonists activate specific Ca^{2+} entry pathways in the same cell type. In fact, the downstream Ca^{2+}-responsive pathways activated by I_{LRC} in vascular SMCs are distinct from those activated by I_{CRAC} (106). Unlike PDGF, stimulation of vascular SMCs with thrombin failed to induce NFAT nuclear translocation (106). On the other hand, knockdown of Orai3 or LTC_{4}S caused a more robust and sustained phosphorylation of Akt1 and Akt2 on Ser^{473}/Ser^{474} after serum stimulation, and this was accompanied by a decrease in SMC migration in vitro, suggesting an inhibitory effect of Akt signaling on vascular SMC migration. In vivo, Orai3 and LTC_{4}S proteins, as well as I_{LRC} densities, are increased in medial and neointimal SMCs acutely isolated from rat carotid arteries 14 days after balloon injury (30, 106). In vivo lentiviral transduction of injured carotid arteries with shRNA against Orai3 or LTC_{4}S to prevent Orai3 and LTC_{4}S upregulation inhibited I_{LRC} in acutely isolated SMCs and reduced neointimal hyperplasia 14 days postinjury compared with injured vessels infected with control nontargeting shRNA (30, 106). Interestingly, Akt activity in acutely isolated SMCs, as assessed by phosphorylation on Ser^{473}/Ser^{474}, was abrogated in neointimal and medial SMCs at 14 days after carotid injury and...
was rescued when vessels were infected with lentiviral particles encoding shRNA against LTC4S or Orai3 (106). In a recent work, we used mouse embryonic fibroblasts isolated from Orai1 knockout mice and HEK-293 cells where endogenous Orai1 expression was silenced by siRNA (17). In each case, Orai1 expression was restored with the use of cDNA plasmids that exclusively express Orai1α or Orai1β driven by a weak promoter to recapitulate the low physiologically relevant levels of Orai1 expression and prevent the dominant-negative effects associated with Orai1 overexpression (17). We discovered that IcCRAC could be recapitulated with Orai1α or Orai1β, while IARC/IIRC requires the long exclusively mammalian isoform Orai1α (17). Although it is expected that IARC/IIRC in vascular SMCs would also require Orai1α, this specific experiment remains to be performed.

Airway smooth muscle. Recently, Thompson et al. (89) used fura 2 imaging to describe AA-mediated Ca2+ oscillations in human airway SMCs when 1–10 μM AA was used. These AA-activated Ca2+ oscillations depended on Ca2+ entry from the extracellular space, while other fatty acids, including the DAG analog 1-oleoyl-2-acetylglycerol, oleic acid, and palmitic acid, at 10 μM were unable to produce Ca2+ signals. Surprisingly, pharmacological inhibition of ryanodine receptor (with 10 μM ryanodine) or IP3 receptor (with 1 μM xestospongic C) to inhibit Ca2+ release from sarcoplasmic reticulum reduced the frequency and amplitude of AA-activated Ca2+ oscillations, while the SOCE inhibitor SKF-96365 did not, leading Thompson et al. to suggest that AA-activated Ca2+ influx could trigger sarcoplasmic reticulum Ca2+ release through Ca2+-induced Ca2+ release to regulate Ca2+ oscillations (89).

Thompson et al. (89) showed that siRNA knockdown of STIM1 and Orai3 inhibited the amplitude and frequency of AA-activated Ca2+ oscillations in human airway SMCs. Interestingly, similar results were obtained when caveolin-1 was knocked down with siRNA, suggesting that these channels are located within caveolin-rich regions of the PM (89); it is noteworthy that the long isoform of Orai1, Orai1α, uniquely involved in store-independent Orai1/Orai3 channels, contains a caveolin-binding region in the 63 NH2-terminal amino acids that are lacking in Orai1β (17, 26) (Fig. 1). Thompson et al. analyzed airway SMCs obtained from asthmatic patients and showed enhanced amplitude of AA-activated Ca2+ oscillations compared with controls (89), suggesting a role for this pathway in asthma pathology. Clearly, extensive whole cell electrophysiological recordings are warranted to define this AA-activated Ca2+ entry in airway SMCs. Furthermore, the specific downstream signaling mechanisms controlled by AA-activated Ca2+ oscillations in asthmatic airway SMCs and their contributions to manifestations of asthma are important issues that warrant additional investigations.

Concluding Remarks

In general, Orai-mediated store-dependent and -independent Ca2+ entry pathways (Fig. 2), which are activated by a variety of growth, migratory, and inflammatory agonists, are upregulated during phenotypic modulation of vascular and airway smooth muscle associated with vascular and airway pathophysiology. In animal models of disease, when in vivo upregulation of these molecules is prevented, SMC dedifferentiation and remodeling are inhibited. This causal link between Orai channel function and disease is of major interest in the development of agents that target Orai-mediated pathways in prevention and treatment of vascular occlusive disease and airway obstructive disease. For example, the effective antiproliferative agent sirolimus (rapamycin), which is clinically used in drug-eluting stents to prevent postoperative vascular remodeling, has been shown to prevent proliferation of human coronary artery SMCs, at least in part through inhibition of the SOCE pathway and downstream CREB-mediated proliferative signaling (39). Novel drugs targeting specific isoforms of Orai, especially the exclusively mammalian Orai3 proteins or the heteromultimeric store-independent Orai1α/Orai3 channels, hold the promise of specific targeting of smooth muscle remodeling with limited side effects.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

A.M.S. and M.T. prepared the figures; A.M.S. and M.T. drafted the manuscript; A.M.S. and M.T. approved the final version of the manuscript; M.T. edited and revised the manuscript.

REFERENCES


Themes

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76. Song MY, Makino A, Yuan JX. STIM2 contributes to enhanced store-operated Ca entry in pulmonary artery smooth muscle cells from patients with idiopathic pulmonary arterial hypertension. Palm Circ 1: 84–94, 2011.


