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Chapter 4

The Interplay between Cytoskeleton and Calcium Dynamics

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Additional information is available at the end of the chapter

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Abstract

Cell motility is a complex cellular event that involves reorganization of cytoskeleton. This reorganization encompasses the transient polarization of the cell to facilitate the plasma membrane ruffling, a rearrangement of cortical actin cytoskeleton required for the development of cellular protrusions. It is known that extracellular Ca^{2+} influx is essential for cell migration and for the positive-feedback cycle that maintains leading-edge structures and ruffling activity. The aim of this review is to summarize our knowledge regarding the Ca^{2+}-dependent signaling pathways, Ca^{2+} transporters and sensors involved in cell migration. Also, we show here reported evidences that support for a crosstalk between Ca^{2+} transport and the reorganization of the cytoskeleton required for cell migration. In this regard, we will analyze the role of store-operated Ca^{2+} entry (SOCE) as a modulator of cytoskeleton and cell migration, but also the modulation of this Ca^{2+} entry pathway by microtubules and the actin cytoskeleton. As a main conclusion, this review will show that data reported in the last years support a role for SOCE in shaping cytoskeleton, but at the same time, SOCE is strongly dependent on cytoskeletal proteins, in an interesting interplay between cytoskeleton and Ca^{2+} dynamics.

Keywords: calcium, microtubules, actin, STIM1, ORAI1, cell migration, cortical cytoskeleton

1. Introduction

Calcium ions (Ca^{2+}) are essential intracellular transducers for cell signaling because of their role to bind Ca^{2+}-sensitive proteins that mediate key activities in signaling pathways. Upon cell stimulation through a variety of receptors and other types of physicochemical stimulations such as depolarization of plasma membrane, changes in osmolarity, physical distortion

of cell surface, temperature, etc., a number of intracellular "second messengers" transmit the initial stimulation into the cell to trigger a proper response to these stimuli. The response is attained in some cases by transiently altering ion transport through plasma membrane as well as intracellular membranes, posttranslational modifications of proteins, changes in gene expression, or reshaping the cytoskeleton in a second-messenger-dependent manner.

To properly act as a second messenger the concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) is substantially different across plasma membrane. While the physiological extracellular medium contains 1–3 mM Ca^{2+}, the level of the cytosolic milieu is within a narrow range, 80–120 nM [1, 2]. Considering this large chemical gradient of free Ca^{2+} concentration, cells can trigger a rapid and transient increase of $[Ca^{2+}]_i$ by increasing Ca^{2+} transport through plasma membrane. This transient Ca^{2+} increase triggers the action of a wide range of Ca^{2+}-dependent mediators that modify signaling pathways. Because Ca^{2+} is involved in numerous signaling pathways, differential features of Ca^{2+} increase are required to respond to diverse initial stimuli. This is achieved by the spatiotemporal control of the Ca^{2+} increase, i.e., the localization of the cytosolic Ca^{2+} spike within the cell, as well as the rate of the increase, the extent of the transient increase and the kinetics of the final decrease to basal levels [3].

This Ca^{2+} transport is therefore tightly regulated by specific channels, which are sensitive to hormones, cytokines, small molecules and other extracellular stimuli. The features of the transporters shape the characteristics of Ca^{2+} current (Ca^{2+} influx), modeling the kinetics of Ca^{2+} entry. Because a specific distribution of Ca^{2+} channels and transducers is required the cytoskeleton has been involved in the spatial regulation of Ca^{2+} entry. Interestingly, Ca^{2+} signaling strongly influences cytoskeleton dynamics, in an interesting interplay that is currently under study. In order to assess the crosstalk between Ca^{2+} signaling and cytoskeleton we will review here the recent literature regarding this topic, with special focus on the role of store-operated Ca^{2+} entry (SOCE) in cell migration, focal adhesion turnover and actin filaments reorganization. In addition, we will review the role of the microtubule cytoskeleton in the normal function of SOCE. A major regulator of SOCE is the protein STIM1, an endoplasmic reticulum resident protein that serves as intraluminal Ca^{2+} sensor and plasma membrane Ca^{2+} channel modulator. STIM1 is known to be a plus-end microtubule binding protein (+TIP). As a +TIP, STIM1 is transported throughout the cell while bound to microtubules, but it is released upon activation. The molecular basis of this regulation and the role of posttranslational modifications in STIM1 that are known to underlie this regulation, will be also described.

2. Ca^{2+} transporters and Ca^{2+} influx pathways

In eukaryotic cells plasma membrane (PM) contains different Ca^{2+} transport systems to control Ca^{2+} influx as well as Ca^{2+} extrusion. Because the temporal control of Ca^{2+} signaling is also strictly controlled, transporters are highly coordinated to let the Ca^{2+} spikes/waves last for a precise time, but this time ranges from microseconds (as in exocytosis) to hours, as observed during mammalian oocyte fertilization. To understand this control, we summarize here the

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most important Ca^{2+} transporters in eukaryotic cells. Ca^{2+} entry channels are divided into the following:

Voltage-operated channels (VOCs) are regulated by the net electric charge across the plasma membrane in the way that they open upon depolarization. This family consists of three different groups of channels, Ca_v1 (L-type channels), Ca_v2 (N-, P/Q and R-types) and Ca_v3 (T-type channels) [4].

Receptor-operated channels (ROCs), which are gated upon binding to agonists such as ATP, glutamate, or acetylcholine [5]. Within this group we should highlight the transient receptor potential (TRP) ion channel family, a large family of channels involved in sensory perception, smooth muscle contraction-relaxation cycles and cell proliferation [6]. Members of the TRPC (transient receptor potential canonical) family are also involved in the organization of heteromeric Ca^{2+} channels that respond to intracellular Ca^{2+} store depletion [7]. This is the case for TRPC1 that has been described to be part of complexes together with members of the ORAI family [8]. Other important members of this family are P2X receptors, which are Ca^{2+} channels gated by extracellular ATP [9] and glutamate receptors, all of them reviewed in Ref. [10].

Second-messenger-operated channels (SMOCs) are members of an important family of channels gated by intracellular second messengers, such as arachidonic acid-regulated Ca^{2+} (ARC) channel or TRPC6, a member of the TRPC which is sensitive to diacylglycerol (DAG) [11].

Store-operated channels (SOCs) are channels that are regulated by the filling state of intracellular Ca^{2+} stores, mainly the sarco(endo)plasmic reticulum (ER). Their activity is actually the result of an initial Ca^{2+} release from the ER, mainly through the activation of the phosphoinositide pathway. A wide range of stimuli triggers the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which activates IP3 receptors (IP3R) at the ER, leading to the transient release of Ca^{2+}. Most of this Ca^{2+} is taken back to the ER by the sarco(endo)plasmic Ca^{2+} reticulum ATPase (SERCA), the Ca^{2+} pump in the ER membrane. However, some of this Ca^{2+} is extruded out of the cell by plasma membrane Ca^{2+} ATPases (PMCA), leading to the partial depletion of Ca^{2+} within the ER when the stimulation is repetitive. Thus, a system is required to replenish this Ca^{2+} and this action is mediated by SOCs that activate the Ca^{2+} influx pathway known as the store-operated Ca^{2+} entry (SOCE), described for the first time in the late 1980s by James W. Putney [12].

As stated above, there are some transporters considered as OFF systems, i.e., they are designed to decrease $[Ca^{2+}]_i$ to basal levels (to approximately 100 nM) and therefore to shut down Ca^{2+} signaling. These are mainly Ca^{2+} pumps, ATP-consuming transporters that pump Ca^{2+} ions against the Ca^{2+} gradient concentration [13]. PMCAs and SERCAs extrude Ca^{2+} from the cytosol to extracellular milieu and into the ER vesicles. In addition to these systems, the plasma membrane Na^+/Ca^{2+} exchanger contributes to Ca^{2+} extrusion in a Na^+-gradient-dependent manner and it is particularly important in cardiac cells and neuronal tissues [14]. Finally, a mitochondrial Ca^{2+} uniporter (MCU) and the secretory-pathway Ca^{2+}-ATPase (SPCA) are additional and widespread systems that restore cytosolic Ca^{2+} levels [15].

In summary, the combination of the activities of channels and pumps, either at the plasma membrane or at subcellular organelles and the particular expression profile of those transporters in different cells and tissues, makes possible for a cell-specific response to a wide range of stimuli.

3. Store-operated Ca^{2+} entry

As stated above, store-operated Ca^{2+} entry (SOCE) is a Ca^{2+} influx transport system that maintains the permanence of the Ca^{2+}-dependent signaling, because it preserves the intraluminal Ca^{2+} levels in the ER in the high micromolar range. For this extraordinary role, SOCE is a ubiquitous mechanism and is one of the most important pathways for Ca^{2+} entry in both excitable and nonexcitable cells [16]. However, the molecular nature of the members that control this Ca^{2+} influx pathway remained elusive until the description of two proteins, STIM1 and ORAI1, 10 years ago [17–22]. The mechanism that links luminal Ca^{2+} levels with plasma membrane Ca^{2+} entry is mediated by STIM1, a transmembrane protein located at the ER that acts as a Ca^{2+} sensor. STIM1 contains a Ca^{2+}-sensitive EF-hand domain at the intraluminal domain and activates plasma membrane Ca^{2+} channels (SOCs) upon Ca^{2+} depletion within the intracellular stores. This depletion triggers the oligomerization of STIM1 and the relocalization in ER-PM juxtapositions required for the binding and activation of plasma membrane SOCs.

One of the most important SOCs is ORAI1 (also known as CRACM1), although the ORAI family contains two additional members, ORAI2 and ORAI3. In addition to ORAI1, some of the transient receptor potential canonical (TRPC) channels can function in a STIM1-dependent mode. STIM1 directly activates TRPC1, TRPC4 and TRPC5 channels that can therefore act as SOCs [23]. TRPC1 also binds to ORAI1 and the TRPC1-ORAI1-STIM1 ternary complexes can be therefore considered SOCs [24]. STIM1 also activates TRPC3 and TRPC6, not by direct interaction, but mediating the heteromultimerization of TRPC3 with TRPC1 and TRPC6 with TRPC4 [23].

The mode of STIM1-dependent gating for ORAIs and TRPCs is also different. STIM1 gates ORAI1 by direct binding through the STIM1-ORAI1-activating region, or SOAR, also called CRAC-activating domain or CAD. In contrast, the C-terminal polybasic domain of STIM1 activates TRPC1 by an electrostatic gating mechanism that results in SOC channel activation [25, 26].

STIM1 can bind other types of Ca^{2+} channels, such as $Ca_v1.2$ channels, but this binding leads to the suppression of the activation of these channels [27, 28]. Interestingly, this inhibitory action is mediated by the domain of STIM1 that activates ORAI1 (SOAR or CAD) and this direct binding to $Ca_v1.2$ causes also the internalization of the channel, which explains the coordinated regulation of Ca^{2+} entry through VOCCS and SOCs. Thus, the combination of different Ca^{2+} channels provides a diversity of Ca^{2+} conductances in response to a wide variety of stimuli. However, the molecular mechanisms underlying the expected differential localization of STIM1 within cells are not clear. In this regard, the cytoskeleton seems to be a requirement for STIM1 function and binding to ORAI1 and therefore for the activation of SOCE. It was early observed that SOCE is sensitive to drugs that modify cytoskeletal components, such

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as cytochalasin D or nocodazole in NIH 3T3 cells [29], or vascular endothelial cells [30] and later confirmed in RBL-2H3 cells, bone marrow-derived mast cells [31], HEK293 cells [32] and platelets [33–35].

From the information given above, one can conclude that cytoskeleton is not only a critical modulator of SOCE, but also one can hypothesize that cytoskeleton might underlie the differential preference of STIM1 for different Ca^{2+} transporters.

4. Cytoskeletal components involved in the control of SOCE

After the molecular description of STIM1 and ORAI1 as the two major elements controlling SOCE, it was earlier found that the microtubule cytoskeleton was involved in the regulation of this Ca^{2+} entry pathway. The group of James W. Putney described that STIM1 tagged with fluorescent proteins colocalized with endogenous tubulin and that the treatment with nocodazole, which induces tubulin depolymerization, triggered the loss of this colocalization in HEK293 cells. Moreover, nocodazole had an inhibitory effect on SOCE that could be reverted by the overexpression of YFP (yellow fluorescent protein)-STIM1 [32], suggesting that the microtubule cytoskeleton has an important role in the activation of SOCE by facilitating the appropriate localization of STIM1 to activate SOCs. Similar findings were reported in COS-7 cells, where the treatment with nocodazole triggered the retraction of tubulin filaments from cell periphery leading to the progressive loss of SOCE, similarly to what is found in mitosis. On the contrary, placlitaxel, which stabilizes microtubules, enhanced SOCE [36], supporting for a role of microtubules in the normal function of SOCE.

It was later demonstrated that STIM1 directly binds to EB1, a microtubule plus-end binding (or tracking) protein (+TIP) and that STIM1 is transported through the surface of the ER network by this microtubule-dependent mechanism [37]. In addition, it was observed that the overexpression of ectopic STIM1 led to the stimulation of the ER extension, an effect that was explained by the direct attachment of the ER to the growing ends of microtubules, which was stimulated by the accumulation of STIM1. Therefore, this observation also revealed that STIM1 concentration could regulate ER extension and remodeling. In addition, Luis Vaca group reported that STIM1 binds to EB1 when traveling through the ER under resting conditions and that there is a dissociation of this STIM1-EB1 complex upon Ca^{2+} depletion of the ER, an event that facilitates the clustering of STIM1 in the periphery of the cell [38].

STIM1-EB1 binding was studied further and it was found that the sequence Thr-Arg-Ile-Pro (TRIP) in the C-terminus of STIM1 is responsible for the direct binding to EB1 [39]. This sequence belongs to a short polypeptide motif, S/TxIP, found in several +TIPs. For those +TIPs with an S/TxIP motif, a phospho-regulation of the binding with microtubules has been reported. The +TIPs, APC [40, 41], MCAK [42] and CLASP2 [43, 44] are phosphorylated in the vicinity of the S/TxIP sequence, negatively regulating their interaction with microtubules. In these +TIPs we find a high number of proline, serine and basic residues that give a positive charge to the surroundings of the EB1 binding domain. This is why the negative charge of the phosphorylation of residues in the surroundings of the S/TxIP motif explains the blocking of the binding to

microtubule ends. In this regard, our group reported that, as it was reported for those +TIPs, the binding of STIM1 to the tip of microtubules growing ends is regulated by phosphorylation. Near the S/TxIP sequence, which in human STIM1 is found in residues 642–645 (Thr-Arg-Ile-Pro), our group have described three phosphorylatable residues, Ser575, Ser608 and Ser621, which are target for ERK1/2 activity *in vitro* and *in vivo* [44, 45]. Using overexpressed STIM1 mutated at ERK1/2 target residues, we reported that dephosphorylated STIM1 (i.e., with Ser-to-Ala substitution mutations) remained bound to EB1, whereas constitutive phosphorylated STIM1 (mimicked by Ser-to-Glu mutations et ERK1/2 target sites) detached from EB1 [46, 47]. By means of the generation of phospho-specific antibodies directed against the three individual residues, we reported that there is a dynamic phosphorylation of STIM1 during its activation. Thus, activation of SOCE by thapsigargin or 2,5-di-ter-butyl-1,4-benzohydroquinone, two SERCA inhibitors that trigger ER emptying, is accompanied by the increase of phosphorylation at the ERK1/2 target Ser residues (575, 608 and 621). Moreover, the washout of the inhibitor with a Ca^{2+}-containing medium, in order to let the refilling of the ER, carried out in parallel to STIM1 dephosphorylation [46]. In addition, Ser/Ala mutation inhibited SOCE and impaired the binding STIM1-ORAI1. In contrast, Ser/Glu mutations enhanced SOCE, whereas facilitated the clustering of STIM1 in response to store depletion, as well as the binding to ORAI1. Later, we reported that IGF-1 [48] and EGF [49] also trigger Ca^{2+} release from the ER and phosphorylation of STIM1, an effect that has been proven to be essential for the dissociation from EB1 and finally for the activation of SOCE (see **Figure 1**).

Taken together, those results support a mechanism that explains the reversible interaction of STIM1 and EB1 [46, 50]. This mechanism predicts that those stimuli that induce store depletion and ERK1/2 activation lead to phosphorylation of STIM1 at Ser575, Ser608 and Ser621, an event that promotes the dissociation of STIM1 from EB1. This dissociation enables STIM1 clustering and the binding to SOCs, in order to activate STIM1-dependent Ca^{2+} entry (SOCE). In addition, the activation of Ca^{2+} entry and the subsequent refilling of Ca^{2+} stores induce STIM1 dephosphorylation, promoting the association of STIM1 with EB1 and microtubules.

In addition to EB1, other members of the cytoskeleton have been involved in SOCE regulation. For instance, it has been reported that the microtubule-binding protein adenomatous polyposis coli (APC) is required for STIM1 puncta near ER-PM junctions, because reduced STIM1 was observed at these junctions in APC-depleted cells and this effect correlated well with the inhibition of SOCE [51]. The APC-binding domain was found in the C-terminus of STIM1 (residues 650–685), similarly to what it has been reported for STIM1-EB1. Thus, it can be assumed that upon depletion of the ER, STIM1 dissociates from EB1 and associates to APC to form puncta near ER-PM junctions and to activate ORAI1 and SOCE.

A downstream effector of SOCE is the transcription factor NFAT (nuclear factor of activated T-cells). Once Ca^{2+} entry becomes activated, the increase of $[Ca^{2+}]_i$ activates the Ca^{2+}-dependent phosphatase calcineurin (or protein phosphatase 2B), which dephosphorylates NFAT, promoting the nuclear translocation of NFAT that can be easily monitored using GFP-tagged NFAT. By means of this well-established feature of SOCE, Sharma et al. [52] designed a genome-wide RNA interference screen for NFAT activation in HeLa cells and they identified septin proteins as key regulators of SOCE. Knockdown of SEPT4 gene expression reduced SOCE

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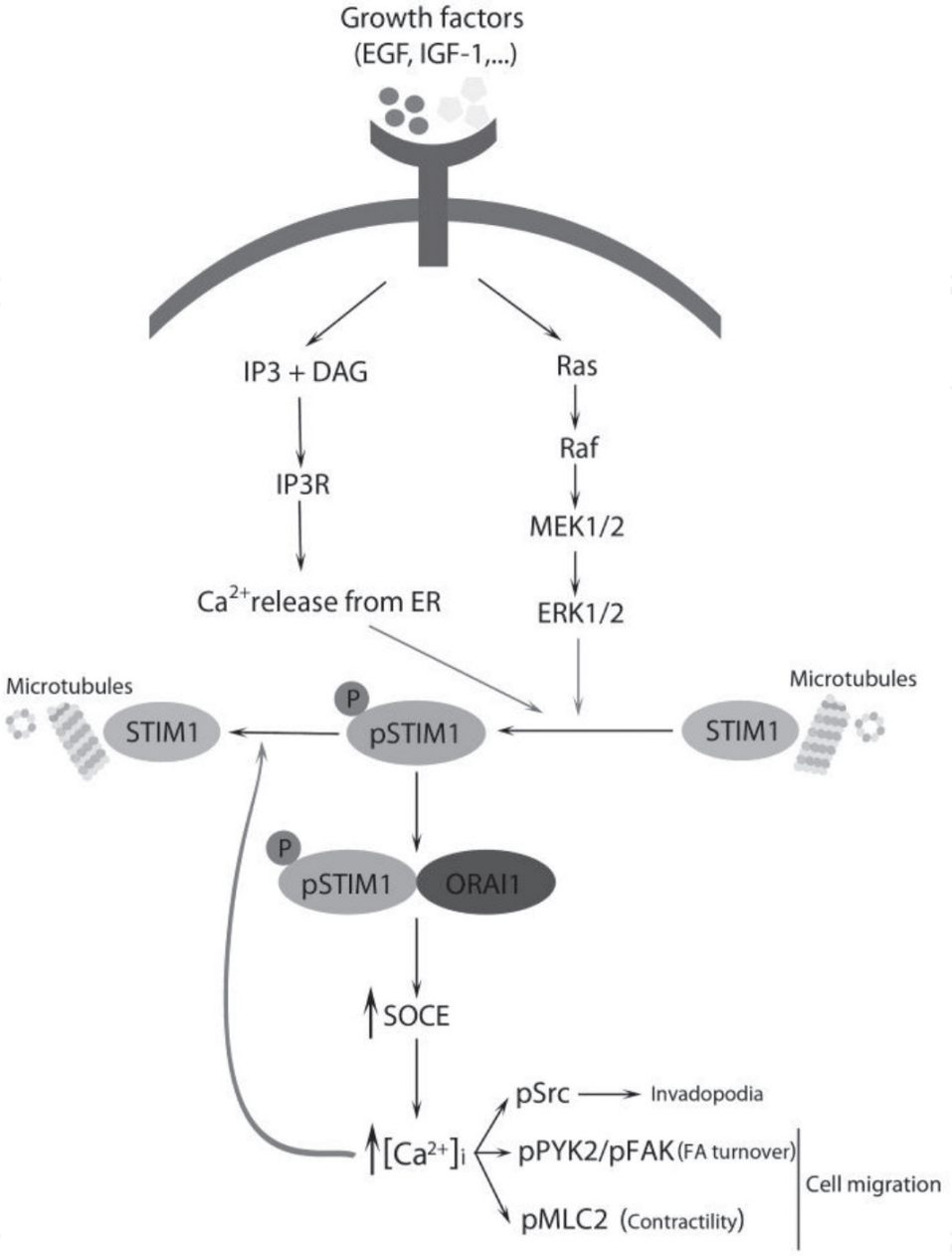

Figure 1. Involvement of SOCE in cell migration. The binding of ligands to some plasma membrane receptors activates the phosphoinositide pathway, releasing IP3 and activating Ca^{2+} release from the ER. In parallel, receptor tyrosine kinases trigger the activation of the MAPK pathway, via activation of Ras-Raf-MEK-ERK. Ca^{2+} mobilization from the ER and phosphorylation of STIM1 are two essential events for the activation of STIM1. Phospho-STIM1 releases from microtubules facilitating STIM1 clustering and translocation to PM-ER junctions to activate ORAI1. This activation increases $[Ca^{2+}]_i$ locally, an event required for the phosphorylation of Src, PYK2 and FAK kinases, as well as MLC2, which are well-known modulators of invadopodia formation, focal adhesion turnover and actomyosin contractility.

without affecting ER-Ca^{2+} release and without inhibiting SERCA or PMCAs. This knockdown also decreased Ca^{2+}-induced NFAT translocation by >95%. Septins were found to be required for proper organization of ORAI1 in the plasma membrane even before depletion of ER-Ca^{2+} stores. Septins also promoted the targeting of STIM1 to ER-PM junctions and the formation of

stable ORAI1 clusters after store depletion. Because septins are considered scaffold proteins that recruit other proteins, preventing diffusion, septins should be considered promoters of the stable recruitment of STIM1-ORAI1 at ER-PM junctions. More recently it was found that Septin 7 inhibits the activation of Orai channels in Drosophila neurons (dOrai) and that the depletion of Septin 7 levels results in higher dOrai-mediated Ca^{2+} entry, independently of the filling state of the ER. In fact, overexpression of Septin 7 reduced Ca^{2+} entry, suggesting that, in Drosophila neurons, Septin 7 is a negative regulator of dOrai channel function [53]. These authors stated that hetero-hexamers septin filaments closely associate with the PM and near the ER in resting neurons and that the reduction of SEPT7 results in breaks in the linear septin filaments present in wild-type cells, leading to the formation of shorter septin filaments. ER-Ca^{2+} store depletion reorganizes these filaments, moving STIM1 to the peripheral ER, promoting the coupling of STIM/dOrai and the activation of Ca^{2+} entry through dOrai (SOCE). Shorter septin filaments due to SEPT7 knockdown leads to STIM1 recruitment to the peripheral ER in resting neurons and the activation of dOrai, resulting in a store-independent activation of dOrai [53].

Homer1 is another scaffolding protein that binds to TRPC channels through a PPPF sequence of the Ca^{2+} channel. However, this sequence is close to the STIM1 binding sequence, thus suggesting that Homer1 and STIM1 could be competitors for binding to TRPC. Yuan et al. [54] proposed that Homer couples TRPC channels to IP3 receptors (IP3R) to keep these channels closed, but dissociation of the TRPC-Homer-IP3R complexes gives STIM1 access to TRPC binding to gate these channels. A similar proposal was later reported for STIM1 and $Ca_v1.2$ channels in HEK293 cells [55], where the treatment with thapsigargin induces coimmunoprecipitation of Homer1 with STIM1 and the $Ca_v1.2$ $\alpha 1$ subunit. Impairment of Homer1 function with the peptide PPKKFR or by siRNA specific for Homer1 reduced the association of STIM1 to $Ca_v1.2$ $\alpha 1$, adding a new scaffolding protein to the list of regulators of Ca^{2+} entry.

In summary, the increasing number of members of the cytoskeleton involved in the specific interaction of STIM1 with different plasma membrane Ca^{2+} channels leads to the conclusion that the cytoskeleton strongly modulates SOCE, as well as the activation and inhibition of others Ca^{2+} transport systems.

5. How SOCE modulates cytoskeleton dynamics and cell migration

Because the localization and function of STIM1 and ORAI1 are strongly dependent on components of the actin and tubulin cytoskeleton, the question of how SOCE and Ca^{2+} dynamics influence cytoskeleton-dependent events, such as cell adhesion and migration, was rapidly considered by many groups.

Focal adhesions are complexes of macromolecules that serve as mechanical links between the extracellular substrate and the cytoskeleton. These molecular assemblies are highly dynamic. However, the molecular mechanism by which Ca^{2+} regulates focal adhesion turnover is still unclear. A few proteins that regulate focal adhesion assembly or disassembly

are sensitive to changes in the intracellular free Ca^{2+} concentration. For instance, the Ca^{2+}-dependent protease calpain [56], which could cleave talin at focal adhesion sites, leads to an increase of focal adhesion disassembly rates. Indeed, disassembly of other focal adhesion components, like paxillin, vinculin and zyxin, has been shown to be dependent on the cleavage of talin by calpain, suggesting a role for the Ca^{2+}-dependent talin proteolysis in the regulation of focal adhesion turnover [57]. In this regard, it has been found that the reduction of STIM1 or ORAI1 gene expression, by RNA interference, dramatically affected the rate of focal adhesion turnover, slowing down cell migration *in vitro* and inhibiting metastasis of MDA-MB-231 cancer cells in nude mice [58]. The treatment of cells with SKF96365, a SOC inhibitor, also induced large focal adhesions *in vitro*, due to defective focal adhesion turnover. In addition, this blocking agent also inhibited tumor progression in mice [58].

Focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 beta (PTK2B or PYK2) are two well-known kinases involved in focal adhesion assembly [59]. Because focal adhesion targeting of PYK2 is required for the turnover and the Tyr402 autophosphorylation is required for PYK2 targeting, Chen et al. studied the correlation between EGF stimulation, PYK2 phosphorylation and STIM1 levels in SiHa cells [60]. EGF activates PYK2 phosphorylation at Tyr402, but this phosphorylation has been shown to be inhibited by the silencing of STIM1 [60]. In addition, STIM1 knockdown induced large focal adhesions, independently of the stimulation with EGF, further confirming the role of Ca^{2+} entry in facilitating the turnover of focal adhesions. Similarly, EGF triggered the phosphorylation of FAK at Tyr397, which is required for focal adhesion turnover and it was demonstrated that this phosphorylation is inhibited by STIM1 knockdown [60].

In this regard, it is known that EGF activates the phosphoinositide pathway, generating IP3 and activating Ca^{2+} release from the ER. EGF also activates de MAPK (mitogen-activated protein kinases) pathway. As a consequence STIM1 becomes phosphorylated at ERK1/2 target sites (Ser575, Ser608 and Ser621) upon EGF stimulation, leading to the dissociation from microtubules (i.e., EB1) and activating SOCE [49]. Casas-Rua et al. demonstrated that non-phosphorylatable mutants of STIM1, such as STIM1-S575A/S308A/S621A, blocked EGF signaling pathway, inhibiting cell migration in the endometrial adenocarcinoma Ishikawa cell line [49]. The impact of STIM1 was observed also at the genomic response level, because ectopic overexpression of STIM1-S575A/S608A/S621A blocked the epithelial-mesenchymal transition (EMT) of Ishikawa cells treated with EGF. The stimulation of cells with EGF induced a significant switch in E-cadherin localization from subplasma membrane region to a diffuse localization throughout the cytosol, as described for other epithelial cells [61]. EGF also triggered an increase of vimentin expression in well-defined cytoskeletal localization, as for other cells upon EGF stimulation [61, 62]. However, Ishikawa cells overexpressing STIM1-S575A/S608A/S621A-mCherry did not show significant increase in vimentin expression, nor E-cadherin relocalization [49], supporting for a role for phospho-STIM1 in the regulation of cell migration and cell transformation into a mesenchymal phenotype.

Cytosolic Ca^{2+} levels also regulate actomyosin, the macromolecular complex of actin and myosin that drives the mechanical forces for cell contractility during migration. Nonmuscle

cell contractility is controlled by nonmuscle myosin II, through the phosphorylation of its regulatory light chains (MLC2) at Ser19 in a Ca^{2+}/calmodulin-dependent manner [63]. The contractile force that moves the cell body is then transmitted to focal adhesions by phospho-myosin II-based actomyosin contraction. This phosphorylation was abolished by the Ca^{2+} channel inhibitor SKF96365, or by knocking-down STIM1 expression [64], indicating that STIM1-dependent Ca^{2+} entry has a significant role in MLC2 activation and in the reorganization of the actin-myosin cytoskeleton in migrating cells.

In addition to focal adhesions, podosomes and invadopodia are also adhesion structures regulated by Ca^{2+} signaling. Sun et al. reported that melanoma invasion is promoted by STIM1- and ORAI1-mediated Ca^{2+} oscillations, which promote invadopodia formation and extracellular matrix (ECM) degradation [65]. These authors found that Ca^{2+} signaling mediated by STIM1 and ORAI1 is essential for invadopodia formation over a collagen matrix and that addition of SKF96365, or chelation of extracellular Ca^{2+} with EGTA, blocked invadopodia assembly. More interestingly, ectopic expression of STIM1 or STIM1+ORAI1 increased phosphorylation levels of Tyr416 in Src kinase, without affecting phospho-FAK. This effect was revealed for MCF-7 cells (human breast cancer cells), NMuMG (mouse mammary epithelial cell line) and WM793 melanoma cells and the activation of Ca^{2+} entry with thapsigargin or ionophore A23187 led to the same result, i.e., a rapid increase of pY416 Src levels. These data, together with the fact that STIM1 shRNA, chelation of extracellular Ca^{2+}, or the inhibition of SOCE with 2-APB, reduced pY416 Src levels, strongly suggesting a direct role of STIM1-ORAI1-mediated Ca^{2+} influx in the preservation of Src activity and invadopodia formation [65].

From the information given above it is now widely accepted that one of the major targets for SOCE is the cytoskeleton and that the dynamics of the focal adhesion assembly as well as the dynamics of actin and tubulin cytoskeleton are strongly influenced by the kinetics of Ca^{2+} entry through store-operated Ca^{2+} channels.

6. Localization and polarization of SOCE in migrating cells

From the given information arises the question about the spatial control of STIM1-ORAI1 localization. The polarization of Ca^{2+} entry pathways has been described in several cell types, especially in exocrine gland cells. In pancreatic acinar cells under stimulation of Ca^{2+} mobilizing receptors, the ER Ca^{2+} release is detected in the apical region of the cell and the $[Ca^{2+}]_i$ increase remains restricted at this region [66], although the signal propagates to basolateral regions at high concentration of agonists. In salivary gland acinar cells, $[Ca^{2+}]_i$ increase is also detected in the apical region and then propagates to the basal region [67]. Indeed, several studies have demonstrated that agonist-stimulated Ca^{2+} signaling in exocrine gland cells is highly polarized. TRPC1 is a key factor for SOCE in salivary gland acinar cells and pancreatic acinar cells. In addition, TRPC3 contributes to SOCE and contributes to the receptor-stimulated Ca^{2+} influx in exocrine pancreatic acinar cells [68]. In addition, SOCE is the major contributor to Ca^{2+} influx in salivary gland and pancreatic acinar cells, so it is expected an asymmetric distribution of STIM1, ORAI1 and TRPC1-3, some of the most widely studied members involved in the control of SOCE.

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As expected, a polarized localization has been shown for all Ca^{2+} signaling proteins in exocrine acinar cells: IP3Rs, SERCAs and PMCA pumps, GPCRs, TRPC channels, ORAI1 channels and STIM1 [66, 69, 70], an asymmetric distribution required for the directed secretion carried out by these cells. However, little is known about the mechanisms involved in the targeting of Ca^{2+}-signaling complexes to these regions.

Because migrating cells are polarized cells, with polarized distribution of receptor tyrosine kinases, the study of Ca^{2+} entry in migrating cells is a task of particular importance. In this regard, Tsai et al. reported recently that STIM1 is enriched at the leading edge of migrating cells, measured with YFP-STIM1 and CFP-tagged ER marker [71]. The authors concluded that the polarization of STIM1 is microtubule-plus-end dependent, because STIM1-I644N/P645N, a mutant STIM1 that does not bind to EB1, failed to polarize in migrating cells. Because of the enrichment of receptor tyrosine kinases at the front of migrating cells, local Ca^{2+} pulses were observed with higher frequency at the leading edge, accompanied by a lower level of luminal ER Ca^{2+} and increased levels of PMCA activity at the front. In this report the authors also described that a DAG gradient is the result of the asymmetric activity of phospholipase C (PLC) at the leading edge of migrating cells, leading to the recruitment and activation of PKCbeta, a kinase involved in migration [72] by phosphorylating myosin [73], or other substrates of the cytoskeleton, such as GAP43, adducin, or fascin [74]. However, the precise mechanism that assembles de STIM1-ORAI1 and/or TRPC1 in a polarized distribution in cells is still unclear and it is expected that additional members of the cytoskeleton will solve this open question.

7. Conclusions

In conclusion, cytosolic-free Ca^{2+} concentration regulates the reorganization of cytoskeleton, focal adhesion turnover, invadopodia formation, actomyosin contractility and it is critical to trigger the development of lamellipodia as the leading structure during migration. But this dependence is reciprocal and Ca^{2+} influx through store-operated Ca^{2+} channels at the plasma membrane is fully dependent on the formation of endoplasmic reticulum-plasma membrane juxtapositions that are shaped by the reorganization of the cytoskeleton. In the last few years valuable knowledge has been gained regarding the activation of STIM1 by Ca^{2+} store depletion and how STIM1 relocalizes and activates ORAI1 at the PM-ER junctions. Here we have shown that both STIM1 and ORAI1 are involved in many aspects of cell migration and that gene silencing and specific inhibitors point out these two proteins in the regulation of Ca^{2+} influx pathways involved in supporting efficient cell adhesion and migration. However, a major lack of knowledge regarding the polarization of the signaling profile persists. Recent advances in genome editing will be valuable tools to knockout and knockin *STIM* and *ORAI* genes, as well as genes coding for cytoskeletal proteins involved in the reorganization of SOCE. With this coming era we will be able to monitor and study the behavior of tagged proteins at endogenous levels, as well as to study the loss of function of certain genes in any cell type, in an attempt to solve this open question in cell biology and signaling.

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