Chapter 23
The TRPC Ion Channels: Association with Orai1 and STIM1 Proteins and Participation in Capacitative and Non-capacitative Calcium Entry

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Abstract Transient receptor potential (TRP) proteins are involved in a large number of non-selective cation channels that are permeable to both monovalent and divalent cations. Two general classes of receptor-mediated Ca^{2+} entry has been proposed: one of then is conduced by receptor-operated Ca^{2+} channels (ROC), the second is mediated by channels activated by the emptying of intracellular Ca^{2+} stores (store-operated channels or SOC). TRP channels have been presented as subunits of both ROC and SOC, although the precise mechanism that regulates the participation of TRP proteins in these Ca^{2+} entry mechanisms remains unclear. Recently, TRPC proteins have been shown to associate with Orai1 and STIM1 in a dynamic ternary complex regulated by the occupation of membrane receptors in several cell models, which might play an important role in the function of TRPC proteins. The present review summarizes the current knowledge concerning the association of TRP proteins with Orai and STIM proteins and how this affects the participation of TRP proteins in store-operated or receptor-operated Ca^{2+} entry.

Abbreviations

$[Ca^{2+}]_c$	cytosolic free Ca^{2+} concentration
ARC	arachidonic acid-activated
CAD	CRAC-activating domain
CMD	CRAC modulatory domain
CRAC	Ca^{2+} release-activated Ca^{2+} channel
DAG	diacylglycerol
ER	endoplasmic reticulum
FRET	Forster resonance energy transfer
IP_3	inositol 3,4,5-trisphosphate
IP_4	inositol 1,3,4,5-tetrakisphosphate
MBCD	methyl-β-cyclodextrin

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OASF	Orai-activating small fragment
PIP_2	phosphatidilinositol 4,5-bisphosphate
PLC	phospholipase C
RACK1	receptor for activated C-kinase-1
ROCE	receptor-operated Ca^{2+} entry
SCID	severe combined immune deficiency
SERCA	sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase
SOC	store-operated channel
SOAR	STIM1 Orai-activating region
SOCE	store-operated calcium entry
STIM1	stromal interaction molecule 1
TRP	transient receptor potential
TG	thapsigargin.

23.1 Introduction

Regulation of the changes in cytosolic Ca^{2+} concentration is a point of convergence of many signal transduction pathways and modulates a variety of cellular functions ranging from fertilization to cell death. Changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$), also known as Ca^{2+} signals, are characterized by sudden and transitory increases in the concentration of free calcium ions [1]. Cell-generated Ca^{2+} signals require both internal and external Ca^{2+} sources. In most cell types, the major internal Ca^{2+} store is the endoplasmic reticulum (ER)/sarcoplasmic reticulum, where Ca^{2+} is stored by SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase). Due to the finite amount of Ca^{2+} accumulated in the ER the entry of extracellular Ca^{2+} is necessary to achieve full activation of a number of cellular functions. Store-operated Ca^{2+} entry (SOCE), also known as capacitative Ca^{2+} entry, a process regulated by the filling state of the intracellular Ca^{2+} stores, is a major mechanism for Ca^{2+} entry in non-electrically excitable cells [2]. There is a body of evidence supporting an important role for SOCE in Ca^{2+} signalling and intracellular homeostasis under physiological conditions, such as supporting Ca^{2+} oscillations [3]. In addition, SOCE has been reported to be required for a number of cellular processes, including cell proliferation, muscle contraction, platelet aggregation and secretion [4, 5]. Finally, SOCE serves as a mechanism to allow ER Ca^{2+} refilling, necessary for protein synthesis and post-translational modifications [6].

The nature of the channels that conduct SOCE has been a matter of intense investigation and debate. Two types of store operated Ca^{2+} channels have been described so far, which, although show distinct biophysical properties, are activated by depletion of intracellular Ca^{2+} stores with agonists, inhibitors of SERCA and/or strong Ca^{2+} chelators. First of all, Ca^{2+} release-activated Ca^{2+}-selective (CRAC) channels have been found and extensively described on the level of whole-cell current in a variety of non-excitable cells, including mast cells, Jurkat T-lymphocytes and RBL cells [7–9]. The current through CRAC channels (I_{CRAC}) is non-voltage activated,

inwardly rectifying, and selective for Ca^{2+} [10, 11]. While I_{CRAC} was the first store-operated Ca^{2+} current identified, it is not the only store-operated current, and SOCE has also been reported to include a family of Ca^{2+}-permeable channels, with different properties in different cell types known as SOC channels, which conduct the non-voltage activated, non-selective I_{SOC} current of small, but resolvable 0.7-11 pS conductance [12]. SOC channels have been found and described on single channel and whole-cell current levels in different cell types [13–16]. The mammalian homologues of the *Drosophila T*ransient *R*eceptor *P*otential (TRP) channels were initially presented as candidates for the conduction of SOCE and, more recently, the protein Orai1 has been proposed to form the pore of the channel mediating I_{CRAC}.

Despite intense investigations over the last two decades, the mechanisms of activation and the identity of the key molecular players conducting Ca^{2+} entry during SOCE have long remained elusive. However, in the last few years, the improvements of gene silencing protocols combined with high throughput platforms have provided important breakthroughs, especially with the identification of STIM1 (stromal interaction molecule 1) as the ER Ca^{2+} sensor and Orai1 as the pore-forming subunit of the archetypical capacitative channel, CRAC. STIM1 is a Ca^{2+}-binding protein located both in intracellular membranes, including the ER, and the plasma membrane with a single transmembrane region and an EF-hand domain in the N-terminus. STIM1 located in the ER shows the EF-hand domain in the lumen of the ER, which, by following different experimental manoeuvres, has been suggested to function as a Ca^{2+} sensor that communicates the filling state of the Ca^{2+} stores to the plasma membrane Ca^{2+} permeable channels [17, 18]. In addition, plasma membrane-resident STIM1, which shows the EF-hand domain facing the extracellular medium, has been reported to modulate the function of the capacitative channels [19, 20], probably acting as an extracellular Ca^{2+} sensor.

The involvement of Orai1 in I_{CRAC} has been identified by gene mapping in patients showing an inherited disorder called severe combined immune deficiency (SCID) syndrome attributed to loss of I_{CRAC}, which results in extreme vulnerability to infectious diseases. The ORAI1 gene located on chromosome 12 has been found to be mutated in SCID patients, and I_{CRAC} has been shown to be restored by expression of wild type Orai1 in T cells [21]. The role of Orai1 in I_{CRAC} was confirmed in a whole-genome screen of *Drosophila* S2 cells by Feske and coworkers [21], with other groups reporting similar results at the same time [22, 23]. Orai1 is a small protein with four transmembrane domains and both N- and C-terminal tails located in the cytosol. The Orai1 protein has been demonstrated to form multimeric ion channel complexes in the plasma membrane [24–29].

In addition to their involvement in receptor-operated Ca^{2+} entry (ROCE), there is now considerable evidence supporting a role for TRP proteins in the conduction of Ca^{2+} entry during SOCE. Particular attention has been paid to members of the TRPC subfamily. Using different approaches, from overexpression of specific TRP proteins to knockdown of endogenous TRPs and pharmacological studies, it has been suggested that most of the TRPC proteins can be activated by Ca^{2+} store depletion [12, 30, 31]. Among TRP proteins, the role of TRPC1 in SOCE has been extensively investigated in different cell types. TRPC1 has been reported to

be involved in SOCE by antisense experiments in human salivary glands [32] and vascular endothelial cells [33]. In support of this, antibodies directed to the pore-forming region of TRPC1 have been shown to reduce SOCE in vascular smooth muscle cells and human platelets [34, 35] and TRPC1-depleted myoblasts present a largely reduced SOCE [36]. Different TRPC associations appear to give rise to channels with distinct biophysical properties. In addition, association of TRPC proteins with STIM1 and Orai1 seems to play an important role in the participation of TRPCs in different mechanisms for Ca^{2+} entry in a number of cell types, although the association between these proteins still remains controversial and further studies are required to fully understand the process.

23.2 Transient Receptor Potential (TRP) Proteins: TRPCs

TRP proteins are ion channel subunits non-selective for monovalent and divalent cations, including Na^+ and Ca^{2+} that were initially identified in the *trp* mutant of *Drosophila*. The light-sensitive current in *Drosophila* photoreceptors is conducted by two Ca^{2+}-permeable channels encoded by the *trp* and *trpl* genes [37, 38]. The *trp* mutant is characterized by transient, rather than sustained, light-sensitive depolarization due to Na^+ and Ca^{2+} influx [39]. Later on, *Drosophila* TRP channels were shown to be gated by diacylglycerol (DAG) or a metabolic byproduct, synergistically with phosphatidylinositol 4,5-bisphosphate (PIP_2) depletion [40].

The identification of mammalian homologues of *Drosophila* TRP channels raised interest in TRP proteins as candidates for Ca^{2+} entry channels. The first mammalian TRP protein, TRPC1, was identified in 1995 in human [41, 42] and mouse [43]. Since their identification, a number of TRP proteins have been found, which are grouped into seven major subfamilies: four are closely related to *Drosophila* TRP (TRPC, TRPV, TRPA and TRPM), two more distantly related subfamilies (TRPP and TRPML), and finally the TRPN group expressed so far only in fish, flies and worms [44]. The canonical TRP (TRPC) subfamily comprises seven members (TRPC1–TRPC7, which, in turn, can be divided into four groups: TRPC1, TRPC2, TRPC3/6/7 and TRPC4/5), the vanilloid TRP subfamily (TRPV) consists of six members (TRPV1–TRPV6), the TRPA (ankyrin) subfamily includes only one mammalian member, TRPA1, and the melastatin TRP subfamily (TRPM) groups eight different channels (TRPM1–TRPM8). The TRPP (polycystin) and the TRPML (mucolipin) subfamilies include three channel members each, and finally, the TRPN has no mammalian members [45].

All members of the TRP family share a common architecture: they are proteins that contain six transmembrane domains, with different cytoplasmic N- and C-termini depending on the subfamily, and a pore loop region between the transmembrane domains 5 and 6 [46]. Many TRP proteins possess long N-terminal regions with several protein–protein interaction domains known as ankyrin repeats, a coiled coil region, and a putative caveolin-binding domain. On the other hand, the C-terminus includes the TRP signature motif (EWKFAR), a proline-rich motif and

different functional regions that facilitate their interaction with calmodulin or inositol 1,4,5-trisphosphate (IP_3) receptor [47–49]. For further information concerning the structure of TRP proteins the reader is referred to Chapter 1.

As reported above, most TRP channels are nonselective for monovalent and divalent cations with Ca^{2+}:Na^+ permeability ratios <10 [50]. There are a number of exceptions, such as TRPM4 and TRPM5, which are selective for monovalent cations, and TRPV5 and TRPV6, which have a Ca^{2+}:Na^+ permeability ratio > 100. Among the TRP channels expressed in mammals, the role of the TRPC subfamily members on agonist-evoked Ca^{2+} entry has focused much attention, and, therefore, this review present an overview of the mechanisms involved in the participation of TRPC in Ca^{2+} entry.

The TRPC members form Ca^{2+}-permeable cation channels and have been presented as candidate subunits for the channels conducting both SOCE and ROCE [30, 35, 51–53]. By means of different experimental manoeuvres, from gene inactivation to gene expression silencing using siRNA or shRNA and the use of neutralizing antibodies, all the members of the TRPC family have been reported to be activated by store depletion or to be involved in SOCE both in excitable and non-excitable cells, including TRPC1 [32, 34, 35, 54], TRPC2 [55], TRPC3 [56, 57], TRPC4 [58, 59], TRPC5 [59, 60], TRPC6 [61–63] and TRPC7 [64]. However, the participation of TRPCs in SOCE depends on special circumstances, such as the expression level. Thus, at low expression levels TRPCs are activated by depletion of the intracellular Ca^{2+} stores, while at relatively high levels of expression TRPCs are not longer sensitive to store depletion but activated by phospholipase C (PLC) or its metabolites [56]. Furthermore, it has been reported that TRPC channels might participate in SOCE or ROCE in the same cell type depending on their mode of expression. In HEK-293 cells, TRPC7 is activated by PLC-stimulating agonists and not by Ca^{2+} store discharge when transiently expressed; in contrast, stably expressed TRPC7 gating can be regulated by either Ca^{2+} stores or PLC activation [64]. Although the reason for this phenomenon has not been determined it might be attributed to the association of TRPC proteins with regulatory subunits that confer store depletion or receptor sensitivity and then participation in SOCE or ROCE. The identification of STIM1 and Orai1 as essential components of SOCE may uncover the mechanism underlying the participation of TRPC subunits in SOCE or ROCE, as reported below.

23.3 STIM and Orai Proteins

Probably one of the most significant advances occurred in the last 5 years on the intracellular Ca^{2+} homeostasis has been, together with the determination of the structure of Orai, demonstrating that STIM1 is the ER sensor that report its Ca^{2+} filling state, essential for Ca^{2+} store depletion-triggered Ca^{2+} influx across de plasma membrane. Although two single transmembrane-spanning domain stromal interaction molecules with no known catalytic activity (human STIM1 and STIM2

containing 685 and 833 amino acids, respectively, and differing primarily in the lengths of their N- and C-terminal tails) have been described, STIM1 is the most interesting for the purposes of this chapter as it was found to act not only as a sensor within the stores [17, 18, 65] but also to play a role in the plasma membrane [17, 20] to activate I_{CRAC}.

From a structural point of view, STIM1 is a Ca^{2+}-binding protein (within either the ER lumen or extracellular space) that includes a number of functional domains described in Table 23.1. Both STIM1 and STIM2 can be phosphorylated predominantly on serine and threonine residues. In addition, STIM1 contains an additional N-linked glycosylation site within the SAM domain itself [66]. STIM1 has been reported to be expressed at the cell surface, as well as in the ER membrane, while STIM2 is expressed only intracellularly, likely reflecting an ER-retention signal (KKXX) present in STIM2 but not in STIM1 [67].

Knockdown of STIM1 by siRNA or functional knockdown of STIM1 by electrotransjection of neutralizing antibodies reduces SOCE in different cell types [20, 68] and I_{CRAC} in Jurkat T cells [20]. Evidence supporting the role of STIM1 in SOCE reports that mutation of the Ca^{2+}-binding EF-hand domain of STIM1 leads to constitutive SOC channel activation, and subsequent entry of Ca^{2+} into the cytoplasm, even without any detectable change in the content of the Ca^{2+} stores [17].

It is noteworthy to mention that, in addition to its role as an ER Ca^{2+} sensor, STIM1 has been found in the plasma membrane in a number of cells, expressing the EF-hand domain in the cell surface and acting as an extracellular Ca^{2+} sensor, where it has been demonstrated to modulate the operation of CRAC and SOC channels. External application of an antibody addressed towards the STIM1 N-terminal EF-hand region has been reported to block both CRAC channels in hematopoietic cells and SOC channels in HEK293 cells [20]. In addition, external application of the anti-STIM1 antibody blocks the inhibition of SOCE induced by increasing extracellular Ca^{2+} concentrations in human platelets, revealing a role for plasma membrane-resident STIM1 in the modulation of SOCE by extracellular Ca^{2+}, probably through its interaction with Ca^{2+} channel subunits such as Orai1 [19]. The pool of STIM1 that resides in the plasma membrane has also been reported to play a key role in other mechanisms of Ca^{2+} entry different from SOCE, such as the store-independent, arachidonic acid-activated, ARC channels, which show high Ca^{2+}-selectivity and low conductance and co-exist with CRAC channels [69].

In the 2006, Vig and co-workers demonstrated that the Ca^{2+} release-activated Ca^{2+} channel protein 1 (CRACM1) is a plasma membrane protein essential for SOCE. Although overexpression of the CRACM1 did not affect CRAC currents, RNAi-mediated knockdown disrupted its activation. Also, they reported that CRACM1 could be the CRAC channel itself, a subunit of it, or a component of the CRAC signalling machinery [22]. Few months later, the same group demonstrated that STIM1 and CRACM1 interact functionally; the overexpression of both proteins greatly potentiated I_{CRAC}, suggesting that STIM1 and CRACM1 mutually limit store-operated currents and that CRACM1 may be the long-sought CRAC channel [70]. Today, CRACM1 is best known for the romantic name of Orai1 (in Greek mythology, the "Orai" are the keepers of the gates of heaven). The mammalian

Table 23.1 Main functional domains of STIM1

Region	Location	Function	References
EF-hand domain	aa 67–95	Ca^{2+} binding domain that senses ER Ca^{2+} concentration	[17, 129]
SAM motif	aa 132–200	Sterile-α motif involved in protein-protein interaction	[129]
Transmembrane region	aa 215–234	A single transmembrane segment	[130]
Coiled-coil regions	aa 238–343 aa 363–389	Include the regions involved in Orai1/CRAC channel activating domains and overlap with the ERM-like domain	[66]
ERM-like domain	aa 251–535	Ezrin-radixin-moesin (ERM)-like domain. Includes de Orai1/CRAC channel activating domain	[131, 132]
CAD	aa 342–448	Orai1/CRAC activating domain	[83]
SOAR	aa 344–442		[81]
OASF	aa 233–450/474		[82]
CCb9	aa 339–444		[84]
Homomerization domain	aa 400–474	Clusters STIM1 into regions close to the plasma membrane	[130, 133]
STIM1 inhibitory domain	aa 445–475	Inhibits the Orai1/CRAC activating domain at rest	[134]
CMD	aa 474–485	CRAC modulatory domain that induces Orai1/CRAC cannel closure	[85]
Serine/proline-rich region	aa 600–629	Localization of STIM1 into ER–PM junctions	[89, 135]
Polybasic region	aa 672–685	Involved in puncta formation	[81]

Table 23.2 Predicted functional domains of Orai1

Region	Location	Function	References
Arginine/proline-rich region	aa 3–8 aa 28–33 aa 39–47	Orai1 assembly	[130, 136]
Arginine/lysin-rich region	aa 77–88	Orai1 assembly	[130, 136]
Transmembrane regions: TM1 TM2 TM3 TM4	aa 88–105 aa 118–140 aa 175–197 aa 236–258	Four transmembrane segments	[130]
Selectivity filter	aa 106–114 E190	Pore-forming domain	[22, 23, 73]
Coiled-coil region	aa 265–294	Involved in protein-protein interactions (STIM1-Orai1 interaction)	[79]

Orai family has two additional homologs, Orai2 and Orai3. Orai proteins share no homology with any other known ion channel family or cellular proteins.

Orai1, a Ca^{2+} selective ion channel, is a 301 amino acids protein with four transmembrane domains and a number of functional regions depicted in Table 23.2. Maruyama et al. [71] have purified Orai1 in its tetrameric form and have reconstructed the three-dimensional structure from electron microscopic images, providing the first depiction of an Orai family member. According to these authors, Orai1 is a teardrop-shaped molecule 150 Å in height, 95 Å in side length, and 105 Å diagonally at the widest transmembrane region.

The structure of Orai2 and Orai3 is similar to that of their homolog Orai 1 [72, 73]. All three Orai isoforms constitute Ca^{2+} selective plasma membrane channels, whose currents have been shown to be inhibited by extracellular Ca^{2+} [74]. The three Orai isoforms can be activated by store depletion when co-expressed with STIM1 although the amplitude of the currents generated are smaller for Orai2 and Orai3, which might reflect that they interact with STIM1 with less efficiency [75, 76]. Orai isoforms show slightly different selectivity for Na^+ (being Orai3 more permeable for Na^+) and distinct sensitivity to the pharmacological agent 2-aminoethoxydiphenyl borate (2-APB) [75]. While Orai1 currents are stimulated by low concentrations of 2-APB and abolished by high 2-APB concentrations, Orai2 currents are only partially sensitive to this inhibitor and Orai3 is stimulated by 2-APB [75, 77, 78].

23.4 STIM1-Orai1-TRPC Communication

The nature of the interaction between STIM1 and the plasma membrane Ca^{2+} channel subunits is currently under intense investigation by a number of research teams in order to determine the mechanism underlying the activation of capacitative channels by STIM1. In 2008, Romanin's group demonstrated a dynamic interaction between STIM1 and Orai1 involving the C-termini of both proteins using Forster resonance energy transfer (FRET) microscopy. Interestingly, the Orai1 R91W mutant associated to SCID syndrome did not impair the interaction with STIM1 but altered the activation of Ca^{2+} currents [79]. The coiled-coil C-terminal domain of STIM1 has been reported to trigger dimerization of Orai dimers resulting in the formation of tetrameric Orai1 channels to activate I_{CRAC} [80].

Four research groups have identified in parallel that a cytoplasmic STIM1 region composed of an ezrin-radixin-moesin domain is essential for the activation of Orai1. This region has been named SOAR (STIM1 Orai-activating region) [81], OASF (Orai-activating small fragment) [82], CAD (CRAC-activating domain) [83] and CCb9 [84]. The four regions, SOAR (including the STIM1 amino acid residues 344–442), OASF (amino acids 233–450/474), CAD (amino acids 342–448) and CCb9 (amino acids 339–444), are located within STIM1 C-terminus and comprise two coiled-coil domains and an amino acid sequence that enhances interaction with Orai1, resulting in increased Ca^{2+} currents. These studies have reported several features of the Orai1-STIM1 interacting region: OASF has been reported to be able to homomerize by a novel assembly domain that occurred subsequent to the coiled-coil domains. In addition, STIM1 oligomerization has been shown to be required for CAD exposure. Furthermore, the SOAR region is able to activate all known Orai isoforms although with different conductances being greater for Orai1 than for Orai2 or Orai3 [81, 82].

In addition, a regulatory domain at aminoacids 474–485 of the cytosolic STIM1 region, containing 7 negatively charged residues, known as CMD (CRAC modulatory domain)/CDI (Ca^{2+}-dependent inactivation, reported as residues 470–491), has recently been described. This domain generates a signal that promotes Orai/CRAC channel closure in a Ca^{2+} concentration-dependent manner, a process known as fast, Ca^{2+}-dependent inactivation of the Orai channels [85–87].

The interaction of STIM1 with Orai1, following depletion of the intracellular Ca^{2+} stores, results in a conformational change in Orai1, as determined by FRET, that might be important for CRAC channel activity [88]. FRET analysis between STIM1-YFP and Orai1-CFP has revealed that STIM1 and Orai1 approach within 100 Å or less after treatment with thapsigargin to induce store depletion. Simultaneously, the interaction Orai1-Orai1 is reversibly reduced upon depletion of the stores or application of extracellular Ca^{2+}, both inducing CRAC channel activation, thus suggesting that Orai1 is subjected to a conformational rearrangement that is relevant, although not sufficient, for CRAC channel function [88].

In 2006, Huang and coworkers reported that STIM1 is able to gate TRPC1 [89]. The association of STIM1 with the TRPC proteins has been shown to be

mediated by the STIM1 ERM domain [89]. More recently, the SOAR region, which has been shown to interact with Orai1 (see above), has been presented as the domain that binds to the TRPC channels [90]. The initial studies by Huang and coworkers reported that the STIM1 K-domain plays an important role in TRPC1 channel gating, although is not necessary for the interaction between STIM1 and TRPC1 [89]. Muallem's team has recently reported that STIM1 gates TRPC1 through the interaction between two conserved, negatively charged, aspartates in TRPC1 ((639)DD(640)) with the positively charged lysine residues in STIM1((684)KK(685)) located in the C-terminal polybasic region. Different charge swapping experiments confirm that STIM1 gates TRPC1 by intermolecular electrostatic interaction [91]. A similar activation mechanism has been reported for TRPC3 mediated by the negatively charged 697 and 698 aspartate residues [91]. However, STIM1 operates Orai1 by a different mechanism since the C-terminal polybasic and serine-proline rich region of STIM1 are not required for activation of Orai1 [91]. Functional association between STIM1 and TRPC1 has been reported in a number of endogenously expressing and transfected cell types, including HEK-293 cells [89, 92–95], Jurkat T cells [89], human platelets [68], salivary gland cells [96], mesangial cells [97], mouse pulmonary arterial smooth muscle cells [98], the hepatic cell line HL-7702 cells [99] and human parathyroid cells [100]. It is noteworthy to mention that association of STIM1 with TRPC1 has not been found in HEK-293 cells co-transfected with both proteins, where STIM1 overexpression has not reported an increase in the activity of different TRPCs in these cells [101], and vascular smooth muscle cells [102]. The reason of this discrepancy, which might reside on the different transfection levels or the idiosyncrasy of the cell type, is still unclear and requires further studies to fully understand.

23.5 Calcium Entry Pathways Mediated by STIM1-Orai1-TRPC Complexes

The nature of the capacitative channels, as well as the mechanisms that gate them after Ca^{2+} stores have been depleted, have been a matter of intense investigation since the identification of SOCE. One of the earliest hypotheses was formulated in sea urchin eggs, where inositol 1,3,4,5-tetrakisphosphate (IP_4) was suggested to modulate Ca^{2+} entry into the IP_3-sensitive pool by physical interaction between IP_3 and IP_4 receptors located in the plasma membrane and the ER membrane, respectively [103, 104]. The role of IP_3 receptors in SOCE has been widely investigated and a relationship between TRP proteins and IP_3 receptors has been demonstrated in different cell types, including HEK 293 cells, where exogenously expressed TRPC3 can be activated by an IP_3 receptor-dependent physical coupling mechanism [105], T3 cells stably expressing epitope-tagged TRPC3 or TRPC6, where IP_3 receptor is detected in TRP immunoprecipitates [106], and human platelets, where endogenously expressed type II IP_3 receptor has been found in TRPC1 immunoprecipitates only after depletion of the intracellular Ca^{2+} stores and independently of rises in

$[Ca^{2+}]_c$, which indicates that this interaction is capacitative in nature [107–110]. IP_3 receptors, such as the type I IP_3 receptor, have also been reported to participate in agonist-induced, probably non-capacitative, Ca^{2+} entry by interaction with TRPC3, the scaffold protein RACK1 (receptor for activated C-kinase-1), STIM1 and Orai1 [111, 112]. However, although the IP_3 receptors might play an important role in agonist-induced Ca^{2+} entry, they lack Ca^{2+} sensing capability.

With the identification of STIM1 as the ER Ca^{2+} sensor, studies concerning the communication between the ER and the plasma membrane channels focused on this protein. It is widely accepted that a functional protein-protein interaction between STIM1 and Orai1 results in the activation of SOCE. STIM1 enhances SOCE when co-expressed with Orai1 [70, 113, 114], as well as with Orai2 [113] and Orai3 [75, 78], which suggests that these combinations of proteins are sufficient to mediate the process of SOCE, although with distinct inactivation profiles and permeability properties. Special attention has been focused on the study of the interaction between STIM1 and Orai1. In HEK-293 cells, which show significant SOCE while the level of endogenous CRAC is extremely low, expression of Orai1 alone clearly reduced SOCE; however, when co-expressed with STIM1, Orai1 induces a dramatic gain in the amount of SOCE [114]. The inhibition of SOCE by Orai1 overexpression suggests that an adequate stoichiometrical relationship between STIM1 and Orai1 is necessary for this process [114]. Consistent with this, store depletion has been reported to lead to aggregation and translocation of STIM1 in close apposition to the plasma membrane in order to recruit Orai1 and assemble functional units of CRAC channels in a stoichiometric manner [115]. Studies based on electrophysiology, single-molecule fluorescence bleaching methods and FRET have demonstrated that the CRAC channels are formed by four Orai1 monomers assembled to form a tetrameric structure, which is associated to two STIM1 molecules [24, 80, 116] (Fig. 23.1). However, it remains unclear whether this is the only configuration that results in CRAC channel activation. In fact, studies in cells expressing Orai1 and STIM1 at different ratios (from 4:1 to 1:4) have reported that low Orai1:STIM1 ratios results in I_{CRAC} with strong fast Ca^{2+}-dependent inactivation, while high Orai1:STIM1 ratios produce I_{CRAC} with strong activation at negative potentials. In addition, the Orai1:STIM1 expression ratio affects Ca^{2+}, Ba^{2+} and Sr^{2+} conductance; thus suggesting that the biophysical properties of the channels formed by Orai1 depend on the stoichiometry of its interaction with STIM1 [117].

Soon after the identification of STIM1, Huang and coworkers reported that the cytosolic C-terminus of STIM1 is sufficient to activate TRPC1 channels and SOCE [89]. The association of STIM1 and TRPC1 has been reported in a number of cell types and models including human platelets endogenously expressing TRPC1 and STIM1 [68, 118, 119], rat basophilic leukemia cells [96] or HEK293 cells [120]. In addition, STIM1 has been reported to associate with other members of the TRPC family including TRPC2 [93], TRPC4, TRPC5 [92] and TRPC6 [63], although the interaction with TRPC6 has been challenged by Yuan and coworkers, suggesting that STIM1 regulates its function indirectly by promoting the heteromultimerization of TRPC6 with TRPC4 [92]. The direct or indirect association between STIM1 and TRPC6 observed in human platelets [63] and HEK293 cells [92] might be due to

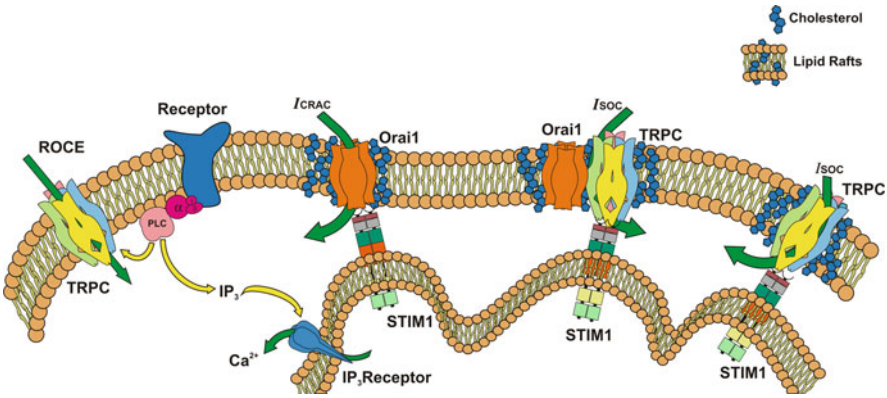

Fig. 23.1 Calcium entry into cells across the plasma membrane might occur through a variety of TRPC-dependent and – independent mechanisms. The Ca^{2+} selective capacitative current I_{CRAC} involves the activation of Orai1 forming channels by STIM1. The non-selective capacitative current I_{SOC} requires the interaction of STIM1 with either TRPC-Orai1 complexes or TRPC containing channels. Lipid raft domains have been shown to be important for capacitative channel activation. In the case of ROCE (including second messenger-operated Ca^{2+} entry) PLC metabolites activates TRPC containing channels independently of STIM1 and the plasma membrane lipid raft domains. ROCE, receptor-operated Ca^{2+} entry, TRPC, canonical transient potential receptor protein; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; STIM1, stromal interaction molecule 1

the idiosyncrasy of the cells or the different expression of the proteins investigated, endogenous in human platelets, although whether the interaction between TRPC6 and STIM1 is mediated by TRPC4, or other TRPC family members, has not been investigated in these cells yet.

Interestingly, the association of STIM1 to TRPC1 has been reported to recruit TRPC1 into lipid rafts, where TRPC1 functions as a SOC channel, while in the absence of STIM1, TRPC1 interacts with other TRPC family members resulting in the formation of receptor-operated Ca^{2+} (ROC) channels (Fig. 23.1); thus providing evidence for a role of STIM1 in the regulation of TRPC1 participation in SOCE or ROCE and highlighting the role of lipid rafts in the modulation of TRPC1 channel function [121]. Lipid rafts are plasma membrane domains that contain high concentrations of cholesterol and sphingolipids. Lipid rafts recruit certain signalling molecules while excluding others. For instance, in human platelets, only TRPC1, 4 and 5 were found to associate with plasma membrane lipid rafts, while TRPC3 or TRPC6 were not found in these domains [59]. A number of studies based on the use of methyl-β-cyclodextrin (MBCD), a compound that forms soluble complexes with cholesterol and thus deplete membrane cholesterol [122], have reported that lipid raft domains are essential for the assembly of signalling complexes, although it should be taken into account that cells might restore the cholesterol level in the plasma membrane by mobilising cholesterol from intracellular cholesterol stores [123]. Therefore, MBCD might reduce intracellular cholesterol levels, and sphingolipids also participate in lipid rafts and might maintain certain

raft structure [124, 125]. Lipid rafts provide a favourable environment necessary for clustering of STIM1 at ER-plasma membrane junctions upon store depletion, facilitating the Ca^{2+} store-dependent interaction between STIM1 and TRPC1 and subsequent SOCE [126]. Lipid rafts have also been reported to play a crucial role in the association between STIM1 and the plasma membrane channel subunits TRPC1 and Orai1 after depletion of the intracellular Ca^{2+} stores and is also necessary for thapsigargin-induced Ca^{2+} entry in human platelets [119].

Recent studies have presented evidence for the existence of functional interactions between Orai1 and TRPCs under the influence of STIM1, and propose that SOC channels are composed of heteromeric complexes that include TRPCs and Orai proteins [25, 26, 96] (Fig. 23.1). Knockdown of Orai1 significantly reduces I_{SOC} in human salivary gland cells [96], where TRPC1 has been demonstrated to be a major SOC channel subunit [127]. Consistent with this, Orai proteins have been reported to confer STIM1-mediated store depletion sensitivity to TRPC channels recruiting TRPC channels for the conduction of SOCE. In HEK293 cells overexpressing store-depletion insensitive TRPC3 or TRPC6, these TRPCs become sensitive to store depletion upon expression of exogenous Orai [26]. These observations suggest that the involvement of Orai proteins in SOCE might be well explained either by a model in which Orai1 are self-contained ion channels activated by STIM1, the proposed CRAC channel hypothesis [23, 70, 73, 128], or a model in which the SOC channels are formed by a combination of TRPCs and Orai proteins [26]. In the latter model, Orai proteins would communicate the information concerning the filling state of the intracellular Ca^{2+} stores from STIM, located in the ER, to TRPC proteins located in the plasma membrane. In support of a role for Orai conferring store-depletion sensitivity to TRPCs, these complexes have been found in non-transfected cells. In human platelets endogenously expressing STIM1, Orai1 and TRPC1, where electrotransjection with anti-STIM1 antibody, specific for the EF-hand domain, both prevented the interaction of STIM1 with hTRPC1 and reduced thapsigargin-evoked SOCE [68], a functional interaction between STIM1, Orai1 and TRPC1 in the activation of SOCE has been demonstrated [118]. In these cells, impairment of the interaction between STIM1 and Orai1, results in disruption of the association of STIM1 and TRPC1, and subsequently alters the behaviour of TRPC1 being no longer involved in SOCE but in ROCE mediated by DAG [118]. Similar results have been observed for TRPC6, in human platelets naturally expressing TRPC6 we have found that the participation of TRPC6 in SOCE or ROCE is regulated through its interaction with the Orai1-STIM1 complex or hTRPC3, respectively, in human platelets [63]; thus STIM1 located in the ER functions as a switch that communicates the filling state of the stores to SOC channels, involving TRPC proteins, through Orai1.

Interestingly, a number of reports have strongly suggested that Orai and TRPC proteins might form complexes that participate both in SOCE and ROCE. A study has reported that expression of Orai1, under experimental conditions that enhance SOCE, leads to the activation of ROCE. In addition, the R91W Orai1 mutant, responsible for SCID, has been shown to block both SOCE and DAG-activated ROCE into cells that, stably or transiently, express TRPC3 proteins [27]. To

integrate these results with current data concerning Orai, TRPCs and STIM, it has been postulated that Orai-TRPC complexes recruited to lipid rafts mediate SOCE, whereas the same complexes mediate ROCE when they are outside of lipid rafts [27], which is consistent with previous studies reporting a role for lipid rafts in the modulation of TRPC function by STIM1 [121]. Therefore, there is a body of evidence supporting that TRPCs might be involved in the formation of ion channels responsible for ROCE or SOCE by receiving information from either PLC or STIM1-Orai, respectively [25–27, 63, 118, 120]. The activation of TRPCs by STIM1 has been challenged in a recent study, although, as reported by the authors, more complex combinations of STIM1, Orai1 and TRPCs, as described in Cheng et al. [120], Jardin et al. [63, 118] and Liao et al. [25–27] have not been addressed in that study [101]. Despite the current knowledge concerning Orai-TRPCs-STIM interactions, further studies are required to describe more accurately the molecular composition of the channels mediating SOCE and ROCE and to clarify whether the channel components are the same when Orai-TRPC complexes mediate ROCE or SOCE.

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